mucosal cell is a diffusion-limited process.

Summary. Evidence of the alteration of the unidirectional, mucosa to serosa, transmural potassium flux by ouabain has been presented. This evidence suggests several conclusions concerning the transport of potassium by the small intestinal mucosal cell. It seems likely that potassium actually passes through the cell, rather than passing through extracellular channels between cells as is commonly suggested. This alteration in cellular potassium transport would appear to be carrier linked, and the carrier involved in the ouabain-enhanced transmural flux is on the serosal surface of the mucosal cell. Because potassium cannot be shown to be transported against an electrochemical gradient, and potassium transport has a low Q_{10} suggestive of a diffusion process, the carrier process involved in potassium transport may be an example of an equilibrating carrier system. Whether potassium has a carrier mechanism regularly available, or whether it is able to use the sodium carrier when the latter is altered by the addition of ouabain to the system is an important question which cannot be answered by these experiments.

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Received March 21, 1967. P.S.E.B.M., 1967, v125.

Inhibition of Enzymes by the Anthocyanin Malvidin-3-Monoglucoside.*† (32171)

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Partially demethylated malvidin was demonstrated to be bactericidal toward *Escherichia coli*(1,2). Pratt *et al*(3) and others (4,5,6) established that anthocyanin compounds influence growth and glucose oxidation by many organisms. Somaatmadja *et al* (7) showed that cyanidin and leucocyanidin inhibited reproduction of *Staphylococcus aureus, Lactobacillus casei,* and *E. coli.* Keith and Powers(8) found that phenolic acids and esters derived from anthocyanin compounds inhibited respiration of *E. coli, Proteus vulgaris, Aerobacter aerogenes,* and *Pseudomonas aeruginosa* and that in absence of other carbon sources these compounds were utilized by the test organisms. Hulme and Jones(9) established that anthocyanins and anthocyanidins inhibited succinic oxidase, whereas leucoanthocyanins inhibited both succinic oxidase and malic acid dehydrogenase. Powers(10) demonstrated that malvidin-3-monoglucoside inacivated yeast hexokinase. The present studies demonstrate the mechanism by which malvidin-3-monoglucoside affects enzyme system of *Salmonella enteritidis* and enzymes from other sources.

* This investigation was supported by Research Grant EF-00349-07, Division of Environmental Engineering and Food Protection, USPHS.

† Journal Series Paper 33, Univ. of Georgia, College of Agri. Exp. Stations, College Station, Athens.

Materials and methods. Isolation of anthocyanin pigment. Malvidin-3-monoglucoside (M-3-G) was isolated from Cabernet Sauvignon grapes as described previously (11).

Cultures. Flasks of brain heart infusion (Difco) were inoculated with an active culture of *S. enteritidis* (obtained from Department of Microbiology, University of Georgia) and incubated with occasional shaking for 18 hours at 37°C. The cells were harvested by centrifugation, washed twice with distilled water, lyophilized, and subjected to two methods of fractionation to obtain the various cell fractions. The first method was a slight modification of that reported by Kanai and Youmans(12) using mechanically disrupted cells. One gram of lyophilized cells were ground vigorously for 5 minutes with the aid of 2 g levigated alumina and cold phosphate buffer (pH 7.0) in a mortar kept in an ice bath. Approximately 50 ml cold phosphate buffer was added to decrease viscosity, and the cell homogenate was then centrifuged at 800 × *g* for 10 minutes to remove alumina, intact cells, and debris. The supernatant was then fractionated as diagrammed in Fig. 1. Three cell fractions were isolated: cell wall (pellet obtained at 3,000 × *g*), particulate fraction (pellet 136,212 × *g*), and a cytoplasmic fraction (supernatant). The second method was that of Burrous and Wood(13) using cells lysed with lysosome. Three cell fractions were also isolated: cytoplasm (obtained after centrifugation of disrupted protoplasts at 34,500 × *g*), membranes (bottom layer 109,045 × *g*), and DNA (top layer 109,045 × *g*). The protein content of the various fractions was estimated by the method of Gornall *et al*(14).

