

Host-Pathogen Interactions¹

XXIII. THE MECHANISM OF THE ANTIBACTERIAL ACTION OF GLYCINOL, A PTEROCARPAN PHYTOALEXIN SYNTHESIZED BY SOYBEANS

Received for publication November 8, 1982 and in revised form March 9, 1983

LAWRENCE I. WEINSTEIN² AND PETER ALBERSHEIM³

Department of Chemistry, University of Colorado, Boulder, Colorado 80309

ABSTRACT

The biochemical basis for the ability of the pterocarpin phytoalexin glycinol (3,6a,9-trihydroxypterocarpin) to inhibit the growth of bacteria was examined. Glycinol at bacteriostatic concentrations (e.g. 50 micrograms per milliliter) inhibits the ability of *Erwinia carotovora* to incorporate [³H]leucine, [³H]thymidine, or [³H]uridine into biopolymers. Exposure of *Escherichia coli* membrane vesicles to glycinol at 20 micrograms per milliliter results in inhibition of respiration-linked transport of [¹⁴C]lactose and [¹⁴C]glycine into the vesicles when either D-lactate or succinate is supplied as the energy source. The ability of *E. coli* membrane vesicles to transport [¹⁴C]α-methyl glucoside, a vectorial phosphorylation-mediated process, is also inhibited by glycinol at 20 micrograms per milliliter. Furthermore, exposure of membrane vesicles to glycinol (50 micrograms per milliliter) at 20°C results in the leakage of accumulated [¹⁴C]α-methyl glucoside-6-phosphate. The effects of the phytoalexins glyceollin, capsidiol, and coumestrol, and daidzein, a compound structurally related to glycinol but without antibiotic activity, upon the *E. coli* membrane vesicle respiration-linked transport of [¹⁴C]glycine and of [¹⁴C]α-methyl glucoside was also examined. Glyceollin and coumestrol (50 micrograms per milliliter), but not daidzein, inhibit both membrane-associated transport processes. These data imply that the antimicrobial activity of glycinol, glyceollin, and coumestrol are due to a general interaction with the bacterial membrane. Capsidiol (50 micrograms per milliliter) inhibits D-lactate-dependent transport of [¹⁴C]glycine but not vectorial phosphorylation-mediated transport of [¹⁴C]α-methyl glucoside. Thus, capsidiol's mechanism of antimicrobial action seems to differ from that of the other phytoalexins examined.

Phytoalexins are low-mol-wt antibiotics produced by plants in response to various biotic and abiotic elicitors (1, 2, 20, 27, 31, 39). The dominant antibacterial phytoalexin isolated from soybean (*Glycine max* [L.] Merr., cv Wayne) cotyledons inoculated with the bacterium *Erwinia carotovora* has been identified as (6aS, 11aS)-3,6a,9-trihydroxypterocarpin, to which the trivial name glycinol has been assigned (45). Glycinol has been shown to be a static agent that is effective against a broad spectrum of microorganisms (45). Bacteria and fungi are inhibited by similar concentrations of this phytoalexin. Glycinol extends the lag phase of growth of both *E. carotovora* and *Saccharomyces cerevisiae* in liquid culture-inhibition assays (45). It has been hypothesized that

glycinol may inhibit the growth of widely diverse microorganisms by a similar mechanism of action (45).

Previous studies on the mechanism of action of structurally diverse phytoalexins have implicated the membrane as the site of antimicrobial action (19, 23, 29, 30, 34, 42). The research detailed in this paper was undertaken to determine the locus of action of glycinol. A comparison of glycinol's effect on bacterial membranes with the effect on the membranes of other phytoalexins has allowed us to examine the question of whether the mode of action of glycinol is representative of other phytoalexins.

MATERIALS AND METHODS

Materials. L-Leucine (3,4,5-³H[N]), uridine (5,6-³H), thymidine (methyl-1',2'-³H), and glycine were purchased from New England Nuclear. Lactose (D-glucose-1-¹⁴C) and methyl-α-D-glucopyranoside ([U-¹⁴C]glucose) were obtained from Amersham. Glycinol and glyceollin⁴ were purified as described (2, 45) and stored as ethanolic solutions under N₂ at -20°C. Crystalline capsidiol was obtained from Dr. E. W. B. Ward, Agriculture Canada, Research Institute, London, Ontario, Canada. Synthetic coumestrol and daidzein were obtained from Dr. K. Kolonko, Department of Chemistry, University of Colorado, Boulder, CO. Polymyxin-B sulfate, chloramphenicol, and rifampicin were purchased from Sigma. Actinomycin-D was purchased from P-L Biochemicals. All other chemicals and solvents used were reagent grade or better.

Microorganisms. *Erwinia carotovora* (Jones) Holl. (ATCC 4950) was obtained from the American Type Culture Collection, Rockville, MD, and was maintained on TSB (Baltimore Biological Labs) agar slants and plates. *Escherichia coli* ML 308-225 (i⁻z⁻y⁺a⁺), a gift of Dr. R. Kaback, Roche Institute of Molecular Biology, Nutley, NJ, was maintained on nutrient agar (Difco) slants and plates. The *E. coli* used in the preparation of membrane vesicles was grown on *Escherichia* medium 63 (9).

General. Liquid culture assays of the ability of glycinol to inhibit bacterial growth were performed as described (45). Glycinol concentrations were determined by *A* at 287 nm in absolute ethanol (ε₂₈₇ = 5800). Membrane vesicles were prepared from *E. coli* following the method of Kaback (14), except that a 20-cc syringe equipped with an 18-gauge needle, rather than a Teflon and glass homogenizer, was used for resuspension of the membrane pellet (H. R. Kaback, personal communication). The membrane-vesicle preparations were shown to be free of bacterial contamination by incubation on nutrient agar. Protein concentrations were determined by the procedure of Bradford (7), using reagents obtained from Bio-Rad. BSA (Sigma) was employed as a standard for protein determination. Radioactivity on filters was

¹ Supported by the Department of Energy (Contract No. DE-AC-76 ERO-1426) and The Rockefeller Foundation (RF 78035). Based on PhD thesis, Chapter III, University of Colorado, Boulder, CO 1982.

² Current address: Monsanto Agricultural Products Co., 800 N. Lindbergh Blvd., St. Louis, MO 63166.

³ To whom correspondence should be addressed.

⁴ Abbreviations: glyceollin, refers, in this paper, to a mixture of glyceollin isomers that are not separated by the TLC procedures employed; TSB, trypticase soy broth; EMM, *Erwinia* defined medium.

determined by suspending each filter in 14 ml of Biofluor LSC cocktail (New England Nuclear). After the filter had become transparent in the cocktail, radioactivity was measured in a Beckman LS-250 liquid scintillation counter equipped with a $^3\text{H} + ^{14}\text{C}$ plug-in module. Radioactivity (counts/min) was monitored for 20 min for all samples assayed.

