number of aberrant colonies as a function of incubation time. The frequency of altered colonies increased from 0.15 percent (control) to 2.63 percent after D5 cells were treated with 100 μg of AF-2 per milliliter for 6 hours. This is about an 18-fold increase over the spontaneous frequency. If the increase in aberrant colonies is based only on mitotic crossing-over events which generate twin-spotted colonies, however, the frequency is 65 times higher than that of the controls. It is interesting to note that under our experimental conditions no cell inactivation was detectable at either of the two concentrations of AF-2 used.

Our results indicate that AF-2 causes genetic alterations in eukaryotes and that this food additive is a potent mutagen. On a per mole basis, the mutagenic activity of this compound in *N. crassa* is higher than that of many well-known chemical mutagens, such as nitrous acid, ethyl methanesulfonate, methyl methanesulfonate, hydroxylamine, ethylenimine and hycanthone. In yeast AF-2 had a higher genetic activity than hycanthone or SQ18,506 (*6*). Studies by others have shown that AF-2 induces mutations in bacteria (*7*) and causes chromosome aberrations in human lymphocytes (*8*). Although Miyaji (*9*) reported that AF-2 failed to increase tumor incidence in mice fed with this compound, he cautiously indicated that his study was not adequate to draw general conclusions on tumorigenicity of AF-2 and stated that more suitable studies need to be carried out. Present knowledge of the genetic activity of AF-2 emphasizes the need for information relating to the possible mutagenic, carcinogenic, and teratogenic hazards of this compound in man and the need for reevaluation of the use of this compound as a food additive.

TONG-MAN ONG
M. M. SHAHIN
Environmental Mutagenesis Branch, National Institute of Environmental Health Science, Research Triangle Park, North Carolina 27709

References and Notes

1. F. J. de Serres, *Genetics* **50**, 21 (1964); *J. Cell. Comp. Physiol.* **64** (Suppl. 1), 33 (1964).
2. ——— and B. B. Webber, *Neurospora Newslett.* **3**, 3 (1963).
3. T. Ong and F. J. de Serres, *Mutation Res.* **13**, 276 (1971).
4. F. J. de Serres and H. G. Komalk, *Nature (Lond.)* **182**, 1249 (1958).
5. F. K. Zimmermann, *Mutation Res.* **21**, 263 (1973).
6. M. M. Shahin, in preparation.
7. T. Kada, *Jap. J. Genet.* **48**, 301 (1973); S. Kondo and H. Ichikawa-Ryo, *ibid.*, p. 283.
8. A. Tonomura and M. S. Sasaki, *ibid.*, p. 291.
9. T. Miyaji, *Tohoku J. Exp. Med.* **103**, 331 (1971).
10. AF-2 was supplied by Dr. T. Matsushima, Institute of Medical Science, University of Tokyo, Tokyo, Japan, through the courtesy of Dr. T. Sugimura, National Cancer Center Research Institute, Tokyo, Japan.

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Plant Species Intermediate for C_3, C_4 Photosynthesis

Abstract. Mollugo verticillata *is the first plant species reported which has characteristics of both C_3 (Calvin-Benson pathway) and C_4 (Hatch-Slack pathway) plants. This plant species is intermediate between C_3 and C_4 plants in at least four features generally used to separate those two plant groups: leaf anatomy, cell ultrastructure, photorespiration, and primary photosynthetic products.*

Prior to 1965, all green plants were thought to assimilate atmospheric CO_2 via the Calvin-Benson or reductive pentose phosphate pathway (*1*). The first stable product of those reactions is the 3-carbon compound, phosphoglyceric acid (PGA). A series of reports by Kortschak and co-workers beginning in 1954 indicated that PGA was not a primary product of photosynthesis in sugar cane (*2*). A complete report on this alternative pathway of CO_2 fixation was described by Kortschak *et al.* in 1965 in which the 4-carbon acids malic and aspartic are the primary products of photosynthesis (*3*). Similar results were independently reported by Karpilov in 1960 (*4*). As a result of this discovery, flowering plants have been divided into C_3 plants (those with PGA as the first stable intermediate) and C_4 plants (those with 4-carbon acids as the first stable intermediates). The C_4 plants are also distinguished from C_3 plants by several other specific physiological and antomical features such as leaf anatomy, organelle structure, low photorespiration rates, high photosynthetic efficiency, and reduced discrimination of ^{13}C (*5*).

