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Testing for Tumor Promoters in *Euphorbia lathyris:* Analysis of Possible Health Hazards

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The growing of green plants as a renewable energy source is attracting increasing interest as a viable source for liquid fuels or chemical feedstocks (CALVIN 1980; COFFEY and HALLORAN 1979). Euphorbia lathyris has been considered as one of the potential hydrocarbon producing plants, and the production of two liquid fuels, gasoline and ethanol, from the crude plant extracts has been demonstrated (NEMETHY et al. 1981). This latex-producing plant grows wild in California and has also been successfully cultivated.

The milky latex of E. lathyris is rich (50% of dry weight) in reduced triterpenoids (NIELSEN et al. 1979); however, it also contains a minor amount (0.1% of dry weight) of esters of the tetracyclic diterpenol, ingenol, which are closely related to the most active components of croton oil, 12-O-tetradecanoyl-phorbol-13-acetate (TPA)* (ADOLF and HECKER 1975). These ingenol esters have been shown to be cocarcinogenic in the mouse skin test (ADOLF AND HECKER 1975). At that time ADOLF and HECKER cautioned that the plant's products, or food contaminated with the plant, should be considered as a potential cocarcinogenic risk.

Taking advantage of the known effects of TPA on cells in culture (BLUMBERG 1980), we have recently developed a sensitive test system for detection of unknown tumor promoters. In this paper, we describe the use of the system in detecting promoter activity in Euphorbia lathyris extracts before and after treatment with organic solvents in the preparation of synthetic fuels.

MATERIALS AND METHODS

Preparation of Latex and Plant Extracts. Three sets of Euphorbia lathyris extracts were prepared for comparative testing: a latex-derived active fraction from the fresh plant, composed of ingenol esters; two fractions of the dried plant obtained via hot

*Abbreviations: TPA, 12-O-Tetradecanoyl-phorbol-13-acetate; RSV, Rous sarcoma virus; PAT, primary avian tendon; CEF, chick embryo fibroblasts; wt and ts RSV, wild type and temperature-sensitive mutants of RSV; 2-dg, 2-deoxy-D-glucose; Tdr, thymidine; HPLC, high pressure liquid chromatography; MeOH, methanol.

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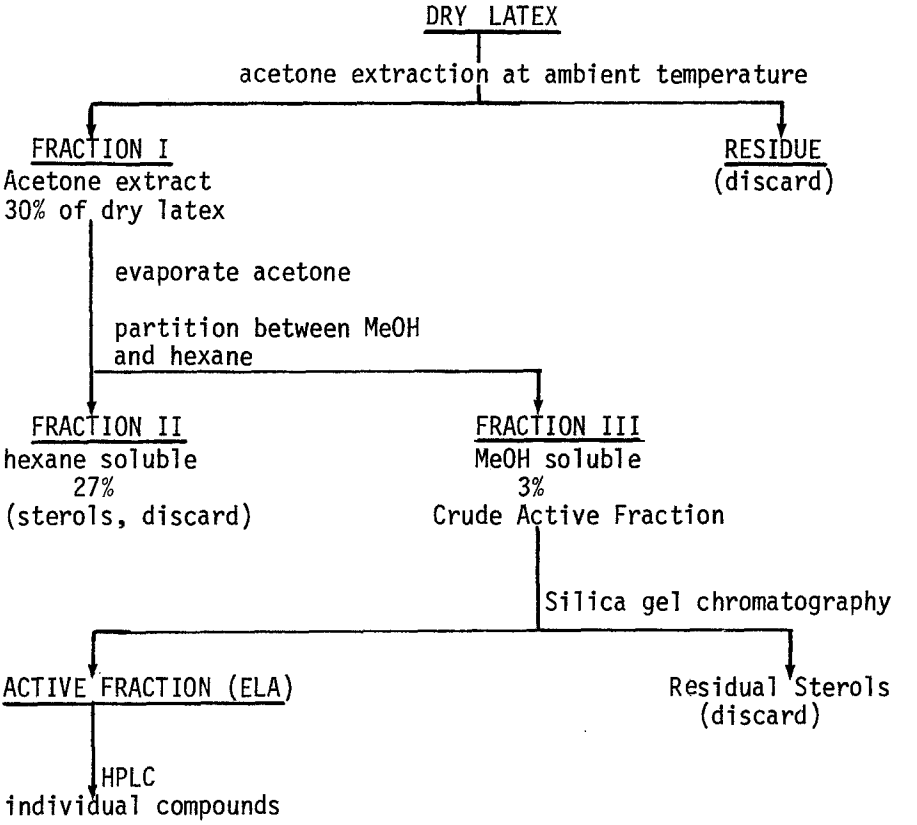
solvent extraction; and two analogous fractions of the dried plant obtained via the same solvent extraction, but at ambient temperature. The organic solvent extractions were performed at ambient temperature in order to test which processing step - the drying of the plant or the boiling solvent extraction destroys the cocarcinogenic activity. The dried plant extracts are the potential source of fuels; gasoline can be prepared from the heptane extracts by catalytic cracking and ethanol can be obtained from the methanol extract by fermentation (NEMETHY et al. 1981).

