Isolate from the Annelid, *Reteterebella queenslandia* (Australia), Active against Ehrlich Ascites Tumor

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Abstract □ A fraction, isolated by dialysis and gel permeation chromatography from an aqueous ethanolic extract of the tentacles of *Reteterebella queenslandia*, controlled and cured Ehrlich ascites tumor in mice in microgram doses. It is effective by either intraperitoneal or subcutaneous injection.

Keyphrases □ *Reteterebella queenslandia*—isolation, antitumor activity against Ehrlich ascites tumor in mice □ Annelids, extract of *Reteterebella queenslandia*—isolation, antitumor activity against Ehrlich ascites tumor in mice □ Antitumor activity—fraction isolated from annelid *Reteterebella queenslandia* □ Gel permeation chromatography—isolation of extract from *Reteterebella queenslandia*

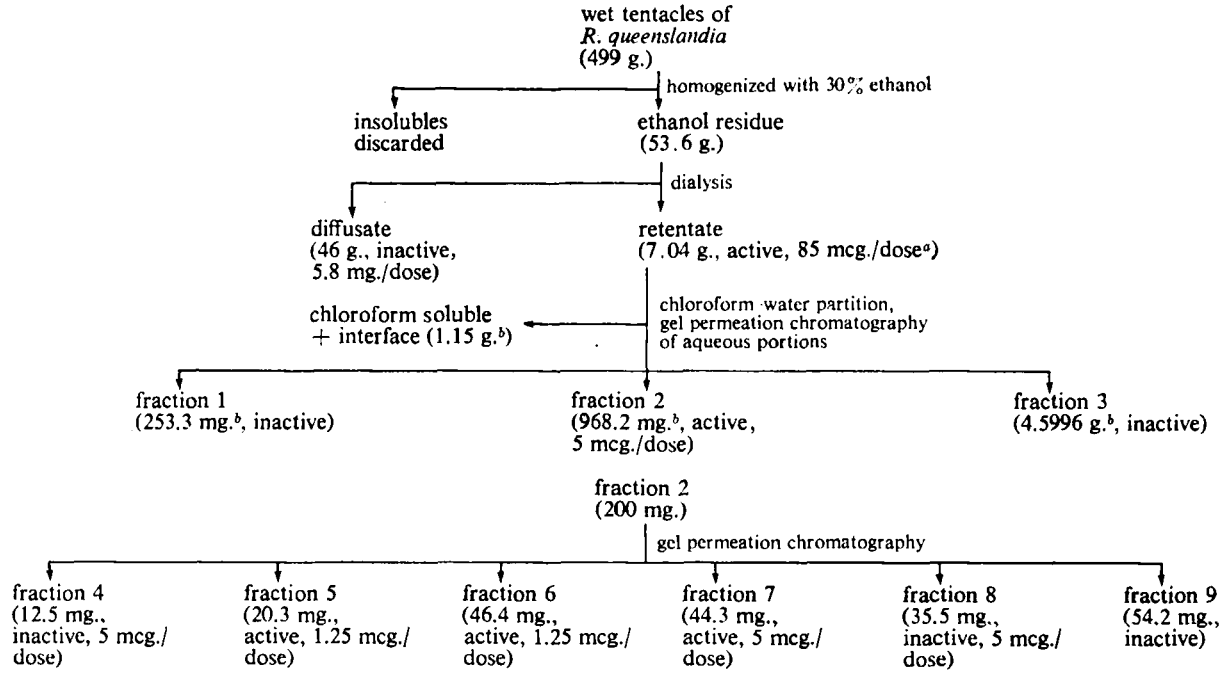
During the pharmacological evaluation of ancient Polynesian medicines, the polychaetous annelids *Lanice conchilega* and *Reteterebella queenslandia* were studied. The Kaunaoa (*L. conchilega*) was reported by Tabrah *et al.* (1) to have been used by the Hawaiians for cancer control in humans. Aqueous ethanolic extracts of both related annelids showed significant antitumor activity against Ehrlich ascites tumor in mice.

