Antimicrobial Activity of Dimethyl Sulfoxide Against *Escherichia coli, Pseudomonas aeruginosa,* and *Bacillus megaterium*

HOWARD C. ANSEL, WILLIAM P. NORRED*, and IVAN L. ROTH†

Abstract □ A study has been made of the antimicrobial activity of dimethyl sulfoxide (DMSO) against three organisms, *Escherichia coli, Pseudomonas aeruginosa,* and *Bacillus megaterium.* Growth was inhibited by increasing DMSO levels and was virtually eliminated for each of the species at approximately 15% DMSO. *P. aeruginosa* demonstrated the least resistance to DMSO inhibition, with over 90% inhibition occurring in the presence of 8% DMSO. The growth rates of each of the microorganisms were shown to be decreased with increasing concentrations of DMSO. Electron microscopy revealed increased cytological alterations with increased concentrations of DMSO.

Keyphrases □ Dimethyl sulfoxide—antimicrobial activity □ Antimicrobial activity—DMSO solutions □ Electron microscopy—cells exposed to DMSO □ Turbidimetric analysis—bacterial suspensions

Dimethyl sulfoxide (DMSO) has in recent years been the subject of numerous studies by pharmaceutical researchers and manufacturers, and several review papers on its activity have been published (1–5). Included in these publications are reports of the value of DMSO as a local analgesic, diuretic, anti-inflammatory adjunct, tranquilizer, and bacteriostatic agent. DMSO has been the center of a controversy ignited by conflicting reports of its ability to hasten or potentiate the action of drug substances by increasing penetration across biologic membranes (6–11). Claims of DMSO's ability to greatly enhance drug penetration (6–8, 10 have been challenged by reports indicating penetrating properties similar to those of more common vehicles (9, 11). DMSO has found other uses, including that of an endocellular cryophylactic agent for the preservation of blood and other cells (12–14), and as a solvent for certain biochemical procedures (8, 15).

In this laboratory the hemolytic activity of antibacterial preservatives commonly employed in the preservation of pharmaceutical products has been investigated (16–21). It was found in the majority of cases that the concentration of a preservative required to induce the total hemolysis of erythrocytes was similar to the concentration required for antibacterial preservation. It was also determined that even though DMSO freely penetrates the erythrocyte membrane, and at concentrations of 40% causes irreversible damage to the cell, low concentrations tend to protect the red blood cell from hemolysis induced by the preservatives (16, 21). It is believed that this interference is cellular-based, rather than the result of an interaction between the preservative and DMSO (21).

The purpose of the present investigation was to study the effects of DMSO against certain common Gram-negative and Gram-positive microorganisms. This type of information would be essential to the effective preservation of future pharmaceutical products employing DMSO as a primary pharmacologic agent or pharmaceutical adjunct. It was hoped that this initial study would provide valuable basic information on the ability of DMSO to act as a preservative itself, and that later studies would relate this activity to preservative-DMSO combinations.

The scope of the present study was to determine the bacteriostatic and bactericidal concentrations of DMSO, to study the effect on the growth curve during the log phase, and to examine by means of electron microscopy any structural changes occurring with the microorganisms.

EXPERIMENTAL

Materials—The DMSO used for this study was experimental drug grade (Crown Zellerbach Corp., Camas, Wash.). The sterile growth medium employed was nutrient broth (Difco Corp., Detroit, Mich.). The cultures of *Escherichia coli, Pseudomonas aeruginosa,* and *Bacillus megaterium* were laboratory stock strains obtained from the Department of Microbiology, University of Georgia.

Inhibition of Growth by DMSO—An inoculum was prepared by introducing a loopful of bacteria into a test tube containing 10 ml. of 1.6% nutrient broth. This mixture was incubated at 37° for 24 hr., after which time 1.0 ml. of the suspension was pipeted into 99.0 ml. of the nutrient broth. The 1:100 dilution thus formed was used as the inoculum for the experimental tubes.

Duplicate pairs of tubes, each containing 2.5 ml. of a combination of DMSO and distilled water were prepared such that the final volume (after the addition of inoculum) contained DMSO concentrations ranging from 0 to 20%, at 1.0% increments. The tubes were plugged with cotton and autoclaved at 15 p.s.i. for 15 min. After cooling, each of the experimental tubes was inoculated with 2.5 ml. of the prepared inoculum. Tubes containing sterile media and the various percentages of DMSO were concurrently run with the inoculated tubes and served as experimental blanks. Inoculated tubes containing growth media but no DMSO served as experimental controls. All tubes were incubated for 24 hr. at 37°.

