writhed after histamine. The histamine releaser I-1935 produced immediate writhing in the mouse but its action was only partially blocked by chlorpheniramine during 10 minutes of observation. Compound 48/80, also a histamine releaser in the rat was inactive in the mouse. These observations are not consistent with the hypothesis that histamine release (as judged by data on such release in rats to be sure) may be an inciting cause of writhing in the mouse. However, it is apparently unknown whether L-1935 can release substances other than histamine. Compound 48/80 was only partially effective as histamine releaser in the mouse(6).

Since phenylquinone and hydrochloric acid were the only effective writhing producers in the rat, it would seem that release of serotonin or histamine is not to be considered in causation in this species.

Writhing in the mouse was blocked in all cases by morphine, LSD, and aminopyrine except that LSD did not block writhing produced by phenylquinone or hydrochloric acid. Chlorpheniramine partially blocked writhing produced by histamine, serotonin, or L-1935. It did not block writhing produced by phenylquinone or hydrochloric acid.

Summary. 1. Histamine is probably not a basic cause of writhing in the mouse. 2. It is possible that 5-HT may be a basic etiologic agent but, if true, the usual releasers of 5-HT do not so function in the mouse. 3. Acidity of injected solution *per se* is probably not etiologic. 4. It is worth considering that phenylquinone, HCl, serotonin, and L-1935 may release some substance other than histamine, serotonin, heparin, or bradykinin which in turn may incite writhing. 5. Direct irritation of nerve endings remains as a distinct causative possibility. 6. In screening for analgesics, both opiate and non-opiate types, phenylquinone and HCl are most useful of writhing agents tried, in eliminating the possibility of false positives. Phenylquinone may be preferable because of its greater duration of action.

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Determination of Cell Viability. (23985)

JOHN H. HANKS AND JOHN H. WALLACE

Leonard Wood Memorial Laboratory, Department of Bacteriology and Immunology, Harvard Medical School, Boston, Mass.

The difficulties which arise from attempts to assess viability of tissue cells solely in terms of reproductive capacity include: suitability of substrates and circumstance; necessity of employing population data to deduce what has happened to individuals; ultimate impossibility of distinguishing "static" from "cidal"; and the fact that certain types of tissue cells have not been induced to proliferate *in vitro*. Ideally, viability should be determined by methods which are simple, rapid, and applicable to the non-proliferating individual as well as to cultivable populations. If diverse factors required for reproduction are set aside, the capacity to control internal ionic environment is a universal characteristic of living beings. Many observations concerning exclusion of dyes by living yeasts(1,2), bac-

TABLE I. Reagents and Materials.

Bacto-Eosin Y (DE-5): 1.0% in H_2O
Idem : 0.15% in BSS
Levy-Hausser hemocytometer
Clean glass slides, 1 × 3″
Standard platinum loops:

Diam., mm	AHT* No.	Vol† delivered, ml	Approx. vol ratios
4	7433B	.01750	80
2	7433	.00250	10
1	7433	.00038	2
.7	8304-G	.00022	1

* Arthur H. Thomas.
† When filled by lifting horizontally through surface of liquids. If withdrawn vertically, the loops transfer approximately ½ the stated volumes.

teria(3) and tissue cells(4,5,6) have readily been confirmed. In view of materials, time and attention required to assess viability of tissue cells and cell cultures by growth determinations, and the ultimate uncertainties of interpretation, it seems useful to emphasize and illustrate the convenience with which the more fundamental criterion of dye exclusion may be applied.

