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.

1. Rahn, O., Barnes, M. N., *J. Gen. Phys.*, 1932-1933, v16, 579.
2. Devereux, E. D., Tanner, F. W., *J. Bact.*, 1927, v14, 326.
3. Knaysi, G., *ibid.*, 1935, v30, 193.
4. Rous, P., Jones, F. S., *J. Exp. Med.*, 1916, v23, 601.
5. Pappenheimer, A. M., *ibid.*, 1917, v25, 633.
6. Schrek, R., *Am. J. Cancer*, 1936, v28, 389.
7. Eagle, H., *J. Biol. Chem.*, 1955, v214, 839.
8. Waymouth, C., *J. Nat. Cancer Inst.*, 1956, v17, 315.

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

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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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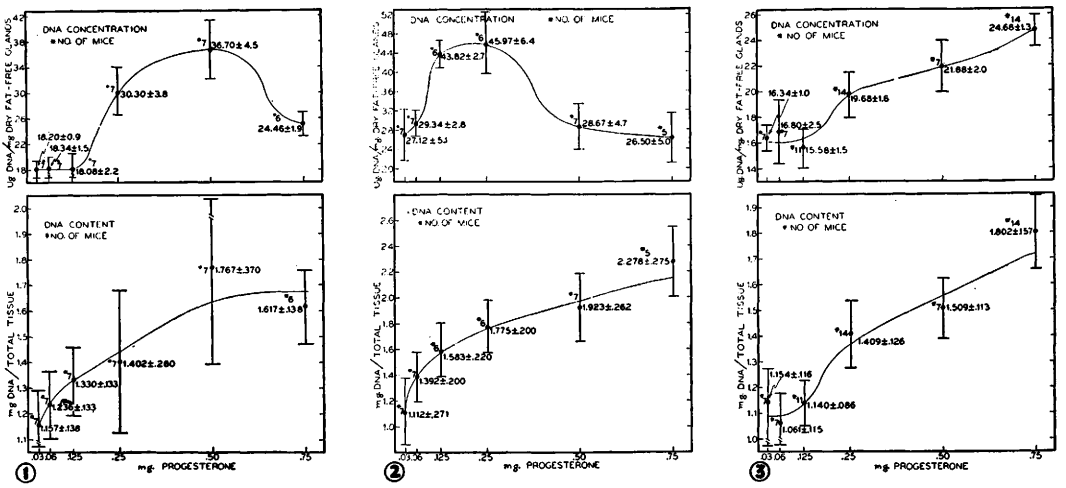

FIG. 1. Relative amounts of DNA in mouse mammary gland at various stages of lobule-alveolar growth, stimulated by inj. of various levels of progesterone plus a constant amount of estrogen (.75 μg)/day for 5 days. Range = ± stand. error.

FIG. 2. Same as Fig. 1 but for 10 days.

FIG. 3. Same as Fig. 1 but estrogen .50 μg/day for 10 days.

terone for 5 or 10 days.

Materials and methods. Mammary gland duct growth was stimulated in male albino mice weighing 16-18 g as described previously (1). In order to estimate the effects of two different levels of synergizing estrogen on promotion of lobule-alveolar growth, mice were subsequently injected daily with .50 or .75 μg estradiol benzoate (E.B.) with various amounts of progesterone (P)/day (.03, .06, .125, .5 and .75 mg). Hormones were dissolved in olive oil in concentrations such that .1 mg solution was injected daily/mouse. Mice receiving .75 mg E.B./day were divided into 2 lots, one was injected for 5-day period, the other for 10 days. Preliminary study revealed lyophilization is unnecessary provided tissues were kept in deep freeze for 4 days and then thawed in 95% ethanol. Further, cold 10% trichloroacetic acid (TCA) removed negligible amounts of nucleotides and nucleosides from small quantities of dry, fat-free pooled mammary tissues, indicating that this step could be eliminated safely. DNA was determined by Webb and Levy(2) procedure with above modifications. Mice were sacrificed on day following last injection, skinned rapidly and ¾ of subcutaneous fascia containing mammary glands was removed and frozen. The DNA of the total tissue was determined to indicate extent of gland hyperplasia and the DNA/mg was calculated to indicate rate of hyperplasia (speed of growth) during the growth process(1).