Enzyme assays. Each cell fraction was assayed for malate and glycerol dehydrogenase, hexokinase, and glutamate decarboxylase. For comparative purposes the same assay procedures were conducted on the following enzymes purchased from Worthington Biochemical Corp.: malate dehydrogenase (MADH) (EC 1.1.1.37 pig heart), glycerol dehydrogenase (GDH) (EC 1.1.1.6 *A. aerogenes*), glutamate decarboxylase (GLD) (EC 4.1.1.15 acetone powder of *E. coli*), and hexokinase (HX) (EC 2.7.1.1 yeast).

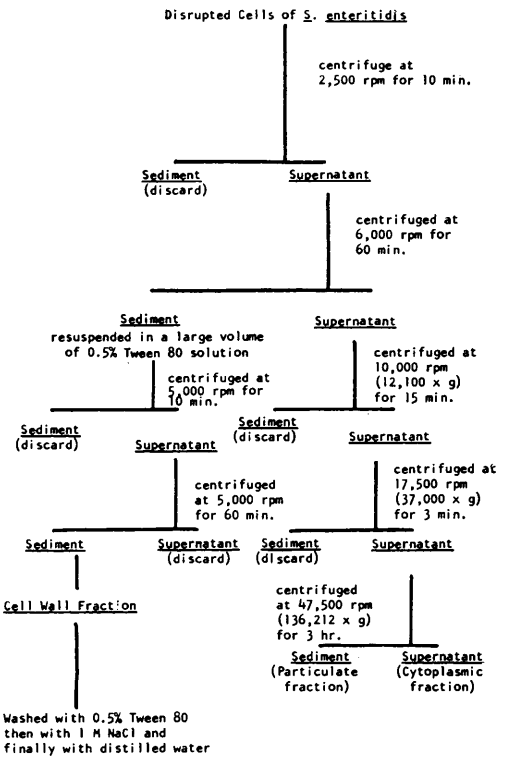


FIG. 1. Flow diagram for isolation of cell fractions from *S. enteritidis*.

MADH was assayed using the method of Mehler *et al*(15). The incubation mixture contained 75 μM phosphate buffer (pH 7.4), 0.14 μM of $NADH_2$, and 0.76 μM of oxalacetate, and the enzyme preparation (2.5 μg of protein) or the cell fraction in a total volume of 3 ml. When this assay was conducted on cell fractions, the following protein concentrations were used: cell wall (0.22 mg), particulate (0.005 mg), and cytoplasm (0.022 mg).

GDH was assayed using the method of Lin and Magasanik(16). The reaction mixture had 11 μM of ammonium sulfate, 1 μM of NAD, 300 μM of carbonate buffer (pH 10), 300 μM glycerol, and the enzyme preparation (0.011 mg of protein) or the cell fractions in a total volume of 3 ml. Protein concentrations in cell fractions used were: cell wall (0.22 mg), particulate (0.118 mg), and cytoplasm (0.22 mg).

HX was determined by the method of Darrow and Colowick(17). The assay mix-

ture consisted of 2.5 ml of the Darrow and Colowick stock solution, 80 μg glucose and 28.5 μg purified enzyme preparation or cell fraction in a total volume of 3 ml. Protein concentration of cell fractions used were: cell wall (0.22 mg), particulate (0.118 mg), and cytoplasm (0.22 mg).

GLD was measured using the Warburg manometric technique. The μl CO_2 released per unit time at 37°C and pH 5.0 was followed using a Gilson Differential Respirometer. Incubation mixtures contained 16.6 μM glutamic acid, 100 μM acetate buffer (pH 5.0), and enzyme preparation (0.414 mg protein) or cell fractions in a total volume of 3.1 ml. The protein concentrations in the cell fraction studied were: cell wall (2.2 mg), particulate (1.18 mg), and cytoplasm (2.2 mg).

