treated and control cultures, especially those \(\approx 60\) days in vitro.

Like the neurons, satellite and Schwann cells of deuterated cultures (particularly at 25 percent \(D_2O\)) are notably rich in formed elements. Their nuclei contain numerous granules (\(\approx 120\) to 150 \(\AA\)) and short filaments, some of which are clustered along the nuclear envelope; within the cytoplasm, ribosomes, microtubules, and fibrillagranular structures occur in abundance. Vacuoles and lipid formations also appear sporadically in \(D_2O\)-treated nervous tissues. The proclivity of deuterated neurons and their supporting cells to display a greater quantity and variety of components (especially nuclear) than the controls is evidenced in cultures fixed in 3.5 percent glutaraldehyde or 2 percent osmium tetroxide, or in both, prepared either with normal water (\(H_2O\)) or with a portion of the \(H_2O\) replaced by an amount of \(D_2O\) equivalent to that in the culture medium. Previous work on microtubule-containing structures, such as the mitotic spindle, has shown that \(D_2O\) may influence their formation through solvent primary or secondary isotope effects, or both (13, 14). Deuterium isotope effects may induce significant conformational and functional changes in nucleic acids, proteins, and other cellular constituents (3, 5, 14). These actions of deuterium might be expected to lead to alterations in cytological and reproductive patterns of the tissues affected, such as those occurring characteristically in our isolated sympathetic ganglia exposed to \(D_2O\).

Pilot studies on organized cultures of developing brain tissues from hypothalamus, cerebellum, and cerebral cortex (which differ from the sympathetic, in details of their early development, in the varying types of neurons involved and in the fact that they give rise to myelinated fibers in culture) indicate that these kinds of nervous tissues also are accelerated in growth and maturation by \(D_2O\). Neurons are larger; both neurons and glia suffer fewer population losses than are normal in culture; myelin sheaths develop earlier and in greater quantity and extent. For central nervous tissue the optimal \(D_2O\) concentration appears to be less than 25 percent. Explanted murine submandibular gland dies in a medium containing 25 percent \(D_2O\); 5 percent is unfavorable but not immediately lethal. Explanted parotid glands thrive at both exposure levels.

Although we can only surmise what was happening in the adult mice which exhibited symptomatic nervous disturbance during \(D_2O\) replacement in previous experiments (1–4), we conclude that some direct action was being exerted by deuterium oxide on their autonomic systems; our experiments do not support our original guess that these symptoms might have been induced by release of unphysiological amounts of nerve growth factor through structural deterioration which was occurring in the submandibular gland. In our hands, \(D_2O\) appears to afford a more potent stimulus to growth in sympathetic nervous tissue developing in isolation than nerve growth factor does. It also has an activating effect upon central nervous tissues. Nerve tissue is able to tolerate for periods of several months substantial amounts (5 to 25 percent) of \(D_2O\) in its ambient medium, while remaining within normal structural and functional limits as broadly defined. Specific metabolic pathways involved in this stimulative action of deuterium deserve investigation, especially in relation to the various unique aspects of neurochemistry.

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Photoinduced DNA-Protein Cross-Links and Bacterial Killing: A Correlation at Low Temperatures

Abstract. The increased sensitivity of Escherichia coli to killing by ultraviolet irradiation when frozen and the variation in this sensitivity as a function of the temperature during irradiation have been correlated with changes in the amount of DNA that was cross-linked to protein by ultraviolet light. These variations in sensitivity to killing do not correlate with the production of thymine dimers.

The sensitivity of Escherichia coli (to killing (1) and to mutation (2) by ultraviolet light increases if the cells are irradiated while they are frozen, the relative sensitivity varying as a function of the temperature at which they are irradiated. It has been suggested (1) that a photochemical lesion, less amenable to repair than the thymine dimer, may be produced in \(E.\) coli irradiated at \(-79^\circ\)C. Since the biological importance of the photochemical cross-linking of DNA with protein has been documented (3), we investigated the possibility that this lesion may be responsible for the enhanced killing of \(E.\) coli by irradiation while frozen. We therefore determined the sensitivity of cells of \(E.\) coli \(B/r\) to killing by ultraviolet light, the tendency of their DNA to become cross-linked to protein, and the production of thymine dimers at \(+21^\circ\), \(-79^\circ\), and \(-196^\circ\)C.

