Table 2. Distribution of eosinophils labeled with ^{3}H-cytidine. Background less than four grains per 1000 μ^2. Cells with five or more grains were scored as labeled. The vast majority of cells had grain counts of 15 or more. All counts are expressed in percentage and are based on 100 cells counted.

Case	Nucleus only	Cytoplasm only	Nucleus and cytoplasm	Not labeled
3	56	2	38	4
6	60	4	34	2

suggested as a potential stem cell for eosinophils, though no experimental proof has been offered (*7*). When lymphocytes were cultured without specific antigen for 72 hours (Table 1), we found eosinophils only in 12 out of 72 cultures, and then their concentration never exceeded 0.5 percent. Moreover, only an ocacasional eosinophil incorporated ^{3}H-cytidine, and none was labeled with ^{3}H-leucine. Also, in cultures of lymphocytes incubated with precursors of RNA and protein immediately after separation, labeling of whatever eosinophils were present was minimal. These considerations suggest that the enrichment of eosinophils in the cultures of combined neoplastic cells and lymphocytes resulted from an active proliferation of this cell type.

The significance of the observed eosinophilic response remains obscure.

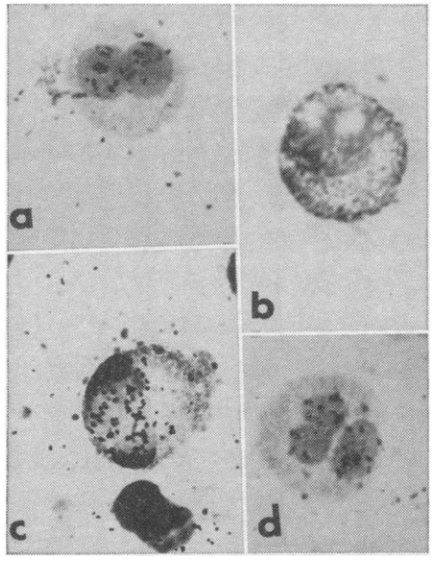

Fig. 1. Eosinophils from cultures of mixed tumor cells and lymphocytes stained with Giemsa (× 1200). (a) Juvenile form labeled with ^{3}H-cytidine (case 3). (b) Immature form with ovoid nucleus. No uptake of ^{3}H-leucine (case 3). (c) Immature form with ovoid nucleus labeled with ^{3}H-cytidine (case 3). (d) Abnormal form with three nuclear segments labeled with ^{3}H-cytidine (case 2).

Eosinophils play an important role in immune reactions in vivo (*8*); however, the exact function of these cells is not clear. Our observations could support the assumption that eosinophils participate in immune reactions involving tumor cells and autologous lymphocytes, because in all mixed cultures transformation of lymphocytes into "blast-like" forms was observed; the incidence of these forms ranged from 4 percent to 11 percent. This finding indicates an immune reaction in which the lymphocytes respond by transformation to "recognized" neoplastic antigenic groupings to which they had been sensitized while circulating (*9*). The fact that a significant eosinophilia in the mixed cultures occurred only in three out of six cases would indicate that such a response is not an essential factor in the interaction between tumor cells and autologous lymphocytes in vitro. It is noteworthy that we have seen marked eosinophilia in sediments of spinal fluid from patients with intracerebral neoplasms, including one case of glioblastoma multiforme. This fact suggests that an eosinophilic response to neoplastic processes is not restricted to a milieu in vitro (*10*).

J. CIEMBRONIEWICZ
O. KOLAR

Division of Neuropathology,
Indiana University Medical Center,
Indianapolis 46207