After incubation the tubes were agitated to insure a uniform bacterial suspension. The turbidity of each tube was measured using a colorimeter[1] with a blue filter which allowed light of a wavelength of approximately 540 mμ to pass through the bacterial suspensions (22).

For each set of duplicate pairs of tubes, the colorimetric reading of the optical density of the blank tube was subtracted from the average reading of the inoculated tubes. The value thus obtained for the control tubes (containing 0% DMSO) was assumed to represent 100% growth of the bacteria. The ratio of the average value of each of the other duplicate pairs of tubes to the value for the control tubes was used to calculate percentage growth. From this the percent inhibition attributable to the presence of DMSO was determined.

Growth Curves—An inoculum was prepared by introducing one loopful of bacteria into 10 ml. of nutrient broth, and incubating 24 hr. at 37°.

Immediately, and at various intervals (usually every 2 hr. during a 6-hr. period), 1.0-ml. portions of the inoculated broth were withdrawn. Appropriate dilutions (from 1:100 to 1:10,000,000) were made using sterile distilled water, and the organisms plated on 100 × 15-mm. Petri plates containing nutrient broth with 1.5% agar. The plates were incubated 24 hr. at 37°, after which time the

[1] Klett-Summerson Photoelectric colorimeter.

colonies were counted. The number of colonies on each plate were multiplied by the dilution factor to obtain the number of viable bacteria per milliliter.

Determination of Bactericidal Concentration of DMSO—An inoculum was prepared by introducing one loopful of bacteria into 10 ml. of nutrient broth, and incubating 24 hr. at 37°. Sterile solutions of various concentrations of DMSO and distilled water were prepared. Small volumes of the inoculum (0.5 ml.) were pipeted into 10-ml. tubes containing the various DMSO solutions, and the tubes agitated thoroughly in order to insure complete exposure of the bacterial cells to the DMSO solutions. The tubes were then incubated for 20 min. at 37°.

At the end of the incubation period the tubes were centrifuged for 5 min. at about 2,500 × *g*, the supernatant liquid removed, and the cells washed twice with 10-ml. portions of sterile saline solution. Finally the cells were resuspended in 1.0 ml. of saline solution and plated in Petri dishes containing nutrient agar. The plates were incubated for 24 hr., and examined for the presence of growth.

Electron Microscopy—Cultures of *E. coli* grown in or exposed to various concentrations of DMSO were prepared for electron microscopy according to the Kellenberger method (23). Thirty milliliters of a suspension of the bacteria was mixed with 3 ml. of Kellenberger fixative, which contains 1.0% osmium tetroxide in a veronal-acetate buffer.[2] The mixture was centrifuged at once for 5 min. at 1,800 × *g*. The pellet produced was then suspended in 1.0 ml. of Kellenberger fixative containing 0.1 ml. of 1.0% tryptone medium, and allowed to remain overnight at room temperature. The suspension was then diluted with 8.0 ml. of Kellenberger buffer,[3] and centrifuged 5 min. at 1800 × *g*. The pellet was resuspended in 1 or 2 drops of warm 2.0% agar, and the drop quickly poured onto a glass slide. After the drop hardened, the agar containing the fixed bacteria was cut into 1-mm.[3] blocks, and washed for 2 hr. in a solution containing 0.5% uranyl acetate in Kellenberger buffer.

The blocks of agar were next dehydrated by exposure to baths of increasing concentrations of alcohol. After final dehydration by exposure to absolute alcohol, the blocks were treated with propylene oxide, infiltrated with a 50–50 mixture of propylene oxide and a condensation resin,[4] and finally embedded in the resin with polymerization carried out at 60° for 48 hr.

The hardened blocks were sectioned with an ultramicrotome (LKB 4801-A), and sections mounted on copper grids. An electron microscope (JEOL 6C) was used for examination of sections. Pictures were photographically enlarged as noted in the figures.

RESULTS AND DISCUSSION

Bacteriostatic Activity of DMSO—Table I shows the inhibition of growth of three species of bacteria by various concentrations of DMSO. Inhibition increased sharply as percent DMSO was increased. Thirteen percent DMSO virtually eliminated growth of each of the bacterial species, and for *Pseudomonas aeruginosa*, about 92% inhibition occurred at a DMSO concentration as low as 8%.

