Antitumor Agents LIV: The Effects of Daphnoretin on *In Vitro* Protein Synthesis of Ehrlich Ascites Carcinoma Cells and Other Tissues

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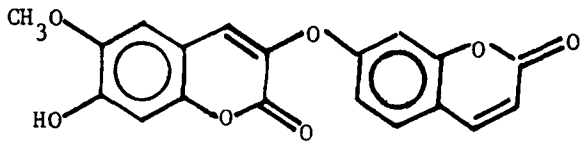
Abstract □ Daphnoretin has been shown to suppress Ehrlich ascites carcinoma growth in mice. One of the modes of action of the drug is to block protein synthesis; however, the inhibition of protein synthesis requires a higher concentration of drug than the inhibition of DNA synthesis. The inhibition of protein synthesis appears to occur during the elongation step with the drug preferentially binding to free ribosomes not engaged in active protein synthesis.

Keyphrases □ Daphnoretin—effects on *in vitro* protein synthesis of Ehrlich ascites carcinoma cells □ Antitumor agents—daphnoretin, effects on *in vitro* protein synthesis of Ehrlich ascites carcinoma cells □ Protein synthesis—effects of daphnoretin on *in vitro* protein synthesis of Ehrlich ascites carcinoma cells

Daphnoretin dicoumaryl ether, has been isolated from *Daphne mezereum* and *Wikstroemia indica* (Thymelaeaceae) (1–4). Daphnoretin, 3 to 12 mg/kg, has been demonstrated to significantly inhibit Ehrlich ascites carcinoma growth. Daphnoretin inhibited leucine incorporation into protein by 77% after administration of the drug at 6 mg/kg/day for 3 days. *In vitro* studies of leucine incorporation into protein of Ehrlich ascites cells demonstrated an $ID_{50} \cong 0.340$ m*M* (5). A more detailed study of the mechanism of protein synthesis inhibition is reported at this time.



Daphnoretin

EXPERIMENTAL

The Ehrlich ascites tumor cell line was maintained in CF_1 male mice (~30 g). The tumor cells were harvested from mice on Day 10, which were inoculated on Day 0 with 2×10^6 Ehrlich ascites cells. The Ehrlich ascites lysates were prepared by an analogous method as outlined by Kruh *et al.* (6). The following were isolated from Ehrlich ascites lysates by literature techniques: runoff ribosomes (7), pH 5.0 enzymes (6), and uncharged transfer RNA (8). The Ehrlich ascites carcinoma initiation factors for protein synthesis were prepared as described by Majumdar *et al.* (9). The [^{3}H]methionyl tRNA was prepared from Ehrlich ascites cell tRNA according to the method of Takeishi *et al.* (10).

The effects of daphnoretin on endogenous protein synthesis of Ehrlich ascites cells (11) was carried out in a reaction medium (0.5 ml) containing 10 m*M* tromethamine (pH 7.6), 76 m*M* KCl, 1 m*M* ATP, 0.2 m*M* guanosine triphosphate, 15 m*M* creatine phosphate, 2 m*M* $MgCl_2$, 1 m*M* dithiothreitol, 0.1 m*M* of each of the 19 essential amino acids, 0.9 mg/ml of creatine phosphokinase, and 20 μCi of [^{3}H]leucine (56.6 Ci/mmole). An aliquot of the reaction mixture was incubated at 30°. After 5 min of incubation, test drugs or the standards, pyrocatechol violet or emetine, were added with a final concentration of 50, 100, and 500 μ*M*. At 1-min intervals, 50-μl aliquots were removed from the reaction tubes and spotted on filter papers[1], which were treated for 10 min in boiling 5% trichloroacetic acid, followed by 10 min in cold 5% trichloroacetic acid and washed with cold 5% trichloroacetic acid, ether–ethanol (1:1), and finally, ether. The filter papers were dried and counted in scintillation fluid[2].