Methods. Useful solutions and materials are indicated in Table I. The 1% stock solutions of eosin are self-sterilizing, or may be autoclaved in water or balanced salt solution (BSS). Since known, reproducible volumes can be transferred by platinum loops, these are used to measure solutions and cell aliquots. If stock solutions are prepared at appropriate concentrations, any desired combinations or dilutions can be prepared in droplets on glass slides. Also, small loops of a reagent may be mixed with the contents of a larger loop, and aliquots dispensed from the latter. Transfer of alkali or metal residues is prevented by swishing the loops in water before drying or flame sterilization. Dilution of cells in dye solutions prior to hemocytometer counts is obviated by drying 1mm loops of aqueous eosin 1% on the inclines to the chamber, and by use of a stippling motion to dissolve the dye uniformly in a 2mm loop of cell suspension before sweeping the mixtures into the counting chambers. As a general method it is more convenient to store clean slides on which 1mm loop-spots of aqueous eosin 1% have been dried (usually 3 horizontal rows of 5 spots each). During tests, a slide is laid in a Petri dish containing (in both the top and bottom) 90mm circles of moistened filter paper. One or more eosin spots are rehydrated with 2mm loops of cell suspension, stirring or stippling to ensure prompt mixture. In the case of dense cell suspensions, the eosin is rehydrated with a 2mm loop of test solution or serum 2% in BSS (without bicarbonate), and a wire or a 0.7mm loop of cells is added. The slide is held in the moist chamber at room temperature for 2 minutes before observations on 200 cells/spot are recorded. If care is taken to transfer uniform aliquots and cells are not too numerous, it is also practical to enumerate total cells per droplet. Nicely stained cells are seen in air-dried droplets which have been extracted for 2 minutes in 95% alcohol or in 10% formalin, rinsed in water, and again dried.

Addition of small loops of test solutions to 2mm loop-droplets of cells in the absence of eosin will be recognized as a means of imposing experimental variables. If counts are not to be made immediately, the droplets are prepared with minimal spreading. The slide is held in a moist Petri dish or in a horizontally placed Coplin jar into which CO_2 can be exhaled and trapped. While the 1mm loop of eosin 1% is being added to assess the results, the droplets are spread to diameters of 6-7mm. For direct observation of viability among cells growing on glass in liquid media, it is simplest to remove the medium to 0.1 ml, to add one 4mm loop of eosin 1%, and to wash this solution over the cells for 2 minutes. After gross or microscopic observation, the cultures are extracted twice for 5 minutes with BSS before adding the renewal medium. Most observations have been made on clone 929 of Earle's L strain of mouse fibrocytes derived from a medium comprised of horse serum 2%, Eagle's supplement(7) and Bacto-peptone 0.5% as recommended by Waymouth(8). If experimental background is not stated, these and other cells were suspended in serum 2% in BSS without added bicarbonate during tests for dye exclusion.

Results. The simplest evidence that eosin distinguishes live from dead cells is as follows. Cells which exclude eosin in moist preparations are seen to be plump and smooth.

TABLE II. Effect of Variable Serum Concentrations.

Counts at	Serum:	% cells white .04%*	.2%	2%	20%
2′		92	90	90	96
15′		85p	95	90	94
30′		54p	90	92	90
Avg % white			92	91	93

Continuous exposure to 0.15% eosin in loop spots on 3 slides in moist Petri dishes.
One slide examined at each interval.
* Concentration of serum in "BSS controls."
p = cells acquiring pink tinge of eosin.

Those which admit dye resemble flattened spheroids. With appropriate illumination, they exhibit the roughened surfaces which always are seen in motion photomicrographs of cells which undergo a fantastic boiling and bubbling just as they expire. Since these death throes of the cell are as well known, and as readily interpreted as the failure of heart beat or respiration in larger animals, the association between eosin staining and death of the cell seems to be a direct one.

Major variables which might influence the validity of methods based on the percentages of cells capable of dye exclusion are: serum concentration, dye concentration, and time of exposure before counting. Because of inevitable pH drift and dehydration, these factors might be expected to cause difficulties in small droplets on glass slides.

As shown in Table II, simple assessments of viability of the L cells (counts within 2-5 minutes) were not modified by a 500-fold range of serum concentrations. Results remained constant during exposure to 100-fold variations in serum background for 30 minutes. Since yeasts are not dependent on serum for maintenance, their use permitted further clarification of this question. Following 5 hours of continuous exposure to 0.15% eosin in serum 0% and 20%, the percentages of white cells were 86% and 88% respectively. Thus, no evidence was obtained that serum binding of eosin alters its availability at pH values above 7.