Results. Results of the 5-day injection period on mammary growth (Fig. 1) indicate that low doses of progesterone have very small effect on total DNA (reported in figure as DNA/total tissue), the growth "spurt" coming with larger doses. This is reflected in DNA concentration curve (DNA per mg dry, fat-free tissue) which indicates a faster rate of growth in mammary glands of mice receiving higher doses of P. The 10-day injection period (Fig. 2) using synergizing dose of .75 μg E.B. and varying doses of P indicates greater total growth (total DNA) produced by higher levels of P, while the greatest growth increments (faster rate of growth) occurs in mammary glands of mice receiving lower doses of P. This is reflected by changes in DNA concentration (growth rate) curve (Fig. 2). Mice injected with synergizing dose of .50 μg E.B. for 10 days (Fig. 3) gave total growth and growth rate curves not dissimilar to those obtained with 5-day injection period and .75 μg E.B. (Fig. 1). Total DNA remained at lower level in mammary glands of mice receiving lower amounts of P, while rapid increases are noted in sets of tissues from mice injected

TABLE I. Comparison of Log Dose *vs* Mean Response Regression Lines.

Daily dose	No. days of inj.	No. of mice	No. of means	b	S_b	Syx	Lambda
.75 μg E.B., varying P	5	41	6	.3992	.0818	.0989	.248
.75 " " "	10	31	6	.7479	.0704	.0852	.114
.5 " " "	10	60	6	.4695	.116	.1404	.299

with higher amounts.

When means of sets of total DNA data concerning effects of time and dose were plotted against log-dose P (Table I), it was found that slope (b) of the line obtained with .75 μg E.B. and varying doses of P for 10 days was significantly greater than obtained by injection for 5 days ($p<.0125$). It was also significantly greater than b obtained ($p<.05$) with .50 μg E.B. plus varying P for 10 days. Lambda (λ), ratio of Syx (standard error of line around the point of means) to b and a good inverse measure of precision of a standard line for an assay procedure(3), was smaller in group receiving .75 μg E.B. and varying P for 10 days. Therefore, the line produced by latter dose and length of injection had greatest precision. All slopes deviated with high significance from the intercept (line) at zero dose of P (the effect of the synergizing dose of E.B. remaining alone), as is evidenced by the small S_b in each case (Table I).

Discussion. An assay technic for determination of mammary gland growth promoting potency of various substances may be developed if some suitable standard curve can be established. It was demonstrated that DNA serves as a suitable index of mammary gland growth produced by injections of various levels of E.B. and P, if a constant ratio of 1:1000 of these substances is maintained. However, in practical procedure it would seem necessary to use a constant dose of estrogen synergizing with various levels of the mammary growth promoting substance to be assayed. The investigation of the effects of progesterone, a highly purified mammary gland growth promoting substance, would yield most information regarding utility of log-dose growth-response lines. Lines produced by injection for either 5 or 10 days with 0.75 μg E.B. synergizing dose would be acceptable in an assay procedure according to this method of determination of precision (Table I) although sensitivity would be lacking in the 5-day procedure due to smaller slope. Moreover, a 10-day procedure is considerably more precise than 5-day injection.

That DNA concentration, expressed as μg DNA/mg dry, fat-free mammary tissue, is an index of growth rate (as opposed to absolute amount of growth) has been proposed previously(1). DNA concentration curves of 5- and 10-day data with .75 μg E.B. (Fig. 1,2) reflect rate changes in growth, thus corroborating this idea.

The synergizing dose of 0.75 μg E.B./day has previously been reported(4,5) to be optimal in the 10-day production of mammary gland growth. Injection of a lesser amount (.50 μg E.B./day) produced a significantly smaller slope, and a much higher lambda. DNA concentration again approximates a lagging differential of the DNA content curve and indicates that no plateauing of the growth rate had occurred in the higher dose of P.