The effects of daphnoretin, pyrocatechol violet, cycloheximide, and emetine on the ribosome profile and leucine incorporation (11) of Ehrlich ascites cell lysates were assayed using a reaction medium (500 μl) described previously. Following drug addition with a 100 μ*M* final concentration, the reaction was incubated for 4 min at 37°. The reaction was terminated by placing the tubes in ice and gradient buffer (1 ml of tromethamine, pH 7.6; 10 m*M* KCl; and 1.5 m*M* $MgCl_2$-$6H_2O$) was added. The mixture was layered over 36 ml of 10–25% linear sucrose gradient (11), prepared in gradient buffer, and centrifuged for 165 min at 25,000 rpm[3] at 4°. The absorbance profile at 260 nm was determined using a flow cell (light path of 0.2 cm) attached to a spectrophotometer[4]. Each gradient was fractionated into 1-ml aliquots. The protein was precipitated by trichloroacetic acid and counted.

The reaction medium for the polyuridine directed polyphenylalanine synthesis (12) contained 50 m*M* tromethamine (pH 7.6), 12.5 m*M* magnesium acetate, 80 m*M* KCl, 5 m*M* creatine phosphate, 0.05 mg/ml of creatine phosphokinase, 0.36 mg/ml of polyuridine[5] ($A_{280}/A_{260} = 0.34$), 0.5 μCi of [^{14}C]phenylalanine (536 mCi/mmole), 75 μg of uncharged Ehrlich ascites cell tRNA, 70 μg of Ehrlich ascites pH 5 enzyme preparation, and 0.9 A_{260} of Ehrlich ascites cell runoff ribosomes. Daphnoretin was present from 0.038–0.570 mmole final concentrations. Incubations were for 20 min at 30° after which a 35-μl aliquot was spotted on filter paper[1] and processed as indicated previously.

The reaction medium (200 μl) used to measure the formation of the 80S initiation complex and the methionyl puromycin reaction (13) contained 15 m*M* tromethamine (pH 7.6), 80 m*M* KCl, 1 m*M* ATP, 0.5 m*M* guanosine triphosphate, 20 m*M* creatine phosphate, 0.2 mg/ml of creatine phosphokinase, 3 m*M* magnesium acetate, 0.1 m*M* edetic acid, 1 m*M* dithiothreitol, 0.1 m*M* of each of the 19 essential amino acids, 3 mg of Ehrlich ascites cell lysates, 100 μg/ml of chlortetracycline[6], 3×10^5 cpm

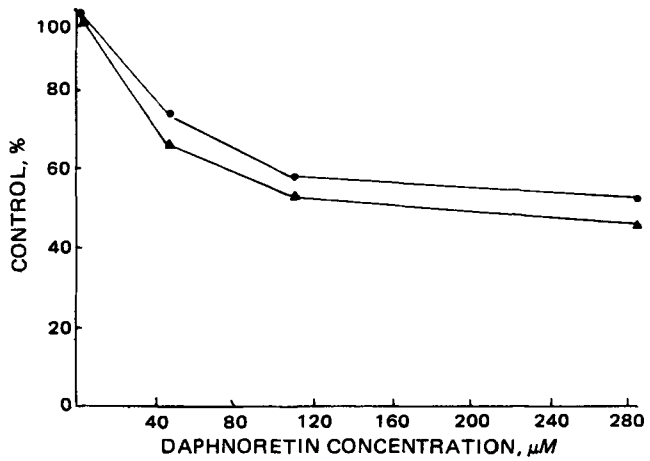


Figure 1—In vitro *effect of daphnoretin on whole cells of Ehrlich ascites protein synthesis. Key: (●), 60-min incubation; (▲), 90-min incubation.*

[1] Whatman No. 3.

[2] Scintiverse, Fisher Scientific Co.
[3] Beckman SW27 rotor.
[4] Gilford.
[5] Miles Laboratory, Inc.
[6] Sigma Chemical Co.

