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# Isolation of Toxic Metabolites of *Phomopsis leptostromiformis* Responsible for Lupinosis

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*Abstract*

Two metabolites of *P. leptostromiformis* (phomopsins A and B) have been isolated as a crystalline mixture from a culture of the fungus on lupin seed. The mixture has been shown to be capable of inducing lupinosis in sheep and in young rats. Key steps in the isolation were the transfer of the phomopsins from crude aqueous solution to tetrahydrofuran and chromatography on macroreticular polystyrene resin. The bioassays used in monitoring fractions were based on inhibition of cell cultures and the mitosis-arresting effect of the metabolites on liver cells *in vivo*.

## Introduction

Lupinosis is a chronic liver disease which commonly afflicts animals grazing lupin stubbles (Gardiner 1967*a*). It is a mycotoxicosis caused by the fungus *Phomopsis leptostromiformis* (Kuhn) Bubak ex Lindl. [syn. *P. rossiana* (Sacc.) Sacc. et D. Sacc] growing on the lupin roughage (van Warmelo *et al.* 1970; Gardiner and Petterson 1972; Marasas 1974). Studies aimed at isolation and chemical identification of the toxin have been in progress for some time and Petterson and Parr (1970), who achieved a measure of purification, concluded that it was probably phenolic or acidic, or both, in character.

This paper describes the chemical fractionation and bioassay of extracts of the fungus grown on lupin seeds. The methods described have led to the isolation of crystalline material with a strong mitosis-arresting effect in the liver of the nursling rat and able to induce the signs and pathology of acute lupinosis when administered to sheep by intraperitoneal injection. The crystalline toxin was shown by electrophoresis to be a mixture of two closely related compounds present in a ratio of approximately 4 : 1. The names phomopsin A and B are proposed for these two compounds, each of which was active in the nursling rat assay.

## Materials and Methods

*Fungal Cultures*

*Phomopsis leptostromiformis* (isolate WA1515 provided by P. McR. Wood, Department of Agriculture, W.A.) was maintained by periodic subculture on 1·5% malt extract agar. Toxic material for chemical isolation was produced by growing the fungus on sterile moist lupin seed (*Lupinus angustifolius* cv. Uniwhite) for 28–30 days at 24–25°C (cf. van Warmelo *et al.* 1970). Sterile distilled water was added at intervals during the incubation period to maintain a suitable seed moisture content.

The toxicity of the cultures was established by force-feeding a slurry of minced culture material to sheep. Four daily doses of 40 g of culture caused jaundice, anorexia and death within 6 days in sheep of weight 30–35 kg. Liver sections showed histopathological changes characteristic of acute lupinosis (Gardiner 1967*b*).

*Bioassays Used in the Monitoring of Chemical Fractionation*

In the early stages of the investigation, the mouse assay was the only method used. Cell culture assays were developed to allow larger numbers of samples to be assayed in a shorter time. The mouse assay was then used as a reference assay to confirm major steps indicated by the results of the cell culture assays. The nursling rat assay was developed as an improvement on the mouse assay for this purpose. It uses the high level of mitotic activity in the liver of the growing rat to enhance the mitosis-arresting effect and a shorter time interval between injection of the toxin and killing the animal (Peterson, unpublished data). It was available for routine use only at a late stage in the investigation. Unless otherwise indicated, the results described are for the bovine kidney cell assay, quoted in cell culture units, and the nursling rat assay, quoted in nursling rat units.

(i) *Mouse liver assay*

The methods of Gardiner and Petterson (1972) and Beck (unpublished data), based on the mitosis-arresting effect of toxic lupin extracts, were modified as follows. Adult mice were injected intraperitoneally with extracts and killed 6 days later. Liver sections were prepared by standard histological methods and stained with haematoxylin and eosin. Parenchymal cell mitoses were counted in 50 fields (× 600) representative of the total area of section. The mouse unit of toxin (m.u.) was defined as the amount giving rise to two mitotic figures per 100 parenchymal nuclei. A threefold dilution series of extracts was used, five mice per dilution.