Within a taxonomic category, plants with C_3 photosynthesis are considered to be ancestral to those with C_4 photosynthesis; the latter having a diverse taxonomic and geographic distribution. In spite of the multiple evolution of C_4 photosynthesis, no plant species has been reported which possesses features intermediate between the syndrome of features characteristic of either C_3 or C_4 plants. All species investigated so far have been entirely C_3 or C_4. This is strange, because the distribution of C_4 plants among flowering plant families does not follow strict taxonomic lines. Even within a genus, not all species need be C_3 or C_4 (*6*). The genus *Atriplex*, for example, has a C_4 species (*A. rosea*) and a C_3 species (*A. patula*). An intermediate hybrid of these two species has been created, but the hybrid, while intermediate in leaf anatomy and many biochemical characteristics, was not intermediate in "photosynthetic performance" (*7*).

The Aizoaceae is known to contain C_3 plants, C_4 plants, and crassulacean acid metabolism (CAM) plants. Previous work with the genus *Mollugo* (Aizoaceae) indicated that it might contain a species which was intermediate between C_3 and C_4 plants (*8*). The cytology of one member of this genus, *M. cerviana*, was wholly C_4, whereas another member, *M. verticillata*, appeared to have characteristics of both C_3 and C_4 plants. This report shows that *Mollugo verticillata* possesses features intermediate between C_3 and C_4 plants in leaf anatomy, cell ultrastructure, photorespiration, and primary photosynthetic products.

Fully expanded leaves of *Mollugo verticillata* L. and *M. cerviana* L. (Aizoaceae) were obtained from plants grown in environmental chambers. A 16-hour photoperiod was maintained with temperatures of 27°C day and 18°C night. Both plants continually flowered under a light energy of 1.4×10^4 erg cm^{-2} sec^{-1} supplied by fluorescent and incandescent lamps. All $^{14}CO_2$ feeding experiments, extraction, separation, and identification of the products of photosynthesis were conducted as described (*9*). Leaves were exposed to $^{14}CO_2$ for 5 seconds. Preparation of leaf material for light microscopy has been reported (*10*).

Photorespiratory rates were determined with a method adapted from that of Zelitch (*11*). Stomata were opened prior to the assimilation period by passing CO_2-free air over the illuminated leaves for 45 minutes. Air flow was then stopped, and the leaves were allowed to completely assimilate 2 μmole of $^{14}CO_2$ for 45 minutes. The $^{14}CO_2$ evolved in the light was compared to the $^{14}CO_2$ evolved in the dark. The $^{14}CO_2$ trapping agent consisted of 50 ml 1.0*N* KOH with 0.6 percent isoamyl alcohol.

Table 1. Early photosynthetic products, photorespiration ratios ($^{14}CO_2$ released, light/dark), and photosynthetic rates (micromoles per milligram of chlorophyll per hour) (*19*) of *Mollugo cerviana* and *M. verticillata*. Control photorespiration ratios for a C_3 plant, *Hordeum vulgare*, and a C_4 plant, *Zea mays*, are also given.

Plant	Early photosynthetic products (%)*					Photo respiration ratios	Photosynthetic rates (μmole mg^{-1} hr^{-1}
	Aspartate	Malate	Alanine	PGA + sugar phosphate	Other		
Mollugo cerviana	73.7	14.0	2.6	3.3	6.4	1.1	63.7
Mollugo verticillata	14.4	6.2	53.9	17.8	7.7	1.8	65.5
Hordeum vulgare						4.5	
Zea mays						1.05	

* The total $^{14}CO_2$ fixed was 152,323 count/min for *Mollugo cerviana* and 66,300 for *M. verticillata*.

The leaf anatomy of *M. cerviana* and *M. verticillata* is illustrated in Figs. 1 and 2. *Mollugo cerviana* has typical C_4 "Kranz" anatomy (*5*) characterized by concentric layers of chlorenchyma surrounding the vascular bundles. The innermost cell layer, or bundle sheath, contains many large chloroplasts and is surrounded by a single layer of mesophyll cells. The leaf anatomy of *M. verticillata* appears at first to be similar to that of C_3 plants (*5*) since it possesses a distinct dorsiventral arrangement of palisade and spongy parenchyma. Its bundle sheath, however, is like that of C_4 plants, since it contains numerous, well-developed chloroplasts. This is in contrast to the bundle sheath of C_3 plants which has only a few poorly developed chloroplasts (*12*). The leaf anatomy is, therefore, unique in possessing both C_3 and C_4 plant characteristics.