Although *L. conchilega* is found widely in the Hawaiian Islands coastal waters and tidepools, it is not found in large numbers. Because of the great difficulty in collecting, and the possible danger of depleting, *L. conchilega*, the more plentiful and closely related *R. queenslandia* from the Great Barrier Reef was chosen for purification and administration method studies.

RESULTS AND DISCUSSION

The purification of the antitumor agent is summarized in Scheme I. The indications of activity are based on >50% survivors at 30 days using 20 doses over 10 days. Controls had a mean survival time of about 17 days and never survived for 30 days, based on more than 2000 diseased control mice used during the past 4 years. Intraperitoneal drug inoculation was started 20 hr. after intraperitoneal inoculation of 5×10^6 Ehrlich ascites tumor cells. The details of the Ehrlich ascites tumor testing were described previously (1).

The fresh tentacles of the *R. queenslandia* were preserved in ethanol and shipped by air from Heron Island[1] to Honolulu where they were kept at −18° until processed. The tentacles were homogenized with 30% ethanol, and the mixture was centrifuged. The crude extract was dialyzed and the active retentate was defatted with chloroform before gel permeation chromatography[2]. The active fraction was eluted[3] at void volume, indicating a molecular weight above 30,000 (if globular protein), so that G-75 was used. All of the antitumor activity was found in fraction 2 (V_e/V_0 = 1.34-2.34). A small sample of fraction 2 was chromatographed on both anionic and cationic cellulose at pH 7.0 with 0.02 *M* phosphate buffer. The active material was not retarded by either ion-exchange column. Fraction 2 was rechromatographed[2] with peak antitumor activity being found



Scheme I—Procedure for purification of the antitumor agent in R. queenslandia. (*[a] Since 25-g. mice were used, 1 mcg./dose was equivalent to 40 mcg./kg./dose. Doses were 0.1 ml. in volume. [b] These weights are extrapolated (1.637 factor) from the somewhat smaller scale run described in the* Experimental *section.*)

[1] Supplied by Reginald McMahon, Heron Island Marine Station, Queensland, Australia.
[2] Sephadex G-75, Pharmacia Fine Chemicals, Inc., Piscataway, N. J.
[3] Sephadex G-50, Pharmacia.

