Spectrophotometry in the Far Ultraviolet

Although the use of the photomultiplier as a detecting device has greatly increased the resolving power of the spectrophotometer, it has also magnified certain potential errors because of its sensitivity (*1*). Photomultiplier types vary in their response to light of different wavelengths. The R.C.A. 1P28 photomultiplier, commonly used in commercial instruments, has a spectral response of between 2000 and 7000 A, with a maximum at 3500 A. Consequently, it "sees" all of the light which is not absorbed by the chromophore—namely, (i) the stray light inherent in the monochromator (*2*) (the amount varies greatly with the design and condition of the instrument used and the nature of the light source); (ii) those extraneous or unwanted wavelengths that appear in the spectral band isolated, on either side of the central "monochromatic" wavelength measured (the amount varies with the width of the slit); (iii) any fluorescent light emitted by the chromophore; and (iv) the fluorescent light emitted by fused silica cells.

Since Beer's law is valid only when monochromatic light is used, spectrophotometric measurements become increasingly subject to error with decreasing wavelengths for the following reasons. In the far ultraviolet, not only does the angle of dispersion of the prism of the monochromator increase rapidly but also the brilliance of the usual light source and the sensitivity of the photomultiplier decline simultaneously (manifestations caused largely by their fused silica envelopes) with the result that the slit must be opened more rapidly than theory indicates to balance the photoelectric bridge circuit. Consequently, with decreasing wavelengths, the ratio of stray light to monochromatic light increases rapidly, and the spectral band isolated is no longer effectively monochromatic.

Attempts (*3*) have been made to correct for stray light in this region by measuring the unabsorbed light emerging from concentrated solutions from which all the monochromatic light presumably had been selectively absorbed by the chromophore. Such corrections are themselves subject not only to the errors which are inherent in measurements of optical density in concentrated solutions but also to those arising from the fact that the incident monochromatic light is never completely absorbed.

The ideal solution to the problem would be the complete elimination of the stray light inherent in the monochromator as well as that caused by excessively large slit widths. Since the far ultraviolet is a spectral region in which many biochemically important compounds show characteristic absorption, it seemed worth while to develop a technique for the accurate study of this property. Such a technique might serve as a means of identifying these compounds, give information concerning their structure, and provide a tool for following the enzymic reactions in which they participate.

The technical details of a spectrophotometer for use between 195 and 230 mμ have been described (*4*). The instrument was designed to minimize the abovementioned sources of error. Stray light of wavelengths longer than 280 mμ has been eliminated by the use of a "solar blind" photomultiplier (R.C.A. developmental type C7180) whose photocathode has a high photoelectric work function (*5*). Also, the slit width has been kept sufficiently small to give a satisfactory approximation of monochromatic light. This has been achieved by a proper balance between a brilliant light source (a Hanovia mercury-xenon arc, 250 w) and the photoelectric systems. The monochromator used was a Leiss single monochromator (*6*). Double monochromation in this system would serve only to reduce the optical efficiency of the instrument.

For work in the far ultraviolet, especially designed crystal quartz cells (*7*) were used for two reasons. First, the transmission of light in the far ultraviolet was demonstrated to be significantly greater with crystal quartz cells than with fused silica cells of the best available quality. With the solar blind photomultiplier and mercury-xenon arc, it was found that crystal quartz cells transmitted, at 195 mμ, 22 percent more energy than did fused silica cells; at 210 mμ, 4 percent more; at 220 mμ, 2 percent more; at 240 mμ, 6 percent more; and at 260 mμ, 1 percent more. Second, all of the many fused silica cells tested, and other samples of highly purified silica as well, showed a characteristic fluorescence which was not found in crystal quartz cells. The increased absorption observed at 240 mμ is caused by the excitation of fluorescence in silica, the maximum point of excitation being found at 244 mμ. The emission of this energy is said to be in the violet (*8*). This fluorescence will introduce an error into absorption spectra taken in fused silica cells when a photomultiplier employing either an S-5 or an S-13 surface is used.