Kinetic measurements. Kinetic studies were performed on GLD (pH 5.0) to determine the type of inhibition caused by M-3-G. The velocity (v) of the reaction over a range of substrate concentrations in the presence of different levels of M-3-G was determined. The concentrations of glutamate used were 16.6, 24.9, 33.2, and 41.5 μM in the presence of 2 and 4 μM of M-3-G, respectively, and plots of reciprocals of velocity (1/v) against reciprocals of substrate concentration (1/S) were made. Three different procedures were followed by adding M-3-G to incubation mixtures: (a) The enzyme together with M-3-G was equilibrated for 15 minutes at 37°C and then substrate added at zero time. (b) The enzyme, M-3-G and substrate were equilibrated separately for 15 minutes at 37°C and all mixed at zero time. (c) The glutamate and M-3-G were equilibrated together for 15 minutes at 37°C and then enzyme added at zero time. Using Linweaver-Burk(18) plots the Michaelis constant (K_m) was determined. Inhibitor constant (K_i) was also obtained by plotting (1/v) against M-3-G concentration at the two extremes of substrate concentration.

Results and discussion. Enzymatic activities in disrupted cell fractions of S. enteritidis. Various concentrations (ranging from 0.005 to 2.22 mg of protein) of cytoplasmic, particulate, and cell wall fractions isolated from mechanically disrupted cells of *S. enteritidis* (Method 1) were assayed for GDH, MADH, GLD, and HX. Results showed the absence of all these enzymes in cell wall; the absence of GLD and HX in all fractions, and the presence of both MADH and GDH in the particulate and cytoplasmic fractions. Salton(19) listed the enzymatic activities so far known to be located in the envelope fraction of Gram-negative bacteria. Among those listed were hydrogenases, dehydrogenases, ATPase, $NADH_2$ oxidase, and malic oxidase. The aforementioned enzymes were again assayed in the cytoplasmic, membrane, and DNA fractions isolated from lysozyme treated cells (Method II). No enzymatic activity was found in the DNA fraction; MADH was active in both cytoplasmic and membrane fractions, but GDH, GLD, and HX were absent in all fractions. The absence of enzymatic activity in the DNA fraction is consistent with results obtained by Burrous and Wood(13) who showed that both cytoplasmic and membrane fractions of *Ps. fluorescens* contained 9 different oxidative and non-oxidative enzymes while the DNA fraction was essentially devoid of enzymatic activity. The absence of HX in all cell fractions may have been due to dilution effect during the fractionating procedure of the cells. Berger *et al*(20) reported that HX undergoes instantaneous loss of up to 50% of its activity in very dilute solution. This decrease in activity may be prevented by dilution in the presence of various proteins, but in the present study the fractions that were diluted with 1% albumin(21) showed no hexokinase activity. Both lyophilized cells and cell fraction of *S. enteritidis* had no GLD activity. A requirement for pyridoxal phosphate in cell free enzyme preparations has been suggested by Gale and Epps(21) and Shukuya and Swhert(22); however, since no activity was found in whole cell preparations this requirement was believed not to be the limiting factor.

Effect of M-3-G on MADH and GDH. After establishing the presence of enzymes in both particulate and cytoplasmic fractions, it was decided to examine the effect of M-3-G on the activities of these enzymes. Inhibition of

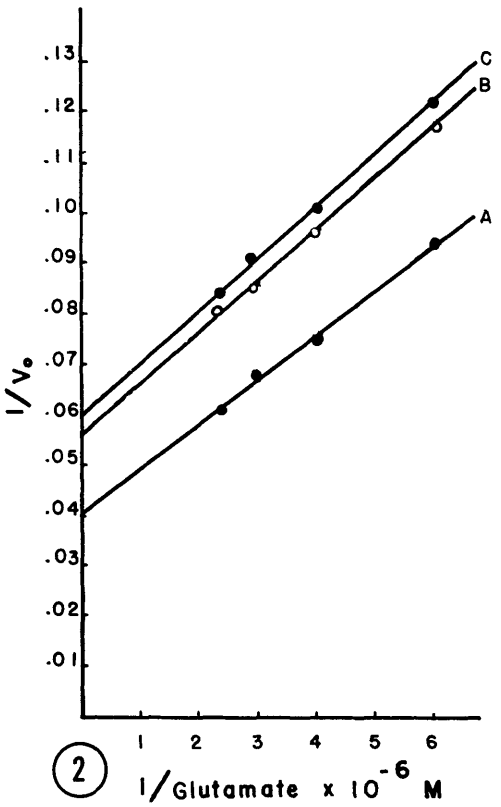
TABLE I. Inhibition of Glycerol (GDH) and Malate Dehydrogenases (MADH) in Cell Fractions of *S. enteritidis* and in Purified Enzyme Preparation by Malvidin-3-Monoglucoside (M-3-G).*