References and Notes

1. All experiments were done in TC Medium 199 with 10 percent fetal calf serum (Microbiological Associates, Bethesda, Md.) to which Streptomycin was added to give a final concentration of 50 mcg/ml.
2. L. Brandt and J. Borgeson, *Acta Med. Scand.* **172**, 459 (1962). (Glass wool–pyrex fiber No. 3950, Corning Glass Works, Corning, N.Y.; 6 percent Dextran in isotonic saline solution, type 100 C, Sigma Chemical Co., St. Louis, Mo.)
3. Bacto Phytohemaglutinin M, Difco, in a concentration of 0.1 ml of rehydrated PHA in 10 ml of culture medium.
4. ^{3}H-Cytidine (specific activity, 5.87 c/mmole) and L-leucine-4, 5-^{3}H (specific activity, 60.0 c/mmole) from New England Nuclear Corp. at a concentration of 2 μc/ml of culture medium.
5. B. M. Kopriva and C. P. Leblond, *J. Histochem. Cytochem.* **10**, 269 (1962).
6. U. Castaris, *Lancet* **1962-I**, 829 (1962); C. A. Caron and I. Sarkazy, *Nature* **210**, 1648 (1964); A. A. MacKinney, F. Stohlman, G. Brecher, *Blood* **19**, 349 (1962); *Clin. Res.* **9**, 163 (1961).
7. J. H. Robbins, *Science* **146**, 1648 (1964).
8. L. Martimer, *J. Cell Biol.* **23**, 355 (1964); R. E. Speirs and F. F. Speirs, *J. Immunol.* **90**, 561 (1963); R. E. Speirs, *Nature* **181**, 681 (1958).
9. W. Boyd, *The Spontaneous Regression of Cancer* (Thomas, Springfield, Ill., 1966), p. 86.
10. Supported by grants from the United Health Foundation of Elkart County, from the American Cancer Society (institutional grant IN46H), and from PHS (NB 02960 and CA 06145). J.C. holds PHS training grant NB 5450. We thank Dr. W. Zeman for help and encouragement, and Mrs. D. Meyer for technical assistance.

19 April 1967

Zeatin and Zeatin Riboside from a Mycorrhizal Fungus

Abstract. *The puffball fungus* Rhizopogon roseolus *produces and releases three cytokinins when it is cultured in liquid media. Two of these compounds have been isolated in crystalline form from such media and were found to have properties identical to those of synthetic zeatin and zeatin riboside.*

Zeatin, a substituted purine which belongs to the group of plant growth substances known as cytokinins, is extremely effective in promoting cell division in plant tissue cultures (*1–3*) and also displays activity in various (*4*) developmental tests. The compound and its ribose and ribosephosphate derivatives occur in young maize kernels (*1, 3, 5, 6*), and the free form has been isolated from plums (*2*). The picrate derivative of zeatin (*1*) and the barium salt of the monophosphate nucleotide have been obtained in crystalline form (*6*). My report is concerned with the production of cytokinins by the mycorrhizal fungus *Rhizopogon roseolus* (Cda.) Th. Fr. (*7*), the isolation of a substituted purine base and its riboside in crystalline form from media in which the fungus was grown, and identification of the compounds as zeatin and zeatin riboside.

The idea that mycorrhizal fungi might produce cytokinins came from the realization that the hypertrophy of root cortex cells that is common to ectotrophic mycorrhizal associations bears some resemblance to the hypertrophy that may result in cortex cells when roots are treated with kinetin, another cytokinin (*8*, for example). *Rhizopogon roseolus* forms mycorrhizal associations with trees such as pines, and assays for cytokinins in liquid media in which this fungus had been growing revealed a high level of cytokinin activity. Soybean cells (var. Acme) that do not grow in the absence of cytokinins (*9*) were used in the assay. Paper chromatography of the filtrate and subsequent assay with the soybean tissue showed that most of the cell division activity was located at the positions to which synthetic zeatin and its riboside migrated. A third active component was detected in much smaller amounts at the position where zeatin ribose-monophosphate would be expected. The activity was therefore tentatively assumed to be due to cytokinins of the substituted purine type, and an isola-

tion procedure was designed accordingly.

Fungus mycelia were cultured in either 10-liter or 15-liter batches at 27°C. The crystalline materials discussed below were obtained from fractions pooled from 13 batches with a total volume of 195 liters. Optimum conditions for production of the cytokinins were not determined since yield appeared to be good in several media. The medium that offered a workable combination of fairly high yield and ease of purification was a modification of Hagem's medium (*10*) on which the fungus ordinarily is grown. This modification contained (in milligrams per liter): sucrose, 10,000; KH_2PO_4, 500; NH_4Cl, 500; $MgSO_4 \cdot 7H_2O$, 500; and ferric citrate, 5. Mycelial mats from a solid agar medium were chopped in a sterile blender at low speed, transferred to a carboy containing sterile medium, and agitated with a magnetic stirring bar for the period of culture. Cultures were incubated for periods ranging from 6 to 27 days, with yields increasing very gradually with the longer periods of incubation. In all experiments, the two most abundant active materials were easily detected in the soybean assay at a concentration representing a 200-fold dilution of the fungal culture medium.