(ii) *Nursling rat liver assay*

Intraperitoneal injection of low doses of toxic extract into 2-week-old rats causes an increase in the number of liver parenchymal cells in mitosis. In these cells there is a high proportion of abnormal figures characterized by scattered chromosomal material indicative of disrupted spindles (Peterson, unpublished data). In an assay based on this, we used a twofold dilution series and five rats of $25 \pm 5$ g per dilution. The rats were killed 18 h after injection. Liver sections were prepared by standard procedures, and abnormal mitotic figures were counted in 100 fields (× 600). The nursling rat unit (n.r.u.) of toxin was defined as the minimum amount that would produce five 'scattered chromosome' mitotic figures in less than 20 fields (× 600).

(iii) *Ovine kidney cell assay*

Ovine kidney cells grown on cover-glasses, when exposed to toxic extract, undergo marked morphological changes (Petterson and Coackley 1973). An assay based on this effect was used in the early stages of this investigation. Fifty fields (× 600) were counted in haematoxylin- and eosin-stained preparations. Cells showing any two of the following features, (1) abnormally dark, eosinophilic colour, (2) abnormal nuclear morphology, (3) abnormal or bizarre shape, were counted. The ovine kidney cell unit of toxin was defined as the amount in 1·0 ml of medium, that produced two such cells per field after 24 h exposure at 37°C.

(iv) *Bovine kidney cell assay*

Secondary cultures of calf kidney cells in Hank's medium with 7% added foetal calf serum are inhibited by a toxic extract. When growth was measured by solubilizing the adherent cell layer with 0·2 M NaOH and determining the protein content of the solution by the method of Lowry *et al.* (1951), the reduction in growth increment bore a relationship to the log of the toxin concentration which was approximately linear between 20 and 80% inhibition. The cell culture unit (c.c.u.) of toxin was defined as the amount required per millilitre of medium to cause 50% reduction, compared with untreated controls, in protein increase in a standard cell sheet in 72 h at 37°C.

(v) *Mouse mastocytoma cell assay*

Cells of the P815 line (obtained from Commonwealth Serum Laboratories, Melbourne) when cultured in Dulbecco's Minimum Essential Medium with 15% foetal calf serum added, were inhibited by a toxic extract in a dose-dependent manner. A toxin assay was based on the reduction in the number of live cells produced in 24 h at 37°C, under standard conditions. Cells were counted in a haemo-

cytometer, live cells being defined as those which were impermeable to the water-soluble dye, nigrosin. The unit of toxin (P815 c.u.) was defined as the amount required per millilitre of medium to cause 50% reduction in number of live cells produced in 24 h at 37°C.

*Chromatography*

Materials used were several grades of Sephadex (Pharmacia), Amberlite XAD-2 macroreticular polystyrene resin (BDH Chemicals), Amberlyst A29 macroreticular ion-exchange resin (BDH Chemicals), Porasil A-60 (Waters Associates), and activated charcoal (Merck, washed and fines removed).

*Microanalyses and Nuclear Magnetic Resonance Spectra*

Microanalyses were performed by the Australian Microanalytical Service. Proton magnetic resonance spectra were measured with a Varian A60 spectrometer operating at 60 MHz with tetramethylsilane as internal reference.

*Methods Used in the Isolation of the Crystalline Toxin*

(i) *Extraction of crude toxin*

An approximately 5-kg batch of a culture of *P. leptostromiformis* on lupin seed was coarsely minced, slurried with 10 litres methanol and packed into a 150 by 10 cm diameter glass column. After percolation of the excess methanol, a further 25 litres methanol–water (80 : 20 v/v) was run through the column. The eluate was brought to pH 8·5 with 2·5 M NaOH and concentrated under vacuum at 35°C until all methanol had been removed. The concentrate was washed with diethyl ether, brought to pH 3·5 with 1 M $H_2SO_4$ and extracted three times with an equal volume of n-butanol. The combined butanol extracts were mixed with an equal volume of light petroleum (b.p. 30–40°C) and the lower of the two phases formed was removed and set aside. The upper phase was extracted repeatedly with small volumes (approximately 10% of that of the upper phase) of 0·1 M NaOH until four successive extracts remained alkaline. The aqueous extracts were combined with the original lower phase, and the resulting alkaline solution brought back to pH 8·5 with 1 M $H_2SO_4$ and evaporated to give the crude extract as a brown gum.