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Table I—Effects of Daphnoretin on Ternary and 80S Complex Formation

	Concentration, μM	Complex Formation, pmoles	Control, %
Ternary Complex Formation			
Control		0.84	100
+ Aurintricarboxylic Acid	100	0.04	5
+ Emetine	100	0.74	89
+ Pyrocatechol Violet	100	0.10	12
+ Daphnoretin	280	0.78	94
+ Daphnoretin	560	0.76	90
80S Initiation Complex Formation			
Control	—	0.44	100
+ Aurintricarboxylic Acid	100	.05	11
+ Emetine	100	.44	100
+ Pyrocatechol Violet	100	.15	35
+ Daphnoretin	280	.42	96
+ Daphnoretin	560	.41	94

of methionine tRNA and 20 μg/ml of polyadenosine–uridine–guanosine and daphnoretin (560 μmole). The incubation was carried out at 23° and after 2 min, aliquots were withdrawn to analyze for the 80S complex formation. Puromycin (10 μg/ml) then was added to the reaction medium. The incubation was continued for another 6 min, and aliquots were withdrawn to analyze for the reaction of the 80S complex with puromycin. All aliquots (50 μl) were diluted to 250 μl of buffer (20 mM tromethamine, pH 7.6; 80 mM KCl; 3 mM magnesium acetate; 1 mM dithiothreitol; and 1 mM edetic acid), layered on 11.8 ml of 15–30% linear sucrose gradient and centrifuged for 3 hr at 36,000 rpm[7]. Fractions (0.4 ml) were collected and precipitated with 10% trichloroacetic acid on filter papers and counted.

The reaction mixtures (75 μl) for the ternary complex formation (14) contained 21.4 mM tromethamine (pH 8.0), 80 mM KCl, 0.26 mM guanosine triphosphate, 2.14 mM dithiothreitol, 10 μg of bovine serum albumin, 5 pmoles of Ehrlich ascites cell [^{3}H]methionyl-tRNA (met-tRNA$_f$) (1×10^4 cpm), 100 A_{260}/ml of crude Ehrlich ascites cell initiation factors, and 560 μM of drug or standards. The incubation was conducted for 5 min at 37° and terminated by the addition of 3 ml of cold buffer (21.4 mM tromethamine, pH 8.0, 80 mM KCl, 2.14 mM dithiothreitol). The samples were filtered through 0.45-μm nitrocellulose filters, washed twice in buffer, and counted.

The reaction mixture (75 μl) for the 80S initiation complex formation (14) contained, in addition to the components necessary for the ternary complex formation reaction, 1.9 mM magnesium acetate, 5 A_{260}/ml polyadenosine–uridine–guanosine and 100 A_{260}/ml of 80S Ehrlich ascites ribosomes. Incubations were conducted for 10 min at 37° and were then cooled to 4° and titrated to 5 mM with magnesium acetate. After 5 min at 4°, the samples were diluted with cold buffer (21.4 mM tromethamine, pH 8.0, 80 mM KCl, and 5 mM magnesium acetate and 2.14 mM dithiothreitol), and filtered as indicated for the ternary complex formation experiment.

Amino acid-tRNA activation steps were determined by the method of Moldave (15). The reaction medium contained 0.1 mM tromethamine (pH 7.4), 0.2 mM ATP, 0.3 mg/ml of pH 5 enzyme from Ehrlich ascites cells, and 2.5 μCi of [^{14}C]phenylalanine (536 mCi/mmole), [^{3}H]leucine (56.5 Ci/mmole) or [^{3}H]methionine (80.0 Ci/mmole) in a total volume of 1 ml. After incubation at 37° for 20 min, 2 ml of ice cold 10% trichloroacetic acid was added and the activated amino acid-tRNA were collected on nitrocellulose filters by suction.

P-388 lymphocytic leukemia cells and L-1210 lymphocytic lymphoma cells were obtained from DBA/2 male mice donors (~30 g). B-16 melanoma and Lewis lung tumor cells were obtained from C_{57}B1/6 male mice donors (~30 g). Antineoplastic tumor screens were conducted according to National Institutes of Health protocols (16). The Ehrlich ascites carcinoma tumor was maintained in CF_1 male mice.

