tional advantage is the favorable geometry of the fadeometer where the tablets are always in a fixed distance from the light source; in the light cabinet, a slight error may be involved since all tablets cannot be placed exactly in the center of the tray.

CONCLUSIONS

1. An apparent first-order rate of fading was established by earlier investigators for colored tablets in conventional light cabinets and in natural light. In this study, it was shown that a similar rate is valid for samples exposed to the carbon arc light source of a fadeometer.

2. In the initial (K_1) phase, color fading is approximately 50–100 times faster in the fadeometer than in the light cabinet. Therefore, in each experiment, a 24-hr. exposure in the fadeometer was found sufficient to compare the fading behavior of tablets and to predict color stability under environmental conditions. (Followup testing under normal conditions for 2 years demonstrated good agreement with predicted stability.)

3. The rapid fadeometer test is a valuable tool which aids the pharmaceutical formulator in the application of optimal colorants and the packaging engineer in the selection of containers most suitable for protecting colored tablets from the effects of light.

REFERENCES

(1) L. Lachman, S. Weinstein, C. J. Swartz, T. Urbanyi, and J. Cooper, *J. Pharm. Sci.*, **50**, 141(1961).

(2) C. J. Swartz, L. Lachman, T. Urbanyi, and J. Cooper, *ibid.*, **50**, 145(1961).

(3) L. Lachman, T. Urbanyi, S. Weinstein, J. Cooper, and C. J. Swartz, *ibid.*, **51**, 321(1962).

(4) R. Kuramoto, L. Lachman, and J. Cooper, *J. Amer. Pharm. Ass., Sci. Ed.*, **47**, 176(1958).

(5) E. R. Garrett and R. F. Carper, *ibid.*, **44**, 515(1955).

(6) L. Lachman, C. J. Swartz, T. Urbanyi, and J. Cooper, *ibid.*, **49**, 165(1960).

(7) M. E. Everhard and F. W. Goodhart, *J. Pharm. Sci.*, **52**, 481 (1963).

(8) F. W. Goodhart, M. E. Everhard, and D. A. Dickcius, *ibid.*, **53**, 338(1964).

(9) R. F. Hoban, *Plating*, **54**, 183(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received May 3, 1972, from the *Pharmacy and Analytical Research Department, Sandoz-Wander, Inc., East Hanover, NJ 07936*

Accepted for publication July 5, 1972.

Presented to the Industrial Pharmaceutical Technology Section, APhA Academy of Pharmaceutical Sciences, Houston meeting, April 1972.

The authors thank Mr. Joshua B. Monego for his advice and technical assistance in the fadeometer experiments and Mr. Donald F. May for formulating the tablets used in this project.

▲ To whom inquiries should be directed.

NOTES

Purification of a Potent Antitumor Agent from a Tahitian Sea Anemone and Methods of Administration Studies with Ehrlich Ascites Tumor in Mice