	% GDH activity				% MADH activity			
	Concentration of M-3-G (μM)							
Source of enzyme	0	1	2	4	0	1	2	4
S. enteritidis								
Particulate	100	79	65	74	100	100	90	80
Cytoplasm	100	48	62	24	100	94	96	96
Cell wall	N.A.†	—	—	—	N.A.†	—	—	—
Purified								
A. aerogenes	100	68	63	15				
Pig heart					100	38	13	18

* In all instances final reaction volume was 3 ml, and the activity of the control was determined in absence of M-3-G. Other enzymatic activities are expressed as percent of control.
† No activity.

MADH and GDH by M-3-G was observed in particulate and in cytoplasmic fractions, and the inhibition was, in general, concentration dependent (Table I). In particulate fraction, M-3-G inhibited GDH to the extent of 21% as compared with the control when 1 μM of M-3-G was used, and 35% and 27% respectively when 2 and 4 μM were used. In the cytoplasmic fraction, the inhibition was 52% with 1 μM, 38% with 2 μM, and 76% when 4 μM was used in incubation mixtures. Varying concentrations of M-3-G inhibited MADH of the particulate fraction, and the inhibition was 10 and 21% in presence of 2 and 4 μM of M-3-G respectively. No effect was noted on MADH in cytoplasmic fraction when 1 to 4 μM of M-3-G was used, whereas maximum inhibition of GDH in particulate fraction was 35% of control with 2 μM of M-3-G and in the cytoplasmic fraction maximum inhibition (76%) was noted with 4 μM of M-3-G. Inhibition of MADH in the particulate fraction was maximum (20%) in the presence of 4 μM of M-3-G, but in cytoplasm the inhibition was 5% for all concentrations of pigment used. Carpenter *et al*(23) established that increasing concentrations of anthocyanins inhibited both MADH and GDH. For comparative purposes these experiments were conducted using purified preparations for MADH and GDH obtained from different sources. Results (Table I) indicated that GDH was inhibited 32% with 1 μM M-3-G, 37% with 2 μM and 85% when 4 μM M-3-G were added. MADH was also inhibited 62, 87, and 82% in presence of 1, 2 and 4 μM of M-3-G respectively.

Effect of M-3-G on HX and GLD. When varying concentrations of M-3-G (1 to 4 μM) were added to yeast hexokinase both stimulation and inhibition were observed. One μM of M-3-G stimulated the activity 21% as compared with the control; 2 μM M-3-G had no effect, whereas 4 μM M-3-G gave a 6% inhibition. GLD activity on the other hand decreased in the presence of all concentrations of M-3-G. After 30 minutes incubation the inhibition was 12, 17, and 17% in presence of 1, 2, and 4 μM M-3-G respectively, whereas after 90 minutes incubation the inhibition was 8, 12, and 12% for 1, 2, and 4 μM of M-3-G respectively. Several possible explanations for the mode of action of anthocyanin compounds on enzyme systems were reported. Chelation of essential metal ions was considered by Somaatmadja *et al*(24) who suggested 3 different possible structures for the chelation of copper by M-3-G. Their conclusion was that the methoxyl group was a chelatogenic site of the malvidin molecule. Such chelation of divalent metal ions could perhaps inhibit enzymatic activity of HX which requires Mg^{++} for its activity. However, in this study, Mg^{++} ions were present in excess in incubation mixture and, therefore, inhibition by chelation seems unlikely. Metzler *et al*(25) and Snell(26) proposed a mechanism for amino acid decarboxylation to include the formation of a metal chelate of a Schiff base with a metal ion chelating amino group with -OH on C-3 of pyridoxal.