Normal tissues (*i.e.*, liver, lung, brain, and kidney) were excised from CF_1 male mice (~30 g) and homogenized in 0.25 M sucrose and 0.001 M edetic acid.

[6-^{3}H]Thymidine (21.5 Ci/mmole) or [4,5-^{3}H]leucine (52.2 Ci/mmole) was incorporated *in vitro* into normal tissues and tumors using 1 μCi of labeled precursor and minimum essential medium with 10% fetal calf serum with drug concentrations varying from 0.042–0.340 mM. The tubes were incubated for 60 min at 37° (11). The acid insoluble DNA was collected on glass filter discs[8] and protein was collected on nitrocellulose filters by vacuum suction. Results are expressed as disintegrations per minute of incorporated precursors per hour per 10^6 cells or 10 mg of wet tissue.

[7] Beckman SW 40 rotor.

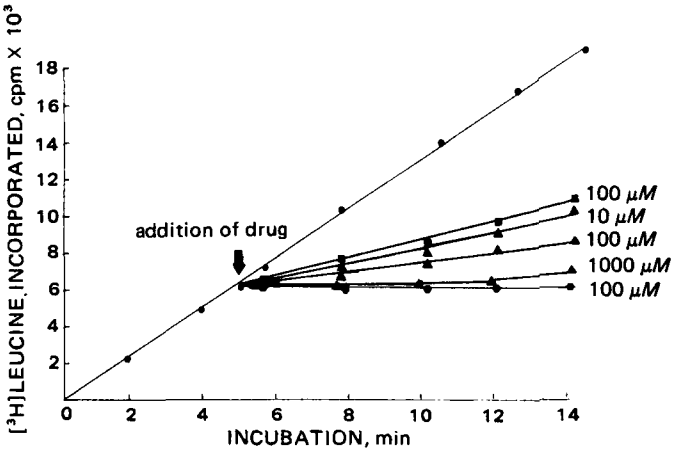

Figure 2—*Effect of pyrocatechol violet, emetine, and daphnoretin on the protein synthesis of Ehrlich ascites homogenates using endogenous messenger RNA. Key: (●) control; (■) pyrocatechol violet; (▲) daphnoretin; (●) emetine.*

RESULTS

The Ehrlich ascites whole-cell incubation studies demonstrated that daphnoretin, after 60 min of incubation, caused 50% inhibition at 240 μM and, after 90 min of incubation, 50% inhibition was achieved at 182 μM (Fig. 1). Twenty-eight percent suppression could be achieved at 560 μM concentration after 90-min incubation. The effect of daphnoretin on protein synthesis was compared to known inhibitors of protein synthesis (pyrocatechol violet is an initiation inhibitor that allows completion of the ongoing round of protein synthesis and causes accumulation of free 80S ribosomes, whereas emetine and cycloheximide are elongation inhibitors which freeze the ribosomes of the mRNA). When daphnoretin (50–500 μM) is added to the Ehrlich ascites lysate actively engaged in protein synthesis, there is a slight lag before inhibition of protein synthesis is effected (Fig. 2). Pyrocatechol violet demonstrated an even longer lag time, whereas emetine caused immediate inhibition of protein synthesis. The effect of daphnoretin, pyrocatechol violet, and cycloheximide on the ribosome profile and leucine incorporation can be seen in Fig. 3. Emetine induces the breakdown of the 80S polysome similar to the control, whereas pyrocatechol violet and daphnoretin allow the accumulation of the 80S ribosomal peak. Daphnoretin at 100 μM after 4-min incubation of lysate caused the release of radioactive peptides from the ribosomes similar to pyrocatechol violet. The results suggest that daphnoretin allows completion of the ongoing chain elongation steps and then causes the release of polypeptides from polyribosomes before it inhibits protein synthesis.

