INHIBITION OF PROTEIN SYNTHESIS IN EHRLICH ASCITES-TUMOUR CELLS BY THE PHENANTHRENE ALKALOIDS TYLOPHORINE, TYLOCREBRINE AND CRYPTOPLEURINE

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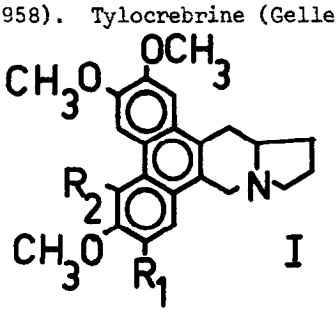
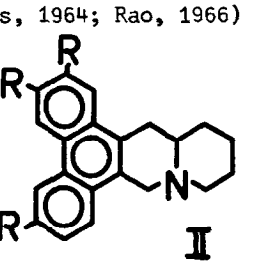
Summary: The phenanthroindolizidine alkaloids tylophorine and tylocrebrine and the phenanthroquinolizidine alkaloid cryptopleurine inhibit incorporation of leucine into protein in Ehrlich ascites-tumour cells by 50% at 1 x 10^{-6} *M*, 2 x 10^{-7} *M*, and 2 x 10^{-8} *M* respectively, but do not inhibit incorporation of uracil into nucleic acids at 10^{-5} *M*. Cryptopleurine inhibits leucine incorporation into protein in a cell-free system from Ehrlich cells, but at 10^{-5} *M* does not inhibit protein synthesis in *Escherichia coli*.

The toxic alkaloids tylophorine (I, R_1 = $-OCH_3$, R_2 = -H; for references to earlier work cf. Gellert *et al.* 1962) and tylocrebrine (I, R_1 = -H, R_2 = $-OCH_3$ Gellert *et al.* 1962), are vesicants (Ratnagiriswaran and Venkatachalam, 1935; Gellert *et al.* 1962), as is the closely-related alkaloid cryptopleurine (II, R = $-OCH_3$; de la Lande, 1948; Gellert and Riggs, 1954; cf. Bradsher and Berger, 1958). Tylocrebrine (Gellert and Rudzats, 1964; Rao, 1966) and to a



I



II

Tylophorine; R_1 = $-OCH_3$, R_2 = -H

Tylocrebrine, R_1 = -H, R_2 = $-OCH_3$

Cryptopleurine, R = $-OCH_3$

Phenanthro[9,10-b]quinolizidine, R = -H

lesser extent tylophorine (personal communication from Professor E. Gellert; cf. Rao, 1966) inhibit growth of L1210 leukaemic cells in mice and HeLa cells in culture; for details of clinical tests see Goldin, Serpick and Mantel (1966). In our own studies, cryptopleurine caused 50% inhibition of tumour growth at a dose of 3 μmoles/kg. body weight when injected intraperitoneally twice daily from day 3 to day 10 after inoculation of female Swiss albino mice with Ehrlich ascites-tumour cells.

Cryptopleurine inhibits growth of yeast and oyster embryos in the range 10^{-6} - 10^{-3} *M* (Cleland, 1950) and during maintenance of human lymphocytes in culture for 24 hr. 10^{-8} *M* cryptopleurine inhibits incorporation of leucine into protein (Forbes, Smith and de la Lande, 1968).

In an attempt to find how these phenanthroindolizidine (I) and phenanthroquinolizidine (II) alkaloids inhibit cell division, their effects on incorpor-

TABLE 1

INCORPORATION OF [^{14}C]URACIL INTO NUCLEIC ACIDS OF EHRLICH ASCITES-TUMOUR CELLS

	Counts/min./extinction unit at 260 nm		
	30 min.	60 min.	120 min.
Control	-	2060	3900
10^{-6} *M* Cryptopleurine	-	2520	4200
10^{-5} *M* Cryptopleurine	-	2150	3930
10^{-4} *M* Cryptopleurine	-	1350	2700
Control	2880	-	-
10^{-5} *M* Tylophorine	2830	-	-
10^{-5} *M* Tylocrebrine	2880	-	-