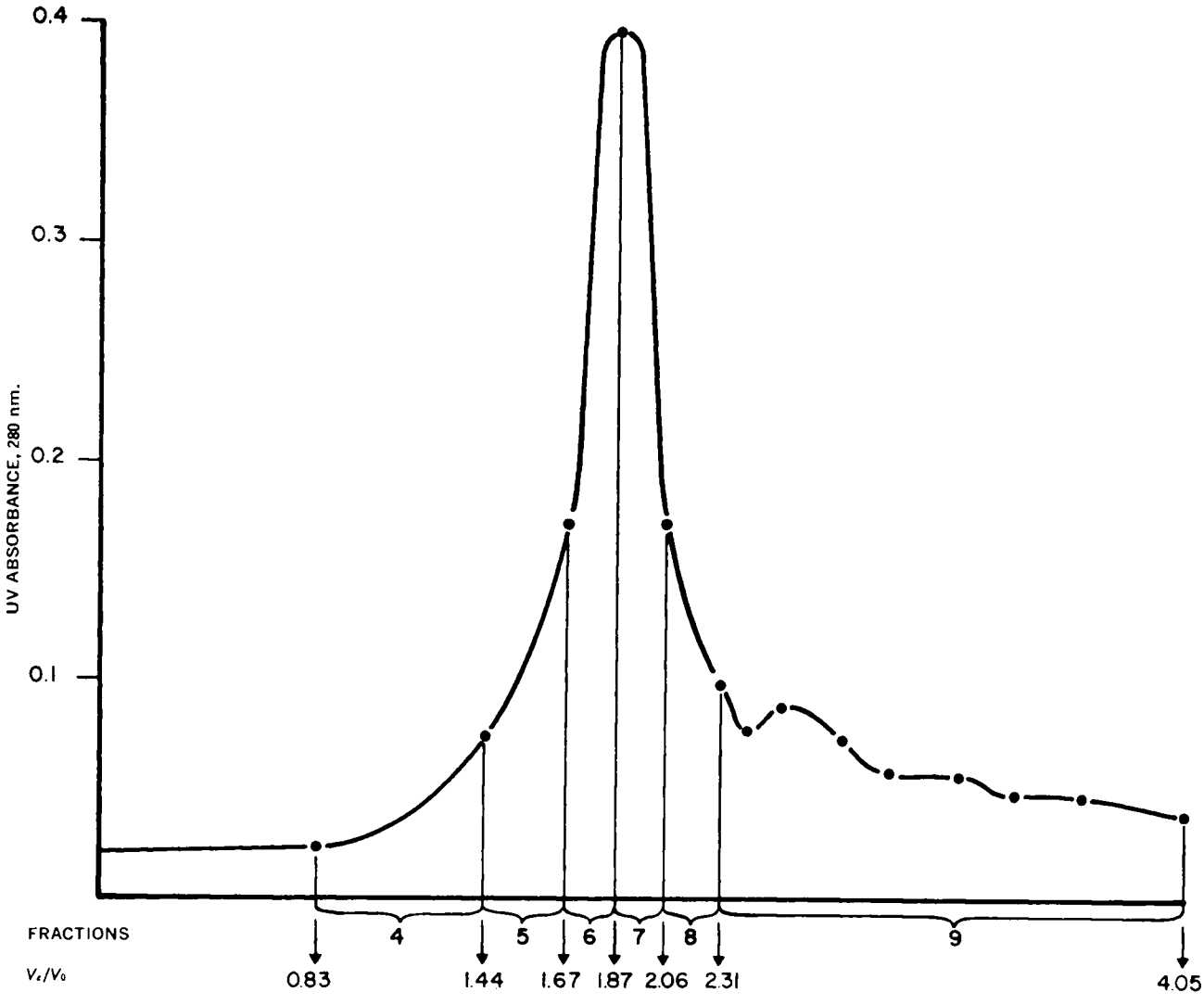


Figure 1—*Gel permeation chromatography of fraction 2 (on Sephadex G-75).*

in fractions 5 and 6 (V_e/V_0 = 1.44–1.87), which did not coincide with the UV absorption peak at 280 nm. (V_e/V_0 = 1.87). Figure 1 illustrates the UV absorption curve, and Table I lists the bioassay data.

In an attempt to correlate the antitumor activity of the gel permeation fractions with some physical or chemical parameter, 17 15-ml. fractions from V_e/V_0 = 0.90–2.47 were assayed for UV absorption at 254 nm., chromate oxidizables, foam stability (height at 48 hr.), dry weight, and anti-Ehrlich ascites tumor activity. The data are plotted in Fig. 2 and described in the *Experimental* section. The activity parameter peak did not correlate with any of these properties.

A sample of fraction 2 run through both cationic and anionic cellulose gave a 37% recovery of nonretarded material, showing a 75% cure rate using Ehrlich ascites at 0.36-mg./kg. doses. A sample of the latter, hydrolyzed for 90 hr. at 110° in 12 *N* HCl, showed an array of amino acids by a ninhydrin test[4] and showed negative tests for sugars. Since the fraction was still impure, this is only an indication that the active substance may be a protein.

Gel electrophoresis of fractions 5 and 6 at pH 4.3 with 15% cross-linked polyacrylamide (5 ma./tube, 200 v., 105 min.) showed two major bands when stained with naphthol blue black at R_f 0.16 and 0.73 (basic fuchsin at R_f 1.00) and six minor bands at R_f 0.41, 0.45, 0.52, 0.57, 0.61, and 0.66. Fraction 5 had a somewhat intensified R_f 0.73 band and a considerably diminished R_f 0.16 band. Fractions 7 and 8 had a considerably diminished R_f 0.73 band and an intense R_f 0.16 band. Based on the bioassays of these fractions, the band at R_f

[4] On Celplate F-22, Brinkmann Instruments, Inc., New York, N. Y.

