nitriles is not disclosed by present studies. Unchanged nitriles and ω-carboxy-nitriles which have undergone no degradation of the carbon chain may represent the major types of urinary excretion products. Identification of α-cyanopropionic acid and unchanged 2-cyanopropylamine as urinary excretion products of 2-cyanopropylamine have been reported(10).

*Summary.* 1. Among weanling rats receiving 0.3% dietary BAPN fumarate those showing less severe skeletal deformities tended to excrete relatively larger amounts of cyanoacetic acid (CAA) in urine, and vice versa. Increasing dietary casein level from 12 to 24% resulted in less severe skeletal lesions and higher urinary CAA excretion for a given dosage of BAPN. 2. Appreciable urinary excretion of CAA followed injection of the following organic nitriles into rats: 2,2′-iminodipropionitrile (IDPN), ethylene cyanohydrin, succinonitrile, 5-aminovaleronitrile, and n-valeronitrile. Traces of CAA resulted from injection of n-hexanenitrile and 4-aminobutyronitrile.

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## Development of Resistance by Cell Cultures to Photodynamic Action. (25379)

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During studies of poliovirus by plaque assay technic(1) it was observed that the uppermost layer of monkey kidney (MK) culture would frequently degenerate within 48 hours after application of overlay medium. The present study was undertaken to determine which factors controlled this cell degeneration.

*Materials and methods. Cell cultures.* Monolayer cultures of MK(2) in 2 oz. bottles were grown in Medium 199 containing 2% calf serum.* *Media.* Four preparations of Medium 199(3) were employed in these experiments—Medium 199 containing no dye, Medium 199 containing 1:400,000 phenol red, plaque Medium 199 containing no dye, and plaque Medium 199 containing 1:50,000 neutral red. *Light source.* In most experiments a fluorescent desk lamp containing two 15 watt daylight-type tubes was placed 9 inches above the cell cultures. Light intensities were measured by a Weston light meter model 715. *Extraction of neutral red from cells.* MK cultures in 2 oz. bottles were fed 5 ml fluid Medium 199 containing 1:50,000 neutral red and incubated in the dark at 36°C for 48 hours. Each culture was rinsed 3 times with BSS, placed in contact with 1.0 ml of 2-fold concentrated Medium 199, frozen and thawed twice and incubated at 36°C for 4 hours. Cell debris was removed by low speed centrifugation and the clarified plaque medium mixed with agar before application to fresh cultures.

*Results.* To determine whether degeneration of the uppermost layer of MK cultures resulted from overheating of top bottles by

* The cultures were prepared by the cell culture laboratory of Division of Biologics Standards, N.I.H. under supervision of Dr. Paul Gerber.

sunlight which entered the laboratory window, thermometers were placed on each of the 5 shelves of metal racks both within and beside culture bottles. Temperatures were recorded every 5 minutes during the routine performance of a plaque neutralization test. This work was done at a laboratory table in front of a window in intermittent sunlight. The highest temperature recorded was 36°C (on the top shelf) which is the temperature at which these tests are usually incubated. Although temperature was never excessive, MK cultures that were on the top shelf showed marked degeneration by the next day. Since excess heat did not cause degeneration of these cells, effect of room light was investigated next. Twenty MK cultures were overlayed with Medium 199 containing 1:50,000 neutral red and exposed to ambient light in the laboratory at 10 minute intervals over a 120 minute period. Cultures were then incubated for 24 hours and observed for degeneration. A 10 minute exposure resulted in no degeneration; a 30 minute exposure resulted in 75% cell destruction and thereafter destruction of the cell sheet was complete, indicating that light was one of the factors which caused cell degeneration. Light meter readings of incident light varied from 75 to 400 foot candles during the experiment. In subsequent experiments cultures were exposed to a fluorescent light source at an illumination of 100 foot candles at the location of the culture bottles.

An experiment was designed to determine (a) minimum exposure time required for complete cell destruction and (b) whether neutral red was required for cell damage. Six MK cultures were overlaid with plaque medium containing neutral red; 6 MK cultures were overlaid with plaque medium without neutral red. A control area of cells was included in each culture by placing opaque masking tape over the lower portion of each bottle. Two cultures with neutral red and 2 without neutral red were exposed to light for 5 minutes. Similar groups of 4 cultures each were exposed for 15 and 30 minute periods. Cultures which were overlaid with medium containing no neutral red did not degenerate after exposure to light, while cultures overlaid with medium containing neutral red showed partial degeneration after 15 minutes exposure and complete destruction after 30 minutes exposure.