After the growth period, the culture was filtered through cheesecloth and the filtrate adjusted to *p*H 2.5. The filtrate was passed through a column containing 600 ml of Dowex 50 W-X8 (H^+, 50 to 100 mesh) ion exchange resin, and the column was washed with 500 ml of H_2O. Active materials were eluted with 800 ml of 6*N* NH_4OH; ammonia was removed in a flash evaporator. The water residue was extracted five times with equal volumes of *n*-butanol, the active material going into the alcohol phase. Most of the butanol was removed by evaporation, and the remainder was streaked onto sheets of Whatman No. 1 filter paper. The sheets were developed with solvent A (*11*), and subsequent observation with short-wave ultraviolet light revealed two quenching compounds (I and II), the positions of which (R_F 0.80 and 0.87) correlated with the cytokinin activity and the positions of zeatin and zeatin riboside, respectively. Further chromatography of these two substances separately in solvents B (R_F 0.88 and 0.79) and then C (R_F 0.64 and 0.52) clearly distinguished them from the related 6-*N*-dimethylallyladenine and its nucleoside. Crystals of both factors were obtained by eluting the active compounds with ethanol and slowly evaporating the solutions to dryness. The crystals were then washed with small volumes of water and ethanol. Less than a milligram of each compound was then available for further analysis.

Compound I showed the following properties: melting point, 207° to 208°C; peaks of absorption in ultraviolet light at 269 mμ in 95 percent ethanol, 273 to 274 mμ in 0.1*N* HCl, and 274 to 275 mμ and 218 mμ in 0.1*N* NaOH; prominent peaks at mass-to-charge ratios (*m/e*) 219, 202, 188, 160, 148, 136, and 135 in a mass spectrum obtained with an MS-9 mass spectrometer (*12*) at 180° to 195°C; apparent breakdown to 6-(*N*-glycinyl)-purine and adenine upon treatment with 100 mg of potassium permanganate per liter; R_F values of 0.51 in 0.03*M* borate buffered at *p*H 8.4, 0.50 in water, and those previously mentioned; formation of a derivative upon treatment with acetic acid anhydride; and measurable cell division activity in the soybean test at a threshold value of about 5×10^{-11} mole/liter. The same properties were displayed by a sample of synthetic zeatin. The melting point (*13*), ultraviolet spectra (*2, 13*), and *m/e* peaks (*1*) agree with those values obtained by other workers. Compound I, therefore, is zeatin, the established structure being 6-(*trans*-3-methyl-4-hydroxy-2-butenyl)aminopurine (*1*).

The properties of compound II were: melting point, 181°C; peaks of absorption in ultraviolet light at 268 mμ in 95 percent ethanol, 264 mμ in 0.1*N* HCl, and 268 and 217 mμ in 0.1*N* NaOH; peaks at *m/e* 351, 334, 331, 320, 262, 248, 228, 219, 202, 188, 178, 164, 160, 148, 136, and 135 in a spectrum obtained at 95°C; apparent breakdown to free zeatin when heated while dissolved in water and mixed with the Dowex 50 ion exchange resin (*3*), breakdown to adenosine and apparently the riboside of 6-(*N*-glycinyl)-purine when treated with permanganate; R_F values of 0.78 in 0.03*M* borate buffered at *p*H 8.4, 0.68 in water, and those for other systems as mentioned above; and a threshold value for promotion of cell division at about 5×10^{-11} mole/liter. The same properties were observed with a sample of synthetic zeatin riboside. The melting point agrees well with the published value (*13*), as do the ultraviolet spectra; the mass spectrum was in extremely good agreement with that reported by others (*14*) for the *cis*-isomer and contained no extraneous peaks. Furthermore, no peak could be seen at *m/e* 192 and peaks at *m/e* 228 and 331 were higher than for the *cis*-isomer; these points are in agreement with the statements made by Hall *et al.* (*14*) regarding differences in the mass spectra of zeatin riboside and the *cis*-isomer. Compound II, therefore, is identical to synthetic zeatin riboside (*13*) and is 6-(*trans*-3-methyl-4-hydroxy-2-butenyl)amino-9-β-D-ribofuranosylpurine.

This is the first demonstration of the production of zeatin and its riboside by an organism other than a higher plant and the first report of crystallization of the riboside from any natural source. The third cytokinin detected at the expected position of zeatin nucleotide has not yet been characterized. Production of zeatin by the fungus permits us to study biosynthesis of the compound more readily than is possible with the green plant. The related compound 6-(γ,γ-dimethylallyl)aminopurine was isolated from cultures of *Corynebacterium fascians* (*15*), a bacterium that invades plants and causes changes in their development. *Uromyces phaseoli* and *U. fabae*, which cause rusts of beans and broad beans, are also known to give off materials having cytokinin activity (*16*). How generally this phenomenon holds for associations of green plants with other invading organisms will be interesting to see.