Bacterial Incorporation Experiments. The effect of various antibiotics on *E. carotovora* incorporation of radiolabeled precursors into biopolymers was determined by the following method. The bacteria were grown in EMM (45) supplemented with unlabeled precursors of RNA, DNA, or protein. Cultures of *E. carotovora* to be used for studies of [^3H]leucine incorporation were grown first in EMM supplemented with equimolar amounts (2 $\mu\text{g}/\text{ml}$) of each of the 20 common amino acids (Beckman). *E. carotovora* cultures employed in studies of [^3H]thymidine- and [^3H]uridine-incorporation experiments were first grown in EMM supplemented with 0.2 $\mu\text{g}/\text{ml}$ thymidine or uridine, respectively.

Radiolabeled metabolite incorporation experiments were performed in 50-ml Erlenmeyer flasks to which a Bausch and Lomb 12- \times 100-mm test tube was permanently attached as a sidearm. The turbidity of the bacterial suspension (10 ml) was measured by tipping the suspension into the sidearm and determining its *A* at 580 nm in a Bausch and Lomb Spectronic 20 colorimeter. Uninoculated medium was used as a blank. Approximately 25 to 30 μCi of the appropriate tritium-labeled metabolite was added to the bacterial suspension when the turbidity of the suspension reached an $A_{580} = 0.10$. The bacteria were allowed to incorporate the radiolabeled metabolite for 5.5 min, and then the antibiotic to be tested was added in a maximum of 20 μl of ethanol. (Rifampicin was added in 5 μl of methanol.) Aliquots (0.5 ml) of the bacterial suspension were removed at various times and immediately added to 5 ml of 20% (w/v) TCA at 0°C. Following incubation for 20 min at 0°C, the 5.5 ml of diluted bacterial suspension was rapidly filtered through a 0.22- μm Millipore filter (type GS, 47-mm diameter). Each filter was then washed three times with 10 ml of 20% TCA at 0°C. The filters were removed and assayed for radioactivity.

Membrane Vesicle Incorporation Experiments. The effect of several antibiotics on respiration-linked active transport into *E. coli* membrane vesicles was examined in the following manner. Membrane vesicles (3.15 mg of protein in 3.0 ml of 10 mM MgSO_4 , 50 mM K-phosphate, pH 6.6) were incubated with aeration for 2 min at 25°C. Glycinol was added in a maximum of 5 μl of ethanol, and incubation was continued for 1 min at 25°C. The energy source (lithium D-lactate or sodium succinate) was then added to the assay mixture at a final concentration of 20 mM. [^{14}C]Lactose or [^{14}C]glycine was introduced immediately following addition of the energy source, and the assay mixture was allowed to incubate at 25°C with aeration. Aliquots (0.5 ml) were removed at various times, rapidly pipetted into 10 ml of 0.10 M LiCl at room temperature, and immediately filtered through a 0.45 μm Millipore filter (type HA, 47-mm diameter). The filters were washed once with 10 ml of 0.10 M LiCl at room temperature and assayed for radioactivity.

The ability of *E. coli* membrane vesicles to transport [^{14}C] α -methyl glucoside, and to accumulate it as [^{14}C] α -methyl glucoside-6-P was verified as described by Kaback (11). Membrane vesicles (3.15 mg protein, in 3.0 ml of 0.3 M LiCl, 10 mM MgSO_4 , 0.10 M P-enolpyruvate, 10 mM NaF, and 50 mM K-phosphate, pH 6.6) were incubated for 15 min at 25°C. KCN (10 mM) was included in the assay mixture to inhibit respiration-linked transport of [^{14}C] α -methyl glucoside. [^{14}C] α -Methyl glucoside (1.6 μCi) was added to the membrane vesicle suspension and incubation continued at 25°C. Aliquots were removed at various times and pipetted into 5 ml of 0.5 M LiCl at room temperature. The diluted suspension was immediately filtered through a 0.45 μm Millipore filter (type HA, 47-mm diameter). The filters were washed once with 5 ml of

0.5 M LiCl to remove any unincorporated [^{14}C] α -methyl glucoside, and then extracted three times with 2 ml of H_2O . The aqueous extracts were pooled, lyophilized to dryness, and redissolved in 50 μl of H_2O . Aliquots (30 μl) were applied to a silica TLC plate (Merck), and chromatographed in a solvent system of chloroform:methanol:water (60:70:26 [v/v/v]). After the plate was dried under a stream of warm air, radioactive spots were located by placing a sheet of Kodak XAR-5 x-ray film (20 \times 20 cm) on the plate and incubating it for 18 h at -70°C . Regions of the TLC plate containing radioactive material were removed, extracted into 3 ml of 50 mM Tris-HCl (pH 8.0), and incubated for 30 min in the presence of 0.10 mg/ml of alkaline phosphatase (Sigma). The reaction was terminated by freezing the samples and lyophilizing them to dryness. The samples were redissolved in 50 μl of H_2O and applied to a silica TLC plate. Chromatography was performed in a solvent system of chloroform:methanol:0.1 M boric acid (60:70:26 [v/v/v]), and radioactive regions on the dried plates were located by autoradiography.

The effect of various phytoalexins on the ability of *E. coli* membrane vesicles to accumulate [^{14}C] α -methyl glucoside-6-P by phosphorylation-linked active transport was examined in the following manner. Membrane vesicles (3.15 mg of protein in 3.0 ml of 0.3 M LiCl, 10 mM MgSO_4 , 0.10 M P-enolpyruvate (Sigma), 10 mM NaF, and 50 mM K-phosphate, pH 6.6) were incubated for 15 min at 25°C in the presence of the phytoalexins of interest (50 $\mu\text{g}/\text{ml}$). KCN (10 mM) was included in the assay mixture to inhibit respiration-linked transport of [^{14}C] α -methyl glucoside (11, 18, 22). [^{14}C] α -Methyl glucoside (0.81 μCi) was added to the assay mixture, and incubation was continued at 25°C. Aliquots (0.10 ml) were removed at various times and added to 5.0 ml of 0.5 M LiCl at room temperature, and the mixture was immediately filtered through a 0.45 μm Millipore filter (type HA, 47-mm diameter). The filters were washed once with 5.0 ml of 0.5 M LiCl and then assayed for radioactivity.

Membrane Vesicle Leakage Experiments. Membrane vesicles were allowed to incorporate [^{14}C] α -methyl glucoside-6-P for 30 min at 20°C, as described above. Following incubation, the vesicle suspension was centrifuged for 15 min at 37,000g. The supernatant was discarded, and the vesicle pellet resuspended in 0.9 ml of 0.1 M P-enolpyruvate, 10 mM MgSO_4 , 10 mM NaF, 10 mM KCN, and 0.25 M K-phosphate, pH 6.6. Glycinol, glyceollin, or daidzein (in a maximum of 5 μl of ethanol) was added to a final concentration of 50 $\mu\text{g}/\text{ml}$, and incubation continued at 20°C. Aliquots (50 μl) were removed at various times and immediately filtered through a 0.45 μm Millipore filter (type HA, 47-mm diameter). The filters were washed once with 5.0 ml of 0.5 M LiCl and then assayed for radioactivity.

RESULTS

All of the experiments reported in this paper were performed a minimum of three times, and all of the data presented represent the average of at least three experimental determinations.