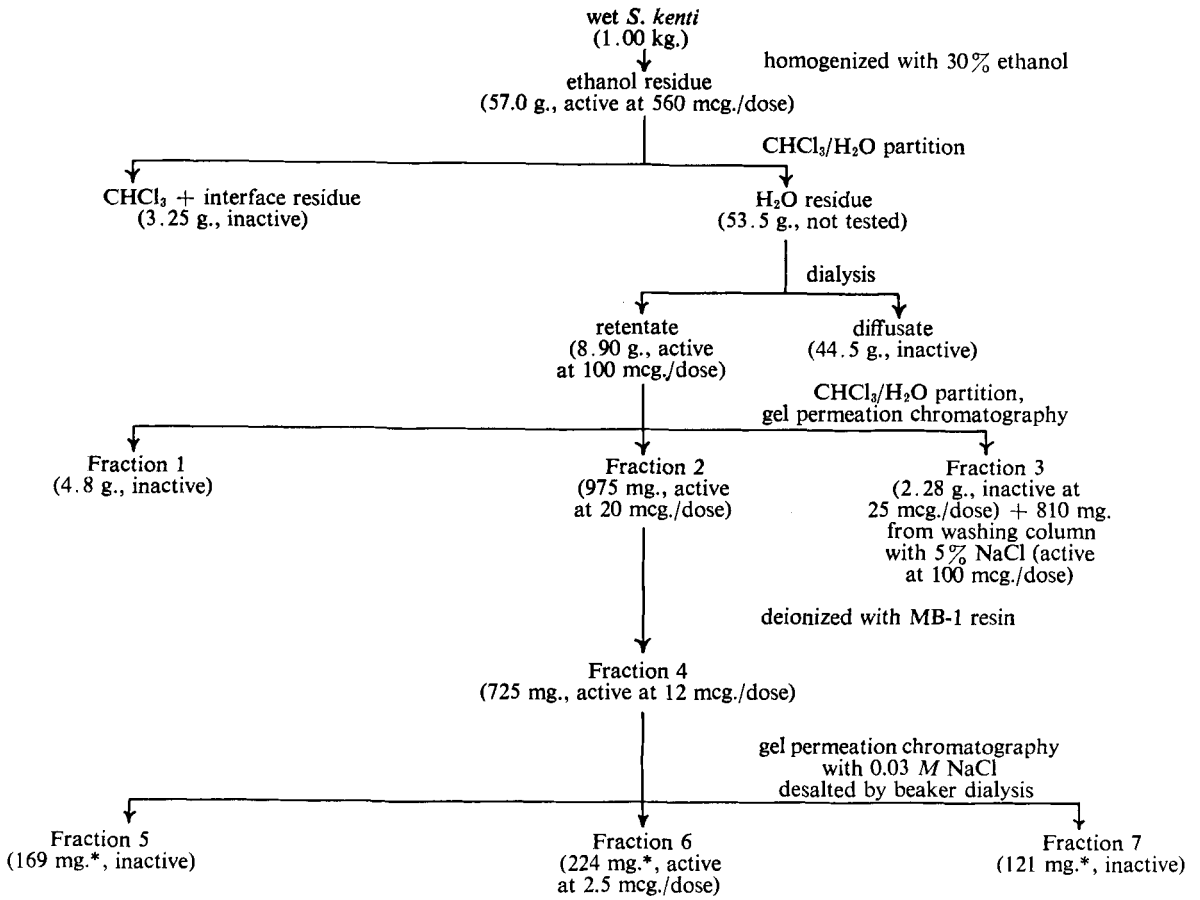
TED R. NORTON▲ and MIDORI KASHIWAGI

Abstract □ An antitumor substance, stoichactin, was obtained from an aqueous alcoholic extract of the sea anemone, identified as most probably being *Stoichactis kenti*, using dialysis and gel permeation chromatography. The drug is effective at very low dosages using several different methods of administration in the control and cure of Ehrlich ascites tumor in mice.

Keyphrases □ Stoichactin—identification and pharmacological studies as an antitumor agent, mice □ Antitumor agents—purification of stoichactin from sea anemone, pharmacological testing with Ehrlich ascites tumor, mice □ *Stoichactis kenti*—isolation and purification of stoichactin, a potent antitumor agent, pharmacological testing, mice

During a rather broad study of pharmacological activity of over 1000 species of natural products from the Pacific basin, it was found that a number of extracts of coelenterates were highly effective in the control of Ehrlich ascites tumor in mice (1). One of these coelenterates was the anemone, identified as most probably being *Stoichactis kenti*[1]. Because of the very high specific activity found in a 30% ethanolic extract of this anemone, purification of the active constituent was undertaken. Partial purification results were recently reported (1). A fraction which deposited crystals on evaporation of an aqueous solution has now been obtained, and the active substance, now essentially pure, is being named "stoichactin." This drug was evaluated

[1] Collected from the reef of Arue Bay in Tahiti by Dr. Frank Tabrah. Dr. Cadet Hand, Director of the University of California Bodega Marine Laboratory, identified this sea anemone as definitely being in the genus *Stoichactis* and most probably as the species *S. kenti*.