Similar results were obtained with plaque medium without protein or containing 2% calf serum or 20% skim milk(4). HeLa(5), HEP 2(6), and continuous human amnion(7) cultures also gave similar results. Fluid medium (Medium 199 or basal medium Eagle) (8) containing neutral red similarly sensitized MK cultures to light while fluid medium without indicator or with phenol red (1:400,000) did not sensitize cultures.

It was then observed that control areas of MK culture bottles (shielded from light by masking tape) which had been stained with neutral red for 2 to 10 days did not degenerate after exposure to laboratory illumination. This observation indicated that the cells did not retain sensitization to light over prolonged periods although they were still stained with neutral red. To determine duration of sensitivity to light after application of neutral red MK cultures were ovarlaid with plaque medium containing neutral red, incubated in the dark at 36°C for various intervals (0.5, 1, 2, 4, 6, 8, 12, 16, 24, and 48 hours) and exposed to light in groups of 2 for 30 minutes. Fig. 1 shows some of these cultures. The lower portion of each bottle was shielded from light and served as control area. Cell destruction became increasingly severe up to 1 hour; between 1 and 6 hours destruction was complete; between 8 and 16 hours cell destruction diminished and after 24 hours the cells were resistant to the action of light. The resistance of cells after incubation with neutral red for 24 hours or longer might be due to a change of cells or to alteration of neutral red. To distinguish between these 2 possible mechanisms 4 MK cultures were overlaid with medium containing neutral red and 4 MK cultures were overlaid with medium containing no dye. After incubation in the dark at 36°C for 48 hours (sufficient time for development of resistance) 0.2 ml of a 1:2000 solution of neutral red was added to half the cultures of each group (each culture contained 5 ml of plaque medium). To allow neutral red to penetrate the cells the cultures were incubated at 36°C for 4 hours and then exposed to light

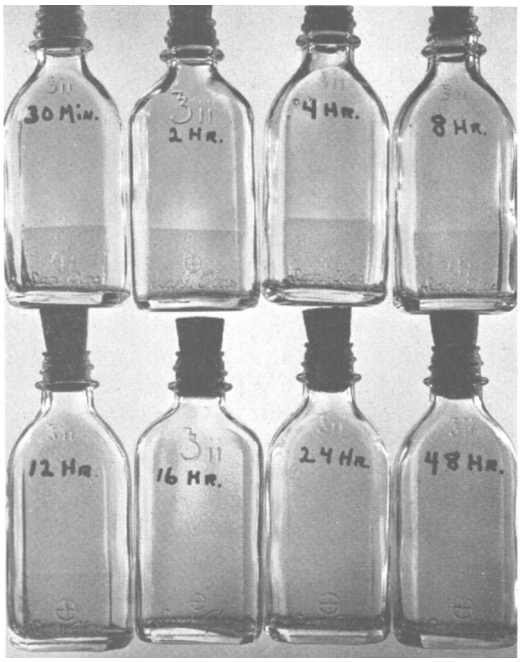


FIG. 1. Photosensitization of MK cultures after preincubation with neutral red for varying periods.

for 30 minutes. Cultures which had developed resistance to light after incubation with neutral red for 48 hours were also resistant to sensitizing action of additional neutral red. Cultures which were incubated for 48 hours in the absence of neutral red were sensitized by addition of neutral red. Control cultures containing a double concentration of neutral red were sensitive to light. It was concluded that loss of sensitization was probably due to a change in the cells rather than alteration of the absorbed neutral red. To determine whether the cell-absorbed neutral red was altered in its ability to sensitize cells, neutral red was extracted from MK cultures which had lost sensitivity to light after incubation with neutral red for 48 hours. Extracted neutral red was applied to fresh MK cultures in plaque overlay and the cultures incubated in the dark at 36°C for 2 hours before exposure to light for 30 minutes. Complete cell destruction was observed 24 hours later, suggesting no alteration of dye.

