the limited frequency response of the recording system, the tracings we obtained may not quantitatively reflect the kinetics of the cellular response to serotonin, but these experiments provide direct evidence of an increase in the intracellular free Ca^{2+} concentration in response to serotonin. They also demonstrate the ability of the cells to buffer increases in free cytoplasmic Ca^{2+} that occur as the result of hormonal stimulation. Whether the cellular Ca^{2+} buffering system involved in the response illustrated by Fig. 3B is mitochondrial or extramitochondrial (*13*) remains to be established.

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6. We pulled micropipettes (tip diameters < 0.4 μm) from Kwik-Fil borosilicate glass tubing (outer diameter, 1.2 mm; inner diameter, 0.68 mm; W-P Instruments, New Haven, Conn.), silanized their inside surfaces with trimethylchlorosilane (Pierce Chemical Co., Rockford, Ill.), in an enclosed chamber under carefully controlled temperature and humidity. We then introduced a column (200 μm to 2 mm long) of the liquid ion-exchanger solution into their tips and filled them with 0.1 *M* $CaCl_2$. Calibration and intracellular recording were performed as described in (*5*).
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Initiation of Sulfate Activation: A Variation in C_4 Photosynthesis Plants

Abstract. *In leaves of plants with C_4 photosynthesis, sulfur assimilation is initiated in bundle sheath cells whereas carbon and nitrogen assimilation are initiated in mesophyll cells. The activation of sulfate by adenosine triphosphate sulfurylase in leaves of C_4 plants occurs in chloroplasts of bundle sheath cells and is effected by two isozymes of approximately equal activities that accounted for 95 to 100 percent of the total leaf activity.*

The assimilatory sulfate reduction pathway in higher plants is thought to be localized within the chloroplast (*1*). However, two distinct types of chloroplasts are present within leaves of plants that assimilate carbon via the C_4 pathway of photosynthesis (*2*). Not only are these chloroplasts localized in two different C_4 leaf cell types, but each is characterized by its own complement of enzymes. The chloroplasts of the mesophyll cells lack ribulose-1,5-bisphosphate (RuBP) carboxylase (*2, 3*) while the chloroplasts of the bundle sheath cells lack nitrite reductase (*4*). Thus, in C_4 plants, the enzymes catalyzing early assimilatory steps of carbon and nitrogen metabolism are compartmentalized within specific cells and chloroplasts. We also have found that the initial enzyme in sulfur assimilation, adenosine triphosphate (ATP) sulfurylase, is compartmentalized. Approximately 95 percent of the activity can be localized in the bundle sheath cell chloroplast. In the 18 C_4 species examined, only trace amounts of ATP sulfurylase activity is in the mesophyll cell. Our findings indicate that in C_4 plants the first step in leaf sulfur assimilation proceeds largely in the bundle sheath chloroplast.

Table 1. Intercellular localization of ATP sulfurylase in the leaves of various plants. Mesophyll protoplasts and bundle sheath strands were obtained from 0.5- to 1.0-mm leaf slices of 2- to 4-week-old seedlings. Incubation was in 0.5*M* sorbitol, 1 m*M* $CaCl_2$. 2 percent (weight to volume) cellulysin, 0.3 percent (weight to volume) pectinase, 0.1 percent bovine serum albumin (weight to volume) and 5 m*M* MES (4-morpholineethane sulfuric acid), *p*H 5.25. In some species 0.25 percent Rohament P (Rohm, G.m.b.H., West Germany) was included. Incubation time at 30°C varied from 1 to 3 hours. Mesophyll protoplasts were harvested in a medium containing 0.5*M* sorbitol, 1 m*M* $CaCl_2$, and 5 m*M* Hepes, *p*H 7.0; they were purified by a method similar to that of method 2 of Edwards *et al.* (*9*). Protein extracts were made in 100 m*M* tris (*p*H 8.2), 2 m*M* β-mercaptoethanol, 1 m*M* EDTA, and 1 percent PVP-40 (polyvinylpyrrolidone). Activity of ATP sulfurylase was determined by a bioluminescence assay (*7*). N.D., not detectable.