Scheme I—Diagram of the procedure for the purification of stoichactin from the sea anemone, S. kenti [**These weights are extrapolated (725/600) from a 600-mg. Fractiyn 4 run described in the* Experimental *section.*]

by several different modes of administration and injection schedules to delineate its antitumor properties.

DISCUSSION

A summary of the purification procedure for 1 kg. of wet *S. kenti* is shown in Scheme I. The presence of activity in the fractions obtained was determined by bioassay against Ehrlich ascites tumor in mice by the method previously described (2). The doses, at which the fraction is described as "active" in Scheme I, were given intraperitoneally 20 times over a period of 10 days and effected an approximate ED_{90} at 30 days after tumor cell administration. The same isolation procedure has been used on 7 kg. of wet anemone[2] with comparable results.

When the anemone specimens were collected, they were drained and fresh frozen, shipped from Tahiti under dry ice refrigeration, and kept frozen until extracted. A freshly thawed sample was diced and homogenized with 30% ethanol. The crude extract was defatted with chloroform and dialyzed. The active retentate was once more defatted and subjected to gel permeation chromatography (Sephadex G-50) using distilled water elution. In this case the bulk of the activity was found in Fraction 2 (V_e/V_o = 1.70–2.20), with a small amount in Fraction 3 and in retained material washed from the column by 5% NaCl after each run. The solid from Fraction 2 was chromatographed on both cationic and anionic cellulose columns, and the active substance was not retarded by either column at pH 7 with 0.01 *M* phosphate buffer eluate. Therefore, Amberlite MB-1 (mixed bed resin) was used to remove ionized impurities. Attempts to rechromatograph the deionized sample using gel permeation chromatography, with water as the eluate, failed, the active substance being completely retarded. It was then found that 0.03 *M* NaCl would break the binding of the active substance to the Sephadex G-50. With the salt present, the active substance in Fraction 6 was eluted earlier (UV absorption maximum at 280 nm. at V_e/V_o = 1.78 compared to 2.06 when water was used on the crude retentate). After desalting by dialysis and subsequent evaporation, Fraction 6 deposited crystals.

The previously described (1) preliminary purification of *S. kenti* extracts was based on work done with specimens collected in 1968. For unknown reasons, the active fraction in this earlier study was retarded more during the gel permeation chromatography of the retentate, appearing in the equivalent of Fraction 3 in the present study. The presence of a lower concentration of ionized organic impurities in the retentate from the 1968 sample may explain this greater retardation of the active substance.

Stoichactin appears to be a neutral compound, stable at pH 0.5 and unstable at pH 13 for 24 hr. at 25°. It does not lose activity after being incubated with pronase, indicating that if the molecule does contain a polypeptide moiety it is most likely either not hydrolyzed by pronase or is not important to its activity. Stoichactin gives stable foams in a 0.1% aqueous solution and causes immediate erythrocyte lysis at 10 p.p.m. Based on the very slight retardation on Sephadex G-25 and moderate retardation on Sephadex G-50, the molecular weight is probably above 5000. Studies on the chemical nature of stoichactin are in progress.

The experimental data from the study of different methods of drug administration in the control of Ehrlich ascites tumor are given in Table I. All experiments were carried out using the methods described previously (1) with modifications indicated in Table I. In this laboratory, during the past 4 years, over 2000 mice have been used as infected controls, and none has survived as long as 30 days. Consequently, the presence of any survivors in the treated groups at 30 days indicates significant activity. The absence of observable abdominal distension from ascitic fluid 30 days after tumor cell inoc-

[2] The authors are indebted to Dr. J. Saugrain, then Director, and Dr. R. Bagnis, in charge of the medical oceanography section of the Institute of Medical Research "Louis Malarde" in Papeete, Tahiti, French Polynesia, for the collection of the 7 kg. of *S. kenti* specimens during the summer of 1971.