The effect of daphnoretin on the initiation steps of protein synthesis was examined using a fractionated Ehrlich ascites system. Table I shows daphnoretin had little or no effect on either the ternary complex[9] or the 80S initiation complex formation[10]. These studies indicated that daphnoretin was not an initiation inhibitor.

Further studies were performed to determine if daphnoretin was an elongation inhibitor. As can be easily seen in Fig. 4, daphnoretin inhibits polyuridine directed polyphenylalanine synthesis of purified runoff ribosomes from Ehrlich ascites tumor cells. Polyuridine-directed polyphenylalanine synthesis does not require the normal initiation and termination reactions; thus, agents that block this step are exclusively elongation type inhibitors.

In an additional study, the formation of the 80S initiation complex[10] and peptide bond formation was examined by treating Ehrlich ascites lysates with the elongation inhibitor, chlortetracycline, which specifically inhibits binding of the aminoacyl tRNA to the ribosome A site but does not inhibit the peptidyl transferase reaction. Thus, when [^{3}H]methionine-tRNA (met-tRNA$_f$) is added to the system, most of the radioactivity

[8] GF/F.
[9] eIF·GTP[^{3}H]methionyl-tRNA.
[10] 80S·adenosine–uridine–guanosine·eIF·GTP[^{3}H]methionyl-transfer RNA.