Table I—Bioassay of Fractions 4–8

Fraction	Dosage[a], mcg. in 0.1 ml.	Number of Mice[b] Inoculated with Ehrlich Ascites Tumor	Number Developing Ascites	Number of Survivors at 30 Days	Percent of Survivors at 30 Days
4	5.0	5	5	0	0
5	5.0	5	0	5 (5[c])	100
	2.5	9	0	9 (9[c])	100
	1.25	10	3	7 (7[c])	70
	0.63	10	10	1	10
6	5.0	5	0	5 (5[c])	100
	2.5	10	0	10 (10[c])	100
	1.25	10	10	5	50
	0.63	10	10	0	0
7	5.0	5	2	4 (3[c])	80
	2.5	10	5	7 (5[c])	70
	1.25	10	10	1	10
	0.63	10	10	1	10
8	5.0	5	5	0	0
Controls	—	10	10	0	0

[a] Twenty of these doses given intraperitoneally over 10 days, starting 20 hr. after 5 × 10^5 Ehrlich ascites tumor cells inoculated intraperitoneally. [b] Swiss-Webster female mice weighing 25 g. [c] Number of "nonascitic" mice at 30 days. Nonascitic means no abdominal distension. See *Reference 3*.

Table II—Effectiveness of Different Modes of Drug Administration Using Fraction 5

Experiment	Experiment Number	Administrative Route	Treatment	Drug, mcg.[a]	Number of Doses	Number of Mice Inoculated with EA[b]	Number developing Ascites	Number of Survivors at 30 Days	Percent of Survivors at 30 Days[c]
Minimum number of doses required	1	EA, 5×10^5 cells, intraperitoneally; drug intraperitoneally[d]	Control	—	—	5	5	0	0
			Drug	2.5	20 (10 days)	9	0	9	100
	2	EA, 5×10^5 cells, intraperitoneally; drug intraperitoneally[d]	Control	—	—	4	4	0	0
			Drug	25.0	4 (2 days)	8	2	8 (6)[e]	100
	3	EA, 5×10^5 cells, intraperitoneally; drug intraperitoneally[d]	Control	—	—	4	4	0	0
			Drug	25.0	1	8	6	5 (2)[e]	62.5
Pretreatment with drug	4	EA, 5×10^6 cells, intraperitoneally; drug intraperitoneally in three doses over 1.5 days, last dose *36 hr. prior to EA cells* inoculation	Control	—	—	5	5	0	0
			Drug	5.0	3 (1.5 days)	10	10	2	20
Drug absorption	5	EA, 1×10^6 cells, intraperitoneally; drug *subcutaneously*	Control	—	—	5	5	0	0
			Drug	10.0	10 (10 days)	9	9	2	22
							Number Developing Solid Tumor	Tumor Weight, mg., Mean (Range) at 9 Days	
	6	EA, 1×10^6 cells, *subcutaneously;* drug intraperitoneally	Control	—	—	10	10	199 (74–388)	
			Drug	10.0	20 (10 days)	10	5	37 (0–98)	

[a] Mice used weighed 25 g., so 1 mcg./ dose was equivalent to 40 mcg./kg./dose. [b] EA = Ehrlich ascites tumor. [c] Mean survival time 17 days. [d] Drug administered 20 hr. after Ehrlich ascites tumor cells inoculated. [e] All nonascitic. The eight nonascitic survivors were kept for 3 months with no signs of abdominal distension.