The preparation of the latex-derived fraction is shown in Scheme 1. The preparation of the dried plant extracts is shown in Scheme 2. The dried, ground plant material was continuously extracted with hot solvents to yield two fractions. Extraction with heptane yields the fraction composed of triterpenoids and sterols (NEMETHY et al. 1979), subsequent extraction with methanol yields a mixture composed mostly of simple sugars. Appropriate controls were prepared for each of the samples to be tested. The positive control for all samples was TPA, the best known and the most active tumor promoter. Since over 50% of the dry weight of the latex is a mixture of plant steroids, the control for the active fraction was chosen to be cholesterol (as a model compound) which was put through the same procedure as the dry latex (Scheme 1). The controls for the dried plant extracts were prepared from a common food crop, corn, which was treated the same as dried E. lathyris (Scheme 2).

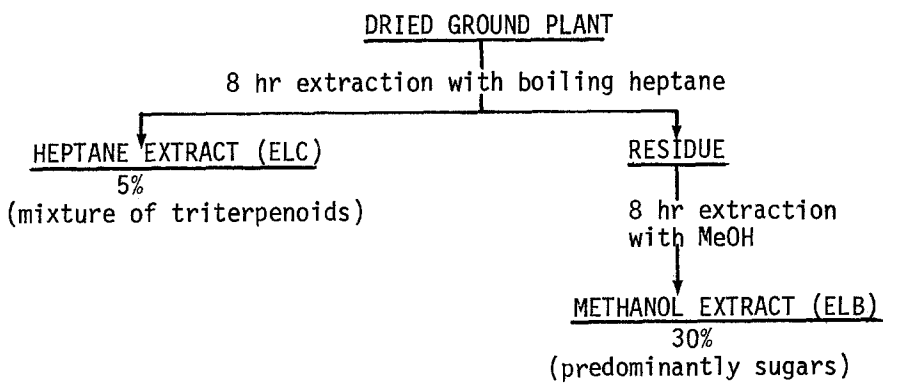
Cell Culture. Chick embryo fibroblasts (CEF) and primary avian tendon (PAT) cells were prepared as described (BISSELL et al. 1977; SCHWARZ and BISSELL 1977). Ascorbic acid was present at 50 g/ml in most experiments with tendon cells. However, since this vitamin has been shown to interfere with RNA virus replication (BISSELL et al. 1980), it was eliminated from experiments utilizing ts mutants until day 5 of culturing.

Virus Infection and Transformation. The wild type and the temperature sensitive mutant of Prague A-RSV (LA24) were focus purified further in our laboratory. The assay of focus forming units (RUBIN 1960) indicated a virus concentration of 10^7 transforming particles per ml of stock medium. Primary CEF cells were infected 4 hrs after seeding with a multiplicity of infection of .1 to .5. Secondary cultures were prepared on day 5 after seeding and cultures were kept at 39°C. In the case of the LA24-infected cells, 24 hrs after secondary seeding cells were moved either to 41° or 35° incubators. Most results reported were done on tertiary cultures seeded at 5×10^5 cells per 35 mm dish and shifted to the appropriate temperatures after 4 hrs at 39°. TPA was added 24 to 48 hrs later in MeOH which was diluted in medium F-12 or 199. The appropriate amount of MeOH was added to control cultures. PAT cells were seeded in the absence of ascorbate as described above and grown at 39°. Twenty-four hrs after seeding, cells were infected with LA24 at a multiciplicity of 1. Medium was changed daily. Cells were shifted to either 41° or 35° on day 5. Experiments were performed 2 to 3 days later.

