For example, in one experiment specimens which had been treated in identical manner by irradiation and isolation were then observed at least five times throughout a 48-hour period after exposure to four different doses, ranging from 85 to 343 kr. The 50 control specimens irradiated for the first time with 343 kr showed a survival rate of 58 percent. In contrast, the 31 specimens from the cumulatively irradiated (1800 kr) clone, after exposure to 343 kr, showed a survival rate of 45 percent 48 hours after irradiation.

X-irradiation has been shown to effect micronuclear number in certain other ciliate *Protozoa.* When specimens of *Paramecium aurelia,* which possess two vesicular micronuclei, were irradiated with single doses of from 200 to 537.4 kr, allowed to multiply for 24 hours, and then killed and stained for micronuclear examination, it was reported that the fraction of specimens with less than two micronuclei increased with increase in dosage while the fraction with two—the normal number—decreased (*4*). In the ciliate *Tetrahymena pyriformis,* which has one micronucleus, haploid exconjugants were produced as a result of crossing one clone that was exposed to 400,000 r with nonirradiated cells of opposite mating type. After two haploids were crossed, a clone resulted which showed 80 to 90 percent amicronucleate animals (*5*). When cultures of two different strains of *T. corlissi* were successively and heavily x-irradiated in a manner somewhat similar to that which I first used in 1954 (*2*), amicronucleate races were also produced (*6*).

During the past 30 years, some paramecia collected in nature revealed the amicronucleate condition (*7*). These naturally occurring amicronucleate forms yielded races which have proved to be as vigorous as their micronucleate sisters. It would appear, at least in these instances, that the micronucleus is unnecessary to the life of ciliates which depend upon asexual reproduction alone. Indeed, amicronucleate races can mate and conjugate with micronucleate ones of opposite mating type.

On the other hand, Miyake (*8*) treated dividing specimens of *Paramecium caudatum*—which has but one micronucleus—with urea and created amicronucleate and bimicronucleate races. He reported that loss of all micronuclei was usually followed by considerable decrease in vitality, fission rate, and body size—all characteristics which I found in the irradiated, amicronucleate animals. Miyake concluded that the micronucleus of *P. caudatum* is a fundamental part of the cell and that the increase or decrease of the number of micronuclei causes a physiological imbalance resulting in the accompanying deleterious effects.

In the heavily irradiated clones of *Paramecium multimicronucleatum* discussed here, the attribution of decrease in vigor, size, reproductive rate, and radioresistance to the loss of micronuclei is untenable, since amicronucleate races of ciliate *Protozoa* which have all the normal and vigorous characteristics of micronucleate races occur in nature. The effects induced by x-irradiation in *P. multimicronucleatum* are more probably caused by the alteration of the sets of genes which are located in the macronucleus (*9*). The experiments suggest that subjecting these organisms and their progeny to repeated exposure of x-irradiation yields mutations which are deleterious to the race (*10*).

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Ozone in High Concentrations as Cause of Tobacco Leaf Injury

Abstract. Evidence obtained by means of rubber strip tests and an ozone recorder indicates the presence of abnormal concentrations of ozone in the atmosphere at times. Excellent correlation was obtained between appearance of "weather fleck" in tobacco and high values for ozone. The great similarity between lesions occurring naturally and those produced by ozone in chambers also indicates that ozone is the probable inciting agent of weather fleck. Varietal differences exist. Study of stomatal action helped to explain variation in leaf injury.

Tobacco leaf injury known as "weather fleck" or "fleck" has become a serious problem in the production of cigar-wrapper tobacco in the Connecticut Valley (*1*). Fleck has been observed on flue-cured tobacco in North Carolina and in Ontario, Canada, and on other tobacco types in some areas. Since 1954, and perhaps earlier, fleck of varying degrees of severity has developed on tobacco in plots at Beltsville, Md., about 6 miles northeast of the District of Columbia.

Research in 1958 at Beltsville and at Riverside, Calif., provides evidence that the inciting agent of tobacco weather fleck is high concentrations of ozone. Formation of ozone by photochemical reactions involving nitrogen dioxide, certain hydrocarbons, or other air-borne chemicals identified in the Los Angeles area is known to occur (*2*). On tobacco, as on bean (*3*) and grape (*4*), the small lesions occur on the upper leaf surface. Primary lesions are restricted to the palisade layer (*1, 4*). After a day the larger lesions, which usually do not exceed 3 mm in diameter, may appear also on the lower leaf surface. Leaf injury progresses from the bottom to the top of the plant. Normally, leaves are not susceptible until they are fully expanded.

