related to the pathological manifestations of radiation injury. If so, determining serotonin content of blood or spleen may be a sensitive index of radiation injury and a useful tool for rapid evaluation of radioprotective agents. Further studies are indicated to determine the time-dose relationship of tissue serotonin levels following radiation and the effects of radioprotective agents thereon.

Summary. Rats exposed to a single dose of 900 r total body x-irradiation exhibited a highly significant reduction 6 days after x-irradiation in the serotonin concentration of the blood and spleen and a marked increment in serotonin concentration of the small intestine compared to values obtained in untreated *ad libitum*-fed and pair-fed controls. A significant reduction in serotonin concentration in the spleen was also observed in rats exposed to gamma radiation. No significant difference in brain serotonin concentration was observed between x-irradiated and gamma-irradiated rats and untreated pair-fed controls with values for all 3 groups, however, reduced below that of untreated, *ad libitum*-fed controls.

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Nicotine Oxidation by *Arthrobacter*.* (26880)

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The metabolism of nicotine by several micro-organisms has been investigated and a number of oxidation products of the alkaloid have been isolated and identified. These products include 6-hydroxynicotine, 6-hydroxypseudooxynicotine, pseudooxynicotine and a group of derivatives in which the pyrrolidine ring of nicotine has been opened and degraded. Rittenberg and co-workers(1,2,3) have obtained cell-free preparations of a soil bacterium, designated as P-34, which catalyzed the oxidation of nicotine successively to 6-hydroxynicotine, 6-hydroxypseudooxynicotine and 2,6-dihydroxypseudooxynicotine. Wada(4,5) reported that a Pseudomonas species, also isolated from soil, could oxidize nicotine to some derivatives in which either the pyridine or pyrrolidine ring or both were oxidized. Frankenberg and Vaitekunas(6) have reported similar nicotine metabolites to be produced by unidentified organisms derived from the surface of tobacco seeds. Wadsworth(7) has shown that a Cory-

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nebacterium species can convert nicotine to a compound resembling 6-hydroxynicotine. Sguros(8) and Hylin(9) have described the cultural and morphological characteristics of *Arthrobacter oxydans,* an organism obtained from air and tobacco leaves, which has the ability to catalyze the oxidation of nicotine. The identity of the oxidation products was not established, however. Decker and coworkers(10) have shown that oxidation of nicotine by a cell-free extract of *Arthrobacter oxydans* proceeds with the formation of 6-hydroxynicotine.

Although 6-hydroxynicotine has been identified as a nicotine metabolite in several types of bacteria, the responsible organisms have not been adequately described in any case. The present paper presents evidence for the formation of 6-hydroxynicotine as an oxidation product of nicotine produced by a species of *Arthrobacter* present on the roots of tobacco plants.

Methods. Isolation and characterization of the microorganism. During a study of nicotine metabolism in the tobacco plant it was noted that washings of tobacco roots had the ability to transform nicotine to a product having an ultraviolet absorption spectrum markedly different from that of the parent compound. The tobacco plants had been raised from seed in Vermiculite† in a greenhouse and were subsequently brought into the laboratory and grown in inorganic nutrient solution(11). Two microrganisms were observed in gram stained preparations and under a phase contrast microscope in the sediment of nutrient solution in which plants had been growing. The first was a non-motile gram negative organism, with darker staining granules, which existed in several morphologies from coccoid to bacilliform. The second was a gram negative, motile, short, slender bacillus. This organism was observed later in mixed colonies with the first organism, but was never isolated.

The first organism was isolated using the nicotine-salt-yeast extract liquid and agar media described by Sguros(8). The liquid medium was also used for observation of the organism's life cycle and nicotine-degrading activity. All cultures were incubated at 24-26°.

† Commercial heat-expanded mica.