Conformity with Beer's law was demonstrated when the optical densities of solutions of adenylic acid, observed at 210 mμ, were plotted as a function of concentration. Many absorption spectra of biochemically important compounds, including the nucleotides and the aromatic amino acids, have been taken; some reveal fine structure previously unobserved in this region (*9*). It is anticipated that the photoelectric technique described will eliminate the difficulties currently being found in following the course of enzymic reactions by observing difference spectra in the far ultraviolet in solutions of high optical density (*10*).

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5. For other regions in the ultraviolet, suitable photosurfaces are described by V. K. Zworykin and E. G. Ramberg [*Photoelectricity and its Applications* (Wiley, New York, 1950), p. 99].
6. Purchased from Carl Leiss, Berlin, Germany, through the agency of the Photovolt Corp. This instrument employs a Littrow prism, together with two parabolic mirrors for monochromating and collimating the light.
7. Made by the Crystal Optics Co., 4319 N. Lincoln Ave., Chicago, Ill.
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9. A paper on these spectra is in preparation.
10. We are indebted to Howard H. Hess, optical engineer for the Crystal Optics Company, for advice on optical problems and to K. G. Benford, electronics engineer of the Research Institute, University of Chicago, for advice on electronic problems. This work was supported by a grant from the National Institutes of Health [A-646(C)].

1 July 1957

Cultivation of Adult Mouse Mammary Gland in Hormone-Enriched Synthetic Medium

As a part of our laboratory research program that is concerned with the factors responsible for the induction, maintenance, and neoplastic transformation of hyperplastic nodules in the mammary gland of mice of the C3H/He CRGL strain, we have been attempting the cultivation *in vitro* of tissues from various growth stages of the adult gland, normal and abnormal. Adult mouse mammary epithelium generally has been difficult to grow *in vitro* (*1*); however, Lasfargues (*2*) has recently obtained successful growth in tissue culture. Moreover, Hardy (*3*) observed some mammary duct growth but no alveolar development in organ cultures of ventral body wall from embryonic mice. This report describes the successful maintenance *in vitro* of adult mammary gland in synthetic medium with added purified hormones.

The Chen adaptation (*4*) to liquid medium of the organ culture method of Fell (*5*) was employed, and the synthetic culture medium "199" (*6*) was used. The cultures were incubated at 37°C, generally for 5 days, with the *p*H of the medium remaining at about 8.4. The hormones used were estrone, progesterone, cortisol, growth hormone, and

mammotropic hormone. The crystalline steroid hormones were dissolved in 100-percent ethanol, and a quantity of this solution was added to medium 199 to give the desired hormone concentration in the final medium and a 0.5-percent ethanol concentration. The same amount of ethanol was added to all other cultures. Progesterone and cortisol remained soluble in medium 199, whereas the concentrations given for estrone represent the amount in solution plus that in fine suspension. The purified protein hormones were added directly to medium 199 and sterilized by passage through a bacterial filter. A certain amount of the proteins did not completely dissolve and did not pass through the filter; thus, the actual concentrations were less than those given.

Mice of the C3H/He CRGL strain, at about the 14th day of pregnancy, provided prelactating mammary tissue with advanced lobuloalveolar development and marked secretory activity. The secretion in the alveolar lumina is often vacuolated, and the cells generally contain large lipid vacuoles. Tissue samples, usually slightly less than 1 mm in diameter, were taken from a single area of one gland and distributed among the various culture media. A sample from the same area was fixed and sectioned to determine the initial histologic state of the gland. At the end of the culture period, the explants were fixed in Bouin's fluid, serially sectioned, and stained with a modified Masson's stain.

In the medium-199 control cultures (Fig. 1*A*), the alveoli and small terminal ducts showed extensive degeneration, and the parenchymal cells were mainly dead or dying. In contrast, the larger ducts retained their organization and consisted of viable cells.