Stretched rubber is used as a specific test for ozone (*5*). To provide tension, the strips of rubber are tied in loop form (*2*). During September, daily exposures of rubber strips were made in the tobacco plots in the hope of correlating fleck appearance with days of high ozone concentration. Ozone concentration based on cracking of rubber strips for each exposure date was evaluated by C. E. Bradley and A. J. Haagen-Smit of California Institute of Technology. From 16 September to 22 October a continuous recorder of atmospheric ozone was operated in the field. This recorder was a prototype of model 725-2 ozone recorder made by Mast Development Corporation (Davenport, Iowa), which was on loan for test purposes to the U.S. Public Health Service (*6*). Although the measurements, based upon the oxidation of potassium iodide solution, are believed to be reliable, further tests are planned to determine the specificity of the instrument for ozone.

Important to these studies were cigar-wrapper varieties of tobacco from the Connecticut Valley. One variety, designated "C" (*7*), is so susceptible to fleck that it cannot be grown for commercial tobacco production. A resistant variety is designated "B" (*8*). Early, medium, and late plantings of the cigar-wrapper varieties were made at Beltsville, both under a cloth shade tent and without a shade tent. During September and October observations were made each day for the appearance of new fleck symptoms, especially in the medium and late plantings. In the early planting, the fleck outbreaks in July and August resulted in lesions on 92 and 64 percent of leaves of varieties C and B, respectively.

Studies conducted in California showed that tobacco was very sensitive to ozone, and the flecks produced by ozone were similar to naturally occurring fleck. The varieties susceptible and resistant to naturally occurring fleck showed 72.0 and 17.5 percent, respectively, of leaves in-

Table 1. Accumulative injury from ozone fumigation on fleck-resistant and fleck-susceptible tobacco varieties, Riverside, California, 1958.

Treatment			Damage	
Date	Hours	Ozone (pphm)	Total leaves flecked (%)	Index of average leaf injury per plant*
		B (fleck-resistant)		
19 Aug.	6.5	21	2.5	0.0
20 Aug.	6.5	26	3.0	0.0
21 Aug.	6.0	29	17.5	0.3
		C (fleck-susceptible)		
19 Aug.	6.5	21	46.0	0.8
20 Aug.	6.5	26	62.5	1.3
21 Aug.	6.0	29	72.0	3.5

* The injury to each leaf was rated from 1 to 10 (mild to severe); the index was obtained by dividing the total of the injury values by the number of leaves per plant. Data were taken 1 day after each fumigation date shown.

Table 2. Ozone concentration for different dates in 1958 in tobacco-breeding plots, Beltsville, Maryland.

Date	Total time above 20 pphm (hr)	Time maximum value was reached	Ozone concentration (pphm by volume)			
			Av. 24-hr period	Av. 8-hr period (9 A.M. to 5 P.M.)	Range in 24-hr period	
					Min.	Max.
23 Sept.	0.8	3:02 P.M.	4.5	12.4	0	31
26 Sept.	1.6*	9:55 A.M.	5.4	14.3	0.5	37
8 Oct.	0.0	3:05 P.M.	1.5	4.4	0	9
9 Oct.	3.3	2:15 P.M.	6.0	16.9	0	38
10 Oct.	1.4*	10:23 A.M.	5.9	12.1	0	50
16 Oct.	1.8*	11:08 A.M.	6.0	15.7	0	43

* Total for two peak periods with values above 20 pphm; on 9 Oct. and 23 Sept. there was only one peak period on each date.

jured after exposure to ozone concentrations of about 25 parts per 100 million (pphm) for about 6 hours on three consecutive dates (Table 1). Results of fumigation experiments indicate that the tobacco is relatively resistant to the reaction products of ozone and hydrocarbons, the toxicants responsible for "smog damage." Smog injury appears primarily on the lower leaf surface and can be distinguished from injury caused by ozone, which appears on the upper leaf surface (*3*).