Table I—Effectiveness of Stoichactin as an Antitumor Agent Using Different Methods of Administration

Experiment	Experiment Number	Administrative Route	Treatment	Dose, mcg.[a]	Number of Doses	Number of Mice Inoculated with EA[b]	Number of Developing Ascites	Number of Survivors at 30 Days	Percent Survivors at 30 Days
Different routes of administration	1	EA, 5×10^5 cells, intraperitoneal; Fraction 2, intraperitoneal[c]	Control	—	—	10	10	0	0
			Drug	20	20 (10 days)	10	0	10[d]	100
							Number Developing Solid Tumor	Number with No Tumor at 60 Days	Tumor weight, mg., Mean Range at 30 Days
	2	EA, 10^6 cells, subcutaneous; Fraction 2, intraperitoneal[c]	Control	—	—	9	9	0	730 (484–1569)
			Drug	80	10 (10 days)	7	4	3	100 (0–314)
							Number Developing Ascites	Number of Survivors at 30 Days	Percent Survivors at 30 Days
	3	EA, 5×10^5 cells, intraperitoneal; Fraction 6, subcutaneous	Control	—	—	10	10	0	0
			Drug	30	20 (10 days)	10	3	7[d]	70
									Number of Survivors at 6 Months
Duration of control	4	EA, 5×10^5 cells, intraperitoneal; Fraction 2, intraperitoneal[c]	Control	—	—	10	10	0[e]	0
			Drug	20	20 (10 days)	6	2	5 (4)[d]	4[d]
									Percent Survivors at 30 Days
Minimum number of doses required	5	EA, 10^6 cells, intraperitoneal; Fraction 2, intraperitoneal[c]	Control	—	—	10	10	0	0
			Drug	40	4 (2 days)	5	0	5[d]	100
	6	EA, 10^6 cells, intraperitoneal; Fraction 2, intraperitoneal[c]	Control	—	—	9	9	0	0
			Drug	40	2 (1 day)	5	3	4 (2)[d]	80
	7	EA, 5×10^5 cells, intraperitoneal; Fraction 6, intraperitoneal[c]	Control	—	—	5	5	0	0
			Drug	50	1	8	1	8 (7[d])	100
	8	EA, 5×10^5 cells, intraperitoneal; Fraction 6, intraperitoneal[c]	Control	—	—	5	5	0	0
			Drug	25	1	8	2	7 (6[d])	87.5
Pretreatment with drug	9	EA, 5×10^5 cells, intraperitoneal; Fraction 2, intraperitoneal, last inoculation 36 hr. prior to EA inoculation	Control	—	—	10	10	0	0
			Drug	40	3 (1.5 days)	10	5	5[d]	50
Delayed treatment with drug	10	EA, 5×10^5 cells, intraperitoneal; Fraction 6, intraperitoneal, 4 days after EA inoculation	Control	—	—	8	8	0	0
			Treatment	20	20 (10 days)	10	2	8[d]	80
Activity of purest fraction	11	EA, 5×10^5 cells, intraperitoneal; Fraction 6, intraperitoneal[c]	Control	—	—	10	10	0	0
			Treatment	2.5	18 (9 days)	10	0	10[d]	100

[a] Mice used weighed 25–30 g., so 1-mcg. dose is equivalent to 33–40 mcg./kg. [b] EA = Ehrlich ascites tumor. [c] Drug administered 20 hr. after Ehrlich ascites tumor cells inoculated. [d] All nonascitic. [e] Mean survival time of 16 days.

ulation was the basis for describing survivors as nonascitic (3). A more reliable indication of cure is survival 2 months or more after inoculation of Ehrlich ascites tumor cells and treatment.