Comparison of the Ability of Glycinol and Other Antibiotics to Inhibit the Rate of Incorporation of Radiolabeled Substrates into Biopolymers of *E. carotovora*. The rate of incorporation of [^3H]leucine into acid-precipitable polymers of *E. carotovora*, under the standard assay conditions, is shown in Figure 1A. Chloramphenicol, an antibiotic that inhibits protein synthesis (8), completely inhibited, within 1 min, the incorporation of [^3H]leucine. Polymyxin-B, an antibiotic that indirectly inhibits protein synthesis by interfering with membrane function (41), required 3.1 min before inhibiting by 77% the rate of [^3H]leucine incorporation. Glycinol was similar to polymyxin-B in that it required 4.0 min before inhibiting by 78% the rate of incorporation of [^3H]leucine into new protein. Thus, glycinol, which was a less effective and slower-acting inhibitor of protein synthesis than chloramphenicol, was about as effective as polymyxin-B.

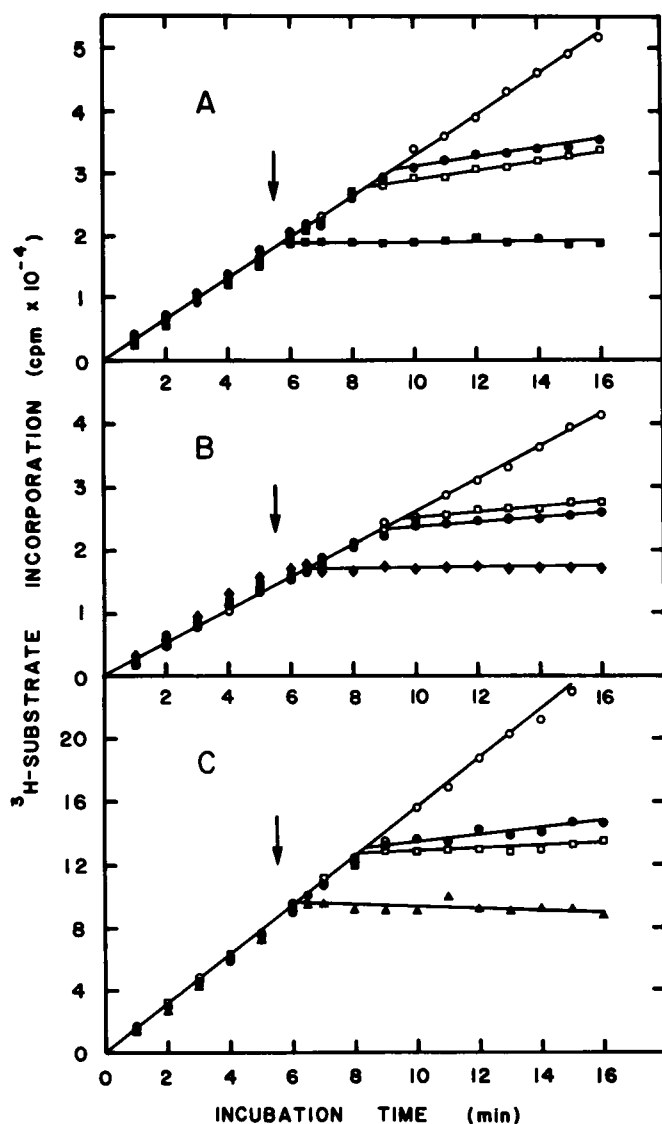


FIG. 1. Effect of 50 $\mu\text{g}/\text{ml}$ of glycitol (\bullet), 5 $\mu\text{g}/\text{ml}$ of polymyxin-B (\square), 10 $\mu\text{g}/\text{ml}$ of chloramphenicol (\blacksquare), 10 $\mu\text{g}/\text{ml}$ of actinomycin-D (\blacklozenge), and 10 $\mu\text{g}/\text{ml}$ of rifampicin (\blacktriangle) on the incorporation of [^3H]leucine (A), [^3H]thymidine (B), and [^3H]uridine (C) into biopolymers of *E. carotovora*. The tritium-labeled substrate was added at the onset of the assay. Antibiotic additions at $t = 5.5$ min are indicated by the arrow.

The rate of incorporation of [^3H]thymidine into the acid-precipitable polymers of *E. carotovora* is shown in Figure 1B. Actinomycin-D, an inhibitor of DNA synthesis (36, 43), completely inhibited, within 1 min, the incorporation of [^3H]thymidine. On the other hand, polymyxin-B required 4.2 min before inhibiting by 96% the rate of [^3H]thymidine incorporation. Glycitol was similar to polymyxin-B, requiring 4 min before inhibiting by 89% the rate of [^3H]thymidine incorporation.

The rate of incorporation of [^3H]uridine into acid-precipitable polymers of *E. carotovora* is shown in Figure 1C. Rifampicin completely inhibited the incorporation of [^3H]uridine within 1 min. Polymyxin-B required 2.8 min before inhibiting by 91% the rate of [^3H]uridine incorporation (Fig. 1C). Glycitol was again similar to polymyxin-B in that it required 3.2 min before inhibiting by 88% the rate of [^3H]uridine incorporation (Fig. 1C). Thus, glycitol was not as effective or as fast-acting in inhibiting RNA synthesis as rifampicin, an antibiotic that is known to act directly upon RNA synthesis (10, 32).

Effect of Glycitol on Active Transport by *E. coli* Membrane

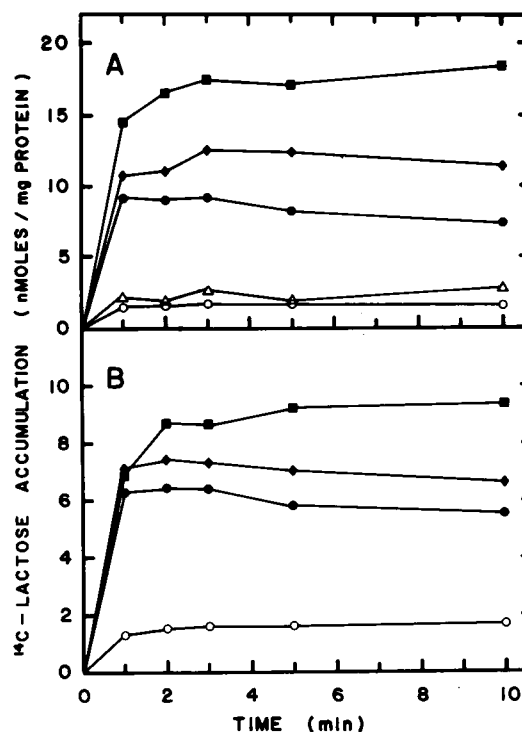


FIG. 2. Effect of 20 $\mu\text{g}/\text{ml}$ (\blacklozenge) and 50 $\mu\text{g}/\text{ml}$ (\bullet) of glycitol and 10 mM KCN (\triangle) upon the respiration-linked accumulation of [^{14}C]lactose by *E. coli* membrane vesicles in the presence of either 0.2 mM D-lactate (A) or 0.2 mM succinate (B). [^{14}C]Lactose accumulation by membrane vesicles not exposed to glycitol and in the presence (\blacksquare) or absence (\circ) of D-lactate or succinate is also shown.