The 199 medium was enriched with a combination of estrone, progesterone, cortisol, growth hormone, and mammotropic hormone (effective *in vivo*) (see 7) in two concentrations. In the "high" concentration, containing 0.2, 2.0, 8.0, 140, and 140 μg/ml, respectively, of the hormones, the explants retained their original appearance in all respects (Fig. 1*B*), including prominent secretory activity, and in several cases appeared to be stimulated beyond their original state. During the culture period, some alveoli became white and opaque, owing to the accumulation of secretory products. After the standard 5-day culture period, some explants were transferred to fresh "high" medium for another 5 days. In general, they maintained their active secretory condition, with only slight evidence of degeneration. In the "low" concentration, wherein the amounts of hormones were 0.02, 2.0, 2.0, 20, and 20 μg/ml, respectively, the alveoli and terminal ducts also maintained their organization. However, the epithelial cells, although viable, were not actively secretory. The adipose and fibrous connective tissues associated with the glandular parenchyma did not appear to respond to the addition of hormones and survived equally well in the presence and in the absence of the several hormonal combinations.

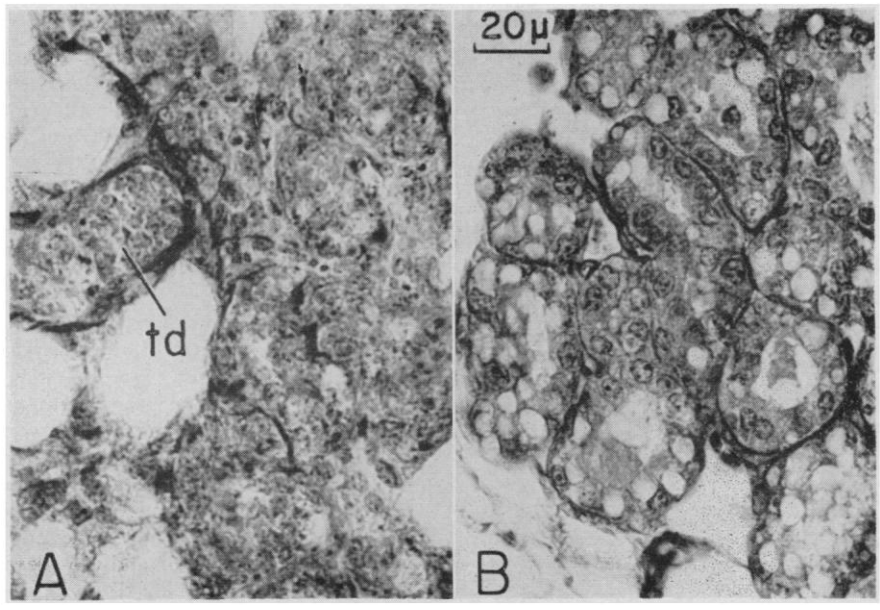

Fig. 1. (*A*) Section of explant from prelactating mammary gland after 5 days in control 199 medium, showing degenerate alveoli and terminal duct (*td*). (*B*) Explant from same gland in medium containing estrone, progesterone, cortisol, growth hormone, and mammotropic hormone in "high" concentration. Note active secretory appearance.

Experiments were then conducted to determine which of the five hormones used were essential for maintenance. The concentration of each hormone was the same as in the original "high" combination. Using media enriched with estrone and progesterone and with cortisol, growth hormone, and mammotropic hormone, it was found that all the activity was in the latter group. Each hormone of this group was then used individually. The results showed that neither cortisol nor growth hormone nor mammotropic hormone alone maintained the tissue. In the final experiment, cortisol and growth hormone, cortisol and mammotropic hormone, and growth hormone and mammotropic hormone media were tested. Of these, only the cortisol and mammotropic hormone combination was effective and showed full activity.