SCHEME 1



SCHEME 2



Biochemical Assays. 2-Deoxy-D-glucose (2-dg) uptake and thymidine (Tdr) incorporation were performed as described previously (BISSELL et al. 1977) using tritiated compounds obtained from New England Nuclear. For collagen assay, cells were labeled with 50 μCi/ml [^{3}H]-proline (New England Nuclear) for 3 hrs and assayed as described (SCHWARZ and BISSELL 1977).

RESULTS

Basic hydrolysis of the active fraction, prepared according to scheme 1, yielded only one compound which was identified as ingenol triacetate by spectroscopic methods. Further analysis of this fraction by HPLC (Fig. 1) revealed a mixture of ingenol esters. This fraction was designated as ELA in our test system.

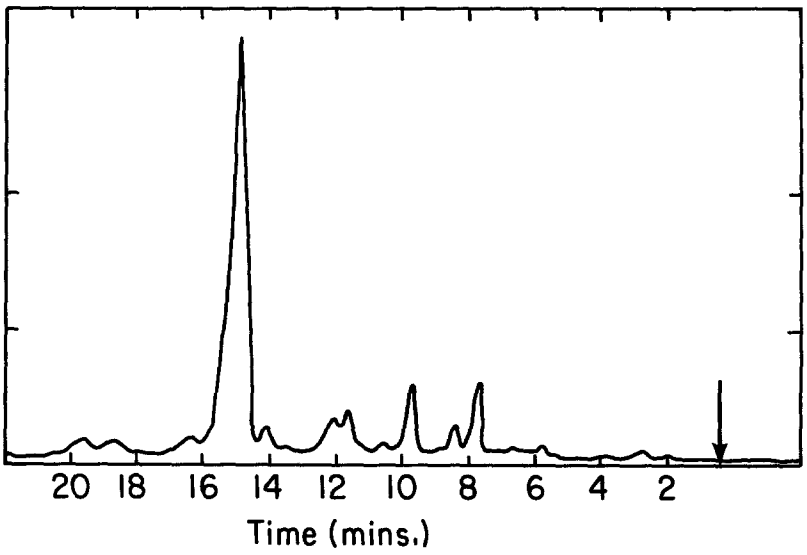


Fig. 1. HPLC trace of latex derived active fraction (ELA). Altex C_8 column. Mobile phase: MeOH:H_2O 1:1, Detection: 290nm.

Table I indicates the various compounds tested and their designations. The morphological effects of ELA and TPA on normal CEF cells are shown in Fig 2. The cross-hatched pattern, characteristic of TPA-treated cells, is seen in ELA-treated cultures as well. ELB and ELC, even at 20-fold higher concentrations, had little or no effect on the morphology of these cells. Thus, by crude morphological cirteria, ELA appears to be active, and ELB and ELC appear inactive. There were no overt toxic effects of these compounds on cells in culture as measured by either slight increase or slight inhibition (varying from experiment to experiment) on the rate of growth at densities higher than 2 x 10^5 cells per cm^2 (results not shown).

TABLE I

Compounds Tested and Their Designations

Compound	Explanation
ELA	Active Fraction I from latex (in MeOH)
ELA_c	Control for ELA, derived from corn (in MeOH)
ELB^c	Hot MeOH extract of dry plant (in MeOH)
ELB_c	Control for ELB, MeOH extract of corn according to Scheme 2
ELC	Hot heptane extract of dry plant (in acetone)
ELC_c	Control for ELC, heptane extract of corn according to Scheme 2
TPA	Positive control in MeOH
MeOH	Solvent control
Acetone	Solvent control
ELB_{cold}	The same as ELB, but extracted with cold MeOH
ELC_{cold}	The same as ELC, but extracted with cold heptane

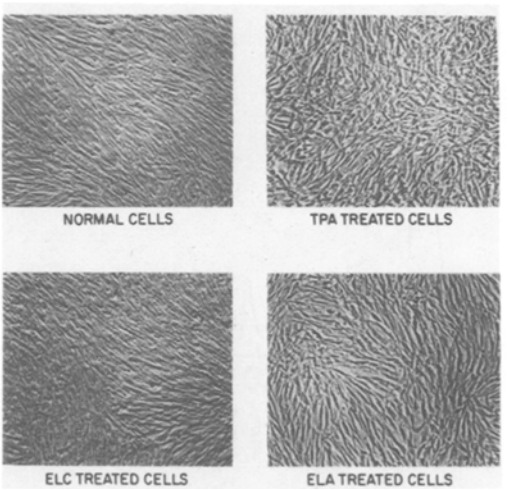


Fig. 2. The morphology of normal and treated cultures.