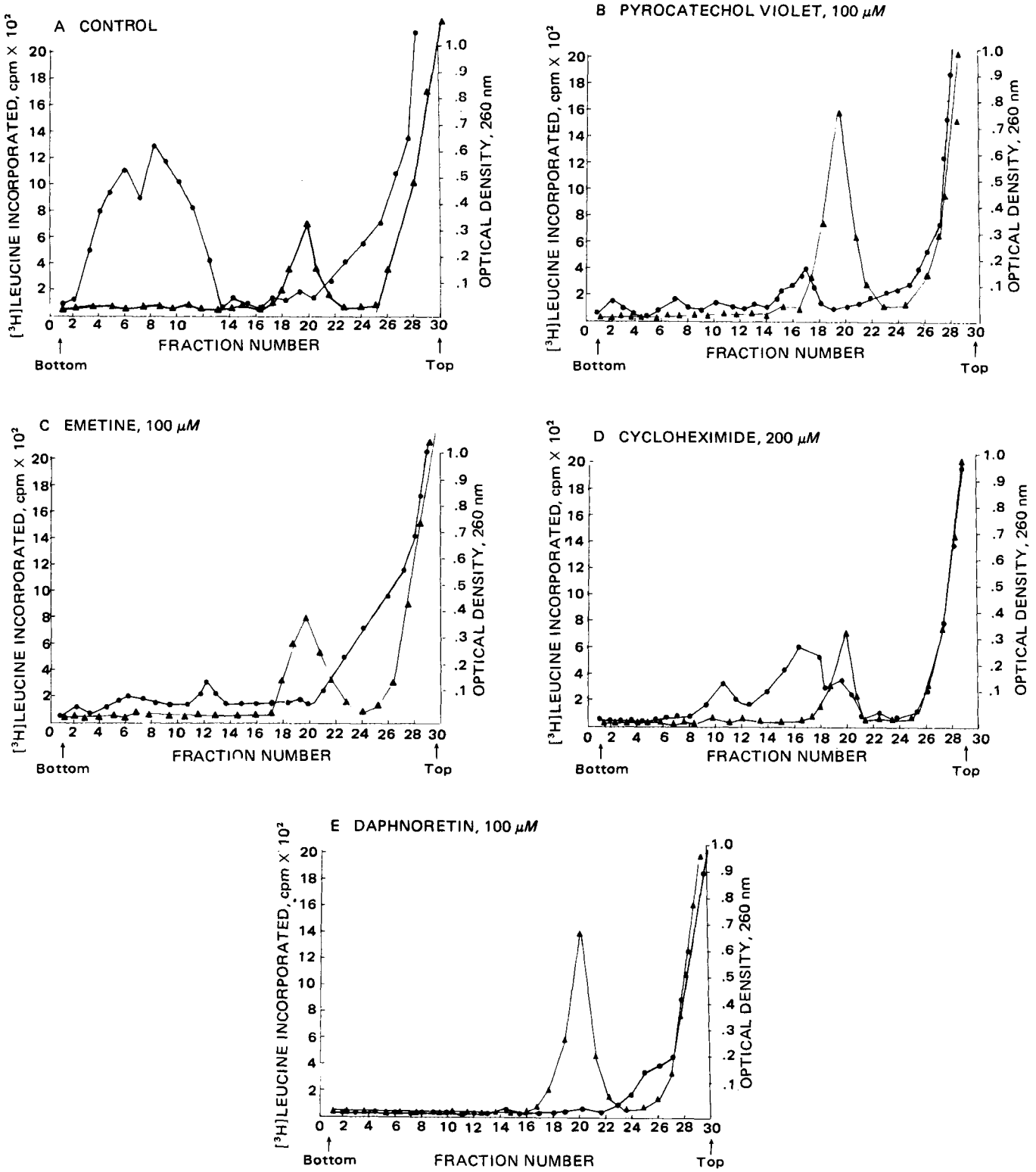

Figure 3—*Fate of nascent protein of protein synthesis in Ehrlich ascites tumor lysate. Key: (●) leucine incorporated; (▲) ribosomal profile.*

is found associated with the 80S initiation complex. Addition of polyadenosine–uridine–guanosine to the chlortetracycline-treated lysate allows formation of an 80S initiation complex (Fig. 5A), which then reacts with puromycin followed by the puromycin-induced release of [^{3}H]-methionine from the 80S complex (Fig. 5B). Daphnoretin (560 μ*M*) does not appear to inhibit the formation of the 80S initiation complex but did inhibit puromycin release of methionine, indicating that daphnoretin inhibits peptide bond formation in Ehrlich ascites ribosomes. Examination (Table II) of the formation of aminoacyl tRNA with phenylalanine, leucine, or methionine indicated that daphnoretin had no effect on the activation of amino acids for incorporation into polypeptides.

Examination of the *in vitro* leucine incorporation into protein for normal tissues of CF_1 male mice demonstrated that daphnoretin at 0.042–0.340 m*M* had no effect on protein synthesis by brain, kidney, or lung but suppressed *in vitro* protein synthesis by liver at low doses (Table III). Concentrations at 0.044 m*M* daphnoretin caused 53% inhibition, at 0.085 m*M* 34% inhibition, 0.177 m*M* 23% inhibition, and 0.340 m*M* 16% inhibition of leucine incorporation into liver proteins. Furthermore, daphnoretin did not suppress the protein synthesis of P-388 lymphocytic leukemia cells, L-1210 lymphocytic lymphomas, B-16

Table II—Effects of Daphnoretin on Amino Acid tRNA Activation in Ehrlich Ascites Cells

Inhibitor	Concentration, μM	Amino Acid Transfer RNA Formation, pmoles	Control, %
[^{14}C]Phenylalanine tRNA			
Control		0.209	100
+ Daphnoretin	10.2	0.205	98
+ Daphnoretin	102	0.201	96
+ Daphnoretin	1020	0.200	95
[^{3}H]Leucyl tRNA			
Control		0.893	100
+ Daphnoretin	10.2	0.848	95
+ Daphnoretin	102	0.830	93
+ Daphnoretin	1020	0.795	89
[^{3}H]Methionyl tRNA			
Control		0.757	100
+ Daphnoretin	10.2	0.741	98
+ Daphnoretin	102	0.719	95
+ Daphnoretin	1020	0.696	92

Table III—*In Vitro* Protein Synthesis of Normal Tissues

CF_1 Male Mouse (n = 6)	Control	Daphnoretin Concentration Control, %		
		0.044 μM	0.088 μM	0.177 μM
Liver (12,202 dpm/hr/10 mg)	100 ± 8	47 ± 4[a]	66 ± 6[a]	77 ± 9[a]
Kidney (8751 dpm/hr/10 mg)	100 ± 6	112 ± 8[b]	116 ± 6[a]	135 ± 7[a]
Lung (6564 dpm/hr/10 mg)	100 ± 5	113 ± 7[a]	129 ± 8[a]	124 ± 5[a]
Brain (6757 dpm/hr/10 mg)	100 ± 6	156 ± 9[a]	146 ± 8[a]	189 ± 6[a]