CARLOS O. MILLER
Botany Department, Indiana University, Bloomington 47401

References and Notes

1. D. S. Letham, J. S. Shannon, I. R. McDonald, *Proc. Chem. Soc.* **1964**, 230 (1964).
2. D. S. Letham, *Phytochemistry* **5**, 269 (1966).
3. C. O. Miller, *Proc. Nat. Acad. Sci. U.S.* **54**, 1052 (1965); *Ann. N.Y. Acad. Sci.*, in press.
4. F. H. Witham, C. O. Miller, *Physiol. Plant.* **18**, 1007 (1965).
5. D. S. Letham, *Life Sci.* **5**, 551 (1966).
6. ——— *ibid.*, p. 1999.
7. *Rhizopogon roseolus* (No. 83), kindly supplied by the U.S. Department of Agriculture, Plant Industry Station, originated from an isolate provided by the Institute of Physiological Botany, Uppsala, Sweden, to Richards and Voigt at Yale University in 1963.
8. N. Arora, F. Skoog, O. N. Allen, *Amer. J. Bot.* **46**, 610 (1959).
9. C. O. Miller, in *Modern Methods of Plant Analysis*, H. F. Linskens and M. V. Tracey, Eds. (Springer-Verlag, Berlin, 1963), p. 194.
10. O. Modess, *Symbolae Bot. Upsal.* **5**, 1 (1941).
11. The solvent systems used were: (A) *tert*-butanol, concentrated ammonium hydroxide, and water (3 : 1 : 1); (B) water-saturated *sec*-butanol; and (C) ethyl acetate, *n*-propanol, and water (4 : 1 : 2, upper layer).

12. Associated Electrical Industries.
13. G. Shaw, B. M. Smallwood, D. V. Wilson, *J. Chem. Soc.* **1966** (c), 921 (1966).
14. R. H. Hall, L. Csonka, H. David, B. McLennan, *Science* **156**, 69 (1967).
15. D. Klämbt, G. Thies, F. Skoog, *Proc. Nat. Acad. Sci. U.S.* **56**, 52 (1966); J. P. Helgeson and N. J. Leonard, *ibid.*, p. 60.
16. Z. Király, B. I. Pozsár, M. El Hammady, *Acta Phytopathol.* **1**, 29 (1966).
17. I thank J. R. Schramm for discussions leading to this work, E. Hacskaylo for a culture of the fungus, Gertrud Cowan for technical assistance, D. S. Letham and G. Shaw for samples of zeatin and zeatin riboside, respectively, and N. J. Leonard for samples of 6-dimethylallylaminopurine and its riboside. The project was supported by grant GE3612 and the mass spectra work by grant GP5234 from the National Science Foundation.

31 May 1967

Induction of Drug-Metabolizing Enzymes in Liver Microsomes of Mice and Rats by Softwood Bedding

Abstract. *Induction of three drug-metabolizing enzymes occurred in liver microsomes of mice and rats kept on softwood bedding of either red cedar, white pine, or ponderosa pine. This induction was reversed when animals were placed on hardwood bedding composed of a mixture of beech, birch, and maple. Differences in the capacity of various beddings to induce may partially explain divergent results of studies on drug-metabolizing enzymes. The presence of such inducing substances in the environment may influence the pharmacologic responsiveness of animals to a wide variety of drugs.*

Approximately 1 year ago during a long-term study of genetic and environmental factors affecting metabolism of hexobarbital (Evipal) in mice, an abrupt decrease in sleeping time was observed. This alteration took place when the animal room, cages, chow, and bedding were simultaneously changed. Because these experiments and those of others indicated that numerous environmental factors significantly affected metabolism of hexobarbital in mice, each of the new conditions was systematically investigated. It was discovered that red-cedar shavings caused decreased sleeping times and increased activity of the liver microsomal enzymes that metabolize hexobarbital. These changes were reversed when a mixture of beech, birch, and maple shavings replaced the softwood bedding (red cedar, white pine, or ponderosa pine). No alteration in the hexobarbital concentration in the brain at the time of restoration of the righting response occurred on any of the softwood beddings tested. Although the mechanism of the effect was not investigated, red-cedar bedding was reported to shorten sleeping time in mice treated with hexobarbital and pentobarbital (*1*).