To quantitate the degree of activity of these fractions, we measured sugar uptake in these cultures. The increase in the rate of sugar uptake has been shown previously to correlate with the tumor promoter activity of TPA and its derivatives (BLUMBERG 1980). A time course of 2-dg uptake after TPA and ELA treatments (Fig. 3A) indicated that 12 hr treatments gave maximal effect. While the exact response curve was dependent on the density, serum concentration and the age of cultures, an 8 to 12 hr time was chosen for subsequent studies. A comparison of the rates of sugar uptake after treatment with ELA, ELB and ELC indicated again that ELA contained tumor promoter activities and that these were lost after drying and extraction (Fig. 3B).

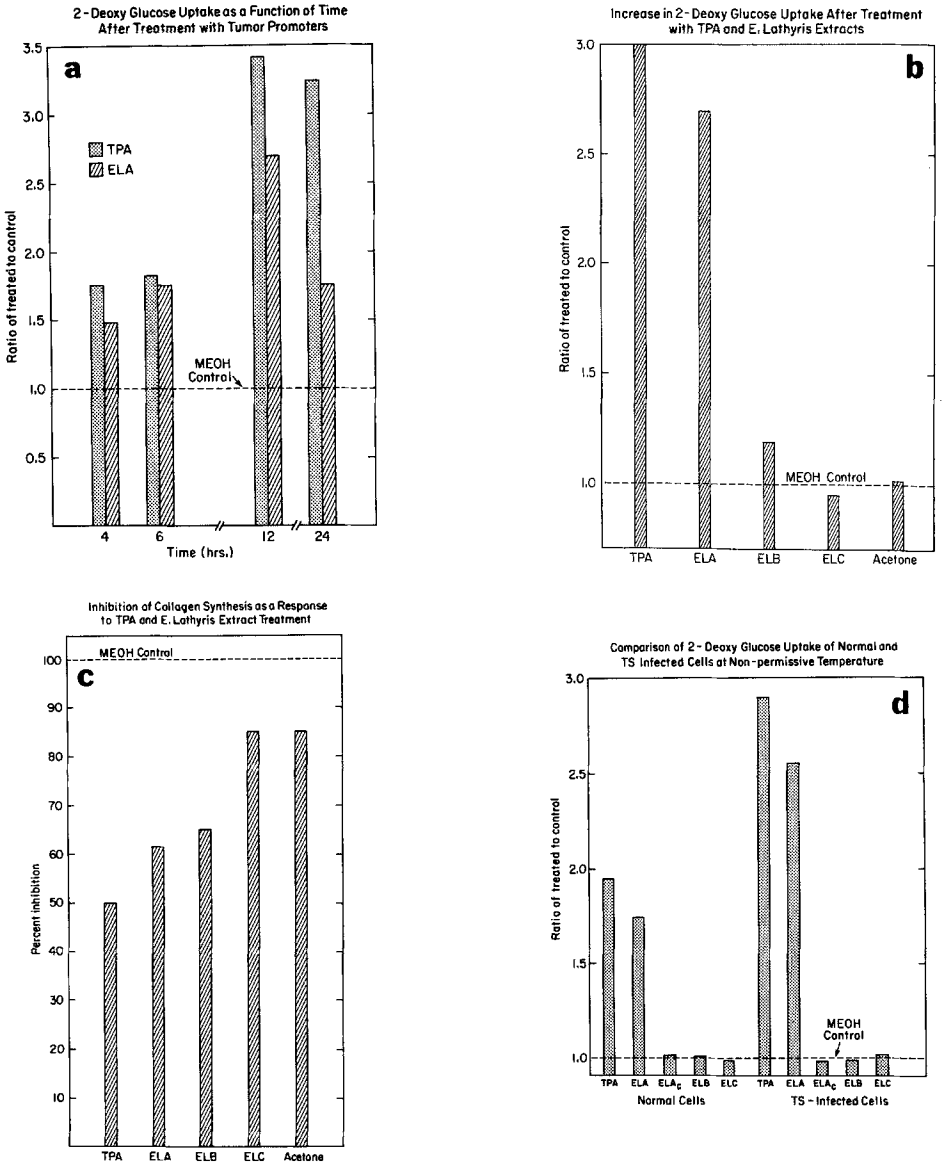


Fig. 3. The rate of glucose uptake and collagen synthesis were measured as described in Methods. Results are average of at least 6 separate experiments on duplicate plates.