[a] $p \leq 0.001$. [b] $p \leq 0.010$.

melanoma and Lewis lung carcinoma as it did Ehrlich ascites carcinoma. Concomitantly, daphnoretin did not possess any antitumor activity in the P-388, L-1210, B-16, or Lewis screen.

Daphnoretin inhibited thymidine incorporation into nucleic acid resulting in an $ID_{50} \cong 0.194$ mM. However, daphnoretin did not suppress *in vitro* DNA synthesis in P-388, L-1210, B-16, and Lewis lung tumor cells. Similarly, daphnoretin had no effect on the *in vitro* thymidine incorporation with DNA of CF_1 mouse liver, lung, kidney, or brain.

DISCUSSION

Daphnoretin has been shown to be an elongation inhibitor of protein synthesis which allows the completion of ongoing polypeptide synthesis and releases the 80S ribosome. These observations suggest that daphnoretin binds to free ribosomes rather than the polyribosomes actively engaged in protein synthesis and similarly to other elongation inhibitors *e.g.* bruceantin, brusatol (17), T-2-toxin (18), verucarin (19), harring-

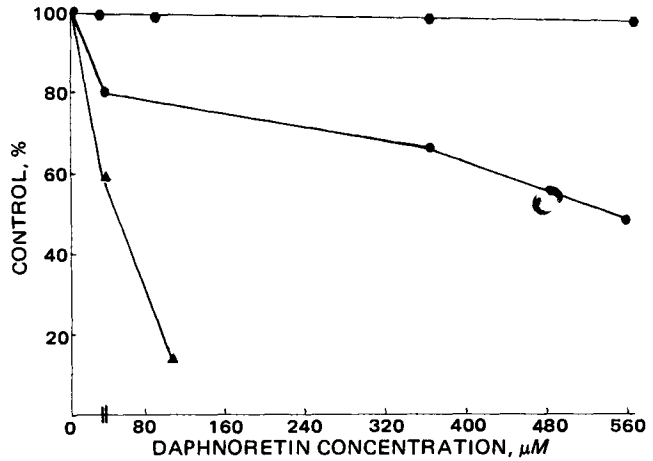

Figure 4—*Effect of daphnoretin on polyuridine-directed poly[^{14}C]-phenylalanine synthesis in Ehrlich ascites tumor cell system. Key: (●) control; (●) daphnoretin; (▲) emetine.*

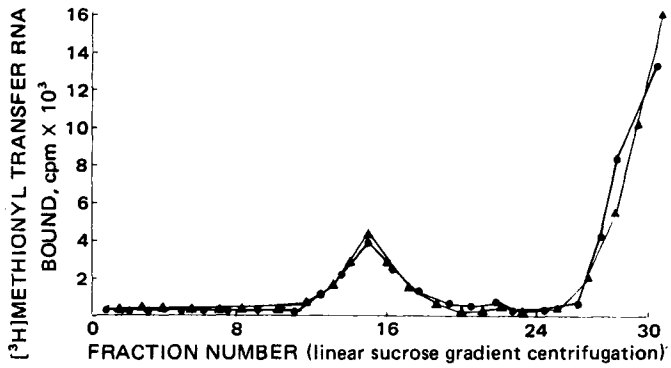

Figure 5A—*The formation of the 80S initiation complex of Ehrlich ascites tumor lysate system. Key: (●) control; (▲) daphnoretin (560 μM).*

