treated and control cultures, especially those $\geqslant$ 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 ($\sim$ 120 to 150 Å) and short filaments, some of which are clustered along the nuclear envelope; within the cytoplasm, ribosomes, microtubules, and fibrillogranular structures occur in abundance. Vacuoles and lipoid 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.

MARGARET R. MURRAY
HELENA H. BENITEZ
Departments of Surgery and *Anatomy, College of Physicians and Surgeons, Columbia University, New York 10032*

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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°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,T⁻ to killing by ultraviolet light, the tendency of their DNA to become cross-linked to protein, and the production of thymine dimers at +21°, −79°, and −196°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 μg/ml, 15.8 mc/mmole; Calbiochem). The cells were harvested and suspended in 0.1*M* phosphate buf-

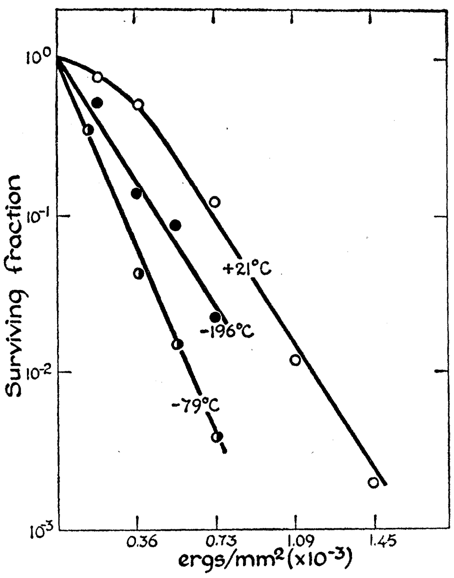

Fig. 1. Survival of *E. coli* B/r,T$^-$ as a function of ultraviolet dose (2537 Å) at different temperatures.

fer (pH 6.8) at a density of about 7×10^8 cell/ml. Ten milliliters of cells in 10-cm petri dishes were irradiated with an unfiltered General Electric germicidal lamp (G8T5) at a distance of 43 cm. The output of the lamp (2537 Å) at this distance was 726 erg/mm^2 per minute. When required, cells were frozen for 30 minutes before irradiation and maintained at the desired temperature during irradiation by placing the petri dish on a large block of aluminum partially submerged in a bath consisting of dry ice and ethylene glycol methyl ether at −79°C or in liquid nitrogen at −196°C.

For measuring survival, the cells were plated on nutrient agar (Difco).

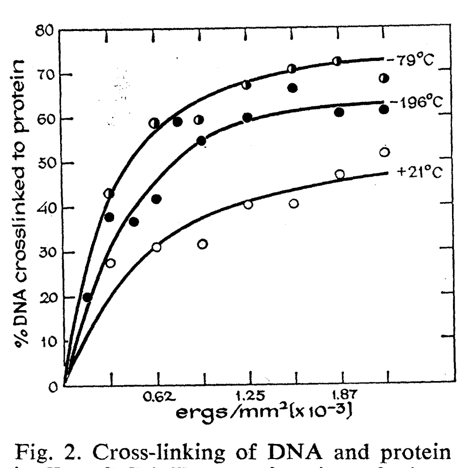

Fig. 2. Cross-linking of DNA and protein in *E. coli* B/r,T$^-$ as a function of ultraviolet dose (2537 Å) at different temperatures. The values plotted are the average values for five experiments at +21°C and two each at −79° and −196°C.

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° to −79°C, an increase in sensitivity to ultraviolet was shown both by a change in extrapolation number (from 4 to 1) and a change in slope in the survival curves [D_{37} (dose for 37 percent survival): 198 erg/mm^2 at +21°C and 129 erg/mm^2 at −79°C]. At −196°C the cells were not as sensitive as at −79°C (D_{37} : 198 erg/mm^2), 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° 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 83 percent, and at −196° 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° 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° and −79°C were either exposed to the same dose of ultraviolet radiation or were killed to approximately the same survival value (Fig. 4). Since current evidence suggests that the photoreactivating enzyme is specific for the repair of cyclobutane-type pyrimidine dimers (*6*), the reduced amount of photoreactivation is consistent with the decrease in the production of thymine dimers.

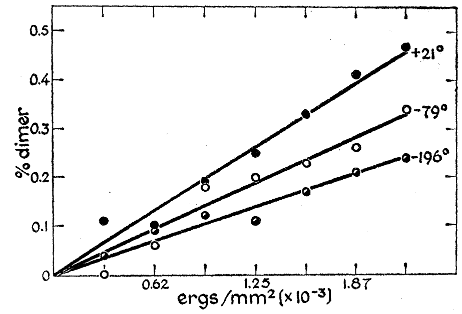

Fig. 3. Formation of cyclobutane-type thymine dimers in *E. coli* B/r,T$^-$ as a function of ultraviolet dose (2537 Å) at different temperatures. Cells labeled with thymine-2-C^{14} were irradiated, hydrolyzed in trifluoroacetic acid, and chromatographed (*9*). The cells used were from certain of the experiments described in Fig. 2. These results are the average of two experiments at +21°C and one each at the other temperatures.

There is no correlation between the

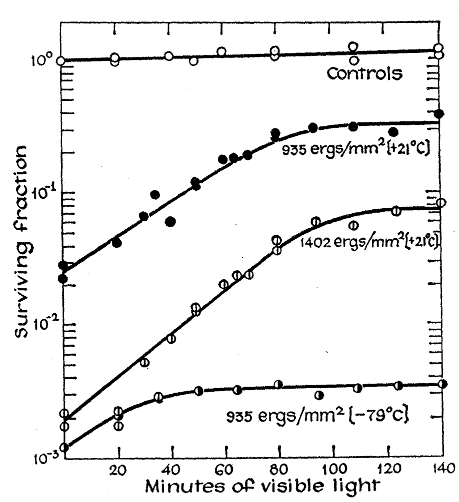

Fig. 4. Photoreactivability of *E. coli* B/r,T$^-$ after ultraviolet irradiation (2537 Å) at +21° and −79°C. Stationary cultures were suspended (5×10^8 cell/ml) in 0.1*M* phosphate buffer at pH 6.8. The suspension was frozen at −79°C for 30 minutes and irradiated, or thawed and irradiated at +21°C. Samples on a shaker table were then exposed to visible light at +21°C for the times indicated, and viability was determined on plates of nutrient agar. The visible light source was two 15-watt Westinghouse Daylight fluorescent bulbs 9 cm above the bottom of the petri dish. The top half of the glass petri dish was inverted and filled with 15 ml of water.

production of thymine dimers and the increased killing of *E. coli* by irradiation at −79° and −196°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 photoproduct 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.

KENDRIC C. SMITH
MARY E. O'LEARY

Department of Radiology, Stanford University School of Medicine, Palo Alto, California 94304

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Nobiletin 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. *Nobiletin 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 nobiletin 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 recrystallization from methanol, this substance melted at 137° to 138°C; its ultraviolet-absorption spectrum exhibited maxima at 248, 272, and 333 mμ. 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_F 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 $FeCl_3$ 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 (R_F, 0.95; *n*-propanol and water at 2:1); it also gave a positive flavone reaction with Mg powder and concentrated HCl in