The cell ultrastructure of *M. cerviana* bundle sheath cells is also characteristic of C_4 dicotyledons since it contains many large mitochrondria and chloroplasts (*8*). Like many C_4 plants, the bundle sheath chloroplasts are centripetally located and much larger than those of mesophyll cells. *Mollugo verticillata* chloroplasts, however, are distributed throughout the bundle sheath cells. This species also seems to be unique in that chloroplasts near the vascular bundle are quite large while those located adjacent to the mesophyll are smaller. The peripheral reticulum, an anastomizing system of tubules in the chloroplasts of C_4 plants (*8*), is also lacking in this species.

Primary photosynthetic products of *M. cerviana* and *M. verticillata* are given in Table 1. Malate and aspartate account for approximately 90 percent of the label incorporated during a 5- to 6-second exposure to $^{14}CO_2$ in C_4 plants (*7, 13*). During a similar exposure period, C_3 plants have more than 90 percent of the ^{14}C located in PGA and sugar phosphates (*7, 13*).

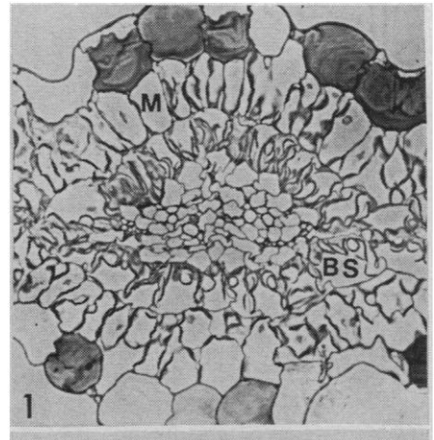
The primary products of *M. cerviana* are consistent with those of C_4 plants. More than 90 percent of the label incorporated is in the C_4 acids malic and aspartic. Activity of the C_3 pathway, as evidenced by the percentage of ^{14}C in PGA and sugar phosphates, is minor. *Mollugo verticillata* gives a strikingly different pattern of primary products. The C_4 acid concentrations approximately equal those of phosphorylated compounds and neither account for a majority of the total ^{14}C fixed. Unlike previously reported C_3 and C_4 plants, *M. verticillata* appears to be actively conducting both C_3 and C_4 photosynthesis during short exposure periods.

Alanine is a heavily labeled primary product in this plant species (Table 1). Photosynthetic production of alanine has been frequently reported both for long-term (*14*) and short-term fixation (*15*), but rarely does it account for more than 10 to 15 percent of the total ^{14}C fixed. We have recently reported that mature leaves of the C_4 dicot, *Portulaca oleracea*, incorporate 38 percent of the total ^{14}C fixed into this amino acid after 10 seconds of photosynthesis.

Another genus of the Aizoaceae, *Mesembryanthemum*, can be wholly C_3 in regard to primary products (*16*). Thus, within one family there exists a gradient with respect to primary photosynthetic products. *Mollugo cerviana* is C_4, *Mesembryanthemum crystallinum* is C_3, and *Mollugo verticillata* is intermediate.

Photorespiration ratios for the two *Mollugo* species, barley (*Hordeum vulgare*, a C_3 plant), and corn (*Zea mays*, a C_4 plant) are presented in Table 1. The photorespiration assay used is sensitive to small differences in photorespiratory activity (*11*). The ratio obtained ($^{14}CO_2$ released, light/dark) for C_3 plants is markedly different from that for C_4 plants. *Mollugo cerviana* gives a ratio nearly identical with that for maize (1.1 and 1.05, respectively). The photorespiration ratio of *Mollugo verticillata* (1.8), however, is significantly higher than that of *M. cerviana* but well below that exhibited by *Hordeum* (4.5). Since a ratio of 3.0 or greater is characteristic of plants with "high photorespiration" (*17*), *Mollugo verticillata* is again seemingly intermediate between C_3 and C_4 plants.