Ehrlich ascites-tumour cells were washed and tested for nucleic acid synthesis at 37° with 5 μ*M* [2-^{14}C]uracil (0.2 μ*C*/ml.) - 5 m*M* inosine as described by Belkohde *et al.* (1967). Alkaloids were included as indicated and 0.5 ml. portions of nucleic acid extracts obtained from samples taken at the times shown were counted in 9 ml. of Bray's solution (Bray, 1960) at 40% efficiency.

ation of [^{14}C]uracil into nucleic acids in Ehrlich ascites-tumour cells were studied. Using the inosine-dependent incorporation system described by Belkohde, Gotto and Touster (1967) cryptopleurine at 10^{-5} *M* and 10^{-6} *M* caused no inhibition of uracil incorporation in whole cells (Table 1); even at 10^{-4} *M* there was only 32% inhibition of incorporation. Incorporation rates were constant for 2 hr. and it was shown in separate experiments that 10^{-6} *M* actinomycin D inhibited incorporation by 80%. In the presence of tylophorine and tylocrebrine at 10^{-5} *M* incorporation was 98% and 100% of that in untreated controls (Table 1). As there was no indication that any of these compounds inhibited RNA synthesis at physiologically-active levels their effects on protein synthesis were studied.

With whole Ehrlich ascites-tumour cells in buffered salts solution containing amino acids and 5 m*M* inosine as described by Belkohde *et al.* (1967) the three alkaloids were found to be strong inhibitors of incorporation of [^{14}C]leucine into protein (Fig. 1); tylophorine, tylocrebrine and cryptopleurine caused 50% inhibition of incorporation of leucine at 1 x 10^{-6} *M* (130 p-moles/mg. of cell protein), 2 x 10^{-7} *M* (26 p-moles/mg. of protein) and 2 x 10^{-8} *M* (2.6 p-moles/mg. of protein) respectively. Unsubstituted phenanthro[9,10-b] quinolizidine (II, R = -H; Westphal, Jann and Heffe, 1961) caused no inhibition at concentrations up to 10^{-5} *M* (Fig. 1) nor did it inhibit growth of Ehrlich ascites-tumour cells at a dose of 20 µmoles/kg. body weight/day. In no case did inhibitors decrease the radioactivity of the amino acid pool (for details of methods see Fig. 1).

In a cell-free system (15,000 *g*/30 min. supernatant) that was prepared from a homogenate of Ehrlich ascites-tumour cells and assayed for incorporation of [^{14}C]leucine into protein as described by Littlefield and Keller (1957) cryptopleurine inhibited incorporation by 1% at 1.4 x 10^{-8} *M*, 32% at 6.8 x 10^{-7} *M* and 78% at 1.4 x 10^{-5} *M*. Incorporation in the test system (0.15 µmoles [^{14}C]leucine (0.5 µ*C*), and 2.8 mg. of protein in 1 ml., incubated for 15 min. at 37°) was 227 c/m in the control. Radioactivity in protein was measured as described in Fig. 1.