Jacob *et al.* (6) claimed that DMSO is bacteriostatic in a 20% concentration against several species of organisms. However, the methods used and the data obtained in these experiments were not published and were not made available upon request. A more recent study by Seibert *et al.* (24) showed DMSO to be bacteriostatic in 25% concentration *in vitro* against a variety of unidentified acid-fast bacteria isolated from leukemic and cancer patients. Pottz *et al.* (25) investigated the effect of DMSO on the antibiotic sensitivity of various microorganisms, including *E. coli*, *P. aeruginosa*, *Staphylococcus aureus*, and others. The bacteriostatic concentrations of DMSO against these organisms were determined by the standard tube dilution method, using brain-heart infusion broth, and found to range from 5 to 10%. It was also found that DMSO does not affect the susceptibility of the microorganisms to various antibiotics, altering neither sensitivity nor resistance of the bacteria.

Table I—Inhibition of Three Species of Bacteria by Various Concentrations of DMSO After 24 hr. at 37°

% DMSO[a]	Average % Inhibition[b]		
	E. coli	*P. aeruginosa*	*B. megaterium*
1	7.63	7.50	14.40
2	18.70	6.70	16.00
3	15.63	26.80	18.70
4	17.25	35.40	25.00
5	23.35	66.90	31.60
6	39.45	71.60	38.17
7	52.70	89.90	43.70
8	68.00	92.17	56.90
9	77.10	92.26	56.90
10	87.15	96.38	81.32
11	87.45	98.07	79.90
12	90.77	98.41	83.07
13	98.16	97.81	90.62
14	99.50	97.53	93.97
15	98.30	97.61	98.30
20	98.50	96.57	100.00

[a] In the presence of 1.6% nutrient broth. [b] Each figure represents the average of two to five experiments.

Ansel and Leake (16), in their study of the hemolytic activity of DMSO, concluded that DMSO freely penetrates the erythrocyte membrane and causes osmotic hemolysis unless the cells are protected by an extracellular material such as sodium chloride. Even then the red blood cells lyse as the DMSO concentration approximates 25%.

In comparing the responses to DMSO of red blood cells and of the bacteria studied in the present report, it can be said that both types of cells are sensitive and can be destroyed by DMSO.

While an explanation of the mechanism of the bacteriostatic activity of DMSO is beyond the scope of this investigation, it appears from the above findings, and from the unique solvent characteristics of dimethyl sulfoxide, that DMSO is capable of penetrating cells, and in some manner altering the bacterial cell so that division of the cells is greatly reduced.

Effect on the Growth Curve of Bacteria—The effect of DMSO on the growth curve of *E. coli* is shown in Fig. 1. Comparable results were obtained using *P. aeruginosa* and *B. megaterium*. At 5% DMSO, only a slight reduction in growth rate was observed over a 6-hr. period. At 10% DMSO, however, the growth rate was substantially decreased, and in fact the number of viable bacteria decreased to a slight extent over a 6-hr. period.

Fowler and Zabin (26), in their investigation of the effects of DMSO on the lactose operon in *E. coli*, found that growth rate and β-galactosidase formation were inhibited to the same extent by DMSO. These results were interpreted as indicating that the differential rate of synthesis of the enzyme remained the same when the cell was exposed to varying low concentrations of DMSO. The activity of another enzyme, thiogalactoside transacetylase, was also unaltered by low concentrations of DMSO. The authors concluded that while the permeability barrier of the bacterial cell is reduced by low concentrations of DMSO, it is probable that irreversible effects on other components of the cell do not occur.

Bactericidal Activity of DMSO—Table II shows that high concentrations of DMSO (70–75%) are required to prevent regeneration of *E. coli* and *P. aeruginosa* exposed to DMSO for 20 min. *Bacillus megaterium* cultures remained viable even after 20-min. exposure to 100% DMSO. Since *Bacillus* species are known to form

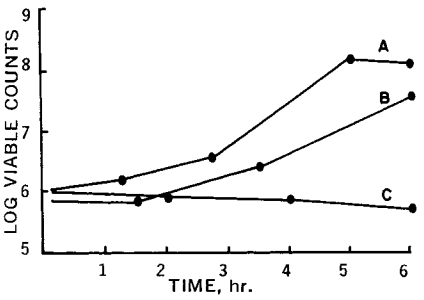

Figure 1—*Effect of DMSO on growth of* E. coli. *Key:* A, *0% DMSO (control);* B, *5% DMSO;* C, *10% DMSO.*

[2] Each 100 ml. of buffer solution contains 2.94 g. of sodium veronal, 1.94 g. of sodium acetate, and 3.40 g. of sodium chloride in distilled water.