At Beltsville on 15 September in the medium planting and on 10 October in the late planting there were outbreaks of fleck. The new lesions were confined to the upper leaf surface and had a water-soaked appearance at first. In about 48 hours lesions changed from brown to white or gray, especially on the most vigorous plants. Peak levels of ozone occurred on the day before each outbreak. The concentration of ozone on 14 September, determined from the cracking of rubber strips, was 2.2 times as high as the average for the 16-day period prior to that date.

With the ozone recorder, highest values were obtained in the middle of the day, and values were zero or very low during the night and early morning hours (Table 2). On 8 October the daytime values also were relatively low and may be considered fairly typical of ozone concentration for most days for which data are available. Data on ozone concentration are also presented, in Table 2, for the five highest-value days. These were sunny, warm days with light winds. The highest average ozone value for the 8-hour period 9 A.M. to 5 P.M. was recorded on 9 October. On this date a maximum of 38 pphm was reached at 2:15 P.M., and concentrations ranged above 20 pphm continuously for 3.3 hours. New fleck symptoms appeared on the morning of 10 October on about half of the leaves in a late planting of the susceptible variety C, both on those grown under the shade tent and on those grown without shade, but none were observed in a comparable planting of the variety B. A small increase in fleck was detected also on 24 September and on 17 October but not on other dates. No new fleck symptoms appeared over a 7-week period except after days with high ozone concentration, but some high-value days were not followed by the appearance of new fleck symptoms.

Examination of ozone values and correlation of these with the appearance of symptoms suggest that a threshold value of approximately 20 pphm may be critical for the development of fleck on variety C. Such values, perhaps, would need to be maintained for about 3 hours to produce easily recognizable new injury on this susceptible tobacco variety grown under conditions of culture such as those found at Beltsville in September and October 1958—months with relatively low rainfall. Higher maximum concentrations than those on 9 October were recorded on 10 October (50 pphm) and 16 October (43 pphm). On these days values above 20 pphm were observed for only 1.4 and 1.8 hours, respectively; these times represent a combination of two peak periods of relatively short duration which occurred on each of these days (Table 2).

The extent of increase in fleck injury following a day of high ozone concentration is determined in part by amounts of susceptible leaf tissue exposed—that is, there would be less damage after the second consecutive day with high values than if 2 high-value days were separated by a period of 2 weeks or more. This phenomenon would account for the relatively large increase in fleck symptoms on 15 September, as new fleck lesions did not appear during September prior to that date.

Most folded leaves had very little or no fleck injury beneath the folds. Tests of stomatal behavior based upon penetration of 100-percent alcohol or benzene (*9*) gave evidence that stomata in the shaded portion of the leaf were closed during bright days. The difference in stomatal behavior during the day, when ozone was high, was sufficiently marked to suggest exclusion of air containing the toxicant, and prevention of injury thereby. Similarly, fleck did not develop after shading with strips of black paper in contact with the upper leaf surface. Fleck development was prevented also by application of lanolin to the lower leaf surface, where stomata are most numerous and active, or by the enclosure of leaves in polyethylene bags to exclude air with high concentrations of the toxicant. More extensive research on the relation of ozone to the tobacco leaf injury is anticipated next season.

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1 December 1958

Possible Biochemical Implications of the Crystal Structure of Biotin

Abstract. An examination of the molecular architecture of biotin, as determined by x-ray crystallographic analysis, has indicated that biotin may be capable of forming an intramolecular hydrogen bond in solution. A review of various chemical analogs of the vitamin has shown a close correlation between the possibility of forming such a hydrogen bond and biotin-like activity.

A recent x-ray analysis of the crystal structure of biotin (*1*) has established the stereochemistry of the molecule and, in particular, shown it to have the *cis-cis* configuration at the three asymmetric carbon atoms (Fig. 1). Indications that both the stereoisomerism (*2*) and the length of the aliphatic chain (*3, 4*) are specific for biological activity prompted a detailed examination of the molecular structure, in the hope that this might throw some light on the mode of action of the vitamin.

An accurate scale model of the biotin structure and—for comparison—analogous models incorporating the alternative configurations at the asymmetric centers and aliphatic chains of several different lengths were constructed. Though the ring portions of the models were rigid, flexibility was allowed in the construction of the side chains so that the effects of rotation about carbon-carbon single bonds might be examined.