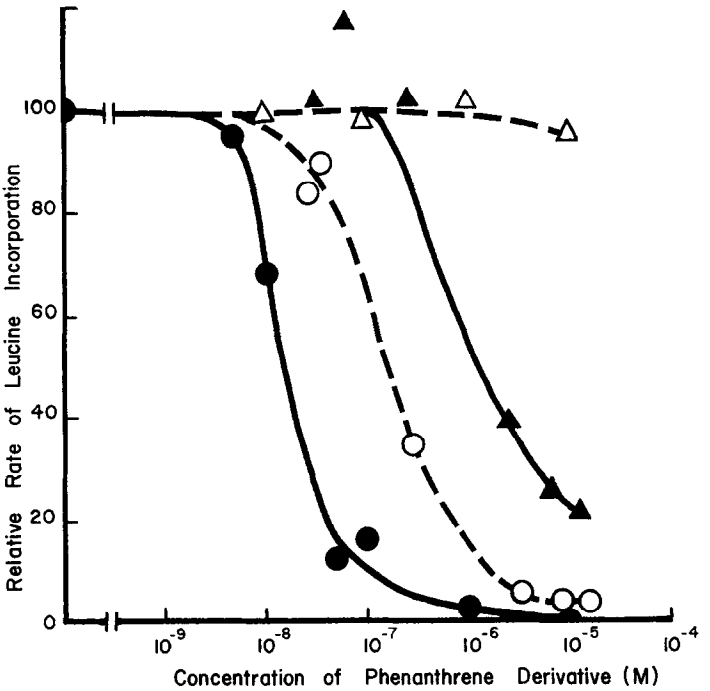


Figure 1. Effects of phenanthroindolizidines and phenanthroquinolizidine derivatives on incorporation of [^{14}C]leucine into protein of whole Ehrlich ascites-tumour cells. Cells were washed and pre-incubated for 30 min. at 37° with 5 m*M* inosine and 20 L-amino acids (each at 1 m*M*; not including leucine) in buffered salts solution (Belkohde *et al.*, 1967). To each 4 ml. of incubation mixture, containing 0.2 ml. packed volume of tumour cells, was added 0.01 ml. of [U-^{14}C] L-leucine (20 μ*C*/ml., 7.9 μ*C*/μmole). Inhibitors and salts solution were added as indicated to a final volume of 5ml. At zero time and at 1 hr. after addition of leucine 2 ml. samples were washed with buffered salts and extracted with 3 ml. of ethanol as described by Belkhode *et al.* (1967). To measure radioactivity in the amino acid pool, 1 ml. portions of this extract were counted in 9 ml. of Bray's solution. After extraction with 2 ml. of 5% trichloroacetic acid (30 min. at 90°), 2 ml. of ethanol-ether (1:1, v/v; 30 min. at 37°), and 2 ml. of ether (15 min. at 25°) the residue was dried and dissolved in 0.5 ml. of 99% formic acid. Portions (0.1 ml.) were counted at 78% efficiency in 10 ml. of 0.5% diphenyloxazole in toluene - ethoxyethanol (2:1, v/v), and protein in 0.1 ml. portions was measured by the biuret method.

Incorporation is expressed as a percentage of the control in the presence of cryptopleurine -●-, tylocrebrine -o-, tylophorine -▲-, and phenanthro[9,10-b]quinolizidine -Δ-. Incorporation in controls was usually about 7000 c/m/mg. of protein at 1 hr. and radioactivity in zero-time blanks was about 100 c/m/mg. of protein.

10^{-5} *M* Cryptopleurine did not inhibit incorporation of leucine into protein in *Escherichia coli* Strain B growing in complete medium (Oxoid Nutrient Broth No. 2; Oxoid Ltd., 1966). With added [^{14}C]leucine (57 n*C*/ml.) incorpor-

ation of radioactivity, (c/m/mg. of protein, measured as in Fig. 1) was 146 and 144 in control and test respectively at 30 min. and 352 and 350 in control and test at 60 min.. In a test with *E. coli* ribosomes and polyuridylic acid (kindly carried out for us by Professor W.H. Elliott and Miss Lyndall Clarke), cryptopleurine at 10^{-5} *M* did not inhibit incorporation of [^{14}C]phenylalanine into protein.

The effect of these inhibitors on protein synthesis has not been tested on a wide range of cells, but it has been found (G.M. Polya and M.R. Atkinson, unpublished results) that cryptopleurine (1.3×10^{-5} *M*) resembles cycloheximide (1.5×10^{-4} *M*) in that these compounds inhibit incorporation of [^{14}C]leucine into protein by 99% and 80% respectively in bootroot slices incubated with a mixture of amino acids (each at 1 m*M*) in 10 m*M* tris chloride (pH 7.8).

The inhibitors described here, like many cytotoxic alkaloids, resemble cycloheximide in the association of an *N*-heterocyclic ring with a hydrophobic homocyclic system, and it will be of interest to find if the alkaloids are competing for sites in protein-synthesizing systems of animal and plant cells that are also able to bind cycloheximide.

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