Summary. The possible use of growth curves in assay procedures for mammary-gland growth promoting substances was investigated. Male mice were fed diethylstilbestrol for 4 weeks to insure duct growth. In addition, one group was injected subcutaneously with 0.75 μg estradiol benzoate (E.B.)/day along with various levels of progesterone (P) for 5- or 10-day periods. Another group similarly received 0.50 μg E.B./day plus various amounts of P for a 10-day period. Desoxyribosenucleic acid determined by a newer, shortened procedure was used as a growth index. Results indicated that best slope with greatest precision was obtained when 0.75 μg E.B./day with various amounts of progesterone was injected for 10-day period.

1. Damm, H. C., Turner, C. W., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 466.

2. Webb, J. M., Levy, H. B., *J. Biol. Chem.*, 1955, v213, 107.

3. Bliss, C. I., Cattel, M. K., *Ann. Rev. Physiol.*, 1943, v5, 479.

4. Yamada, J., Magai, J., Naito, M., *Endocrinologica Jap.*, 1954, v1, 63.

5. Sigel, M. B., 1955 M. S. Thesis, Univ. of Mo.

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Storage of Pyrimethamine in Cells *in vitro*. (23987)

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Residual pharmacological activity of certain insoluble drugs is maintained by injection of these substances, intramuscularly, to form a "depot" from which they are slowly and continuously released. Other drugs manifest prolonged activity without the need for such means of injection, and it is known that in these cases the substances are differentially distributed in the body. The binding of pharmacologically active substances in tissues or cells is not clearly understood and there is little information on occurrence of this phenomenon *in vitro*. Pyrimethamine (Daraprim) manifests prolonged residual activity following oral administration(1). Schmidt *et al.*(2) determined the concentrations of pyrimethamine in plasma, erythrocytes, and other tissues of rats and monkeys. It was shown that all fixed tissues examined contained significantly higher concentrations of drug than blood plasma. Smith and Ihrig(3) have also demonstrated that pyrimethamine persists for considerable periods in the body of both man and monkey. We observed previously(4) that in monkey kidney tissue cultures infected with *Toxoplasma gondii*, treated with pyrimethamine for 7 days, and then incubated for 11 days without drug, cellular degeneration due to proliferation of the parasite did not occur. Nevertheless, by inoculation of mice with these cultures immediately after removal of drug, the presence of toxoplasmas could frequently be demonstrated. These results were explained by possible storage of pyrimethamine, at a level inhibitory to the parasite, within cells growing in tissue culture. Experiments reported here were designed to demonstrate this storage of pyrimethamine in cells *in vitro* and to define the conditions under which it can occur.

Materials and methods. Tissue culture: Monkey kidney tissue cultures were prepared and maintained by the method of Melnick (5). Mouse mince cultures were prepared by the method of Chernin and Weller(6). Chick embryo heart explant cultures were prepared by standard plasma clot technics. HeLa cells, KB cells, conjunctival epithelium (Chang), and human intestinal cells (Henle) were purchased from Microbiological Associates, and maintained in Eagle's medium(7) with 10% calf serum, 100 units penicillin, and 100 μg streptomycin/ml. (HeLa maintenance medium). *Drug Solutions*. Daraprim, a brand of pyrimethamine, was supplied through the courtesy of Dr. G. H. Hitchings of Burroughs & Wellcome Research Laboratories, Tuckahoe, N. Y. Stock solutions of 100 mg % pyrimethamine were prepared by bringing 100 mg of pyrimethamine dissolved in 2 ml lactic acid, U.S.P.-85%, to 100 ml with maintenance medium. This drug solution was sterilized by 02 Selas filtration and stored at 4°C. All other solutions of pyrimethamine were prepared from such stock solutions. *Mice*. NIH general purpose albino female mice of 14-20 g were used. *Protozoa*. The RH strain of *Toxoplasma gondii* was used in all tests. Methods of handling the parasite have been described elsewhere (Cook and Jacobs, in press).

Results. Preliminary experiments to test storage of pyrimethamine in cells growing in tissue culture were performed as follows:

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