[3] Each 100 ml. of buffer solution contains 20 ml. of veronal-acetate buffer, 28 ml. of 0.1 *N* HCl, and 1.0 ml. of 1 *M* calcium chloride in distilled water.

[4] "Epon 812," Shell Chemical Co., New York, N. Y.

Table II—Minimum Bacteriostatic Concentrations of DMSO for Three Species of Bacteria After 20-min. Exposure at 37°

	% DMSO in Distilled Water
Escherichia coli	70
Pseudomonas aeruginosa	75
Bacillus megaterium	—

spores which are generally resistant to heat and chemical action, it is likely that *B. megaterium* is protected from the effects of DMSO by its ability to form spores.

Electron Microscopy—In order to further investigate the actions of DMSO on bacteria, cultures of *E. coli* were exposed to several concentrations of DMSO for various periods of time. Figure 2 shows thin sections of *E. coli* grown in nutrient broth for 24 hr. in the absence of DMSO. The cell wall and plasma membrane are evident, and nuclear material appears as light areas within the cells.

Figure 3 shows a longitudinal and a transverse section of two bacteria which were grown in the presence of 5% DMSO during a 24-hr. period. The extracellular material surrounding the bacteria is believed to be a precipitate resulting from a reaction between DMSO and the fixative used, osmium tetroxide, as noted from the

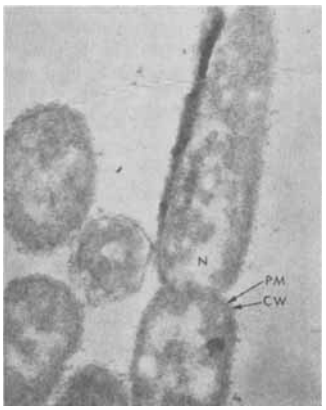

Figure 2—E. coli *grown in absence of DMSO during a 24-hr. period. 122,400×. Key:* CW, *cell wall;* PM, *plasma membrane;* N, *nucleoplasm.*

appearance of a black precipitate when the two reagents were mixed. The most noticeable difference in the appearance of these cells when compared to those in Fig. 2 is increased electron density of the cell wall. Montes *et al.* (27) in investigating ultrastructural changes occurring in the horny layer of guinea pig skin following treatment with DMSO, suggested that osmium binding during fixation might be influenced by DMSO, in that good fixation of the granular layer results with osmium after DMSO application. From Fig. 3 it would seem, then, that dimethyl sulfoxide becomes associated with the cell wall, and that when osmium is added an electron-dense cell wall material-DMSO-osmium complex is formed.

Figure 4 shows the effects of 10% DMSO on *E. coli* after an exposure period of 24 hr. Note that the cell wall is intact, but the differential staining of the wall is not apparent, as it is in Fig. 3. DMSO has been reported to be capable of crossing biologic membranes and causing morphological changes in tissues (1, 27). The cytoplasm of the bacteria in Fig. 4 appears to have been altered. Nucleoplasm is not as apparent as in the control cells, and empty spaces appear where either the cytoplasm has separated from the

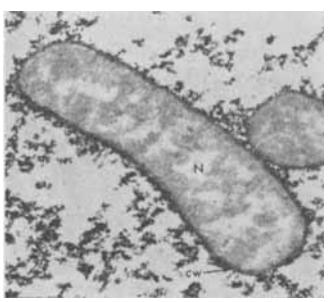

Figure 3—E. coli *grown in the presence of 5% DMSO for 24 hr. 92,400×. Key:* CW, *cell wall;* N, *nucleoplasm.*

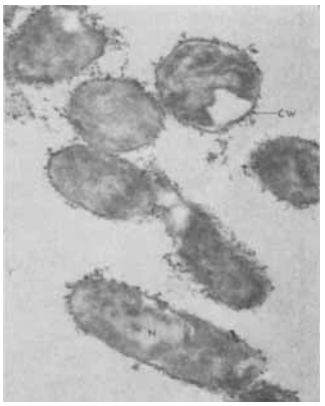

Figure 4—E. coli *exposed to 10% DMSO for 24 hr. 72,000×. Key:* CW, *cell wall;* N, *nucleoplasm.*

plasma membrane, or the membrane has separated from the cell wall.