Organism	Activities*		
	Whole leaf	Mesophyll protoplasts	Bundle sheath strands
	C_4 plants		
NADP⁺-malate enzyme type			
Bothriochloa caucasica	29.3	1.6	54.3
Cymbopogon martini	22.6	2.3	88.8
Digitaria sanguinalis	42.2	6.0	91.9
Echinochloa colonum	23.6	3.8	56.0
Echinochloa crus-galli	18.4	1.4	72.6
Euchlaena mexicana	24.1	2.4	64.4
Pennisetum americanum	79.1	0.7	183.8
Sorghum bicolor	22.4	1.9	93.8
Zea mays	5.3	0.4	16.9
NAD⁺-malate enzyme type			
Chloris distichophylla	26.6	N.D.	36.8
Eleucine indica	16.2	0.8	21.5
Panicum bergii	24.4	0.8	39.7
Panicum miliaceum	10.9	N.D.	15.5
PEP-carboxykinase type			
Brachiaria erucaeformis	20.0	3.2	64.3
Chloris gayana	28.7	1.1	53.2
Panicum maximum	35.5	2.5	37.8
Panicum molle	33.0	4.1	67.2
Urochloa mosambicensis	51.2	0.5	162.9
	C_3 plants		
Avena sativa	26.7	N.D.	N.D.
Triticum aestivum	41.9	N.D.	N.D.
	CAM† plant		
Kalanchoë daigremontiana	11.2	N.D.	N.D.

*Micromoles of ATP produced per milligram of chlorophyll protein per hour. †Crassulacean acid metabolism.

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ATP sulfurylase (E.C. 2.7.7.4) catalyzes the ATP-dependent activation of sulfate to adenosine 5′-phosphosulfate (APS) with the release of pyrophosphate (PP_i). The activity of ATP sulfurylase, determined as APS- and PP_i- dependent ATP synthesis (*5*), was measured in whole leaf, mesophyll, and bundle sheath extracts of various C_4 plants (Table 1). This study included three types of C_4 plants: $NADP^+$-malate (E.C. 1.1.1.40) enzyme, NAD^+-malate (E.C. 1.1.1.39) enzyme, and phosphoenolpyruvate (PEP) carboxykinase (E.C. 4.1.1.32). In all species, more than 95 percent of the activity is associated with the bundle sheath extracts (*6*). This finding is consistent with our report that in *Digitaria sanguinalis* ATP sulfurylase was enriched in the bundle sheath cell fraction (*7*). The whole leaf activities reported in Table 1 are intermediate between the mesophyll and bundle sheath activities, consistent with an enrichment of activity in the bundle sheath. The whole leaf activities also are within the same activity ranges found in C_3 and CAM species reported here (Table 1) and previously (*5*, *8*).

To localize the ATP sulfurylase activity intracellularly, bundle sheath protoplasts were lysed and subjected to differential centrifugation. Bundle sheath protoplasts isolated from *Panicum miliaceum* were fractionated into cytosol, chloroplast, mitochondrial, and peroxisomal fractions as shown by the separation of $NADP^+$-glyceraldehyde 3-phosphate dehydrogenase, cytochrome *c* oxidase, and catalase activities, respectively (Table 2). The ATP sulfurylase activity was in the same fraction as the chloroplast marker enzyme. Similarly, in *Urochloa mosambicensis* ATP sulfurylase activity followed the activity of another chloroplast marker enzyme, RuBP carboxylase, in differential centrifugation of bundle sheath protoplast extracts. Here 98 percent of the chloroplast marker enzyme and 94 percent of the ATP sulfurylase activity were found in the chloroplast fraction sedimenting at 300*g* (Table 2).

ATP sulfurylase activity could not be detected in mesophyll protoplasts isolated from *U. mosambicensis*. A high activity of $NADP^+$ glyceraldehyde 3-phosphate dehydrogenase (E.C. 1.2.1.9) was found in this same preparation, however, which indicates that soluble proteins were not lost during chloroplast isolation. In mesophyll protoplasts isolated from a C_3 plant, *Avena sativa*, 71 percent of the chloroplast marker enzyme and 82 percent of the ATP sulfurylase were found in the fraction sedimenting at 300*g* (Table 2). Collectively, these findings indicate that ATP sulfurylase is chloroplastic in both C_3 and C_4 plants. In C_4 plants, however, the enzyme apparently

