be detected only under anaerobic conditions at pH 9 where the competing reactions leading to the formation of the dioxo compound and the keto acid were suppressed. Quantitative studies of the kinetics of the anaerobic hydrolysis are presently being conducted and will be reported at a later time.

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Proteinaceous Antitumor Substances from Plants II

Mirabilis multiflora

By AYHAN ULUBELEN* and JACK R. COLE

Preliminary screening of the aqueous extract of *Mirabilis multiflora* (*Nyctaginaceae*) has shown antitumor activity against Sarcoma 180 in mice. The isolation, purification, and partial characterization of proteinaceous material is reported. This material has shown activity against Lewis lung carcinoma, Walker carcinosarcoma 256 (intramuscular), and lymphosarcoma.

As a result of a routine screen of southwestern plants for potential antitumor activity, the aqueous extract of *Mirabilis multiflora*[1] (*Nyctaginaceae*) Britton and Rusby demonstrated activity toward the Sarcoma 180 test system in mice.[2] This screening program was carried out by the Cancer Chemotherapy National Service Center (CCNSC), Bethesda, Md. Further fractionation has resulted in activity in the Lewis lung carcinoma, Walker carcinosarcoma 256 (intramuscular), lymphosarcoma, and Sarcoma 180 test systems.

The plant is an herbaceous perennial with a large tuberous root. The above-ground portion of the plant dies during freezing weather or extended drought and regrowth occurs from subterraneous parts of the plant.

Part of the collection used in this study was obtained on rocky slopes about 5 miles south of St. David, Ariz., at an elevation of about 3,700 ft., May 31, 1962. Another part of the collection was obtained at the Boyce Thompson Southwestern Arboretum near Superior, Ariz., on July 1, 1964. It was in cultivation in a sandy loam soil within a grove of eucalyptus.

EXPERIMENTAL

Fresh roots of *M. multiflora* (3.5 Kg.) were extracted with approximately 15 L. of petroleum ether followed by 15 L. of water at room temperature. The water solution was then washed with benzene and chloroform and lyophilized in a Repp Industries model 15 sublimator. The yield was 320 Gm. One-hundred grams of this crude material was dissolved in 1000 ml. of water at room temperature. The insoluble part was separated by centrifuge and discarded (32 Gm.). The remaining aqueous portion was extracted with 5 × 200 ml. of ether to remove the remaining fatty materials. The aqueous solution was increased in volume threefold by the addition of 95% ethanol. A light-colored precipitate formed and was separated by centrifuge. It was dissolved in water and lyophilized. Twenty-one grams of the material was obtained. This crude material showed an activity against the Sarcoma 180 test system of the CCNSC. The requirement for activity in this system is two successive dose response tests showing a reduction of at least 56% in tumor size. The crude material showed a decrease in tumor size of 84% at a dose of 22 mg./Kg. The elementary analysis of this material showed 13.4% inorganic material which consists mostly of magnesium and phosphate ions and also the presence of sulfur and nitrogen. Upon hydrolysis of the material a number of amino acids were obtained. Two grams of the crude material was dissolved in water and dialyzed against distilled water, using a dialyzing tube of $^5/_8$ in. diameter (Arthur H. Thomas Co., No. 4465-A2, Philadelphia, Pa.). After a period of 7 days, the precipitate in the dialysis tube was separated from

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[1] Identification confirmed by Robert Barr, Research Associate, College of Pharmacy, and Dr. Charles Mason, Curator of the Herbarium, Botany Department, University of Arizona, Tucson. A reference specimen was also deposited.
[2] Preparation of the preliminary extraction was carried out by Dr. M. E. Caldwell.