The results in Table III demonstrate that in the presence of serum 2% these cells tolerated twice the recommended concentration of eosin for 30 minutes and that 30 minute exposures usually were required to demonstrate the deleterious effect of 3 times the recommended concentration.

The tolerance of cells to dehydration of small droplets during counting on glass slides is explained by their adaptability to a wide range of osmotic pressures. Dilute suspensions of the L cell in serum 0.4% and 2% were exposed to modified electrolyte concentrations for periods of 1 hour; 2mm loop samples were assayed on eosin slides at 15, 30 and 60 min. to determine the onset of damage; the cells were then washed and replanted in the original tubes to confirm the number and quality of surviving cells. Within less than 3 minutes cells in BSS 200% became much smaller than usual, while those in BSS 20% and 10% were expanded to many times their normal size. Both eosin and cultivation revealed that 200% to 20% of the usual electrolyte concentration was tolerated for 1 hour; also that the onset and degree of damage in BSS 10% water 90% varied with the quality of cells employed in different experiments. In the presence of BSS 10%, the earliest indications of the onset of damage were revealed by the fact that 90-97% of cells excluded eosin for 2 minutes, but were unable to exclude it for 10 minutes.

The practicality of employing dye exclusion to replace cultivation procedures is now to be illustrated by a few examples.

The merits and liabilities of the two customary methods of preparing cell suspensions —scraping or enzyme release—were readily defined by direct observation in loop-spots on slides or by sampling from tubed suspensions which permitted more accurate control of pH

TABLE III. Effect of Increased Eosin Concentrations.

Counts at	Eosin:	% cells white —*	.15%	.30%	.45%
2′		88	91	93	90
15′		93	90	92	88
30′		88	87	89	87p
Avg % white		90	90	91	(89)†

Continuous exposure as in Table II: Serum 2%, eosin variable.
* Control: Eosin added just 2′ prior to each examination.
† Avg after 2′ and 15′ exposure.

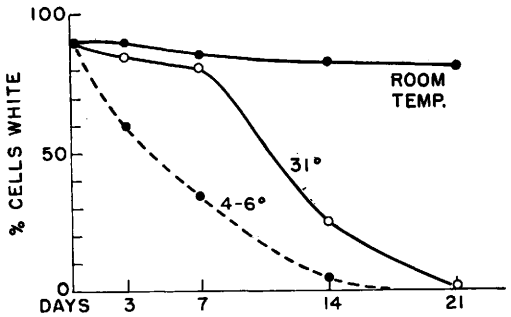

FIG. 1. Viability of dilute suspensions of L cells during storage without pH adjustment or renewal of medium. Cells: 530 × 10^3/ml. Fluid depth: 8 mm. Assays = transfer of 2 mm loops of cell suspension to eosin slides; counts after 2 min.

and evaporation. When approximately half the cells were removed from 5 culture tubes by firm strokes with a rubber policeman the percentages of cells excluding eosin were: 69, 88, 75, 73 and 89% (average 79%). Vigorous, 3x aspiration of these suspensions lowered the average values by 10%. The remaining cells, removed by pancreatin 0.1%,* revealed uniformly high viability, the average value being 96%. Excessive pancreatin concentrations or exposure times, however, were unfavorable. During exposure of suspensions containing some 20% dead cells to pancreatin 0.3% or 0.9% for 30 minutes, the percentages of viable cells rose to 95%, while counts of total cells dropped.

The data in Fig. 1 represent determinations of optimal conditions for the storage of stock cell suspensions in siliconed tubes. It is evident that the simplest alternative (to let the culture tubes stand in a block on the bench) assures useful suspensions over a period of three weeks. In other experiments, it was shown that periodic adjustment of pH improved maintenance at 31°.

The direct simplicity of identifying favorable or unfavorable circumstances for cells residing on glass is emphasized, particularly because observations on dye exclusion can be made *in situ* and without interference with the continuation of the cultures. If supernates are withdrawn to 0.1 ml and a 4mm loop of eosin 1% is added, macroscopic observation reveals within one or 2 minutes any portions of a cell sheet which have been damaged. This permits removal of such areas while rinsing out the eosin in preparation for renewal, and spares viable portions of the culture from contending with dyed cells and their debris. Culture tubes of FJ cells were compared after standing upright for one hour at room temperature, some with tight caps and others improperly sealed. Microscopic examination of the sealed tubes revealed a few dead cells near the upper margins of the culture, while inspection of the others showed that death had occurred halfway downward through the cell populations.