Hyperplastic alveolar nodules characteristic of the mammary gland of C3H/He CRGL mice were also cultured. These nodules are similar to prelactating lobules in organization and histologic appearance (8) and are considered to be precancerous lesions (9). Their reactions in all culture media employed were essentially similar to those of normal prelactating tissue; however, they seemed to be more responsive to the effective hormones. With explants from normal inactive gland, in which alveoli are relatively infrequent and are not organized into lobules, no successful cultures have been obtained to date. Only the larger ducts were maintained.

These initial results indicate that alveoli and terminal ducts are dependent on certain hormones for survival *in vitro*, whereas larger ducts survive in the absence of hormones. This is in accord with the fact that in hypophysectomized-adrenalectomized-ovariectomized female mice of the C3H/He CRGL strain, the alveoli degenerate, whereas the larger ducts remain (10). In our experiments, the hormone environment of the explanted tissue was almost wholly defined; the only questions concern the purity of the hormones used and the substances in the explants themselves. With these limitations in mind, it may be said that the hormonal combination of cortisol and mammotropin, acting directly on the explants, maintains and stimulates the secretory activity of prelactating and hyperplastic mammary lobules. Other steroids and proteins may be capable of producing these effects; experiments to test the specificity of the phenomena observed and to analyze further the hormonal control of mammary growth and function *in vitro* are now in progress (11).

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11. This study was aided by grants (MOR-27) from the American Cancer Society. I am indebted to H. A. Bern and K. B. DeOme for their guidance, to C. H. Li for the purified pituitary hormones, to G. K. Hawkins of Schering Corporation for the estrone and progesterone, and to K. Pfister of Merck, Sharp and Dohme for the cortisol. Mammotropin was from Li preparation No. L2738E and growth hormone from Li preparation No. L2732D. Other preparations of these hormones are currently being employed, and their final activity in the media are being determined by standard bioassay methods.

7 August 1957

Controlling Growth of Test Bacteria for Antibiotic Assays through Anaerobiosis

Sarcina lutea and *Micrococcus pyogenes* are standard Food and Drug Administration test organisms used in assay methods for antibiotics, including penicillin, aureomycin, and chloromycetin. Both bacterial species, if not obligate aerobes, display strong aerobic characteristics.

The inability of these antibiotic-sensitive bacteria to grow well in an oxygen-deficient environment prompted a study of the effects of strict anaerobiosis on (i) growth retardation when strains of these bacteria were imbedded in thin layers of agar, (ii) survival, under this condition, during storage at warm temperatures, and (iii) retention of sensitivity to antibiotics following resumption of growth.

Anaerobiosis was induced either by removal of air from the environment through high vacuum or by saturating the environment with nitrogen. Three milliliters of melted, sterile nutrient agar, seeded with a test organism, were layered into a disposable, plastic petri dish (60 mm in diameter). After solidification of the agar, the seeded plate was inserted into an aluminum-polyethylene pouch with low oxygen-transmission characteristics (*1*). Evacuation of the pouch to 29.0 in. (76 cm) of vacuum and automatic heat-sealing of the open lip was carried out with a high-speed, Flex-Vac controlled-atmospheric packaging machine (*2*). Other pouches were evacuated similarly and then gassed with nitrogen and sealed by heat. All sealed pouches (Fig. 1) were held 1 day at approximately 20°C during transit to the analytical laboratory, and then were stored at 34°C for a period of days. At specific intervals, 1, 3 and 13 days, some pouches were removed for examination of bacterial survival and of sensitivity to antibiotics.

The pouches were torn open to permit re-entry of oxygen. Then paper disks saturated with standard penicillin solutions of various concentrations were applied to the seeded nutrient agar surfaces; the plates were then incubated at 33°C for 6 hours or more to develop zoning.