Cells (\(E.\) coli \(B/r\), \(T\) obtained from D. Freifelder) were grown to stationary phase (16 hours) in a salts-glucose medium (4) supplemented with thymine-2-\(C^{14}\) (2 \(\mu\)g/ml, 15.8 mc/mole; Calbiochem). The cells were harvested and suspended in 0.1M phosphate buf
All operations were performed under yellow light (General Electric Bug-Lite bulbs). The DNA was extracted and analyzed as described (5), except that the cells were treated with the detergent for only 30 minutes and then placed in the cold room overnight. Next morning, the samples were brought to room temperature, heated for 5 minutes at 60°C, and then stirred for 30 minutes at room temperature. Thereafter, the original procedure (5) was followed.

Our variant of E. coli showed differences in survival after ultraviolet irradiation as a function of the temperature at which the cells were irradiated (Fig. 1), in agreement with the results of Ashwood-Smith et al. (1). When the temperature was reduced from +21°C to −79°C, an increase in sensitivity to ultraviolet light was shown both by a change in extrapolation number (from 4 to 1) and a change in slope of the survival curves [D₅₀ (dose for 37 percent survival): 198 erg/mm² at +21°C and 129 erg/mm² at −79°C]. At −196°C the cells were not as sensitive as at −79°C (D₅₀ > 198 erg/mm²), but were more sensitive than at +21°C due to the absence of a shoulder. In some experiments the cells to be irradiated at +21°C were frozen for 30 minutes at either −79°C or −196°C and then thawed and irradiated at +21°C. The survival curves were the same whether or not the cells had been previously frozen. The viability of unirradiated cells frozen for 100 minutes at −79°C was 82 percent, and at −196°C was 75 percent.

A larger percentage of DNA was cross-linked to protein by a given dose of ultraviolet radiation when the cells were irradiated at −79°C or at −196°C as compared to +21°C (Fig. 2). There is clearly a correlation in rank between the several cross-linking curves in Fig. 2 and the survival curves in Fig. 1.

In contrast, the rate of formation of cyclobutane-type thymine dimers decreased when the temperature of the cells during irradiation was varied from +21°C to −79°C and to −196°C (Fig. 3). These curves show no correlation in rank with the survival curves in Fig. 1.

Concomitant with this decrease in yield of thymine dimers in irradiated frozen cells, a decrease in the production of photoreactivable damage also occurred. This was seen when the cells at +21°C and −79°C were either exposed to the same dose of ultraviolet radiation or were killed to approxi-
production of thymine dimers and the increased killing of *E. coli* by irradiation at \(-79^\circ\) and \(-196^\circ\)C. This suggests that cyclobutane-type thymine dimers do not play as significant a role in the events leading to the death of irradiated frozen cells as they appear to play at room temperature (7). These results provide further evidence that the relative biological importance of a given photoprotein can change markedly, depending upon growth or irradiation conditions (8).

The photochemical event that does correlate with viability under the present conditions is the cross-linking of DNA with protein. Freezing produces both a change in the rate of formation and in the yield of DNA cross-linked to protein. The freezing cannot be a simple dose-modifying factor because the final yield of cross-linked DNA is different at the different temperatures. Freezing, however, may alter the configuration or the proximity of the protein and DNA within the cells so that the probability of forming DNA-protein cross-links by irradiation is greatly enhanced, thus leading to the greater lethality observed under these conditions.

