The potencies of the former two mixtures have decreased significantly faster than either of the respective pure species, whereas no difference in assay was observed between that obtained for the pure ampicillin trihydrate and its mixture. The results for dicloxacillin confirmed the original finding. The stability assay showed that the potency of the dicloxacillin stearic acid mixture decreased to 85% whereas pure dicloxacillin decreased to 94%.

Although part of the information gained in this work came as confirmation of an already observed unstable experimental formulation, it did identify the inactivating component. In the latter part of the study, however, data have been obtained that can now be used as a guide for future formulating trials. Since the completion of this study, it has become routine to examine new systems with DTA to provide data relevant to the compatibility of the several materials present in a formulation, and thereby a more rational approach to early formulation designs.

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Differential Agar-Diffusion Bioassay for Cytotoxic Substances

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Abstract □ A differential agar-diffusion bioassay for antitumor compounds was developed based on the cytocidal effects of the test compound on Eagle's KB cells, Earle's L cells, and L-1210 cells in an agar medium. The three cell strains were grown in suspension culture in Waymouth's medium MB 752/1 supplemented with calf serum. Paper disks dipped in test solutions were applied to seeded agar plates and after 18-hr. incubation the cytocidal effects were determined by flooding the surface with the vital stain, 2,6-dichlorophenolindophenol. The diameters of the nonreduced zone around the paper disks were measured. The KB cells were sensitive to more compounds than the Earle's L cells or the L-1210 cells in a survey of 30 compounds with demonstrated antitumor activity *in vivo*. The agar plates were also used for preparing bioautographs of paper chromatograms and thin-layer chromatograms.

Keyphrases □ Cytotoxicity—screening method □ Agar diffusion bioassay, differential—cytotoxic activity □ 2,6-Dichlorophenolindophenol—viable cell detection

The use of an agar-diffusion test with mammalian cells to screen for potential antitumor agents is based either on the loss of the capacity of mammalian cells in agar suspension to reduce an indicator dye to suitable oxidation-reduction potential in the presence of the test compound (1–3) or the inhibition of cell growth in the agar media in the presence of the toxic agent (4, 5). The authors have examined the former method as a screen for compounds of potential interest as antitumor agents and have used cells from three sources: Eagle's KB strain of human epidermoid carcinoma cells (6); Earle's L cells (mouse fibroblast, NCTC 929) (7), and L-1210 leukemia cells in tissue culture (8). A number of compounds were cytotoxic to only one or two of these three cell lines. This differential cytotoxicity may be useful in screening compounds in the future.

METHODS

Earle's L cells and Eagle's KB cells were grown in suspension culture using Waymouth's medium MB 752/1 supplemented with 10% (v/v) calf serum; 0.3 g./l. 4000 cps. methylcellulose,[1]; 1 g./l. nonionic polymer,[2]; and 1 g./l. anionic surfactant[3] in 250-ml. conical flasks on a rotating shaker as previously described (9). The L-1210 cells were grown in this medium without shaking as the cells remained in suspension under these conditions when incubated at 37°. The cells were grown in the shaken culture for 3 days at 37° and then harvested by centrifugation. The cells of each culture were then suspended in fresh medium so that the cell count was 4×10^6 cells/ml. Fifty milliliters of this cell suspension was added to a flask containing 37 ml. of calf serum, 1 g. of glucose, and 3 g. of melted agar (in 120 ml. water) and the resulting suspension poured into a 3-qt. Pyrex baking dish and allowed to solidify. Schleicher and Schuell 12.5-mm. paper disks (holding 0.1 ml. of solution) were dipped in the test solutions and placed on the agar surface. The baking dishes were loosely covered with an aluminum cover and incubated at 37° for 18 to 20 hr. The disks were removed and the agar surface was flooded with 0.05% solution of 2,6-dichlorophenolindophenol and allowed to stain for 5 min. After the dye had been poured off the plates were placed in a 37° incubator for 40 to 60 min. Under these conditions the viable cells reduced the dye while the dead cells did not. The diameters of the zones of toxicity were measured and the appearance of halos or diffuse edges noted.