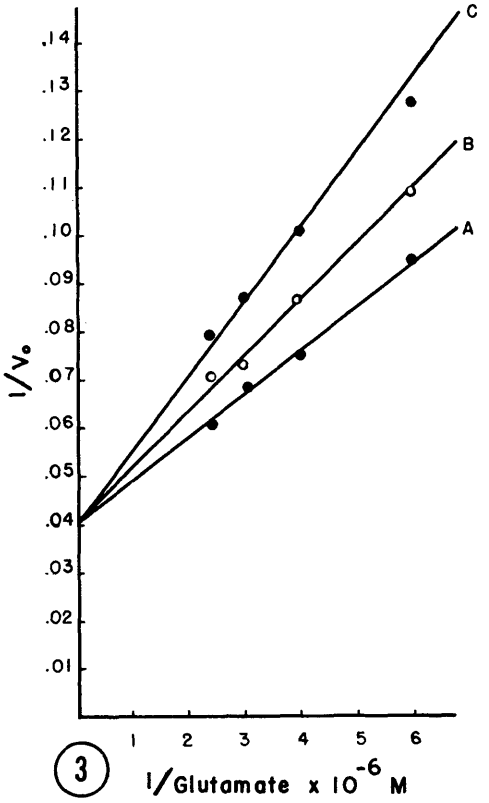


FIG. 2. Lineweaver-Burk plot of glutamate decarboxylase inhibition at 37°C by M-3-G in 100 μM acetate buffer, pH 5.0, with substrate concentrations of 8.3, 16.6, 24.9, 33.2, and 41.5, μM and enzyme preparation (0.414 mg protein). The inhibitor concentrations were: A = 0, B = 2, and C = 4 μM M-3-G. The enzyme and M-3-G were equilibrated for 15 min and the substrate added at zero time and the velocity of the reaction was measured as μl CO_2/min.

FIG. 3. Lineweaver-Burk plot of glutamate decarboxylase inhibition at 37°C by M-3-G in 100 μM acetate buffer, pH 5.0 with substrate concentrations of 8.3, 16.6, 24.9, 33.2, and 41.5 μM and enzyme preparation (0.414 mg protein). The inhibitor concentrations were: A = 0, B = 2, and C = 4 μM M-3-G. The enzyme, M-3-G, and substrate were equilibrated for 15 min separately and mixed at zero time and the velocity of the reaction was measured as μl of CO_2/min.

Kinetic studies. Decarboxylation of glutamic acid was found to follow Michealis-Menton kinetics as indicated from linear relationship in Lineweaver-Burk(18) plots (Fig. 2 and 3). The apparent K_m was found to be 2.3×10^{-5} M (from 8 experiments). This value is somewhat higher than that of 0.82×10^{-5} M reported by Shukuya and Swhert(22) at pH 4.6 and 36°C; however, pH and temperature were different. The kinetic plots of inhibition by M-3-G showed both competitive and non-competitive type inhibition depending on how substrate, inhibitor (M-3-G), and enzyme were mixed. When enzyme and inhibitor were equilibrated together and glutamate added at zero time, the inhibition was non-competitive (Fig. 2); but when all 3 reactants were equilibrated separately and mixed at zero time the inhibition seemed to follow the "fully competitive" type (Fig. 3). When substrate and M-3-G were equilibrated and then the enzyme added at zero time, partial competitive inhibition was noted especially at the concentration levels used. The need for the variation in mixing procedure of enzyme, inhibitor, and substrate was suggested by Clark(27) who reviewed the effects of flavonoids on decarboxylase activity and showed that mixing affects the type of inhibition. The inhibitor constants K_i were 1.9×10^{-7} M for enzyme and inhibitor equilibrated together and sub-

strate added at zero time, 0.86×10^{-5} M for enzyme, substrate, and inhibitor all added at the same time, and 1.18×10^{-5} M for substrate and inhibitor equilibrated together and the enzyme added at zero time. The competitive inhibition of GLD by M-3-G is apparently caused by competition of pigment with the substrate for binding site of the enzyme.