The results are partly presented in Table 1. Strict anaerobiosis induced by automatic vacuum packing of aluminum-polyethylene pouches completely prevented growth of these bacteria at exposure temperatures of 20°C and 34°C. *Sarcina lutea* and *Micrococcus pyogenes* (*3*), under these conditions, survived for at least 12 days at 34°C, and removal of anaerobiosis by tearing open the pouch reinitiated growth in the agar with unimpaired sensitivity to penicillin. Generally, however, growth of bacteria after they had been kept dormant in the warm, airless environment was not as dense or as rapid as in the case of those grown on

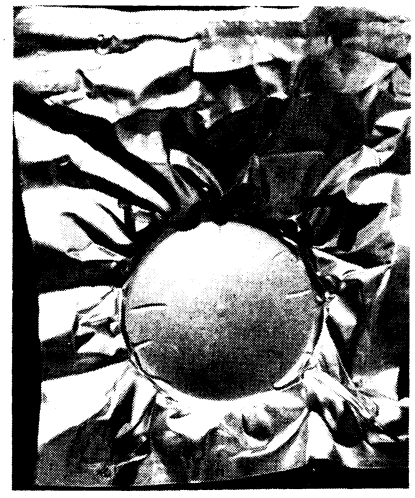

Fig. 1. Aluminum-polyethylene pouch with seeded agar plate under 29-in. (76 cm) vacuum. The concavity of plate is an indicator of vacuum level.

control plates (*4*), but with only a few exceptions, this did not seriously impair disk assay analysis. A few plates, three in this lot of 53 that were tested, failed to show growth after incubation for reasons not readily apparent.

The application of nitrogen to induce anaerobiosis produced results comparable to those obtained with high vacuum. Growth of bacteria held under nitrogen pack after 12 days' storage at 34°C was not as dense and rapid as that on control plates (*4*) that had been kept in cold storage under atmospheric conditions, but no significant loss of sensitivity against penicillin was observed. Carbon dioxide has not yet been tested, but in a well buffered medium, it conceivably would act in the same manner as nitrogen.

It is interesting that *Sarcina lutea* and *Micrococcus pyogenes* in most, but not all, instances survived and also retained sensitivity to penicillin when exposed in the absence of oxygen to long and extremely warm-temperature storage. More research in this area may show an application for these results. If growth control can be standardized with anaerobiosis by using an inexpensive, lightweight, gas-impervious package, a simple field test for the detection of antibiotics in milk and other biological materials might possibly ensue. An urgent need exists for a simple field test that would indicate effectively when milk has become adulterated with antibiotics at the farm. Such adulteration occurs after therapy of mastitis-afflicted dairy cattle with antibiotic infusions of the udder (*5*).

Perhaps, with proper selection of test organisms, a pocket disk or cup assay test for on-the-spot screening of antibiotics from natural sources might be designed. Furthermore, continued study with high-speed, vacuum and gas packaging of bac-

Table 1. Effect of anaerobiosis on growth of antibiotic-sensitive bacteria and on their retention of sensitivity to penicillin. Control plates were seeded and kept under atmospheric conditions for 13 days at 5°C. Vacuum plates were seeded and kept under vacuum for 1 day at 20°C and then 12 days at 34°C. Nitrogen plates were seeded and kept under a nitrogen atmosphere for 1 day at 20°C and then 12 days at 34°C. Measurements of zone diameter and observations of growth were made after 6 to 10 hours' incubation at 33°C.

Penicillin standard* (unit/ml of fluid)	Control plates		Vacuum plates		Nitrogen plates	
	Zone diameter (cm)	Growth†	Zone diameter (cm)	Growth†	Zone diameter (cm)	Growth†
			Sarcina lutea			
0.25	2.1	Excellent	2.2	Good	2.3	Good
0.50	2.4	Excellent	2.4	Good	2.6	Good
			Micrococcus pyogenes			
0.25	2.2	Excellent	2.3	Fair	2.4	Fair
0.50	2.5	Excellent	2.5	Fair	2.5	Fair

* Applied on agar with 1.3 cm. disk.
† No initial growth was apparent when the sealed container was opened, whether the plates were control plates or vacuum or nitrogen packed after storage for 13 days at respective temperatures.