RESULTS AND DISCUSSION

A series of 27 compounds which were found to inhibit tumor growth in experimental animals and/or man or to have cytotoxic activity (as measured by inhibition of growth of KB cells) were

[1] Methocel, Dow Chemical Co., Midland, Mich.
[2] Pluronics, Wyandotte Chemical Co., Wyandotte, Mich.
[3] Darvan No. 2, R. T Vanderbilt Co., Inc., New York, N. Y.

Table I — Differential Agar-Diffusion Assay for Antitumor Compounds

Compd.	Inhibition Zone Diam., mm. KB Cells, mcg./disk 200	100	L Cells, mcg./disk 200	100	L-1210 Cells, mcg./disk 200	100
Bufadieneolide[a]	39	37	No zone		29	26
Crotepoxide[a]	23	20	No zone		15	12.5
Eupacunin[a]	27	24	27	24.5	25	22
Hydroxyeupacunin[a]	23	20	19	16	25	21
Hellebrin[a]	39	35	Trace	No zone	15	12
Hellebrigenin-3-bromoacetate[a]	21	19	23	21	32	29
Pectolinarigenin[a]	27	25	Trace	No zone	Trace	No zone
Podophyllotoxin[a]	No zone		No zone		No zone	
Vernolepin[a]	22	19.5	22		28	26
Vernolepin (methanol adduct)[a]	27	24	25	23	28	24.5
Methotrexate[b]	No zone		Trace	No zone	15	No zone
1,4-Butanediol, dimethane sulfonate[b]	18	16	No zone		Trace	No zone
6-Mercaptopurine[b]	No zone		No zone		18	15.5
Dactinomycin[b]	35	32	18	15	Trace	No zone
5-Fluorouracil[b]	17	15	No zone		17	14.5
5-Fluorouridine[b]	No zone		No zone		18	15
Alanine mustard[b]	18	15.5	Trace	No zone	16	14
Guanidine, 1, 1′-(methylethanediylidene) dinitrilo di-, dihydrochloride[b]	21	19	Trace	Trace	17	14
Butyric acid, 4-*p*-bis(2-chloroethyl) amino phenyl-[b]	23	20	22	20	Trace	No zone
Phosphine, sulfide, tris(1-aziridinyl)[b]	20	17	Trace	No zone	Trace	No zone
Merphalan[b]	24	21	18	16	22	20
s-Triazine, 2, 4, 6-tris(1-aziridinyl)[b]	24	21	24	21	20	18
Mithramycin[b]	26	24	Trace	No zone	17	15
Cytarabine[b]	Trace	Trace	Trace	Trace	16	14
Dibromomannitol[b]	23	20	No zone		19	17
Cyclophosphamide[b]	16	13	No zone		No zone	
Terephthalanilide, 4′, 4″-di-2-imidazoline-2-yl[b]	20	17	No zone		16	14

[a] Kindly supplied by Professor S. M. Kupchan. [b] Kindly supplied by the Cancer Chemotherapy National Service Center.

examined using the agar-plate technique described above. Some of the data collected in this study are summarized in Table I. It is apparent that KB cells were inhibited more often than the other two cell lines in this test system. A number of compounds showing pronounced activity in experimental animal tumors and in treatment of cancer in man were relatively inactive in this differential test system. Therefore, it is not suggested that this test system be used as the only tool in selecting promising antitumor compounds.

However, if an interesting compound is active in the described test system it can be used for a quick assay. Straightline dose-response curves were obtained when the log of the dose was plotted against zone diameter for many of the compounds listed in Table I. Thus a rough quantitative assay can be made of the concentration of the toxic agent in the test solution.

These agar plates can also be used for obtaining bioautographs of paper or thin-layer chromatograms. The chromatograms are placed on the agar surface for 40 min. and then removed. The plates are incubated for 18 hr. at 37°, then flooded with the vital stain, and the dye reduction noted.

This assay technique measures only cytocidal activity and is based on inhibiting the cells from oxidizing glucose. It differs from the methods of Siminoff and Hursky (4) and Renis *et al.* (5) in which cell-multiplication inhibition is the end point of the assay. The agar-diffusion assay described in this paper differs from these other methods in that results are obtained within 18 hr.; it does not measure the effect on cell growth but rather on metabolism.

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