Vesicles. The active transport systems of vesicles of *E. coli* membranes have been well characterized (11–18, 24, 26, 35). Therefore, the vesicles can be used to examine the effect of exogenously supplied compounds upon the transport systems of the membranes. Membrane vesicles of *E. coli* ML 308-225 were prepared to examine the effect of glycitol upon active transport of amino acids and sugars into the vesicles.

The effect of glycitol upon the D-lactate-stimulated transport of [^{14}C]lactose into membrane vesicles of *E. coli* ML 308-225 is shown in Figure 2A. Addition of glycitol at a final concentration of 20 $\mu\text{g}/\text{ml}$ resulted in 41% less [^{14}C]lactose accumulated by membrane vesicles after a period of 10 min than by membrane vesicles not exposed to glycitol. Glycitol, at a final concentration of 50 $\mu\text{g}/\text{ml}$, resulted in the membrane vesicles accumulating 72% less [^{14}C]lactose after a period of 10 min. It was thus determined that bacteriostatic concentrations of glycitol (45) inhibited D-lactate-dependent active transport of [^{14}C]lactose.

The substitution of succinate for D-lactate did not qualitatively alter the inhibitory effect of glycitol upon the accumulation of [^{14}C]lactose by *E. coli* membrane vesicles. Addition of 20 and 50 $\mu\text{g}/\text{ml}$ of glycitol resulted in 36% and 64%, respectively, less [^{14}C]lactose accumulated by succinate-energized membrane vesicles after a period of 10 min (Fig. 2B). Thus, bacteriostatic concentrations of glycitol inhibited active transport of [^{14}C]lactose when either D-lactate or succinate was supplied as an energy source.

Shown in Figure 3A is the inhibitory effect of glycitol upon another respiration-dependent active transport system, the D-lactate-stimulated accumulation of [^{14}C]glycine by *E. coli* membrane vesicles. Addition of glycitol to a final concentration of 20 $\mu\text{g}/\text{ml}$ resulted in 42% less [^{14}C]glycine accumulated by membrane vesicles during a 15-min period than by membrane vesicles not exposed to glycitol. Glycitol, at a final concentration of 50 $\mu\text{g}/\text{ml}$, resulted in the membrane vesicles accumulating 66% less

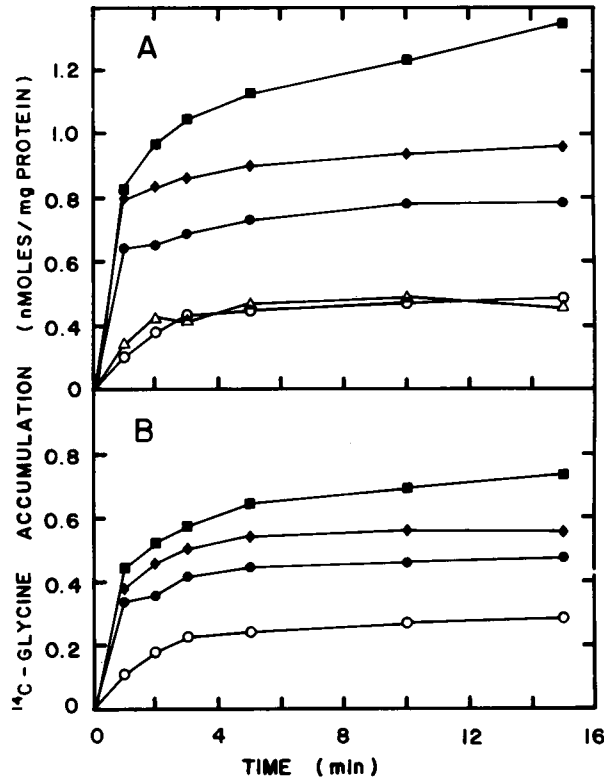


FIG. 3. Effect of 20 $\mu\text{g/ml}$ (\blacklozenge) and 50 $\mu\text{g/ml}$ (\bullet) of glycinol and 10 mM KCN (\triangle) upon the respiration-linked accumulation of [^{14}C]glycine by *E. coli* membrane vesicles using either D-lactate (A) or succinate (B) as the energy source.

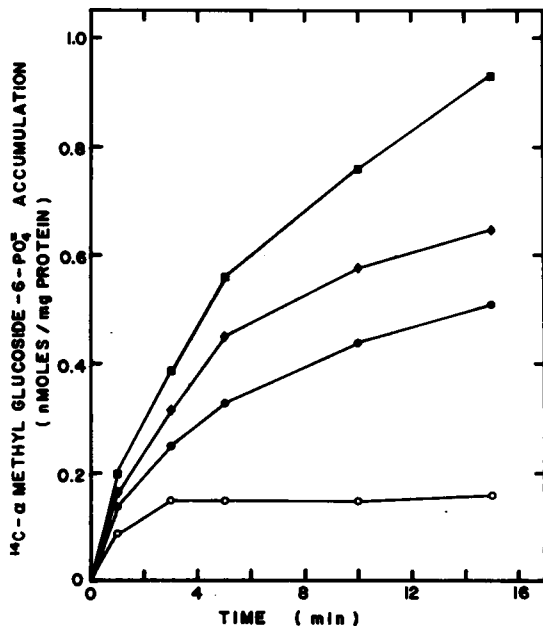


FIG. 4. Effect of 20 $\mu\text{g/ml}$ (\blacklozenge) and 50 $\mu\text{g/ml}$ (\bullet) of glycinol upon the accumulation of [^{14}C]methyl glucoside-6-P by *E. coli* membrane vesicles in the presence of 0.2 mM P-enolpyruvate and 10 mM KCN. [^{14}C] α -Methyl glucoside-6-P accumulation by membrane vesicles not exposed to glycinol and in the presence (\blacksquare) or absence (\circ) of P-enolpyruvate and 10 mM KCN is also shown.

[^{14}C]glycine during a period of 15 min. Thus, bacteriostatic concentrations of glycinol (45) inhibited D-lactate-dependent accumulation of [^{14}C]glycine.