Although such inhibition calls for binding of glutamate and M-3-G on the same site of the enzyme, the possibility cannot be ruled out that the pigment may bind irreversibly to the enzyme through a special type of mechanism leading to the non-competitive type of inhibition noted when the enzyme and M-3-G were equilibrated and the substrate added at zero time. Griesemer *et al*(28) studied the inhibitory effect of 40 compounds on hog kidney 3,4-dihydroxyphenylalanine decarboxylase (DOPA). They found that 30 minutes incubation of enzymes and inhibitor before the addition of substrate increased the inhibition 2 1/2 fold. Some of the most active samples tested were: 2',2,4-trihydroxy-4'-acetoxychalcone (93% inhibition at 0.01 relative molarity (R.M.) expressed as relative to that of substrate), 3',4',7,8-tetrahydroxyflavanone (60% inhibition at 0.1 R.M.), α-methyl-3,4-dihydroxycinnamic acid (55% inhibition at 0.1 R.M.). Clark(27) reported studies on inhibitors of amino acid decarboxylases and stated that when using flavonoid compounds precautions should be taken to prevent oxidation to quinones by adding cysteine to substrate and glutathione to enzyme before reacting under anaerobic conditions. He also pointed out that the Lineweaver-Burk plot showed that the inhibition under these conditions was competitive, whereas without such precautions, the inhibition was non-competitive or pseudo-competitive.

Malvidin-3-monoglucoside (Fig. 4) is an anthocyanin compound structurally related to flavonols, and it is classed as a flavylium cation. Hartman *et al*(29) examined a series of flavonoids for their ability to inhibit dopa decarboxylase including flavone, flavans, flavanones, chalcones, coumarins, and related compounds. They found that the most powerful inhibitor was 5-(3,4,-dihydroxycinnamoyl) salicylic acid and that the inhibition was competitive.

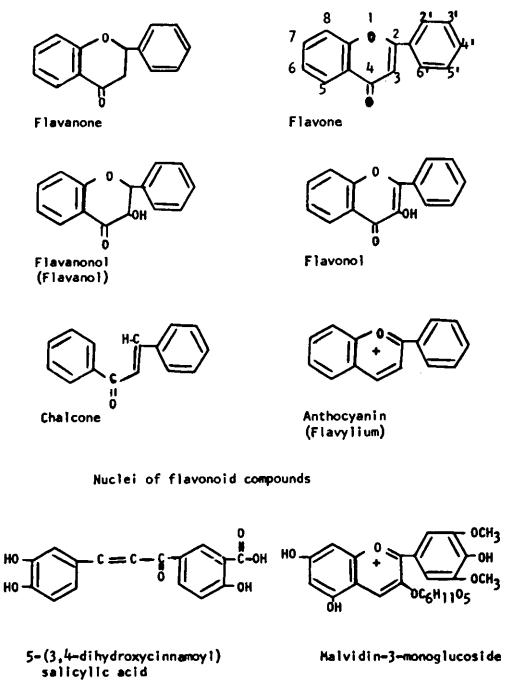


FIG. 4. Structural relationship between Malvidin-3-monoglucoside and other flavonoid compounds.

Cole(30) found that leucoanthocyanins can act as inhibitors of polygalacturonase after they have undergone a quinone polymerization.

Summary. The cytoplasmic, particulate, and cell wall fractions were isolated from disrupted bacterial cells and assayed for MADH, GDH, HX, and GLD enzymes in absence of pigment (control) and presence of various M-3-G concentrations. The magnitude of enzymatic inhibition due to increasing concentrations of M-3-G (1 to 4 μM) was not the same for each enzyme. Two μM of M-3-G inhibited glycerol dehydrogenase (35% of control) in particulate fraction, whereas, 76% inhibition was noted in cytoplasmic fraction with 4 μM of pigment. MADH was inhibited to the extent of 10 and 20% in particulate fraction with 2 and 4 μM of M-3-G respectively and 4% in cytoplasm with both pigment concentrations. For comparative purposes, the effect of M-3-G on the same enzymes obtained commercially from other sources revealed that GDH *(Aerobacter aero-*

genes) was inhibited 85% with 4 μM pigment. Inhibition (87%) of MADH (pig heart) and (17%) GLD *(Escherichia coli)* was noted with 2 μM M-3-G. Stimulation of HX (yeast) (21% over control) was observed with 1 μM M-3-G; 2 μM had no effect, whereas 4 μM caused 6% inhibition. Competitive and non-competitive inhibition of GLD by M-3-G, depending on how substrate, inhibitor and enzyme were mixed, followed Michaelis-Menton kinetics as indicated by the linear relationship in the Lineweaver-Burk plot with $K_m = 2.3 \times 10^{-5}$ M for enzyme and inhibitor equilibrated together and substrate added at zero time.

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