Finally, Fig. 5 shows the effects of the bactericidal concentration of DMSO, 75%, on *E. coli* following an exposure period of 20 min. The upper portion of the cells is partially destroyed, probably as a result of the angle of the cell to the sectioning knife of the ultramicrotome. Again the cell wall appears intact, but the cytoplasm has taken on a granular appearance, and nuclear material is barely discernible.

In conclusion, this study has shown that DMSO possesses antimicrobial activity, and that it is capable of causing ultrastructural changes in bacteria. It is hoped that this information will be useful, not only in the future use of DMSO as a medicinal agent, but also in its ever increasing use as an adjunct in the study of bacteria and other cells.

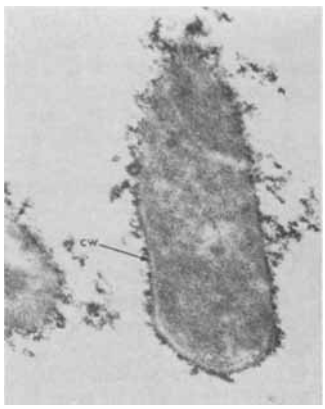

Figure 5—E. coli *exposed to 75% DMSO for 20 min. 122,500×. Key:* CW, *cell wall.*

REFERENCES

(1) G. E. Schumacher, *Drug Intelligence*, **1,** 188(1967).

(2) L. H. Block, *Drug Cosmetic Ind.*, **95,** 342(1964).

(3) "Biological Actions of Dimethyl Sulfoxide," *Ann. N. Y. Acad. Sci.*, **141,** 1(1967).

(4) N. Kharasch and B. S. Thyagarajan, *Quart. Rept. Sulfur Chem.*, **1,** 1(1966).

(5) "Dimethyl Sulfoxide," Bulletin, Crown Zellerbach Corp., Camas, Wash., 1962.

(6) S. W. Jacob, M. Bischel, and R. J. Herschler, *Current Therap. Res.*, **6,** 134(1964).

(7) S. W. Jacob, M. Bischel, G. A. Eberle, and R. J. Herschler, *Federation Proc.*, **23,** 2(1964).

(8) S. W. Jacob, M. Bischel, and R. J. Herschler, *Current Therap. Res.*, **6,** 193(1964).

(9) A. Horita and L. J. Weber, *Life Sci.*, **3,** 1389(1964).

(10) R. B. Stroughton and W. Fritsch, *Arch. Dermatol.*, **90,** 512(1964).

(11) R. L. Dixon, R. H. Adamson, M. Ben, and D. P. Rall, *Proc. Soc. Exptl. Biol. Med.*, **118,** 756(1965).

(12) C. E. Huggins, *Proc. 9th Congr. Inst. Soc. Blood Transf., Mexico*, **1962,** 69(1964).

(13) C. E. Huggins, *Transf.*, **3,** 483(1963).

(14) H. M. Pyle and H. F. Boyer, *Federation Proc.*, **21**, 164(1962).
(15) J. E. Lovelock and M. W. A. Bishop, *Nature*, **183**, 1394 (1959).
(16) H. C. Ansel and W. F. Leake, *J. Pharm. Sci.*, **55**, 687(1966).
(17) H. C. Ansel, *Am. J. Hosp. Pharm.*, **21**, 25(1964).
(18) H. C. Ansel and D. E. Cadwallader, *J. Pharm. Sci.*, **53**, 169(1964).
(19) D. E. Cadwallader and H. C. Ansel, *ibid.*, **53**, 169(1964).
(20) H. C. Ansel, *ibid.*, **54**, 1159(1965).
(21) H. C. Ansel and G. E. Cabre, (1967), unpublished data.
(22) F. Kavanagh, "Analytical Microbiology," Academic Press, New York, N. Y. 1963, pp. 141–159.
(23) D. H. Kay, "Techniques for Electron Microscopy," Davis, Philadelphia, Pa., 1965, pp. 166–212.
(24) F. B. Seibert, F. K. Farrelly, and C. C. Shepherd, *Ann. N. Y. Acad. Sci.*, **141**, 175(1968).
(25) G. E. Pottz, J. H. Rampey, and F. Benjamin, *ibid.*, **141**, 261(1968).
(26) A. V. Fowler and I. Zabin, *J. Bacteriol.*, **92**, 353(1966).
(27) L. F. Montes, J. L. Day, C. J. Wand, and L. Kennedy, *J. Invest. Dermatol.*, **48**, 184(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 24, 1968, from the *School of Pharmacy, University of Georgia, Athens, GA 30601*

Accepted for publication December 11, 1968.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

This investigation was supported by the Office of General Research, University of Georgia.