Stoichactin appears to be systemic in action in that it is effective not only in the screening procedure where Ehrlich ascites tumor cells and drug are both administered intraperitoneally but at higher dosages when Ehrlich ascites tumor cells are injected subcutaneously and drug intraperitoneally (Experiment 2, Table I) and vice versa (Experiment 3, Table I). To determine if any "cures" had been effected using the standard test (2), a treated group of mice was held for 6 months; 100% of the nonascitic mice survived (Experiment 4, Table I). Fewer than the usual 20 doses of drug were found effective; in fact, only one dose of the drug given 20 hr. after Ehrlich ascites tumor cells were injected controlled tumor growth (Experiments 5–8, Table I). When the drug was given in three doses, giving the last dose 36 hr. prior to Ehrlich ascites tumor cells injection, fair control of tumor growth was observed (Experiment 9, Table I). This indicates that either the drug is metabolized slowly or an induced immunity is produced. Also, if the treatment is delayed until 4 days after Ehrlich ascites tumor cells injection, good control is observed

(Experiment 10, Table I). This indicates that even well-established tumor proliferation can be controlled by the drug. The purest fraction obtained (Fraction 6) gave 100% inhibition of tumor growth in the standard test with only 2.5 mcg./dose (100 mcg./kg.), as shown in Experiment 11. Now that the highly purified material is available, further studies of modes of administration, effects on other tumor systems, and mechanism of action on Ehrlich ascites tumor cell cultures *in vitro* are in progress.

EXPERIMENTAL[3]

One kilogram of freshly thawed *S. kenti* was diced into approximately 1-cm. cubes and homogenized in 125-g. batches with 500 ml. of 30% ethanol per batch, using a blender[4] at top speed for 5 min. The 5 l. of suspension was centrifuged in 250-ml. batches at 30,000 × *g* for 20 min. at room temperature. The supernatant liquid was flash evaporated at ≷40° to remove the ethanol and reduce the volume to 500 ml. of aqueous suspension. An equal volume of chloroform was added, and the mixture was shaken thoroughly and then centrifuged at 30,000 × *g* for 20 min. The clear supernatant solution was pipeted from the chloroform and a thin emulsion layer. The aqueous layer was dialyzed against 12.5 l. of distilled water using 10 2.22-cm. (0.875-in.) (flat) dialysis tubes for 24 hr. at 4°. The dialysate was discarded, and 12.5 l. of fresh distilled water was added for an additional 24 hr. of dialysis at 4°.

The combined retentate solution was lyophilized to give 8.90 g. of off-white solid retentate. This solid retentate was subjected to gel permeation chromatography in three separate batches of 3 g. each. Each 3-g. batch was triturated with 50 ml. of water and sonicated until clear (temperature maximum 60°). This solution was thoroughly shaken with 50 ml. of chloroform and centrifuged at 18,000 × *g* for 15 min. The clear slightly amber supernatant solution was placed on a 2600-ml. wet volume of Sephadex G-50 (fine) in an 8 × 52-cm. column and eluted with water saturated with chloroform at a rate of 14 ml./min. (0.28 ml. cm.$^{-2}$ min.$^{-1}$). The void volume, V_o, was previously determined to be 905 ml. using 50 ml. of 0.15% solution of >200,000 mol. wt. blue dextran. The column effluent was monitored using a recording UV absorption cell at 280-nm. wavelength. The first fraction showed a UV absorption maximum of 0.44 absorbance units at V_e/V_o (total eluted volume/void volume) = 1.05 and was cut at V_e = 1540 ml. corresponding to V_e/V_o = 1.70. The second cut of 450 ml. showed a UV absorption maximum of 0.32 absorbance unit at V_e/V_o = 2.06, and the cut was made at V_e/V_o = 2.20. The third cut of 590 ml. showed a sharp peak at V_e/V_o = 2.55 and 0.67 absorbance unit, with a shoulder at V_e/V_o = 2.68 and 0.42 absorbance unit; the cut was made at V_e/V_o = 2.85 (Fraction 3).