Table III –Gel Permeation Chromatographic Fractions from the Retentate

Fraction	V_e/V_0	V_e/V_0 Peak Absorbance, UV at 280 nm.	Weight, g.
1	0.85–1.35	1.03	0.1574
2	1.35–2.30	1.95	0.5914
3	2.30–3.70	3.09	2.8094

Table IV –Gel Permeation Chromatographic Fractions from Fraction 2

Fraction	V_e/V_0	UV Absorbance at 280 nm.	Weight, mg.
4	0.83–1.44	0.04–0.08	12.5
5	1.44–1.67	0.08–0.18	20.3
6	1.67–1.87	0.18–0.41 (max.)	46.4
7	1.87–2.06	0.41–0.18	44.3
8	2.06–2.31	0.18–0.10	35.5
9	2.31–4.05	0.10–0.05	34.2

0.73 (high positive charge to mass ratio) appears to be the active basic protein.

Fraction 5 was used to study the effectiveness of various modes of administration of the drug. It was found to be 100% effective with 20 doses over 10 days at 100 mcg./kg. and 62.5% effective with only one dose of 1.0 mg./kg. The drug given 36 hr. prior to inoculation with Ehrlich ascites tumor cells showed 20% control. Systemic activity was indicated by some control of intraperitoneally induced tumor by subcutaneous injection of drug and by good control of subcutaneously induced solid tumors by intraperitoneally administered drug, with 50% of the mice developing *no* solid tumors (Table II). Antitumor evaluations were carried out using the method described previously (1), with exceptions as noted in Table II.

The drug (fraction 2) showed no activity against P-388 mouse leukemia[5]. Tests on other systems are still in progress.

EXPERIMENTAL

Extraction and Purification—A 499-g. wet drained weight sample of the tentacles of *R. queenslandia*, preserved in 95% ethanol for air shipment, was kept on arrival at −18° until use. One liter of 30% ethanol was used to homogenize the sample at top speed in a blender[6] for 5 min. The solids were removed by centrifugation at 30,000-$\times g$ for 1 min. and then triturated with 200 ml. of 30% ethanol and recentrifuged; the combined supernates were evaporated at ≦50° to 400 ml. volume. Some solid that separated was removed by filtration using a büchner funnel and a small amount of diatomaceous earth[7] filter aid. The solid was washed with 50 ml. of water, and the filtrate was adjusted to 500 ml. (dry weight 53.6 g.).

The 500 ml. of crude aqueous extract was dialyzed using a 1.0-m.[2] hollow-fiber artificial kidney[8]. The extract was passed through the fibers at 10 ml./min. concurrently with 1 l./min. of tap water flowing outside the fibers through the jacket (total of 50 l. of water). After flushing the kidney, the retentate was lyophilized to give 7.0385 g. of solids.

A 4.30-g. sample of this retentate was dissolved in 150 ml. water and thoroughly shaken with 150 ml. chloroform. The emulsion formed was centrifuged for 5 min. at 20,000$\times g$, and the aqueous layer was removed using a pipet. The chloroform and interface were dried and weighed 0.70 g. The clear aqueous layer was evaporated at ≦50° to 50 ml. and chromatographed in four 12.5-ml. batches (total dry weight 3.60 g., 900 mg./batch).

[5] These tests were carried out at the Southern Research Institute with the cooperation of Dr. Jonathan Hartwell, National Cancer Institute.

[6] Waring.

[7] Celite.

[8] Functional Products & Systems Dept., Dow Chemical Co., Midland, Mich.