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Nobilentin Is Main Fungistat in Tangerines
Resistant to Mal Secco

Abstract. *A number of crystalline compounds isolated from peel of tangerines resistant to "mal secco" were characterized and tested for fungistatic activity toward Deuterophoma tracheiphila. Nobilentin exhibited strong fungistatic activity, tangeritin was weakly active, and hesperidin slightly stimulated fungal growth. Rough lemon seedlings, inoculated with the pathogenic fungus, rapidly developed the symptoms of mal secco, whereas continuous infusion of the inoculated seedlings with nobilentin solution largely prevented appearance of the disease.*

The "mal secco" disease of citrus varieties, caused by the pathogenic fungus *Deuterophoma tracheiphila*, is widespread throughout the Mediterranean (Israel, Egypt, Cyprus, Turkey, Greece, Italy, Southern France) and Black Sea areas. The economic importance of the disease derives from the fact that it greatly reduces the life expectancy of affected lemon groves (1); other citrus varieties such as grapefruit also appear to be susceptible. No means of prevention or cure have yet been suggested.

Research on the relation between natural substances and resistance to disease has been carried out mainly with conifers (2). Many bioflavonoid compounds have been isolated from citrus plants (3), but their physiologic roles in the mechanism of resistance to disease are obscure. Certain unidentified substances have been shown to inhibit growth of *D. tracheiphila* in vitro (4), but, as far as I know, no other results have been published. I have tried to identify the substances present in disease-resistant citrus varieties that inhibit the growth of *D. tracheiphila*.

The sources of the isolated substance were dried leaves, bark, or peel of resistant varieties of tangerine (*Citrus reticulata* Bl.) such as Dancy and Cleopatra tangerines and clementines. Water extracts of these materials inhibited growth of the fungus in vitro. The following description refers to dried peel from Dancy tangerines. Initial experiments were made with water extracts, but subsequently I found that methanol extracts had comparable fungistatic activities. Methanol was preferred for preparative work because it extracts less impurities.

Two hundred grams of finely ground tangerine peel were refluxed with 600 ml of methanol for 4 hours. The mixture was filtered, and the residue was similarly extracted four more times. The combined filtrates were concentrated under partial vacuum to 200 ml and left at room temperature for 48 hours. The white precipitate that formed was filtered off (precipitate 1); the filtrate was concentrated to 50 ml, and 200 ml of distilled water was added. The solution was allowed to stand at room temperature for 48 hours longer. A light-brown precipitate was filtered off (precipitate 2); the aqueous methanolic filtrate was extracted with first, 250 ml and then 100 ml of ethyl acetate. The combined ethyl acetate extracts were dried over sodium sulfate and filtered. The filtrate was concentrated to 35 ml and left for 24 hours at room temperature. A white precipitate was filtered off (precipitate 3). The filtrate was concentrated nearly to dryness, and the residue was dissolved in 15 ml of hot 95-percent ethanol and filtered.

After at least 48 hours at 5°C, a light-yellow crystalline material was obtained and dried at 105°C under partial vacuum, yielding 0.7 g of a crystalline substance. After repeated treatment with active charcoal and re-crystallization from methanol, this substance melted at 137° to 138°C; its ultraviolet-absorption spectrum exhibited maxima at 248, 272, and 333 mλ. The crystals showed a yellow fluorescence. Paper chromatography on Whatman No. 1, with a mixture of n-propanol and water (2:1) as the developing solvent, yielded a spot with a bluish-white fluorescence and *R*ₚ of 0.92. The substance gave a positive flavone reaction with magnesium powder and concentrated hydrochloric acid in alcoholic solution. The absence of free OH groups was indicated by insolubility of the compound in dilute alkali and by the negative FeCl₃ reaction. All these characteristics are identical with those reported for 5,6,7,8,3',4'-hexamethoxyflavone, or nobiletin (Fig. 1A; 5, 6).

Chromatographic fractionation of the mother liquor from which nobiletin had been crystallized showed the presence of another substance, with a strong-yellow fluorescence, that moved to the front of the paper (Rₚ, 0.95; n-propanol and water at 2:1); it also gave a positive flavone reaction with Mg powder and concentrated HCl in