The second and third batches gave similar results, and each combined fraction was flash evaporated at ≷40° to approximately 100 ml. volume and lyophilized. Fraction 1 weighed 4.8 g. (inactive), Fraction 2 weighed 975 mg. (active), and Fraction 3 weighed 2.28 g. (slight activity). After each run, 50 ml. of 5% NaCl was put on the column and eluted with water to wash absorbed material from the column. This salt wash cut of 280 ml. removed material at V_e/V_o = 2.3–2.6, with a UV absorption maximum (280 nm.) at 2.50 with 0.30 absorbance. This was immediately followed by the aqueous sodium chloride cut from 2.6 to 2.9 with a maximum Cl^- titer at V_e/V_o = 2.72. The three salt wash cuts were combined and lyophilized, and the dry solid weighed 810 mg.

Fraction 2 (975 mg.) was dissolved in 100 ml. water and passed through 15 ml. of Amberlite MB-1 deionizing resin (20–50 mesh) in an 8 × 195-mm. column at 1 ml./min. and washed with 25 ml. H_2O. The deionized solution was lyophilized, and the colorless solid weighed 725 mg. Six hundred milligrams of this was dissolved in 30 ml. of 0.03 *N* NaCl and put on the same Sephadex G-50 column as already described except that the column was previously equilibrated with 0.03 *N* NaCl by running through the column a 30-ml. sample of 5% NaCl followed by 0.03 *N* NaCl. The sample was eluted with 0.03 *N* NaCl saturated with chloroform. Cuts were obtained at V_e/V_o = 1.0–1.58, 1.58–2.02, and 2.02–2.34. The first cut had a 280-nm. UV absorption maximum at V_e/V_o = 1.08, and the second cut was at V_e/V_o = 1.78 (active cut). Each of the three cuts was evaporated to 90 ml. and desalted in a 0.1-m.^{2} surface area beaker dialyzer[5] by passing distilled water through the fibers at 100 ml./min. for 25 min. Each retentate was dialyzed to give 140 mg. of Fraction 5, 185 mg. of Fraction 6 (active fraction), and 100 mg. of Fraction 7. Recent runs showed that the balance of the solids is eluted at V_e/V_o = 2.34–2.9. When a drop of 0.1% solution of Fraction 6 was placed on a slide, rather clean colorless crystals with a proliferated branching pattern were observed.

[3] The dialysis tubing was obtained from the Food Products Division, Union Carbide Corp., Chicago, Ill.; the hollow-fiber dialyzer was obtained from the Functional Products & Systems Department, Dow Chemical Co., Midland, Mich.; the Sephadex G-50 was obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N. J.; and the Amberlite MB-1 resin was obtained from Mallinckrodt Chemical Works, St. Louis, Mo.

[4] Osterizer Cyclo-trol Eight.

[5] Dow HFD-1.

REFERENCES

(1) F. Tabrah, M. Kashiwagi, and T. R. Norton, *Int. J. Clin. Pharmacol. Ther. Toxicol.*, **4**, 420(1972).

(2) F. L. Tabrah, M. Kashiwagi, and T. R. Norton, *Science*, **170**, 181(1970).

(3) K. Sugiura and H. J. Creech, *Ann. N. Y. Acad. Sci.*, **63**, 962 (1956).

ACKNOWLEDGMENTS AND ADDRESSES

Received March 24, 1972, from the *Department of Pharmacology, University of Hawaii, Leahi Hospital, Honolulu, HI 96816*

Accepted for publication June 2, 1972.

Supported by the Elsa U. Pardee Foundation, Midland, Mich., and Public Health Service Research Grant CA-12623.

The authors appreciate the helpful discussions with Professor Y. Hokama, Pathology Department; Professor S. C. Chou, Pharmacology Department; and Dr. R. Quinn, Chemistry Department; all at the University of Hawaii.

▲ To whom inquiries should be directed.