The concentric arrangement of bundle sheath and mesophyll cells in C_4 plants has frequently been suggested to be responsible for their low photorespiratory activity. It appears not to be that simple since *M. verticillata*



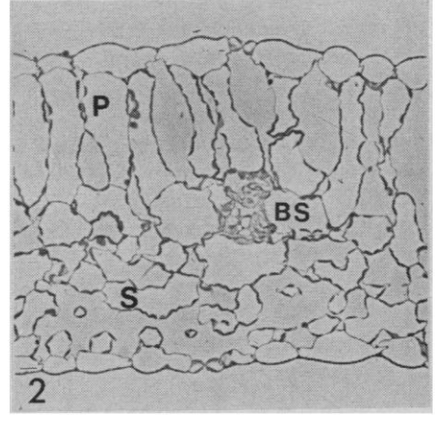


Fig. 1. Cross section of *Mollugo cerviana* leaf. "Kranz" anatomy, composed of a concentric layer of mesophyll cells (*M*) surrounding the bundle sheath cells (*BS*), is present (× 960). Fig. 2. Cross section of *Mollugo verticillata* leaf. A mesophyll of both palisade (*P*) and spongy (*S*) chlorenchyma cells is present. Bundle sheath cells (*BS*) with well-developed chloroplasts surround the vascular tissue (× 960).

lacks the concentric mesophyll layer, but yet has photorespiratory rates below those of C_3 plants. Kranz anatomy may not be a requirement for the low photorespiration observed in C_4 plants.

Photosynthetic rates of the two *Mollugo* species are also given in Table 1. Under the conditions used, the rates are approximately equal. The relatively low photorespiration ratio of *M. verticillata* may be responsible for the nearly identical photosynthetic rates in spite of the different leaf anatomies and primary products of the two species.

Mollugo verticillata is, as we have shown, intermediate in at least four features which are diagnostic for C_4 photosynthesis: leaf anatomy, cell ultrastructure, photorespiration, and primary photosynthetic products. This "continuum" of C_4 expression within closely related species is not surprising, since C_4 photosynthesis probably arose independently in many areas as a result of similar environmental pressures (*18*). Since selective pressures for the development of C_4 photosynthesis exist as gradients, transitional (ecotonal) species are also likely to exist. *Mollugo verticillata* appears to be such a species.

R. A. KENNEDY
W. M. LAETSCH

Department of Botany, University of California, Berkeley 94720

References and Notes

1. J. A. Bassham and M. Calvin, *The Path of Carbon in Photosynthesis* (Prentice-Hall, Englewood Cliffs, N.J., 1957).
2. *Annual Report Hawaiian Sugar Planter's Association* (1954), pp. 1956–1959.
3. H. P. Kortschak, C. E. Hartt, G. O. Burr, *Plant Physiol.* **40**, 209 (1965).
4. Y. S. Karpilov, *Kazakhstan Agric. Inst.* **4**, B.I. 21 (1960).
5. W. M. Laetsch, *Sci. Prog.* **57**, 323 (1969).
6. G. W. Welkie and M. Caldwell, *Can. J. Bot.* **48**, 2135 (1970).
7. C. S. French, *Carnegie Inst. Wash. Year Book* **69**, 623 (1971).
8. W. M. Laetsch, in *Photosynthesis and Photorespiration*, M. D. Hatch, C. B. Hatch, C. B. Osmond, R. O. Slayter, Eds. (Interscience, New York, 1971), p. 323.
9. R. A. Kennedy and W. M. Laetsch, *Planta* **115**, 113 (1973).
10. W. M. Laetsch and H. P. Kortschak, *Plant Physiol.* **49**, 1021 (1972).
11. I. Zelitch, *ibid.* **43**, 1829 (1968).
12. C. C. Black, *Adv. Ecol. Res.* **7**, 87 (1971).
13. T. M. Chen, R. H. Brown, C. C. Black, *Plant Physiol.* **47**, 199 (1971).
14. W. Stepka, A. A. Benson, M. Calvin, *Science* **108**, 304 (1948).
15. H. Usuda, R. Kanai, M. Takeuchi, *Plant Cell Physiol.* **12**, 917 (1971).
16. K. Winter and D. J. von Willert, *Z. Pflanzenphysiol.* **67**, 166 (1972).
17. I. Zelitch, *Plant Physiol.* **52**, 33 (1973).
18. W. M. Laetsch, *Am. J. Bot.* **55**, 875 (1968).
19. We thank Dr. Norman Terry, Department of Soils and Plant Nutrition, University of California, Berkeley, for determining the photosynthetic rates by infrared gas analysis.
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Cyclic Guanosine and Adenosine 3′,5′-Monophosphates in Canine Thyroid: Localization by Immunofluorescence