Though these methods have not been used in a systematic study of the way in which tuberculin initiates damage to sensitive cells, it may be noted that the addition of eosin to cultures of spleen cells from normal and tuberculin sensitive animals revealed the specific damage of sensitive cells by OT within 24 hours, and before direct microscopic examination permitted decision.† Rinsing and renewal of cultures revealed that eosin did not interfere with continued maintenance of the relatively delicate cell types.

Identification of cells subject to physiological deficits. Whether good cells or poor ones are examined within a few minutes after the addition of eosin, differentiation of living from dead cells is simple and decisive. The cells are either white or red. Examination of the more shadowy hinterland of cells which are exposed to eosin while embarrassed in various ways teaches two lessons: (a) the desirability of standardizing serum background and routine working conditions and (b) the possibility that capacity for dye exclusion might replace more time-consuming methods in the study of depleted cells and their nutritional or metabolic requirements. A general state of deficiency in suspensions or cultures which contain a high incidence of viable cells on initial counts is revealed by the fact that almost this exact proportion of cells soon acquires a pink tinge of eosin within the cytoplasm (Tables II and III). Such deficiencies have been observed in cells from cultures which had not been renewed at date, in cells refrigerated

* Difco Pangestin 1:75, Control #419 221.

† Observations with Dr. Marcus Brooke.

several hours in dilute pancreatin and serum digest, and in cells exposed to rigorous washing. If such cells are observed during continuous eosin exposure, there are readily revealed: a greater susceptibility to increased concentrations of eosin, and the beneficial effects of brief prior incubation in serum or a nutritional medium.

Discussion. While theoretical soundness and universal applicability are prime qualifications of the principle of dye exclusion, practicality may determine its general usefulness. Small numbers of cells suffice for microscopic determinations of viability. Replacement of sterile tubes and pipettes with standard loops. counting in eosin-containing droplets on ordinary slides, and direct observation in cell cultures *in situ* facilitate application of this principle to cell suspensions and cultures. The relative non-toxicity of eosin is revealed by the tolerance of cells to unnecessary concentrations of dye and by continuation of epithelial cell, L cell and spleen cell cultures after the recommended exposures. Pappenheimer(5) has stated that the concentrations of trypan blue required for reproducible estimates are roughly proportional to serum concentrations. It is probably an advantage of eosin that minimal binding by proteins occurs in the physiological range of pH values.

Summary and conclusions. Differentiation between life and death in unicellular beings should be based upon criteria which are more convenient and fundamental than measurements of capacity for growth. The principle of ion (eosin) exclusion has been substantiated as a simple, rapid tool for this purpose. The conditions for valid observations in cell and tissue cultures have been delineated in respect to: eosin, serum and electrolyte concentrations. These simplified methods have replaced cultivation procedures in studies on: the effects of exposure to pancreatin and to drying, and the exposure of sensitive cells to tuberculin; storage of stock suspensions of cells without renewal of medium; and use of such methods for investigating nutritional or metabolic requirements.

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Effects of Hormone Dose and Length of Injection on Mouse Mammary Gland Growth.* (23986)

HENRY C. DAMM† AND CHARLES W. TURNER
(With technical assistance of Mary E. Powell)
Department of Dairy Husbandry, University of Missouri, Columbia

Use of desoxyribosenucleic acid (DNA) as index of mammary gland growth in mice has been proposed previously(1). Ultimate practicability of its employment in an objective assay technic for mammary gland growth promoting substances would be aided by elimination of any unnecessary steps in preparation of tissues and extraction of DNA, as well as determination of minimum time and estrogen required to produce an utile standard curve for comparison. We, therefore, determined total mammary gland DNA of mice injected with various levels of estrogen and proges-

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† Public Health Service Research Fellow of Nat. Cancer Inst.