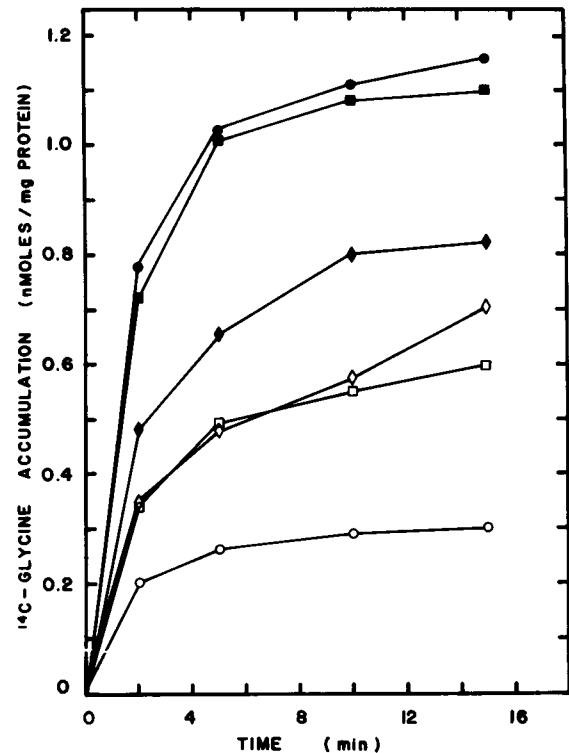


FIG. 5. Effect of 50 $\mu\text{g/ml}$ of glyceollin (\blacklozenge), daidzein (\blacksquare), capsidiol (\square), and coumestrol (\diamond) upon the respiration-linked accumulation of [^{14}C]glycine by *E. coli* membrane vesicles in the presence of 0.2 mM D-lactate. The accumulation of [^{14}C]glycine by membrane vesicles not exposed to glycinol and in the presence (\bullet) or absence (\circ) of D-lactate is also shown.

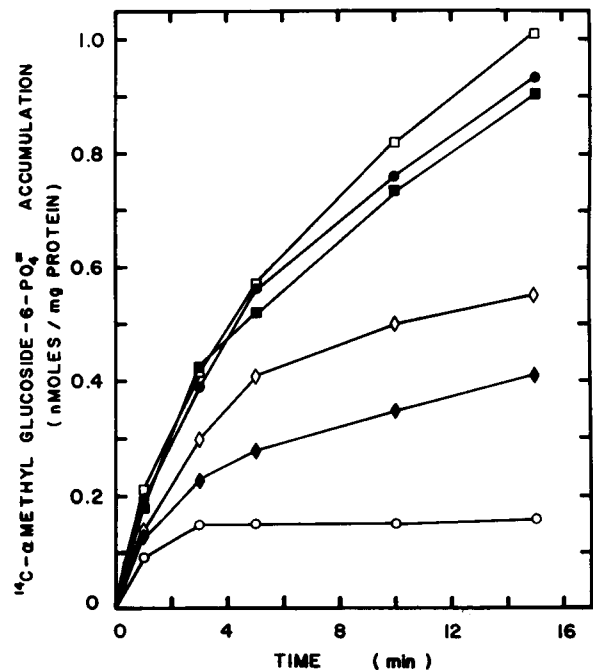


FIG. 6. Effect of 50 $\mu\text{g/ml}$ of glyceollin (\blacklozenge), daidzein (\blacksquare), capsidiol (\square), and coumestrol (\diamond) upon the accumulation of [^{14}C] α -methyl glucoside-6-P by *E. coli* membrane vesicles in the presence of 0.1 M P-enolpyruvate and 10 mM KCN.

The substitution of succinate for D-lactate did not alter the inhibitory effect of glycinol upon the accumulation of [^{14}C]glycine by *E. coli* membrane vesicles. Addition of 20 and 50 $\mu\text{g/ml}$ of

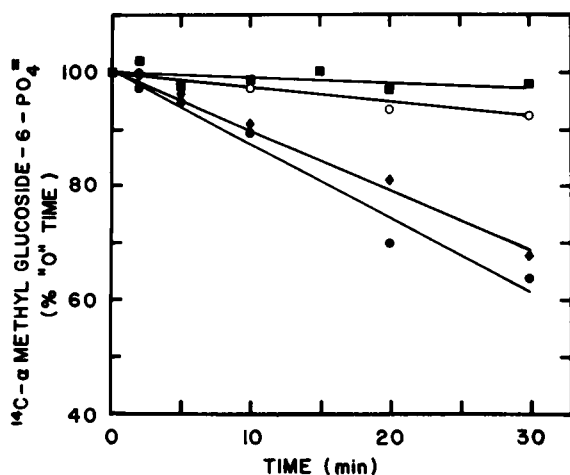


FIG. 7. Effect of 50 $\mu\text{g}/\text{ml}$ of glycinol (●), daidzein (○), glyceollin (◆), and 5 μl of ethanol (■) upon the efflux of [^{14}C] α -methyl glucoside-P from *E. coli* membrane vesicles. Experiments were performed as described in "Materials and Methods."

glycinol resulted in 31% and 56%, respectively, less [^{14}C]glycine accumulated by succinate-energized membrane vesicles during a period of 15 min (Fig. 3B).

These results were all consistent with the possibility that glycinol interfered directly with the respiratory chain in *E. coli* membrane vesicles. Therefore, the effect of glycinol on the phosphorylation-linked transport of [^{14}C] α -methyl glucoside by *E. coli* membrane vesicles, a membrane-bound active transport system whose function is not dependent on respiration, was examined. In the presence of 10 mM KCN, addition of [^{14}C] α -methyl glucoside to membrane vesicles previously incubated in the presence of P-enolpyruvate resulted in the rapid intravesicular accumulation of radiolabeled compounds that remained at the origin of a TLC plate (see "Materials and Methods"). Removal of the radioactive material from the origin, subsequent treatment with alkaline phosphatase, and rechromatography produced a compound that cochromatographed with [^{14}C] α -methyl glucoside. Therefore, in agreement with previous studies (11), this compound was assumed to be [^{14}C] α -methyl glucoside-6-P.

The effect of glycinol on phosphorylation-linked transport of [^{14}C] α -methyl glucoside and accumulation of [^{14}C]methyl glucoside-6-P by *E. coli* membrane vesicles is shown in Figure 4. Glycinol, at 20 $\mu\text{g}/\text{ml}$ and in the presence of 10 mM KCN, inhibited P-enolpyruvate-linked accumulation of [^{14}C] α -methyl glucoside-6-P by 37% after 15 min. Accumulation of [^{14}C] α -methyl glucoside-6-P in the presence of 50 $\mu\text{g}/\text{ml}$ of glycinol was inhibited by 55% after 15 min. Thus, glycinol, at concentrations inhibitory to bacterial growth, inhibited an active transport process that is not dependent on bacterial respiration.

Effect of Glyceollin, Daidzein, Capsidiol, and Coumestrol on *E. coli* Membrane Vesicle Active Transport. The following experiments were designed to compare the effects on bacterial membrane function produced by other phytoalexins with those of glycinol. The effects on membrane vesicle active transport of several other phytoalexins and of daidzein, a compound without phytoalexin activity but structurally related to glycinol, were examined. The object of these experiments was to examine the ability of the compounds to inhibit membrane-dependent active transport systems.

Glyceollin, a mixture of structurally similar 6a-hydroxy pterocarpan phytoalexins from soybean (2, 27), inhibited D-lactate-dependent uptake of [^{14}C]glycine by *E. coli* membrane vesicles (Fig. 5). Glyceollin, at 50 $\mu\text{g}/\text{ml}$, resulted in a 40% inhibition of [^{14}C]glycine accumulated after 15 min (Fig. 5). Glyceollin at 50 $\mu\text{g}/\text{ml}$ also resulted in a 68% inhibition of [^{14}C] α -methyl glucoside-

6-P accumulated after 15 min (Fig. 6). Glyceollin was thus capable of inhibiting both respiration-linked and vectorial phosphorylation-mediated active transport at bacteriostatic concentrations (1, 21, 25).