A comparable pattern was observed when the rate of collagen synthesis was measured. Collagen synthesis in culture has been shown to be inhibited by TPA (DELCLOS and BLUMBERG 1979; BISSELL et al. 1979). While in this assay, ELB and ELC show some inhibition (Fig. 3C), this is probably due to other components in these fractions since treatment with their respective controls also leads to slight inhibition of collagen synthesis (Fig. 3C Nevertheless, ELB shows a higher level of inhibition than ELC. This may indicate either a slight residual activity in the ELB preparation, or the presence of another component which may specifically interfere with collagen synthesis and/or secretion. The latter component, however, is probably not a tumor promoter since ELB is inactive in the other assays for tumor promotion (Figs. 3B and D).

To differentiate further between possible contaminating amounts of tumor promoter activities left in fractions ELB and ELC we utilized a yet more sensitive test system. We have previously shown that chick cells, infected with temperature-sensitive mutants of Rous sarcoma virus and grown at non-permissive temperatures, constitute a more sensitive assay for detection of tumor promoter activity of phorbol esters (BISSELL et al. 1979). This is because these cells appear to be "initiated" and as such may be expected to have a more precise response to tumor promoters. Experiments performed under conditions where untreated controls are still growing (Fig. 3D), indicate that response to TPA and ELA is more pronounced in ts-infected cultures in addition to being above and beyond the growth response. Furthermore, ELB and ELC still appear inactive. The magnitude of response of normal cells in these experiments (Fig. 3D) is lower than the data shown in Fig. 3B. The reason for this apparent reduction is that the untreated control cultures which are still growing have a higher rate of sugar transport than cells which are serum deprived and density arrested. Nevertheless, the pattern of response remains the same.

In order to distinguish whether or not hot solvent extraction is essential for the loss of activity or whether the drying and the extraction procedure per se lead to the destruction (or leaving behind) of the active ingredients, a cold solvent extraction was performed as described in Methods. ELB_{cold} and ELC_{cold} were found also to be inactive in these assays (results not shown).

DISCUSSION

Since E. lathyris is native to California and can be cultivated in this climate, we have studied this member of the large family of Euphorbiaeceae as a liquid fuel producer. There are, however, other plant species which can be and are considered for similar use, some of which are also latex bearing species of the family Euphorbiaeceae. The latexes of several Euphorbias are often rich in reduced terpenoids; however, the irritant and cocarcinogenic principles are also prevalent in this family of plants (HECKER 1978). Euphorbia tirucalli, for example, is being actively investigated as a liquid fuel producer in Japan. The latex of this plant also contains esters of 4-deoxy phorbol which are irritant and cocarcino-

genic (FÜRSTENBERGER and HECKER 1977). Therefore, in view of the proposed utilization of these plants, it is essential to investigate the potential carcinogenic risks of these alternate energy sources.

While there are many systems for detection of primary carcinogens, the systems for detection of potential tumor promoters are scarce and are being developed only now. The classical model used by Berenblum which was influential in formulation of his two-stage model of carcinogenesis is the mouse skin (BERENBLUM 1975). Nevertheless, this system has the usual shortcomings of whole animal studies: it is not sensitive enough and it is extremely time consuming and expensive if one is to perform a statistically meaningful trial. Cell culture has proven a reliable substitute for structure-function studies of phorbol esters and its derivatives. For example, the extent of increase in 2-deoxyglucose uptake by chick embryo fibroblasts in culture correlates directly with the degree of tumor promoting activity on the mouse skin (BLUMBERG 1980). These cells, therefore, not only could be used to understand the mechanism of action of TPA but provide also a simple and reliable assay for detection of unknown tumor promoters.

Using both normal and ts-virus infected cells, we show here that we can detect tumor promoter-like activities in the latex of Euphorbia lathyris. We further show that these activities are absent after extraction of potential sources of fuel. Our results indicate that after mechanical harvesting and drying there should be no toxicological dangers. This finding has much potential significance, both for the further processing of E. lathyris and for other possible sources of energy.

ACKNOWLEDGEMENTS

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