Results. In several respects the organism was similar to *Arthrobacter oxydans*(8). It exhibited a life cycle which was complete in 29 hours in cultures grown with aeration. Aged cultures were in the coccoid cystite form; within 4 hours after inoculation into fresh liquid medium the cystites formed one to 3 germ tubes emerging at obtuse angles to one another. Two or more organisms were often seen linked together in a chain-like arrangement. After 12 hours the organisms appeared to be long bacilli with a number of darker staining granules apparent within them. At about 17 hours the organisms were shorter and the granules were more diffuse, ellipsoidal in shape, and less well defined. At 24 hours the granules again became more spherical and distinct. Finally at 29 hours the organism assumed a homogeneous morphology consisting of cocci, or crystites, singly, in pairs and in clusters as observed in stained preparations.

Crystites were largely gram positive with very little gram negative material apparent within the cells. The germ tubes were faintly gram negative. As the cells matured, they became more strongly gram negative with the granules appearing as darker-staining bodies. At about 26 hours the cells contained distinctly gram positive granules and at 29 hours again had assumed an almost completely gram positive coccoid form.

The organism produced a deep blue diffusible pigment first visible at about 4 hours in aerated liquid cultures. The chemical nature of this pigment was not studied. The intensity of the color increased until at 24 to 36 hours the cultures assumed a blue-black appearance. Upon further aging of the cultures the color became yellow-brown. The production of a blue pigment has also been reported for other nicotine-oxidizing bacteria (2,8,10).

Growth on nicotine-containing agar plates produced small, raised, gray-white mucoid and slightly opalescent colonies. Older colonies exhibited a dense diffusible blue pigment which became yellow-brown in about 10 days.

Proteolytic activity of the organism was demonstrated by its ability to liquefy gelatin.

Liquefaction began at the surface of the medium and proceeded to about ½ the depth of the tube in 10 days and proceeded no further.

Nicotine oxidizing activity of the isolate under consideration was greatly diminished when the cultures were not aerated during incubation, implying a dependence on molecular oxygen.

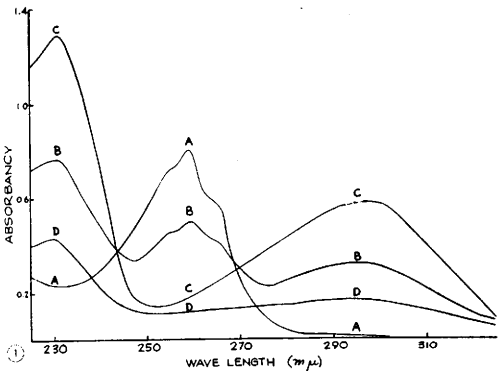
Application of 3% hydrogen peroxide solution to colonies of the organism on nicotine agar plates resulted in immediate vigorous formation of gas, indicating catalase activity.

The isolated organism differed from *Arthrobacter oxydans* in one respect: sucrose and fructose support growth of the organism but no acid or gas are produced either in static or aerated cultures. It has been reported that *Arthrobacter oxydans* produces acid from these substrates(8). Possibly then the isolate under consideration represents a unique species or strain.

Nicotine oxidation. During the various stages of the life cycle of the organism samples of the cultures were taken, and inactivated by addition of a 10% aqueous solution of trichloroacetic acid followed by heating for 5 minutes in a boiling water bath. Insoluble material was removed by centrifugation and aliquots of the samples were assayed on a Cary recording spectrophotometer in the ultraviolet range (220 to 340 mμ). The spectra of several of these samples are shown in Fig. 1.

Nicotine and 6-hydroxynicotine concentrations at different stages in the growth of the organism (Fig. 2), were calculated from molecular extinction values and absorbancies at their characteristic absorption maxima (259 mμ for nicotine, 295 mμ for 6-hydroxynicotine). The nicotine concentration remained relatively constant for 13 hours, after which it rapidly dropped to zero. Formation of 6-hydroxynicotine began at 13 hours. Its concentration reached a maximum at 16.5 hours, then the compound rapidly disappeared. Maximum concentration of 6-hydroxynicotine in the medium was approximately 50% of the starting nicotine concentration on a molar basis, as shown in Fig. 2.