Coumestrol is a phytoalexin accumulated by soybean infected with *Pseudomonas phaseolicola* (28). Coumestrol has been shown to possess both estrogenic and antimicrobial activity (21, 28). The addition of coumestrol (50 $\mu\text{g}/\text{ml}$) to the *E. coli* membrane vesicles resulted in a 53% inhibition in the amount of [^{14}C]glycine accumulated after 15 min (Fig. 5). Coumestrol (50 $\mu\text{g}/\text{ml}$) also resulted in a 50% inhibition of [^{14}C] α -methyl glucoside-6-P accumulated after 15 min (Fig. 6). Thus, coumestrol was capable of inhibiting both respiration-dependent and vectorial phosphorylation-mediated transport at biologically active concentrations (21, 28).

Capsidiol is a sesquiterpene phytoalexin from bell pepper (*Capsicum annuum*) that is elicited by both bacteria and fungi (40, 44). Addition of capsidiol (50 $\mu\text{g}/\text{ml}$) resulted in a 70% inhibition in the amount of [^{14}C]glycine accumulated after 15 min (Fig. 5). However, the addition of capsidiol at the same concentration resulted in an 8% stimulation of the amount of [^{14}C] α -methyl glucoside-6-P accumulated after 15 min (Fig. 6). Therefore, capsidiol, at antifungal concentrations (40, 44), inhibited respiration-linked transport but not vectorial phosphorylation-mediated transport.

Daidzein, an isoflavone that has no reported phytoalexin activity, was also tested for its effects on membrane vesicle active transport (Figs. 5 and 6). Daidzein at 50 $\mu\text{g}/\text{ml}$ exhibited no significant inhibition of either D-lactate-dependent [^{14}C]glycine accumulation (Fig. 5) or P-enolpyruvate-linked [^{14}C] α -methyl glucoside-6-P accumulation (Fig. 6).

Effect of Soybean Isoflavonoid Compounds on the Efflux of Radiolabeled Metabolites from *E. coli* Membrane Vesicles. The effect of glycinol, glyceollin, and daidzein upon the permeability of *E. coli* membrane vesicles is shown in Figure 7. In the absence of added isoflavonoids, membrane vesicles lost, in 30 min at 20°C, about 3% of intravesicular [^{14}C] α -methyl glucoside-6-P (Fig. 7). The addition of daidzein (50 $\mu\text{g}/\text{ml}$) resulted in the loss of only 7% of the radiolabeled metabolite after 30 min at 20°C (Fig. 7). On the other hand, membrane vesicles exposed to glycinol (50 $\mu\text{g}/\text{ml}$) lost 37% of the intravesicular [^{14}C] α -methyl glucoside-6-P after 30 min at 20°C (Fig. 7), and the vesicles lost 30% of [^{14}C] α -methyl glucoside-6-P when exposed, under the same conditions, to glyceollin (50 $\mu\text{g}/\text{ml}$) (Fig. 7). Thus, bacteriostatic concentrations of either glycinol or glyceollin, but not of daidzein, increased the permeability of *E. coli* membrane vesicles, as judged by an increased efflux of intravesicular [^{14}C] α -methyl glucoside-6-P.

DISCUSSION

The results of these studies indicated that glycinol's interaction with *E. carotovora* affected a variety of cellular functions. Results of experiments in which the incorporation of [^3H]leucine, [^3H]thymidine, and [^3H]uridine into *E. carotovora* was examined suggested that the synthesis of protein, DNA, and RNA were all inhibited within 4.5 min of the addition of 50 $\mu\text{g}/\text{ml}$ of glycinol. The ability of glycinol to inhibit the incorporation of radiolabeled metabolites into *E. carotovora* was similar to the inhibition observed upon adding polymyxin-B, a cyclic peptide antibiotic produced by *Bacillus polymyxa* (41) whose site of action in Gram-negative bacteria is the membrane. Polymyxin-B and glycinol required about the same amount of time to inhibit [^3H]leucine, [^3H]thymidine, and [^3H]uridine incorporation into *E. carotovora* biopolymers. Glycinol and polymyxin-B were not able to inhibit [^3H]leucine, [^3H]thymidine, and [^3H]uridine incorporation as rapidly or as completely as were, respectively, chloramphenicol, actinomycin-D, and rifampicin, whose primary mechanisms of inhibition of the protein-, DNA-, and RNA-synthesizing systems have been established (Fig. 1) (8, 10, 32, 36, 43). These results suggested that glycinol and polymyxin-B inhibition of incorpora-

tion of radiolabeled metabolites into protein, DNA, and RNA were of a secondary, rather than a primary, nature.

The respiration-linked accumulation of radiolabeled amino acids and β -galactosides by *E. coli* membrane vesicles was studied in detail by Kaback and his coworkers (13–18, 22, 26). The transport of each of 16 amino acids by membrane vesicles prepared from *E. coli* ML 308-225 exhibited characteristics similar to those of [14 C]lactose transport (13, 17, 26). It was established that D-lactate-dependent amino acid and lactose uptake were inhibited by anaerobiosis and by inhibitors of electron transport, and that their accumulation did not require the supply of a high-energy phosphate source. It was concluded, therefore, that the active accumulation of amino acids and lactose was coupled to electron transport and did not involve the synthesis or direct utilization of high-energy phosphate intermediates (5, 6, 24, 26).

Membrane-vesicle accumulation of [14 C]lactose was characterized by an initial rapid accumulation of [14 C]lactose, followed by steady-state maintenance of an intravesicular concentration of [14 C]lactose that was higher than that in the assay medium (6) (Fig. 2). The higher intravesicular [14 C]lactose concentration, observed 2 min after exposure of the vesicles to [14 C]lactose, was maintained by an equilibrium between the rate of the respiration-coupled influx of the metabolite and the osmotically driven efflux of the radioactive metabolite (5, 6). Exposure of membrane vesicles to glycinol had little, if any, effect on the initial rate of [14 C]lactose transport (Fig. 2). However, the equilibrium concentration of [14 C]lactose maintained by these vesicles was significantly reduced in the presence of glycinol (Fig. 2).

The results of studies of the ability of glyceollin, coumestrol, and capsidiol to inhibit [14 C]glycine accumulation in membrane vesicles suggested that these phytoalexins also inhibited respiration-linked accumulation of amino acids, altering the normal equilibrium of the respiration-linked influx and osmotic efflux of [14 C]glycine. The ability of the phytoalexins examined to reduce the level of [14 C]lactose and [14 C]glycine accumulation by *E. coli* membrane vesicles can be explained by either of two possible mechanisms, that is, specific inhibition of respiration-driven transport or general impairment of membrane function. To differentiate between these two possibilities, we decided to examine the effects of glycinol, glyceollin, coumestrol, and capsidiol upon an active transport system known to be independent of respiration-linked electron transport. The *E. coli* bacterial P-enolpyruvate-phosphotransferase-dependent transport system requires P-enolpyruvate for vectorial phosphorylation-mediated transport of carbohydrates. This system is not dependent upon electron transport (11, 13–15, 33). Therefore, if the primary site of action of these phytoalexins is the electron-transport chain, these phytoalexins would not inhibit the P-enolpyruvate-phosphotransferase-dependent transport of [14 C] α -methyl glucoside.