*Discussion.* The unexpected degeneration of the uppermost layer of MK cultures during plaque assay of viruses was shown to be caused by neutral red and light which acted together to cause cell destruction. Similar degeneration has been observed independently by others(9) and has been demonstrated to be due to neutral red and light(9).

Other investigators have observed damaging effect of vital stains on living cells in the presence of light(10,11,12). The phenomenon of dye-sensitization of living organisms to light has been termed photodynamic action (10). Evidence obtained with microorganisms indicates that dye binds to the organisms, adsorbs light energy and results in a damaging oxidative reaction(10). It is likely that many of the unexplained cell failures during plaque assay may have been due to photodynamic action since there is sufficient illumination in a well lighted laboratory to cause cell destruction after a 30 minute exposure. Damaging effect on cultures used for plaque assay may be avoided if the cultures are placed in the dark within a few minutes of application of overlay medium and not exposed to bright light until 24 hours have elapsed.

The sensitization of cells by neutral red was shown to increase to a maximum at 1 hour after application of overlay medium. Loss of sensitization began at 8 hours and cells were completely resistant to light at 24 hours. Initial increase in sensitization during the first hour may be analogous to a time-dependent sensitization of bacteriophage T2 by toluidine blue(13). To our knowledge, loss of sensitization to photodynamic action has not been reported previously. Results indicate that loss of sensitization was probably due to a change in the cells rather than alteration of absorbed neutral red. One of the possible explanations for development of cellular resistance is an adaptive-like change of the cells(14). Further studies are required to determine the correct explanation.

*Summary.* Unexpected degeneration of uppermost layer of cell cultures was shown to be caused by sensitization of cells by neutral red to damage by light *i.e.*, photodynamic action. Sensitization of cells by neutral red was shown to increase to a maximum at 1 hour. Loss of sensitization began at 8 hours and cells were completely resistant to light at 24 hours. The results indicate that loss of

sensitization was probably due to a change in cells rather than alteration of absorbed neutral red.

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## Utilization and Toxicity of Dialdehyde- and Dicarboxyl-Starches.* (25380)

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The modified physical and taste characteristics of oxidized starch have suggested the possibility of use in foods. A preliminary study of effects on the rat is presented here. Anhydroglucose units of starch can be oxidized by periodic acid to dialdehydes(1). The reaction is stoichiometric so that the number of glucose units thus oxidized can be regulated by amount of periodic acid used. An electrolytic procedure which regenerates the periodic acid as it is used up has recently been introduced(2). Further oxidation by chlorous acid quantitatively converts the aldehyde groups to carboxyl groups(3). The reactions are represented in Fig. 1.

*Materials.* Oxidized cornstarches were obtained from Northern Regional Research Lab., Peoria, Ill. Four dialdehyde starches had 0.05, 2, 40 and 100% of glucose units oxidized. These will be referred to as DAS-.05, -2, -40, and -100, respectively. The dicarboxyl starch had 5% of the glucose units oxidized (DCS-5). *Methods. Acute toxicity* tests were not performed with DCS, because some animals in the subacute study were ingesting daily 16-17 g/kg without apparent ill effects. Acute oral toxicity was studied with DAS-100. The material was dissolved with a minimum of NaOH, neutralized immediately with HCl and given to young adult rats (2 males and 5 females) at 1 g/kg. One male rat died after 11 days with multiple lung abscesses, presumably not due to the material. The others remained alive and healthy during 2 weeks observation. The basal diet for subacute toxicity experiments consisted of corn meal, 51.5; casein, 11.5; linseed oil cake meal, 10; alfalfa meal, 2; cod liver oil, 3; bone ash, 1.5; NaCl, 0.5; and cornstarch, 20%. Experimental diets were made by replacing an equal amount of corn starch with the desired amount of the appropriate oxidized starch. The animals were 30-day-old male rats, except for the DAS-0.05 test, where females were used. They were caged in groups of 4 to 6 animals, with water and food available *ad lib.* Feeding of diets continued for 90 to 100 days except when marked toxicity or limited supply forced earlier termination (lack of sufficient DAS-0.05 ended the feeding of 20% diet after 41 days). Concentrations of oxidized starches ranged from 1 to 20% of the diet for each of the DAS products, and from 0.2 to 20% for DCS-5.

* Histopathological examinations were done by Dr. Wm. E. Ribelin.

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