TABLE I.—*In Vivo* TUMOR INHIBITION[a]

	Dose, mg./Kg.	% T/C[b]
Lewis lung carcinoma	12	38
P-1798 Lymphosarcoma	12	22
	12	22
	8	55
	5.3	72
	3.5	53
	40	...
	27	...
	18	...
	12	33
Sarcoma 180	10	8
	10	11
	4.4	44
	2.9	63
Walker carcinosarcoma 256 (intramuscular)	45	39
	12	58
	8	79
	5.3	73
	3.5	69

[a] "Protocols for Screening Chemical Agents and Natural Products Against Animal Tumors and Other Biological Systems," Cancer Chemotherapy National Service Center, *Cancer Chromatography Rept.*, No. 25, December 1962. [b] The criteria for activity is defined as being a per cent T/C (test/control) value of less than 42 in a satisfactory dose response test.

TABLE II.—TUMOR INHIBITION OF SEPHADEX-TREATED FRACTIONS

Sephadex	Dose, mg./Kg.	% T/C
G-50	10	13
G-100	10	29
G-200a	10	15
G-200b	10	20

the solution. The solution was then lyophilized, with a yield of 957.7 mg. (47.8%) which contained only 2.1% inorganic compounds. The lyophilized material (compound *A*) was subjected to screening against several test systems. The results are indicated in Table I.

A series of column chromatographic purifications were attempted. They included column substrates of DEAE Sephadex A-50, CM Sephadex C-50; G-50, G-100, and G-200 Sephadex. The first two substrates were ineffective, whereas the latter three were successful. A series of fractions were collected and the presence of protein was indicated by means of absorption at 280 mμ in an ultraviolet spectrophotometer. One protein fraction was obtained from the G-50 and G-100 Sephadex columns and two different protein fractions were obtained from the G-200 Sephadex column. After subsequent dialysis and lyophilization, the materials obtained were submitted to tests on Sarcoma 180. The results are tabulated in Table II. Comparison of Tables I and II shows that essentially no increase in activity was obtained by purification techniques.

Physical and Chemical Characteristics of Compound A.—Paper electrophoresis of compound *A* was run utilizing a barbital buffer of pH 8.6, 0.05 ionic strength in a Spinco model instrument, and after 18 hr. of electrophoresis using a 5-ma. current, the papers were developed by two different procedures. Bromophenol blue indicated the presence of two proteins. One appeared at the point of application and the other had a mobility of 6.6×10^{-7} cm.²/sec. v. (Fig. 1). The second development procedure used was periodic acid and Schiff reagent which indicated a glycoprotein at the application point. The closed aldehyde groups in the polysaccharide conjugate are oxidized by periodic acid, then fuchsin sulfite stains the polysaccharides. Other substances such as glucose, glycogen, and the amino acids, serine and threonine, can be stained with fuchsin sulfite, but they are removed in the prerinse.

The above would indicate that compound *A* is a combination of a glycoprotein and protein. The two proteins were separated by a Sephadex G-200 column using a series of phosphate buffer systems pH 8.6, ionic strength 0.075. The compound was then hydrolyzed with 6 *N* hydrochloric acid in a sealed and evacuated glass tube at 105°. The resulting solution was applied on Whatman No. 1 paper using the same solvent system reported in a previous paper (1). Figure 2 shows the presence of 22 amino acids and perhaps some polypeptide residues.

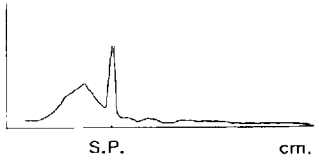

Fig. 1.—Absorbance relationship of the proteins. One unit of integration = 0.1 cm.². Key: SP, starting point.

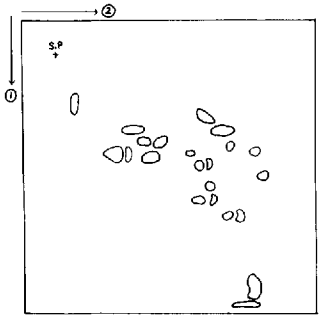

Fig. 2.—Paper chromatography of hydrolyzed compound *A*. Key: SP, starting point; 1, phenol–water (3:1); 2, butanol–formic acid–water (7:1:3).