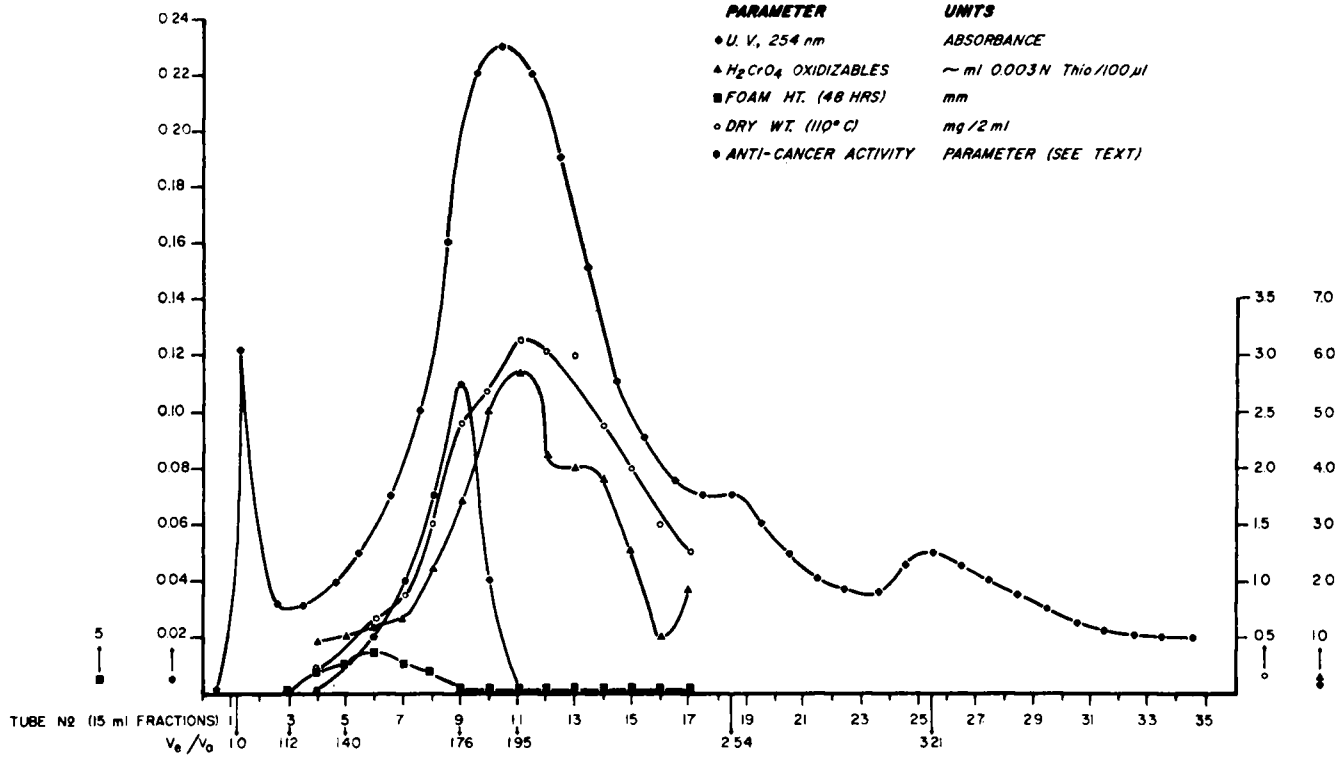


Figure 2—*Gel permeation chromatography of fraction 2, parameter correlations.*

Table V—Antitumor Assay of Tubes 4–11 from Gel Permeation Chromatography of Fraction 2

Tube Number	Number of Survivors at 30 Days		Parameter
	Nonascitic	Ascitic	
4	0	0	0
5	0	1	0.5
6	1	0	1.0
7	2	0	2.0
8	3	1	3.5
9	5	1	5.5
10	2	0	2.0
11	0	0	0

The 12.5-ml. batches were each chromatographed on 550 ml. (wet) resin[9] in a column (44 × 4 cm.) using distilled water for elution at about 3 ml./min. The void volume (V_0) was determined to be 170 ml. using 12.5 ml. of a 0.15% solution of blue dextran of mol. wt. 200,000. The fractions were collected as shown in Table III. Each fraction was lyophilized, weighed, and stored under refrigeration.

A 200-mg. sample of fraction 2 dissolved in 5 ml. water was chromatographed on 585 ml. (wet) resin[9] in a column (47 × 4 cm.) equilibrated with 0.030 *N* NaCl saturated with chloroform and eluted with the same solvent. The void volume was 174 ml. The fractions collected were desalted using a 0.1-m.[2] hollow-fiber beaker dialyzer[8], passing distilled water through the fibers at 80 ml./min. for 31 min. (2.5-l. total), and then lyophilized to give the dried fractions (Table IV).