Isolation and Identification of 6-Hydroxynicotine. The procedure used for isolation of



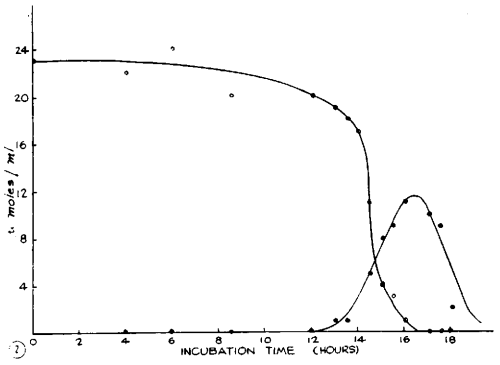


FIG. 1. Ultraviolet absorption spectra showing oxidation of nicotine to 6-hydroxynicotine by an Arthrobacter species in aerated liquid cultures. A, Zero time (nicotine); B, 14.5 hr incubation; C, 17.0 hr incubation; and D, 18.0 hr incubation.

FIG. 2. Relative concentrations of nicotine and 6-hydroxynicotine during oxidation by Arthrobacter in aerated liquid cultures. ○——○, nicotine; ●——●, 6-hydroxynicotine.

6-hydroxynicotine was essentially that described by Hochstein and Rittenberg(1). Two ml of *l*-nicotine in 1.5 l of inorganic nutrient solution(11) were inoculated with organisms, and after 4 days incubation with aeration, 6-hydroxynicotine concentration had reached a maximum. The extended period required for completion of nicotine oxidation in comparison with the studies already described was presumably the result of differences in pH and composition of the medium. The solution was made 0.1 *N* with HCl and heated on a steam bath for 15 minutes. The insoluble material was removed by filtration. A saturated aqueous silicotungstic acid solution was added to the filtrate until no more precipitate formed. After standing overnight, the silicotungstic acid salt was removed by filtration and decomposed with $Ba(OH)_2$.

Excess Ba^{++} was removed as $BaCO_3$. The filtrate was decolorized with a small amount of Norite and evaporated to dryness under reduced pressure at 40°. The solid residue was extracted with boiling ligroin (b.p.60-90°). The product was allowed to crystallize from the solution and was recrystallized several times from this solvent; melting point, 120.5-121.5, melting point of the picrate, 165.5-166, molecular extinction (in 0.1 N HCl) 5440 at 295 mμ and 11,700 at 232 mμ, and a specific rotation (1.5% aqueous solution) of −68.9°.

These data may be compared with similar physical constants for 6-hydroxynicotine (1,10).

A mixed melting point of the picrate prepared from the isolated product with that of the picrate prepared from 6-hydroxynicotine‡ was not depressed.

An elementary analysis of the isolated product gave the following results for $C_{10}H_{14}ON_2$§: Found—67.44% C, 7.90% H, 15.8% N, Calculated—67.42% C, 7.86% H, 15.73% N.

The infrared spectrum of the isolated compound in carbon tetrachloride solution was identical with that of 6-hydroxynicotine.

Discussion. The ultraviolet absorption spectra presented in Fig. 1 show that the characteristic peaks of 6-hydroxynicotine are retained until the ultraviolet absorption disappears. This seems to indicate that no other ultraviolet absorbing compound was produced by the organism. The second product in the metabolic pathway might then be one in which the pyridone ring of 6-hydroxynicotine is altered, since this part of the molecule is largely repsonsible for the ultraviolet absorption. However, since intact cells were used in these experiments no definite conclusion can be drawn. Perhaps the enzyme system catalyzing the conversion of nicotine to 6-hydroxynicotine was present on the surface of the cells; the product would be released into the medium and subsequently enter the cells where further oxidation could take place. In these experiments, therefore, later products might have been removed from the samples along with the intact cells and other trichloracetic acid-insoluble and heat-coagulable material.

