a failure of regulation of FAD synthesis. It seems probable that in *Eremothecium ashbyi* NaF blocks the oxidation of the remaining glucose and therefore the flavin system oxidizing the accumulated pyruvate is strengthened. This is manifested by enhanced riboflavin production. In fact, manometric measurements performed by the direct method of Warburg have shown that 24-h-old washed mycelium is inhibited by NaF in its ability to oxidize glucose and acetate at concentrations of inhibitor which stimulate pyruvate oxidation (Fig. 3). NaF seems to be, therefore, an important factor shifting the metabolism of *E. ashbyi*, and its application in the riboflavin production process based on use of this microorganism should be considered.

Department of Microbiology, Charles University, Viničná, Prague (Czechoslovakia) F. KAPRÁLEK

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The effect of sulphonamides on ferricyanide reduction by illuminated spinach chloroplasts

The enzyme, carbonic anhydrase (EC 4.2.1.1), is present in considerable quantities in leaves[1]. This enzyme catalyzes a rapid exchange of oxygen between carbon dioxide and water. The occurrence of such exchange[2] sets a practical limit to the usefulness of ^{18}O as a tracer to determine the source of the O_2 evolved in photosynthesis. In the course of investigating the source of the O_2 in the Hill reaction[3], using carbonate labeled with ^{18}O, it was noted that spinach chloroplasts catalyzed a rapid exchange of $K_2C^{18}O_3$ with H_2O under experimental conditions where the non-enzymatic rate was very slow[2]. Diamox (5-acetylamino-1,3,4-thiadiazole-2-sulfonamide[4]), a potent inhibitor of animal carbonic anhydrase, was added to the chloroplast, buffer, ferricyanide mixture in hopes of avoiding this difficulty. This compound effected a marked inhibition of the Hill reaction with ferricyanide as the electron acceptor. Other sulfonamides were then tested for their effect on the photoreduction of ferricyanide by spinach chloroplasts and the results are reported here. The sulfonamides which inhibited the Hill reaction are potent inhibitors of animal carbonic anhydrase. The inhibition by the sulfonamides appeared particularly interesting in view of the catalytic requirement for CO_2 in the Hill reaction demonstrated by WARBURG AND KRIPPAHL[5,6] and confirmed by STERN AND VENNESLAND[7,8] and ABELES, BROWN AND MAYNE[9].

MANN AND KEILIN[10] originally observed that sulfanilamide and certain related sulfonamides that contained an unsubstituted sulfamyl group would inhibit animal carbonic anhydrase *in vitro*. MILLER, DESSERT AND ROBLIN[4] found that a number of heterocyclic unsubstituted sulfonamides, in which the sulfur atom of the sulfonamide group is joined to a carbon atom of the heterocyclic ring were particularly effective

TABLE I

THE EFFECT OF SULPHONAMIDES ON FERRICYANIDE REDUCTION BY ILLUMINATED SPINACH CHLOROPLASTS

The reaction mixtures contained 200 μmoles of Tris buffer (pH 8), 10 μmoles of $K_3Fe(CN)_6$, 70 μmoles of NaCl, sulfonamide and chloroplasts equivalent to 0.1 mg of chlorophyll, in a final volume of 2.0 ml. The flasks were illuminated for 10 min at 15° with red light of about 2000 foot candles. Solutions of the sulfonamides were freshly prepared and adjusted to pH 8 with NaOH. Chloroplasts were prepared from spinach leaves by isolation in 0.35 M NaCl (see ref. 8). They were washed once with the NaCl solution. The amount of ferricyanide reduced was determined spectrophotometrically on trichloroacetic acid filtrates of the reaction mixture. The absorbancy at 420 mμ of a solution containing 1 mM of ferricyanide was taken as 1.02

Sulfonamide	*Concentration required for 50% inhibition (M)*
Polythiazide	0.0005*
Neptazane	0.0025
Diamox	0.005
p-Toluenesulfonamide	0.0065
p-(Aminomethyl)benzenesulfonamide	0.015
Sulfanilamide	—**
	Non-inhibitory at (M)
N^1-acetylsulfanilamide	0.02
Sulfabenzamide	0.02
Sulfadiazine	0.02
Sulfathiazole	0.02
p-Carboxybenzenesulfonamide	0.04
2-Amino-ethanesulfonamide	0.02

* Added in 0.1 ml of 50% acetone. The control flasks had the same amount of acetone.
** 20% inhibition at 0.02 M.

as inhibitors of animal carbonic anhydrase. Diamox* and Neptazane (5-acetylimino-4-methyl-Δ^2-1,3,4-thiadiazoline-2-sulfonamide) were reported to be approx. 330 times as active as sulfanilamide *in vitro*[4,11]. Diamox and Neptazane have had extensive clinical use as diuretics because of their ability to inhibit renal carbonic anhydrase[12].