Behavior on Ion Exchange—A 50-mg. sample of fraction 2 was dissolved in 1.0 ml. water and put on a 25 × 1-cm. column of previously conditioned cation-exchange packing[9] (5.0 g. dry and 0.73-meq./g. capacity), which had been equilibrated with 0.02 *M* phosphate at pH 7.0, and was eluted with the same solvent. The peak 280-nm. UV absorption was at V_0 (0.94 absorbance). The fraction from 13–31 ml. effluent (18 ml.) was desalted[10] (sample at V_0, phosphate retarded to V_e/V_0 = 1.8–2.2) and lyophilized to give 51.8 mg. (104% recovery, probably contains a trace of phosphate).

The same procedure was used on this 51.8-mg. sample with the anion-exchange packing[11] (capacity 0.93 meq./g.), providing 18.4 mg. (fraction 10) in the void volume fraction at pH 7 and 0.02 *M* phosphate. Under these conditions the active substance was not retarded by either the anionic or cationic cellulose.

Hydrolysis of Fraction 10—A 1.0-mg. sample of fraction 10 and 1.0 ml. of 12 *N* HCl was sealed in a Pyrex tube under nitrogen and heated at 110° for 90 hr. The acid was removed from the colorless hydrolysate by evacuation over sodium hydroxide pellets in a desiccator. The dry residue was dissolved in 0.5 ml. water, and 1 µl. was spotted[12]; using an *n*-butanol–water–acetic acid (3:1:1) solvent, strong ninhydrin positive spots were observed at R_f values of 0.054, 0.127, 0.17, 0.27, 0.34, and 0.38 with the solvent front 15.75 cm. from the origin.

A test of the hydrolysate for reducing sugars with Fehling's solution was negative.

Correlation of Physical and Chemical Properties with Antitumor Activity—A 241-mg. sample of fraction 2 equivalent (prepared in a different run from above) was dissolved in 10 ml. water and spun at 20,000 × *g* for 5 min. to remove a trace of insolubles. The supernate was put on 550 ml. of resin[9] in a column (44 × 4 cm.) and eluted with water. The void volume (V_0) was 165 ml. A total of 32 fractions of approximately 15 ml. each was collected. The exact total volume eluted (V_0) was noted at the end of each fraction, and the UV absorption at 254 nm. was recorded continuously. Dry weights were determined using 2 ml. of the cut heated at 130° to constant weight. The foam height was determined by giving each tube 30 sharp raps by hand, allowing the samples to stand for 48 hr., and measuring the height of the foam.

To determine chromate oxidizables, a modification of the method of Märki and Witkop (2) was used. The procedure involved carefully adding exactly 2.00 ml. of the chromic acid solution (0.02013 *N* $Na_2Cr_2O_7 \cdot 2H_2O$ in 98% H_2SO_4) to 100 µl. of the fractions and to control blanks and heating for 20 min. in a steam bath. The solution was then diluted to 100 ml., 1 ml. of 5% KI was added, and the solution was allowed to stand no less than 10 nor more than 20 min. (less time gave incomplete I^- to I_2 conversion and more than 20 min. gave a gradual drop in titer). The liberated iodine (I_2) was titrated with the 0.00303 *N* thiosulfate using a starch indicator; the end-point was taken when the starch–iodine color was first completely extin-

[9] Cellex CM, Bio-Rad Labs., Richmond, Calif.
[10] Using Sephadex G-25.
[11] Cellex D, Bio-Rad Labs., Richmond, Calif.
[12] Celplate F-25.

guished (on standing the color reappeared). The blanks required 13.40 ml. thiosulfate (theory = 13.29 ml.).