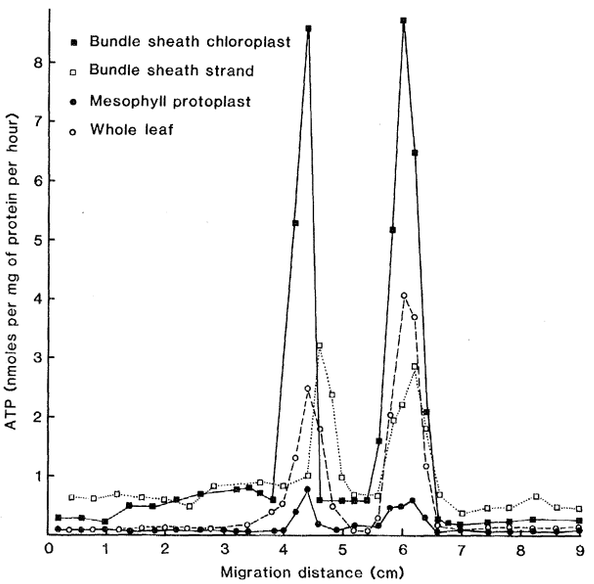

Fig. 1. Isozymes of ATP sulfurylase in different protein extracts of *Panicum miliaceum*. Mesophyll protoplasts, bundle sheath strands, and bundle sheath chloroplasts were obtained as described in the legend to Table 2. Disc electrophoresis was performed on 6 percent polyacrylamide gels (15 mA per gel) under nondenaturing conditions. The gel was run with 68 μg of whole leaf protein, 90 μg of mesophyll protein, 12 μg of bundle sheath strand protein, and 26 μg of bundle sheath chloroplast protein. The activity of ATP sulfurylase in these protein extracts was 0.31, 0.05, 0.44, and 0.53 μmole of ATP produced per miligram of protein per hour, respectively. The gel was sliced into 2-mm sections, and each section was placed into 100 μl of 50 m*M* tris (*p*H 8.2), 1 m*M* EDTA, and 1 m*M* β-mercaptoethanol. After 3 hours at 2°C the fractions were assayed for ATP sulfurylase as indicated in Table 1. Between 10 and 15 percent of the original activities placed on each gel were recovered in assays of the gel slices.

Table 2. Intracellular localization of ATP sulfurylase. Mesophyll (M) protoplasts were obtained as described under Table 1. Bundle sheath (BS) protoplasts from *P. miliaceum* were obtained as described by Edwards *et al.* (*9*) except that the plant material was 5 days old (*10*). Bundle sheath protoplasts were obtained from 10- to 15-days-old *U. mosambicensis* (*11*). Purified mesophyll and bundle sheath protoplasts were ruptured by several passes through a 50-μl Hamilton syringe in a medium containing 0.3*M* sorbitol, 2m*M* EDTA, 1 m*M* $MgCl_2$, 1 m*M* $MnCl_2$, 50 m*M* Tricine (*p*H 8.0), 2 m*M* cysteine, and 1 percent PVP-40 (weight to volume). After breaking, BSA was added to a final concentration of 0.1 percent (weight to volume). The protoplast extracts were centrifuged for 3 minutes at 300*g* to remove the chloroplasts and the supernatant was then centrifuged for 10 minutes at 10,000*g* to sediment the mitochondria and peroxisomes. After centrifugation most of the mitochondria were in the 10,000*g* pellet fraction while a large portion of peroxisomes remained in the supernatant. All fractions were supplemented with Triton X-100 to a final concentration of 0.1 percent prior to assay. ATP sulfurylase was assayed as indicated in Table 1, and the organelle marker enzymes $NADP^+$ glyceraldehyde-3-phosphate dehydrogenase (G3PDH), catalase, and cytochrome *c* oxidase were assayed as described (*12*).

Plant type and species	Cell type	Enzyme	Activities*			
			Whole protoplast extract	300*g* pellet	10,000*g* pellet	Supernatant
			C_4 plants			
Panicum miliaceum	BS	ATP sulfurylase	23	16.2 (71%)	N.D.	6.6 (29%)
		$NADP^+$ G3PDH	468	396 (66%)	110 (18%)	92 (16%)
		Catalase†	53	15 (23%)	12 (19%)	37 (58%)
		Cytochrome *c* oxidase	102	N.D.	67 (100%)	N.D.
Urochloa mosambicensis	BS	ATP sulfurylase	163	145 (94%)	N.D.	10 (6%)
		RuBP carboxylase	212	212 (98%)	N.D.	4 (2%)
Urochloa mosambicensis	M	ATP sulfurylase	N.D.	N.D.	N.D.	N.D.
		$NADP^+$ G3PDH	1218	974 (68%)	78 (5%)	380 (27%)
			C_3 plant			
Avena sativa	M	ATP sulfurylase	10	7.7 (82%)	N.D.	1.7 (18%)
		$NADP^+$ G3PDH	856	497 (71%)	82 (12%)	117 (17%)

*Micromoles per milligram of chlorophyll of the original extract per hour. †Times 10^3.

is restricted to the chloroplasts of the bundle sheath cells.