Abstract. *When guanosine 3′,5′-monophosphate (cyclic GMP) and adenosine 3′,5′-monophosphate (cyclic AMP) are localized in canine thyroid by a fluorescence immunocytochemical procedure, distinct staining patterns for each nucleotide are seen: cyclic AMP is distributed throughout the follicular cell cytoplasm before and after administration of thyroid-stimulating hormone, while cyclic GMP is localized to the follicular cell membrane in the control state, and increased cytoplasmic fluorescence is visualized after acetylcholine. These data provide histological evidence that correlates with cyclic nucleotide tissue measurements, suggesting diverse roles of the two nucleotides in thyroid function.*

Guanosine 3′,5′-cyclic monophosphate (cyclic GMP) is present in almost all mammalian tissues, but its role in cell function is poorly understood. In some tissues such as kidney and heart, the addition of cyclic GMP or its dibutyryl derivative causes effects antagonistic to those of adenosine 3′,5′-cyclic monophosphate (cyclic AMP), although exogenous cyclic GMP acts as a weaker cyclic AMP in most tissues. An agonist-antagonist hypothesis for interreaction of these nucleotides has been suggested and the subject has been reviewed recently (*1*).

Cyclic GMP could also be involved in the action of acetylcholine (ACh) at muscarinic receptor sites. Acetylcholine and other cholinergic compounds cause an increase in the concentration of cyclic GMP in a variety of tissues, and this rise is blocked by prior treatment of the tissues with atropine (*2*).

To gain insight into possible roles of cyclic GMP in cell function and to examine further the relation of cyclic GMP to the action of ACh we have applied the technique of fluorescence immunocytochemistry (*3*) in the localization of cyclic GMP to thyroid tissue and have contrasted the localization of this cyclic nucleotide with that of cyclic AMP. We have used thyroid tissue for these studies because thyroid-stimulating hormone (TSH) has been shown to increase the concentration of cyclic AMP but not that of cyclic GMP, whereas ACh increases concentrations of cyclic GMP but not of cyclic AMP (*4*). We report distinct differences in localization of cyclic AMP and cyclic GMP in canine thyroid under basal conditions and after hormonal stimulation and suggest that the diverse roles of these two nucleotides and their intracellular localization may be related.

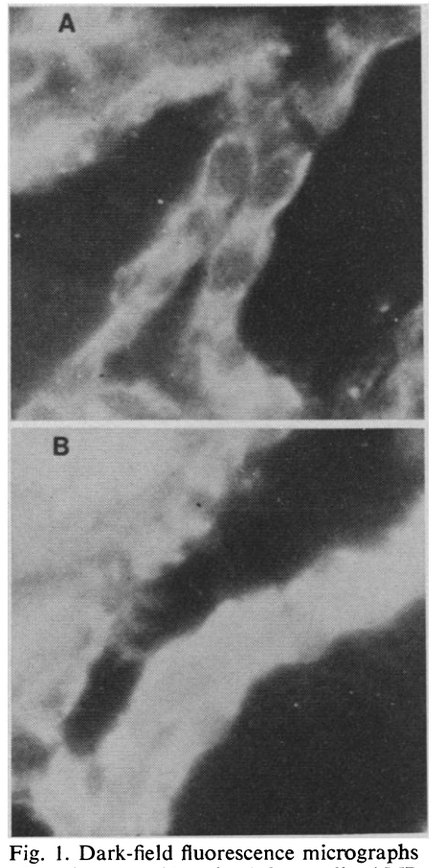

Fig. 1. Dark-field fluorescence micrographs of canine thyroid stained for cyclic AMP (×400). (A) Before TSH stimulation. (B) Twenty minutes of incubation with TSH (50 milliunits per milliliter), illustrating an apparent increase in cytoplasmic staining.

In each flask four thyroid slices (approximately 10 to 15 mg each) were first incubated in 2 ml of Krebs-Ringer bicarbonate buffer, *p*H 7.4, for 10 minutes at 37°C, with agitation and continuous gassing with 95 percent oxygen and 5 percent CO_2. The tissues were then incubated in the presence of $10^{-2}M$ theophylline with and without the addition of 100 milliunits of bovine TSH per milliliter or $10^{-3}M$ ACh or carbachol. An acetylcholinesterase inhibitor, physostigmine, was also added in a concentration of $3 \times 10^{-4}M$ with the cholinergic stimulants.

After a 20-minute incubation, a sam-