The activity parameter was determined as follows. A 5-ml. portion of the fraction was diluted to 60 ml., and a 0.1-ml. dose was given intraperitoneally twice daily for 10 days to eight mice starting 20 hr. after inoculation of 5×10^6 Ehrlich ascites tumor cells. At 30 days, the number of ascitic (abdominal distension) and nonascitic survivors was counted. The activity parameter is the sum of the nonascitic survivors and one-half the ascitic survivors out of the group of eight mice used for each bioassay. Only tubes 5–10 had any survivors at 30 days. The bioassays gave the results shown in Table V. The data on all of the parameters obtained are plotted in Fig. 2.

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Nonsink Dissolution Rate Equation

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Abstract □ An equation was developed which describes the dissolution of monodisperse particles beyond the point where concentrations are small compared to solubility. If it is assumed that a stagnant layer model applies, the thickness of these layers is of the same order of magnitude as calculated *via* the Hixson–Crowell treatment but dissolution rate constants are 1.5–2 times as large. The application of the equation to dissolution of hydrocortisone, levodopa, and *p*-hydroxybenzoic acid is shown.

Keyphrases □ Dissolution rates, monodisperse particles—equation developed for nonsink conditions, applied to hydrocortisone, levodopa, and *p*-hydroxybenzoic acid □ Particles, monodisperse—nonsink dissolution rate equation developed, applied to hydrocortisone, levodopa, and *p*-hydroxybenzoic acid

The equation by Hixson and Crowell (1) has been employed for many years [*e.g.*, Wurster and Taylor (2)] for the purpose of describing the dissolution rates of monodisperse powders. Higuchi and Hiestand (3), Carstensen and Musa (4), and Brooke (5) extended its use to describe the dissolution kinetics of polydisperse powders. The treatment relies on an assumption of sink conditions, a condition that frequently—but not always (particularly for sparingly soluble compounds)—applies. Therefore, it was considered appropriate to seek a solution not relying on sink conditions.

THEORY

When Fick's law (6) is applied to dissolution of a spherical particle under laminar flow conditions, allowing for an adsorbed surface film, it takes the form:

$$dm/dt = (L)\,dC/dt = \frac{DO}{h}[S - C] = LkO[S - C] \quad \text{(Eq. 1)}$$

where m is the mass dissolved, L is the volume of the dissolution medium, C is the concentration in the dissolution medium, t is time, ρ is the density of the solid, D is the diffusion coefficient, O is the surface area, h is the thickness of the adsorbed liquid film, S is solubility, k is the dissolution rate constant[1], n is the number of particles, r is the diameter of each particle, and subscript zero denotes initial magnitudes.

The Hixson–Crowell treatment emanates from the Noyes–Whitney equation, where it is assumed that $C \ll S$; this leads to the well-known cube root equation:

$$m_0^{1/3} - m^{1/3} = Kt \quad \text{(Eq. 2)}$$

where:

$$K = 1.61LkS(n^{1/3})/(\rho^{2/3}) \quad \text{(Eq. 3)}$$

If sphericity is assumed as above, S is assumed to be independent of r, and sink conditions are not invoked, one has the following expression for the concentration in the medium at time t:

$$C = \frac{n\rho}{L}\frac{4\pi}{3}[r_0^3 - r^3] = \alpha[r_0^3 - r^3] \quad \text{(Eq. 4)}$$

where:

$$\alpha = \frac{n\rho}{L}\frac{4\pi}{3} \quad \text{(Eq. 5)}$$

Equations 1 and 4 may be combined in the form:

$$dC = -\frac{n\rho}{L}\frac{4\pi}{3}3r^2\,dr = k(n^{1/3}\pi r^2)[S - \alpha(r_0^3 - r^3)]\,dt \quad \text{(Eq. 6)}$$

or:

$$-\frac{\rho}{L}dr = k[\beta + \alpha r^3]\,dt \quad \text{(Eq. 7)}$$

where:

$$\beta = S - (m_0/L) \quad \text{(Eq. 8)}$$

is positive when:

$$m_0 < SL \quad \text{(Eq. 9)}$$

The assumption is made in the following that the amount of

[1] The dimension of k is cm.$^{-2}$ sec.$^{-1}$. Some authors denote kL (cm./sec.) as the dissolution rate constant.