While in general the various interatomic distances and angles of the biotin molecule conform with those found in similar structures, there are some unusual features near the junction of the ring and chain portions of the molecule. There is a particularly short separation (2.8 A) between atoms C_{10} and N_7, and a C_8–C_9–C_{10} angle of 119°. This unusually large angle, which is presumably the result of repulsion between C_{10} and N_7, would appear to facilitate rotation in solution about the C_9–C_{10} single bond, which would otherwise be restricted by steric hindrance. When the ring and chain portions of the biotin model were folded together, by a rotation about the C_9–C_{10} bond, it was found that the chemically reactive centers in the ureido ring system and the carboxyl group could approach each other closely, while Van der Waal's distances of separation were maintained between the other atoms of the chain and the ring system. In particular it was found that such a folding, together with only small rotations about other single bonds in the chain, would enable the structure to meet the rather stringent physical requirements for hydrogen bonding between O_6 and one of the carboxyl oxygen atoms (Fig. 2) (*5*).

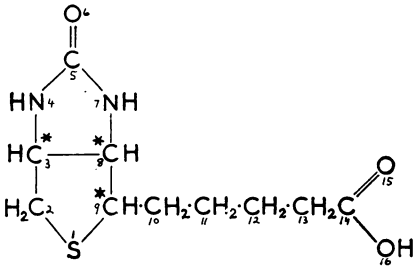

Fig. 1. Structural formula of biotin (atoms numbered arbitrarily).

A study of the various other models indicated that the short C_{10}–N_7 separation in biotin (and presumably the large C_8–C_9–C_{10} angle) is a direct consequence of the *cis-cis* configuration. Furthermore, none of the three stereoisomers of biotin, or molecules with different chain lengths, appear to be capable of forming an intramolecular hydrogen bond, the possibility of which depends critically on both the steric configuration and the chain length.

Supporting evidence for the implication of this type of hydrogen bonding in the biological function of the vitamin appears to be provided by studies of the biotin-like properties of several dozen chemical analogs of the vitamin. These studies have indicated a high degree of biological specificity for the structure of biotin, not only with regard to the steric configuration (*2*) and the length of the aliphatic chain (*3, 4*), but also with regard to the ureido ring system (*6*) and the presence of an oxygen atom at the position of the carboxyl group (*4*). However, it is possible to modify the ring containing the sulfur atom (*7*) and to prepare amides and amino acid derivatives of biotin (*8*)—neither of which need necessarily prevent intramolecular hydrogen bonding—without destroying the biological activity.

It is not quite clear how the formation of an intramolecular hydrogen bond would affect the chemical reactivity of the molecule. In aqueous solution such a hydrogen bond would presumably be unstable, allowing the biotin molecule to alternate between two different states. The formation of the hydrogen bond might be expected to alter the charge distribution in the ureido ring system and to displace the keto-enol equilibrium to enol, resulting in a change of chemical reactivity at the nitrogen atoms, or a system of hydrogen transport along the lines suggested by Lichstein (*9*), whereby the substrate may donate a proton at one point and accept one at another during a keto-enol transition (*10*).

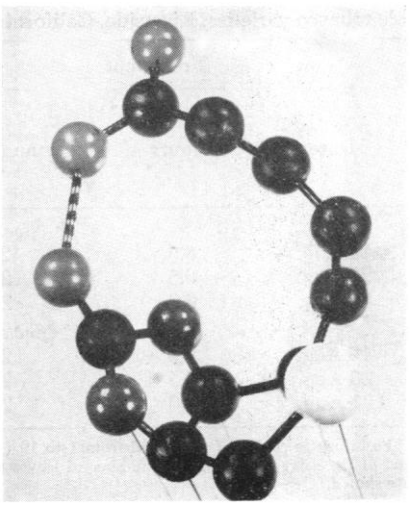

Fig. 2. Possible mode of intramolecular hydrogen bonding in biotin: O_{16} lies in the plane of the ureido ring system; O_6 lies in the plane of the carboxyl group; the distance O_{16}–O_6 is about 2.6 A, and all the other distances between atoms of the chain and those of the ring system (except N_7–C_{10}) are greater than 3.4 A. Angles C_{14}–O_{16}–O_6 and C_5–O_6–O_{16} are both about 120°.

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