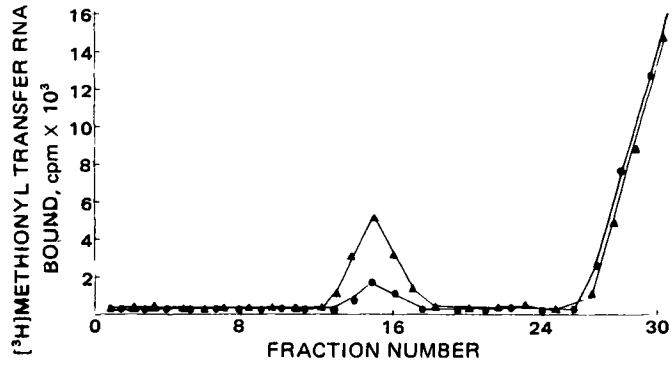

Figure 5B—*The formation of the methionyl puromycin reaction of Ehrlich ascites tumor lysate system. Key: (●) control and puromycin; (▲) daphnoretin (560 μM) and puromycin.*

tonine (20), and fusarenon-X (13). One explanation for this is that peptidyl tRNA or methionyl tRNA lowers affinity binding of drug to ribosome (21). Daphnoretin effectively blocked peptidyl transferase activity of the elongation process. The magnitude of inhibition of protein synthesis is of a sufficient degree to account for the cessation of cell growth. It has been reported previously that daphnoretin suppresses DNA synthesis with an $ID_{50} \cong 0.194$ mM (5). The present studies show that daphnoretin only suppresses protein and DNA synthesis in the Ehrlich ascites carcinoma cells, the only tumor screen in which daphnoretin demonstrated antineoplastic activity at 3–12 mg/kg/day. Protein and DNA synthesis of whole cells was not suppressed in normal mouse tissue, *e.g.*, brain, kidney, and lung; but there was a moderate reduction of protein synthesis in the liver. Daphnoretin was isolated from *Wikstroemia indica* C. A. Mey (Thymelaeaceae), which has been used as a herbal remedy to treat human cancer, arthritis, syphilis, and whooping cough (22, 23). This study of the mode of action of daphnoretin may explain some of the pharmacological properties of the plant.

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Square Root of Time Dependence of Matrix Formulations with Low Drug Content

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Abstract □ One of the conditions of derivation of the Higuchi square root law is that $A/\epsilon > S/2$ where A is drug content per cubic centimeter of matrix tablet, ϵ is the porosity, and S is the solubility of the drug in the dissolution medium. In actuality, A/ϵ should be larger than S. It is shown in this work that a similar square root equation can be derived when $A/\epsilon < S$. Experimental data are presented supporting the equation $Q = A(Dt)^{1/2}$, where Q is the amount of drug released per square centimeter at time t and D is the diffusion coefficient.

Keyphrases □ Matrix formulations—square root of time dependence, with low drug content □ Dissolution—square root of time dependence of matrix formulations with low drug content □ Release rate—square root of time dependence of matrix formulations with low drug content

Many sustained-release products are designed around the principle of imbedding the drug in a porous matrix. Liquid will penetrate and dissolve the drug, which will then diffuse out into the exterior liquid (Fig. 1). In general, for the purpose of derivation, a slab is considered which has a unit cross-section, is infinite to the left (Fig. 1), has a porosity ϵ', contains A grams of drug (of density ρ) per cubic centimeter of matrix, and allows penetration and diffusion through the unit surface only. At time t a depth of h is penetrated.

BACKGROUND

Higuchi (1, 2) was the first to derive the following expression for the amount of material released (Q) through the unit surface. He cautions that "the equation would be essentially valid for systems in which A is greater than the solubility S or ϵS by a factor of three or four. Of course,

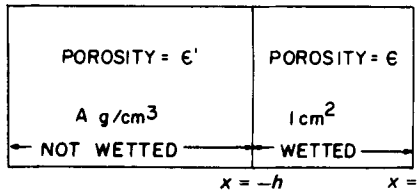

Figure 1—*Schematic of penetration of liquid into a solid matrix. The distance is denoted* x *(cm) and is zero at the surface and* −h *at the border of penetration.*

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