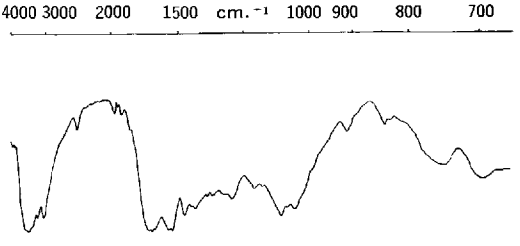

Fig. 3.—Infrared spectrum of compound *A*.

TABLE III.—PRELIMINARY AMINO ACID ANALYSIS OF *Mirabilis* PROTEIN[a]

Lysine	5.74%
Histidine	1.09
Arginine	4.32
Aspartic acid	12.49
Threonine	6.44
Serine	4.95
Glutamic acid	8.00
Proline	3.45
Glycine	6.10
Alanine	2.95
Half-cystine	6.54
Valine	5.24
Methionine	0.89
Isoleucine	6.91
Leucine	4.89
Tyrosine	6.54
Phenylalanine	4.62

[a] Based on three determinations of the dried sample.

One-hundred milligrams of compound *A* was hydrolyzed with 3 *N* hydrochloric acid in a boiling water bath for 24 hr. in order to determine the sugars. Using Whatman No. 1 paper and the solvent system *n*-butanol–pyridine–water (6:4:3) and comparing with standard sugar samples the following were detected: galactose, mannose, galactosamine, and fucose. The latter two were present in very small amounts. Two additional solvent systems were employed to verify the presence of the above sugars. They were (*a*) ethyl acetate–acetic acid–ethanol–benzene–water (325:93:236:200:146) and (*b*) *n*-butanol–acetic acid–water (4:1:2).

Ultraviolet absorption of compound *A* showed maxima at 280, 243, and 210 mμ. Infrared curve shows characteristic peaks at 3350, 3150, 3030, 2900, 1600, and 1535 cm.$^{-1}$. (See Fig. 3.) Nitrogen determination of compound *A* using microkjeldahl method yielded 14.06% nitrogen. Results of a preliminary amino acid analysis utilizing a Beckman model 120B instrument are shown in Table III.

DISCUSSION

In the work being carried out in this laboratory, glycoproteins have been found on several occasions to be antitumor agents.

A review of the literature has shown a number of publications concerning the relationship of malignant tumors and the increase of glycoprotein concentration in the blood of test animals and humans. There are, however, several varied opinions as to the cause of this increase. Seibert and co-workers (2) reported that the source of glycoproteins was the breakdown of the products of tissue necrosis. Several other workers (3–5) suggested complex mechanisms involving increased hepatic synthesis and release of glycoproteins into the circulation in response to an appropriate stimulus which has a tumor or inflammation. Patterson *et al.* (6) suggest the increase in glycoprotein is a function of bacterial contamination. Other groups of workers (3, 7, 8) reported that this increase is due to a liberation of products of the metabolic activity of rapidly reproducing cells. Catchpole (9) has found a high concentration of glycoprotein in the neighboring tissues of a malignant tumor. Therefore, this could be the depolymerization of the ground substance of this adjacent connective tissue with subsequent release into the circulation. Engel (10), Gilmore and Schwarz (11) are in accord with this theory. The carbohydrate portion of these serum glycoproteins has been shown to contain mannose, galactose, glucosamine, fucose, and sialic acid (12, 13). Macbeth *et al.* (14–18), in a private communication (19), indicated that the liver is the most active agent in the synthesis of these glycoproteins. By using isotopic techniques they also have shown that the tumor system is capable of synthesizing and subsequently liberating glycoproteins into the blood stream. This work was done on intact and hepatectomized rats.

The authors believe that further investigation should be carried on to determine whether the introduction of a certain type of a plant glycoprotein into tumor-implanted animals may result in an inhibition of the synthesis and a reduction of the serum glycoprotein followed by a reduction in overall tumor size. In addition to the two plants mentioned above, there are three other plants under investigation in our laboratories which appear to have glycoproteins as the active antitumor agents.

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