Table 1. Sleeping times and activities of hepatic, microsomal hexobarbital oxidase, aniline hydroxylase, and ethyl morphine *N*-demethylase of NIH male mice kept 3 days on hardwood or red-cedar bedding (mean ± standard deviation). All activities are expressed in micromoles of substrates metabolized per gram of liver for 10 minutes, except for hexobarbital which is metabolized for 15 minutes. The number of animals is given in parentheses. All differences are significant ($P < 0.1$).

Bedding	Sleeping times (min)	Hexobarbital oxidase	Aniline hydroxylase	Ethyl morphine *N*-demethylase
Hardwood	35.3 ± 5.5(12)	0.46 ± 0.01(6)	0.35 ± 0.06(6)	3.8 ± 0.6(6)
Red cedar	16.0 ± 3.1(12)	0.95 ± 0.03(6)	1.00 ± 0.20(6)	7.0 ± 0.5(6)

Ten inbred and two outbred strains of adult male and female mice and male Sprague-Dawley rats (200 g) were used. Sleeping times were measured by stopwatch from the time of intraperitoneal administration of sodium hexobarbital (125 mg/kg for mice and 200 mg/kg for rats) to the time of restoration of the righting response. At the time that the righting response was restored, the animals were killed, and the brains and livers were removed.

The hexobarbital content of the brain was determined by extraction of the drug from brain homogenate into spectrally pure heptane containing 1.5 percent isoamyl alcohol according to a modification of the method of Cooper and Brodie (*2*). Next, the heptane was washed with critic acid buffer *p*H 5.5 to remove metabolites of hexobarbital. The hexobarbital in the heptane was then extracted into 0.8*M* phosphate buffer *p*H 11.0. Differences between readings at wavelngths 245 and 260 mμ in a Beckman DU spectrophotometer were related to readings at these wavelengths of a standard solution of hexobarbital. When the brain is homogenized in phosphate buffer *p*H 8 and the above procedure is followed, the blanks are close to zero. Recovery of a known quantity of hexobarbital added to the brain homogenate from a control mouse is approximately 90 percent.

The hexobarbital-metabolizing activity of the supernatant prepared from a 20 percent homogenate of liver centrifuged at 9000*g* was assayed as previously described (*2*). This supernatant (2 ml) was incubated for 15 minutes at 37°C with sodium hexobarbital (1 μmole), glucose-6-phosphate (25 μmole), nicotinamide-adenine dinucleotide phosphate (1.25 μmole), and $MgCl_2$ (50 μmole) in 3 ml of 0.2*M* phosphate buffer *p*H 7.5 to give a final volume of 5.0 ml. The amount of hexobarbital remaining after a 15-minute incubation was determined by the procedure described above. Similar studies were performed on the microsomal fraction prepared by centrifuging this supernatant for 60 minutes at 105,000*g*.

Twenty-four hours after NIH mice were placed on red-cedar bedding, their sleeping times diminished by one-third, and the activity of their hexobarbital-metabolizing enzymes in liver supernatant (centrifuged at 9000*g*) increased correspondingly. By 48 hours, the sleeping time had decreased by 66 percent of initial values, and the liver enzyme activity increased correspondingly (Fig. 1). Concentrations of sodium hexobarbital in the brain on these 3 days were similar: 36 ± 5, 39 ± 6, and 35 ± 5 μg per gram of brain, respectively. Alterations in sleeping time and enzyme activity were reversed when a combination of beech, birch, and maple bedding replaced the red-cedar shavings after 3 days (Fig. 1).

A further decline in sleeping time and increase in enzyme activity occurred after 1 day on the hardwood bedding (Fig. 1), but subsequently the values gradually returned to those existing prior to exposure of the mice to red-cedar bedding. Similar depression of sleeping time and elevation in the enzyme activity of the supernatant (centrifuged at 9000*g*) and microsomal fraction occurred in other strains of mice and in Sprague-Dawley rats when these animals were put on red-cedar, white-pine, or ponderosa-pine bedding. Two other drug-metabolizing enzymes, ethyl morphine *N*-demethylase (*3*) and aniline hydroxylase (*4*), were elevated, both in the supernatant (centrifuged at 9000*g*) and the microsomal fraction, in mice on red-cedar or white-pine bedding, as compared to their activities in mice on a mixture of beech, birch, and maple bedding (Table 1).

In an attempt to isolate the substance or substances responsible for