Glycinol, glyceollin, and coumestrol inhibited the accumulation of [14 C] α -methyl glucoside-6-P by *E. coli* membrane vesicles (Figs. 4 and 6). The presence of 10 mM KCN in the assay mixture, known to inhibit totally *E. coli* membrane vesicle electron transport (6, 12, 26), did not alter the inhibitory effect of these phytoalexins on [14 C] α -methyl glucoside-6-P accumulation. The accumulation of [14 C] α -methyl glucoside-6-P was inhibited by the same concentrations of glycinol, glyceollin, and coumestrol capable of inhibiting respiration-linked transport (*cf.* Figs. 2 through 6). The inhibitory effect of glycinol, glyceollin, and coumestrol on the accumulation of [14 C] α -methyl glucoside-6-P by membrane vesicles demonstrated that these phytoalexins inhibited membrane-associated processes not directly coupled to electron transport as well as processes that were dependent on electron transport.

Additional evidence suggesting that phytoalexins impaired membrane structural integrity came from leakage studies of a radiolabeled phosphorylated metabolite from *E. coli* membrane vesicles (Fig. 7). In contrast to respiration-dependent active trans-

port systems, sugars transported via vectorial phosphorylation accumulated in the intravesicular pool as phosphorylated derivatives (11, 15). The membrane vesicles could not dephosphorylate sugar phosphates, nor were they capable of transporting sugar phosphates (11, 15). Therefore, the retention of metabolites transported by the phosphotransferase system was a measure of passive permeability of the vesicle membrane (11, 14, 15). Bacteriostatic concentrations of glycinol and glyceollin (50 μ g/ml) resulted in an accelerated efflux of [14 C] α -methyl glucoside-6-P (Fig. 7). Therefore, the inhibitory effect of glycinol and glyceollin upon phosphotransferase-mediated transport cannot be attributed solely to inhibition of the phosphorylating system.

The effects of two other phytoalexins—coumestrol and capsidiol—upon the respiration-dependent and phosphotransferase-mediated active transport suggested that these compounds also impaired membrane-associated functions. Coumestrol inhibited both of the membrane vesicle active transport systems studied (Figs. 5 and 6), confirming previous reports of coumestrol inhibition of membrane-associated processes (21, 28). Capsidiol inhibited respiration-linked [14 C]glycine uptake (Fig. 5), supporting observations of sesquiterpene inhibition of bacterial respiration (29, 30). However, capsidiol did not significantly inhibit phosphotransferase-mediated transport of [14 C] α -methyl glucoside-6-P (Fig. 6), suggesting that capsidiol-induced inhibition of microbial growth is different from that of the other phytoalexins examined.

Daidzein, a quantitatively major isoflavonoid present in soybean tissue (21), exhibited no effect on membrane vesicle active transport or membrane vesicle permeability. These results correlate with the observation that daidzein lacks antimicrobial activity (21; L. Weinstein and M. Hahn, unpublished results).

The evidence presented here shows that the effects of glycinol, glyceollin, and coumestrol are of a general nature and not directed at a specific cellular process. Bacteriostatic concentrations of glycinol and of glyceollin inhibited all of the cellular processes examined, regardless of whether or not they were coupled to respiration. The results of this study suggest that glycinol, glyceollin, and coumestrol act as nonspecific membrane antibiotics that alter the structural integrity of the membrane, thereby causing the membrane to be a less efficient matrix for membrane-dependent processes.

Previous attempts to define the mechanism by which glyceollin inhibits the growth of organisms have suggested that glyceollin has a specific site of action. Glyceollin has been shown to inhibit O₂ uptake by isolated mitochondria of etiolated soybean hypocotyls. On the basis of these results, it was proposed that glyceollin specifically inhibited electron transport in soybean mitochondria (23). A similar conclusion was reached from results of studies showing the effect of glyceollin on the root-knot nematode *Meloidogyne incognita* (19). Although many antibiotics do possess definite sites of action, these studies were not extensive enough to present a convincing argument for a specific target for the action of glyceollin. In particular, these reports failed to show that the inhibition of electron transport was the earliest physiological effect produced by glyceollin (19, 23). The observed inhibition of electron transport by glyceollin could, in fact, be one of several secondary effects resulting from the interaction of glyceollin with the cell membranes.

Other evidence contradicting the hypothesis of a single site of glyceollin action comes from studies in which structure-function relationships of the glyceollin isomers were examined (21; L. Weinstein and M. Hahn, unpublished results). These studies showed that a specific pterocarpan structure was not necessary for phytoalexin activity. It is, therefore, unlikely that glyceollin action is based upon interaction with a specific target molecule.

It has been proposed that phytoalexins are multiple-site toxicants affecting a variety of membrane-associated processes (20, 39, 42). The results of other studies on the mechanism of action of

phaseollin and of pisatin action can be interpreted as evidence that these pterocarpan phytoalexins also exert their antibiotic effect by a general disruption of membrane function (3, 30, 34, 37, 42). A mechanism of action in which phytoalexins alter the structural integrity of the membrane would account for the various physiological functions that many of these antibiotics have been observed to affect (20, 23, 29, 30, 34, 38, 39, 42). The ability of phytoalexins to work through such a general chemical process would make it difficult for microbes to evolve phytoalexin-insensitive targets. Furthermore, the ability to affect cells by altering the structural integrity of their membranes would account for the widely observed broad-spectrum antibiotic activity of phytoalexins. The evidence that we have presented supports such a mechanism of action for pterocarpan phytoalexins.