The enzymatic character of nicotine oxidation in these experiments was demonstrated by the fact that samples which were acidified and heated immediately after addition of microorganisms showed no change in the typical nicotine absorption spectrum after 48 hours.

Cell-free protein fractions from the bacillus P-34 have been obtained that catalyze the successive oxidation of nicotine to 6-hydroxynicotine(1), 6-hydroxypseudooxynicotine(2) and 2,6-dihydroxypseudooxynicotine (3). Further oxidation led to production of the unidentified blue pigment(2). The first step in nicotine oxidation by the Arthrobacter species is the same as that of P-34 and the blue pigment is also produced, suggesting that the rest of the pathway might be the same. This possibility is currently being investigated.

The rate of nicotine oxidation was greatest in the Arthrobacter species during the fragmenting stage in the life cycle of the organism. This may reflect the rapid increase in "total cell substance" reported by Sguros(8) for this stage of *Arthrobacter oxydans* growth. It is also possible that the enzymes responsible for catalysis of nicotine oxidation were not elaborated until this stage of maturation was reached.

Maximum concentration of 6-hydroxynicotine detectable in the medium was approximately 50% of initial nicotine concentration. Since the blue pigment appeared to be a nicotine metabolite, its production would account for at least part of the remaining nicotine.

However, the greater share of the remaining nicotine can be accounted for if it is assumed that the 6-hydroxynicotine was taken into the bacterial cells and was further metabolized intracellularly. The extent of conversion of nicotine to 6-hydroxynicotine could then have been greater than 50% if oxidation of nicotine and absorption of the product into the cells were taking place simultaneously.

Summary. A gram negative, non-motile

‡ Dr. Sydney C. Rittenberg kindly supplied a sample of 6-hydroxynicotine produced by the bacterium, P-34.

§ Performed by Spang Microanalytical Laboratory, Ann Arbor, Mich.

bacterium isolated from tobacco plant roots was identified as a member of the genus Arthrobacter. The organism had the ability to convert nicotine to 6-hydroxynicotine. Maximum concentration of 6-hydroxynicotine detectable in the culture medium was approximately 50% of initial nicotine concentration on a molar basis. The identity of the product was established by its melting point, and the melting point of its picrate, its specific rotation, elementary analysis, and ultraviolet and infrared absorption spectra.

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Synergism Between Staphylococci and Proteus in Mixed Infection.* (26881)

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Synergism among bacteria in mixed infection is not a new concept although carefully studied cases of specific synergism *in vivo* appear to be relatively sparse in the literature. Even with the current emphasis on staphylococcal infection few studies on synergism between staphylococci and other organisms have been reported(1-3). It was recently noted in this laboratory that mice receiving mixed infections of *Staphylococcus aureus* and *Proteus vulgaris* died rapidly with fulminant septicemia although neither of these organisms alone appeared to have any deleterious effect on the animals when injected in comparable numbers and by the same routes.

This report presents the preliminary results of an investigation of this phenomenon.

Materials and methods. All mice† used in these experiments were young adult Webster-Swiss females weighing from 17 to 20 g. Prior to use in specific experiments the animals were held in large cages in an isolated stock-room. No evidence of spontaneous infectious disease in the stock animals has been observed even when they have been held up to 3 months in these quarters. (These older mice were not used for the reported experiments). The standard diet was Purina rat chow pellets and water supplied *ad lib.* For infection experiments the mice were transferred to steel box-type cages. Inoculation of these animals was carried out in a room separate from the stock-room where the test mice were then held for observation until experiments were terminated.

The staphylococci‡ used in these observations were isolated from human infections. Both the staphylococci and gram-negative bacteria used were routinely maintained at room temperature on Trypticase-Soy agar slants. Liquid cultures were grown in Trypticase-Soy broth at 37° C under fully aerobic

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