To verify this conclusion, we sought to identify cellular and subcellular activities of the enzyme utilizing preparative gel electrophoresis. Two isozymes could be identified in the whole leaf protein extract (Fig. 1). The activity was approximately equal in the two isozyme fractions. Both isozymes were present in extracts of bundle sheath strands and bundle sheath chloroplasts (Fig. 1). The bundle sheath chloroplasts were enriched in the specific activity of the enzyme, which is consistent with our findings in Table 2. Further, both isozymes appeared in approximately the same ratio as observed in the whole leaf extract. Trace amounts of both isozymes were detected in the extract prepared from mesophyll protoplasts (Fig. 1), presumably because of a minor contamination of bundle sheath protoplasts in this preparation (compare with Table 1). In this experiment (Fig. 1) both protoplast types were isolated simultaneously from very young tissue.

We have found the activity of ATP sulfurylase to be largely in bundle sheath cells of a variety of C_4 plants. Within this cell type two isozymes exist, both of which are chloroplastic. It is not known whether the entire process of sulfur assimilation to the reduced level of cysteine is compartmentalized in leaves of C_4 plants. Recently, thiosulfonate reductase sulfite reductase activity was shown in both mesophyll and bundle sheath cells of *D. sanguinalis*, with the bundle sheath extracts containing two to three times more activity than did mesophyll cell extracts (*7*). While the consequences of cellular compartmentation during sulfur assimilation have yet to be examined fully, it is clear that sulfur activation has been modified in the leaves of C_4 plants, with the initial step of sulfate activation occurring primarily in one chloroplast type of the leaf. The cellular compartmentation of sulfur assimilation in leaves of C_4 plants is analogous to that of carbon and nitrogen assimilation; but it is distinct in that carbon and nitrogen assimilation are initiated in mesophyll cells while sulfur assimilation is initiated in bundle sheath cells.

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Carcinogenic Activity of Particulate Nickel Compounds Is Proportional to Their Cellular Uptake

Abstract. *Particles (≤5 micrometers) of the potent carcinogen crystalline nickel subsulfide were actively phagocytized by cultures of Syrian hamster embryo cells and Chinese hamster ovary cells. Cells did not take up significant quantities of similar-sized particles of the noncarcinogen amorphous nickel monosulfide. The carcinogenic activity of this and other metal compounds appears to be proportional to their cellular uptake.*

Lung, sinonasal, and laryngeal carcinomas in nickel refinery workers have been attributed to the inhalation of nickel compounds (*1*). Rats receiving a single intramuscular injection of crystalline Ni_3S_2 or crystalline Ni_3Se_2 have developed a 65 to 100 percent incidence of sarcomas (*2–4*). However, when amorphous NiS was administered under similar conditions, no cancers were observed to develop (*5*). The differences in carcinogenic activity between crystalline Ni_3S_2 and amorphous NiS have been documented in other experimental animals, including mice and Syrian hamsters, with various administration routes (that is, intrarenal and intratesticular injection) (*2–4, 6*). The inhalation of crystalline Ni_3S_2 has also been shown to induce lung cancer in experimental animals (*7*).

The striking difference in carcinogenic activity between amorphous NiS and crystalline Ni_3S_2 was also observed in a tissue culture cell transformation assay (*8–10*); Ni_3S_2 induced a concentration-dependent incidence of morphological transformation in cultured Syrian hamster embryo cells, whereas amorphous NiS produced little or no change (*10–12*). Colonies transformed by Ni_3S_2 were cloned and derived into immortal cell lines, which are capable of forming three-dimensional colonies in soft agar and of producing tumors when inoculated into nude athymic mice (*8*). Since both Ni_3S_2 and NiS particles have similar water solubility properties and do not readily dissolve in tissue culture media, we were able to study their cellular uptake with light and electron microscopy. Both compounds were ground separately and passed through a 5-μm screen.

Both compounds appeared to be of similar particle size under the light and electron microscopes (≤5 μm). When these compounds were added to cultured cells grown on plastic microscopic slides (Leighton tubes), the cultures actively phagocytized the Ni_3S_2 particles but did not readily phagocytize the NiS particles. Figure 1, A and B, shows light microscope photographs of Chinese hamster ovary (CHO) cells and Syrian hamster embryo (SHE) cells phagocytizing Ni_3S_2 particles. The phagocytized Ni_3S_2 particles were almost always contained in vacuoles. Figure 1C shows an electron microscope photograph of a CHO cell that has engulfed Ni_3S_2 particles. The particles are contained in a vacuole, and these nickel-containing vacuoles are primarily associated with the cytoplasmic compartment (*11*).

Within 30 minutes after the addition of Ni_3S_2 to the media of these cultures, Ni_3S_2 particles were seen entering the cells. The time course of the uptake of

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