LITERATURE CITED

- ALBERSHEIM P, BS VALENT 1978 Host-pathogen interactions in plants. Plants, when exposed to oligosaccharides of fungal origin, defend themselves by accumulating antibiotics. *J Cell Biol* 78: 627-643
- AYERS AR, J EBEL, F FINELLI, N BERGER, P ALBERSHEIM 1976 Host-pathogen interactions. IX. Quantitative assays of elicitor activity and characterization of the elicitor present in the extracellular medium of cultures of *Phytophthora megasperma* var. *sojae*. *Plant Physiol* 57: 751-759
- BAILEY JA, BJ DEVERALL 1971 Formation and activity of phaseollin in the interaction between bean hypocotyls (*Phaseolus vulgaris*) and physiological races of *Colletotrichum lindemuthianum*. *Physiol Plant Pathol* 1: 435-449
- Deleted in proof
- BARNES JR EM, HR KABACK 1970 β -Galactoside transport in bacterial membrane preparations: energy coupling via membrane-bound D-lactic dehydrogenase. *Proc Natl Acad Sci USA* 66: 1190-1198
- BARNES JR EM, HR KABACK 1971 Mechanisms of active transport in isolated membrane vesicles. I. The site of energy coupling between D-lactic dehydrogenase and β -galactoside transport in *Escherichia coli* membrane vesicles. *J Biol Chem* 246: 551
- BRADFORD MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248-254
- BROCK TD 1961 Chloramphenicol. *Bacteriol Rev* 25: 32-48
- COHEN GN, HW RICKENBERG 1956 Concentration spécifique réversible des amino acides chez *Escherichia coli*. *Ann Inst Pasteur* 91: 693-720
- FURESE S 1970 Chemical and biological properties of rifampicin. *Antibiotica Chemother* 16: 316-351
- KABACK HR 1968 The role of the phosphoenolpyruvate-phosphotransferase system in the transport of sugars by isolated membrane preparations of *Escherichia coli*. *J Biol Chem* 243: 3711-3724
- KABACK HR 1969 Regulation of sugar transport in isolated bacterial membrane preparations from *Escherichia coli*. *Proc Natl Acad Sci USA* 3: 724-731
- KABACK HR 1970 Transport. *Annu Rev Biochem* 39: 561-598
- KABACK HR 1971 Bacterial membranes. *Methods Enzymol* 22: 99-120
- KABACK HR 1974 Transport in isolated bacterial membrane vesicles. *Methods Enzymol* 31: 698-709
- KABACK HR, EM BARNES JR 1971 Mechanism of active transport in isolated membrane vesicles. II. The mechanism of energy coupling between D-lactic dehydrogenase and β -galactoside transport in membrane preparations from *Escherichia coli*. *J Biol Chem* 246: 5523-5531
- KABACK HR, LS MILNER 1970 Relationship of a membrane-bound D-(-)-lactic dehydrogenase to amino acid transport in isolated bacterial membrane preparations. *Proc Natl Acad Sci USA* 66: 1008-1015
- KABACK HR, ER STADTMAN 1968 Glycine uptake in *Escherichia coli*. II. Glycine uptake, exchange, and metabolism by an isolated membrane preparation. *J Biol Chem* 243: 1390-1400
- KAPLAN DT, NT KEEN, IJ THOMASON 1980 Studies on the mode of action of glyceollin in soybean. Incompatibility to the root knot nematode *Meloidogyne incognita*. *Physiol Plant Pathol* 16: 319-325
- KEEN NT 1981 Evaluation of the role of phytoalexins. In RC Staples, ed, *Plant Disease Control*. J Wiley and Sons, New York, pp 155-177
- KEEN NT, BW KENNEDY 1974 Hydroxyphaseollin and related isoflavonoids in the hypersensitive resistance reaction of soybeans of *Pseudomonas glycinea*. *Physiol Plant Pathol* 4: 173-185
- KERWAR GK, AS GORDON, HR KABACK 1972 Mechanism of active transport in isolated membrane vesicles. IV. Galactose transport by isolated membrane vesicles from *Escherichia coli*. *J Biol Chem* 247: 291-297
- KOEPPE DE, JD PAXTON 1981 Glyceollin: phytotoxicity at complex I of soybean mitochondrial electron transport. *Plant Physiol* 67: S-108
- KONINGS WM, EM BARNES JR, HR KABACK 1971 Mechanisms of active transport in isolated membrane vesicles. III. The coupling of reduced phenazine methosulfate to the concentrative uptake of β -galactosides and amino acids. *J Biol Chem* 246: 5857-5861
- LEBEN C, GC DAFT, AF SCHMITTENNER 1968 Bacterial blight of soybeans: population levels of *Pseudomonas glycinea* in relation to symptom development. *Phytopathology* 58: 1143-1146
- LOMBARDI FJ, HR KABACK 1972 Mechanisms of active transport in isolated bacterial membrane vesicles. VIII. The transport of amino acids by membranes prepared from *Escherichia coli*. *J Biol Chem* 247: 7844-7857
- LYNE RL, LF MULHEIRN, DP LEWORTHY 1976 New pterocarpanoid phytoalexins of soybeans. *JCS Chem Commun* 423: 497-498
- LYON FM, RKS WOOD 1975 Production of phaseollin, coumestrol and related compounds in bean leaves inoculated with *Pseudomonas* spp. *Physiol Plant Pathol* 6: 117-124
- LYON GD 1978 Attenuation by divalent cations of the effect of the phytoalexin rishitin on *Erwinia carotovora* var. *atroseptica*. *J Gen Microbiol* 109: 5-10
- LYON GD 1980 Evidence that the toxic effect of rishitin may be due to membrane damage. *J Exp Bot* 31: 957-966
- LYON GD, P ALBERSHEIM 1982 Host-pathogen interactions. XXI. Extraction of a heat-labile elicitor of phytoalexin accumulation from frozen soybean stems. *Plant Physiol* 70: 406-409
- MCCABE WR, V LORIAN 1968 Comparison of the antibacterial activity of rifampicin and other antibiotics. *Am J Med Sci* 256: 255-265
- MILNER LS, HR KABACK 1970 The role of phosphatidylglycerol in the vectorial phosphorylation of sugar by isolated bacterial membrane preparations. *Proc Natl Acad Sci USA* 65: 683-690
- OKU H, S OUCHI, T SHIRAIISHI, K UTSUMI, S SENO 1976 Toxicity of a phytoalexin, pisatin, to mammalian cells. *Proc Jpn Acad* 52: 33-36
- OWEN P, HR KABACK 1978 Molecular structure of membrane vesicles from *Escherichia coli*. *Proc Natl Acad Sci USA* 75: 3148-3152
- REICH E 1963 Biochemistry of actinomycins. *Cancer Res* 23: 1428-1441
- SKIPP R, J BAILEY 1976 The effect of phaseollin on the growth of *Colletotrichum lindemuthianum* in bioassays designed to measure fungi toxicity. *Physiol Plant Pathol* 9: 253-263
- SLAYMAN CL, HD VANETTEN 1974 Are certain pterocarpanoid phytoalexins and steroid hormones inhibitors of membrane ATPase. *Plant Physiol* 54: S-24
- SMITH DA 1982 Toxicity of phytoalexins. In JA Bailey, JW Mansfield, eds, *Phytoalexins*. J Wiley and Sons, New York, pp 218-252
- STOESSEL A, CH UNWIN, EWB WARD 1972 Post-infectious inhibitors from plants I. Capsidiol, an antifungal compound from *Capsicum frutescens*. *Phytopathol Z* 74: 141-152
- STORM DR, KS ROSENTHAL, PE SWANSON 1977 Polymyxin and related peptide antibiotics. *Annu Rev Biochem* 46: 723-763
- VANETTEN H, D BATEMAN 1971 Studies on the mode of action of the phytoalexin phaseollin. *Phytopathology* 61: 1363-1372
- Waksman conference on actinomycins: their potential for cancer chemotherapy 1974. *Cancer Chemother Rep* 58: 1-123
- WARD EWB, CH UNWIN, A STOESSL 1973 Post-infectious inhibitors from plants. VI. Capsidiol production in pepper fruit infected with bacteria. *Phytopathology* 63: 1537-1538
- WEINSTEIN LI, MG HAHN, P ALBERSHEIM 1981 Isolation and biological activity of glycinol, a pterocarpan phytoalexin synthesized by soybeans. *Plant Physiol* 68: 358-363