* Participant, Undergraduate Research Program, School of Pharmacy, University of Georgia.

† Department of Microbiology, University of Georgia.

Plant Antitumor Agents III: A Convenient Separation of Tannins from Other Plant Constituents

MONROE E. WALL, HAROLD TAYLOR, LINDA AMBROSIO, and KENNETH DAVIS

Abstract □ Several methods were tested for the nondestructive separation of tannins from other plant constituents which might have antitumor activity. A comparison was made of precipitation of tannins by caffeine or lead acetate and chromatography on polyamide. The caffeine procedure was preferred over lead acetate; both tannins and nontannins were effectively separated and the caffeine conveniently removed. Polyamide was an effective tannin remover; nontannins were easily collected but tannins were irreversibly adsorbed. The application of the caffeine procedure to a variety of plants is described.

Keyphrases □ Tannin separation—antitumor plant constituents □ Caffeine—tannin precipitation □ Lead acetate, caffeine precipitation, chromatography—tannin separation comparison

Tannins are a ubiquitous plant constituent (1) and are quite soluble in ethanol, aqueous 50% ethanol, or water. For some years the Walker 256-carcinosarcoma tumor has been used by groups fractionating plants for antitumor agents under the Cancer Chemotherapy National Service Center program.[1] A variety of alcohol and water-soluble substances inhibit growth of this tumor in rats, including tannins, saponins, and certain cardiac glycosides, and other less well-defined substances. In some cases the initial activity may be due to mixtures of the above substances. Accordingly, a convenient, specific, and nondestructive method which would separate tannins from other constituents would be useful and is the subject of this report.

EXPERIMENTAL

Seven kilograms of air-dried whole plant[2] (root, stem, leaf, and flowers) was continuously extracted with 95% ethanol in a continuous, pilot-plant scale extractor (continuous hot-percolation type) for 48 hr. After concentration of the alcohol *in vacuo* to a syrup, the latter product was partitioned between chloroform and water (4 l. water extracted three times with 2 l. chloroform containing 10% ethanol). The aqueous fraction was freeze dried; the chloroform fraction was evaporated to a syrup *in vacuo*. Figure 1 gives the extraction data and 5WM activities of the various fractions.

Countercurrent Concentration—Two-hundred and fifty grams of the aqueous fraction (Fig. 1) was partitioned in a system consisting of ethyl acetate–methanol–water, 2:1:2; the phases were mutually saturated and equal volumes of upper and lower phase used in a 10-tube countercurrent distribution instrument.[3] A bank of ten, 4-l. separators set up containing 1,800 ml. of upper and lower phase, respectively. The freeze-dried water-soluble fraction from Fig. 1 was dissolved in the lower phase of this system, shaken, and after separation transferred to the next tube containing 1,800 ml. of lower phase. A similar quantity of upper phase was added to the first funnel and the partition continued for a total of 10 tubes. Volatile solvent was removed from upper and lower phases *in vacuo* at 40°; the residual upper-phase material in each tube was added to the corresponding lower phase and water removed by freeze drying. The results are shown in Table I. Fractions 6–9, Table I, all gave strong qualitative tannin tests [ferric chloride, salt-gelatin, method of Wall *et al.* (1)]. Fraction 7 was arbitrarily chosen for comparison of the various tannin-removal procedures.

Lead Acetate Precipitation—Ten grams of Fraction 7 in 100 ml. of water was treated with 30 ml. of neutral lead acetate aqueous solution (23%). The precipitate was centrifuged and suspended in methanol. The suspension was treated with a large excess of H_2S gas and the lead sulfide centrifuged. The residual solution was evaporated *in vacuo* and the residue taken up in water and freeze

[1] The procedure for this assay is described in *Cancer Chemotherapy Rept.*, **25**, 1(1962).

[2] The authors wish to thank Dr. Robert E. Perdue, Jr., New Crops Research Branch, ARS, USDA, Beltsville, Md., for all the plant material described in this paper.

[3] Craig CCD, H. O. Post Scientific Instruments Co.