The effect of various sulfonamides on the photoreduction of ferricyanide by spinach chloroplasts is shown in Table I. The concentration required to reduce the rate of ferricyanide reduction by 50% is given for those compounds which were inhibitory. Polythiazide (2-methyl-3-(β,β,β-trifluoro-ethylthiomethyl)-6-chloro-7-sulfamyl-3,4-dihydro-1,2,4-benzothiadiazine-1,1-dioxide), one of the newest diuretics, is about 1/5 as potent a carbonic anhydrase inhibitor *in vitro* as Diamox and Neptazane[13]. Polythiazide was the most effective inhibitor of ferricyanide reduction that

* The following chemicals were generously donated: Diamox, Neptazane, and *p*-nitrobenzenesulfonamide from Dr. C. WESTMARK of Lederele Laboratories, Chicago, Illinois; *p*-sulfamyl-2-hydroxyethylcarbanilate from Warner-Lambert Research Institute, Morris Plains, New Jersey; 6-chloro-3,4-dihydro-7-sulfamoyl-2H-1,2,4-benzothiadiazine 1,1-dioxide from Abbott Laboratories, North Chicago, Illinois; 6-ethoxybenzothiazole-2-sulfonamide, thiophene-2-sulfonamide and 2-aminoethanesulfonamide from the Upjohn Company, Kalamazoo, Michigan; Polythiazide from the Pfizer Laboratories, New York City, New York; 4,5-dichloro-*m*-benzenedisulfonamide from Merck, Sharp and Dohme, West Point, Pennsylvania; *p*-carboxybenzenesulfonamide was purchased from Aldrich Chemical Company and *p*-toluenesulfonamide from the Eastman Kodak Company. Sulfanilamide, sulfabenzamide, N^1-acetylsulfanilamide, sulfadiazine, sulfathiazole, and *p*-(aminomethyl)benzenesulfonamide were products of Nutritional Biochemicals Company.

was tested. The concentration required for 50% inhibition was 0.5 mM compared to 2.5 mM for Neptazane and 5 mM for Diamox. Sulfanilamide caused an 8% inhibition at 0.01 M and a 20% inhibition at 0.02 M. Lower concentrations did not inhibit.

The N^1 substituted sulfonamides, sulfaacetamide, sulfabenzamide, sulfadiazine, and sulfathiazole did not inhibit ferricyanide reduction at concentrations up to 0.02 M. In fact all of these compounds stimulated ferricyanide reduction 10–30% at 0.02 M. KREBS[14] found that these compounds inhibited animal carbonic anhydrase *in vitro*, but the concentrations required to produce 50% inhibition were 100–10000 times higher than that for sulfanilamide.

p-Carboxybenzenesulfonamide and 2-amino-ethanesulfonamide had no inhibitory effect on the Hill reaction. They have been reported to be inhibitors of animal carbonic anhydrase *in vitro*[14,15].

A number of other carbonic anhydrase inhibitors[1,12] were tested for their effect on ferricyanide reduction. 4,5-Dichloro-*m*-benzenedisulfonamide, *p*-sulfamyl-2-hydroxyethylcarbanilate, 6-chloro-3,4-dihydro-7-sulfamoyl-2H-1,2,4-benzothiadiazine 1,1-dioxide, 6-ethoxy-benzothiazole-2-sulfonamide, *p*-nitrobenzenesulfonamide, thiophene-2-sulphonamide, and benzothiazole-2-sulphonamide all inhibited ferricyanide reduction 10–30% at concentrations of 2.5 mM or lower. It was not possible to test these compounds at higher concentrations because of their limited solubility at pH 8.

It is unlikely that the sulfonamides act by inhibiting the carbonic anhydrase present in spinach chloroplasts. The enzyme from spinach has been extensively purified by KONDO, CHIBA AND KAWAI[16] and has very different properties than the enzyme from animal sources. The plant enzyme contains no zinc. A preparation of the spinach enzyme through the second $(NH_4)_2SO_4$ fractionation described by KONDO *et al.*[15] was not inhibited by 3.3 mM Neptazane nor did the addition of the enzyme overcome the inhibition of ferricyanide reduction by Neptazane.

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Department of Biochemistry University of Chicago, Chicago, Ill. (U.S.A.) BABETTE K. STERN

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