(ii) *Partition into tetrahydrofuran*

Portions of crude extract were dissolved in water (*c.* 15 g/100 ml) and shaken with a four- to fivefold volume of tetrahydrofuran. The aqueous layer was discarded and the tetrahydrofuran layer shaken with one-tenth to one-twentieth of its volume of aqueous potassium chloride (15 g/100 ml). The aqueous layer was removed and washed twice with an equal volume of tetrahydrofuran. The tetrahydrofuran fractions were combined and evaporated to give the toxic fraction.

(iii) *Chromatography on macroreticular polystyrene resin (XAD-2)*

The product from tetrahydrofuran was dissolved in water and applied in approximately 2-g lots to a column of Amberlite XAD-2 resin (400 g; bed 4 by 50 cm) which had been degassed before packing and washed successively after packing with degassed acetone, methanol and finally water. Elution was performed with degassed water (2 litres), aqueous methanol (1 : 1 v/v, 1·5 litres) and methanol (2 litres), the three eluates being collected separately.

(iv) *Paper electrophoresis*

Paper electrophoresis was conducted in an enclosed strip apparatus (Frahn and Mills 1964) using Whatman No. 4 paper in strips 13·5 by 61 cm with 45 cm enclosed under pressure and cooled by the circulation of tap water at about 20°C through the coils of the cooling plate.

Test solutions (*c.* 0·5 μl) were applied to papers impregnated with 0·05 M sodium borate buffer (pH 9·2) and electrophoresis was allowed to proceed for about 30 min at about 20 V $cm^{-1}$. The paper strips were then dried at 100°C, examined under a Hanovia Chromatolite u.v. lamp and sometimes sprayed with potassium permanganate–chromium trioxide–sulphuric acid reagent (Frahn and Mills 1959) to locate substances.

Preparative electrophoresis was conducted in the same apparatus using 3MM paper impregnated with ammonium carbonate buffer (0·1 M; pH 9·5) and a film of Teflon as the insulating envelope (Frahn 1970). Solutions of toxin in the buffer (100 μl) were streaked uniformly across the papers and subjected to electrophoresis for 2 h. The bands, visible on dried papers in u.v. light, were excised, extracted with water, and the extracts evaporated under vacuum.

*Other Separation Procedures which led to a Significant Concentration of Crude Extracts*

(i) *Use of differential solubilities in water and alcohols*

A solution of the crude extract in water at pH 3·5 was extracted with n-butanol, the butanol was evaporated and the residue extracted with n-propanol. The propanol extract was evaporated and the residue extracted with a small volume of water. The water-insoluble material was extracted with propanol–ethanol (4 : 1), leaving some insoluble material of high specific toxicity.

(ii) *Countercurrent distribution*

The crude extract (2 g) was distributed between 20-ml volumes of n-butanol and ammonium acetate buffer, pH 4·5, in seven separating funnels. After the seventh transfer, the total contents of each tube were evaporated. The ammonium acetate is lost during the evaporation.

(iii) *Preparative thin-layer chromatography*

Preparative thin-layer chromatography was carried out on Merck PSC preparative plates using either ethanol–water (80 : 20 v/v) or n-propanol–n-butanol–water–ammonia (sp. gr. 0·880) (65 : 15 : 17 : 3 v/v) as developing solvent. Plates were washed by pre-running several times in the developing solvent to remove elutable material. The material was then applied as discrete spots on one-half of the plate, the other half being used as a blank. The plates were examined under u.v. light, and corresponding bands removed from sample and blank sides of the plate. Substances were eluted from the silica by shaking three times with ethanol–water (50 : 50 v/v), and centrifuging.

## Results

### *Isolation of the Crystalline Toxin*

The crude butanol extracts from two different batches of *P. leptostromiformis* culture amounted to (i) 153 g, total toxicity $1{\cdot}91 \times 10^6$ c.c.u., specific toxicity 12·5 c.c.u./mg, and (ii) 40·7 g, total toxicity $0{\cdot}41 \times 10^6$ c.c.u., specific toxicity 10 c.c.u./mg.

When partitioned into tetrahydrofuran in lots of about 15 g, crude extract amounting to 111 g, $1{\cdot}46 \times 10^6$ c.c.u., 13 c.c.u./mg, was converted into an amber gum, 17·3 g, $0{\cdot}99 \times 10^6$ c.c.u., 57 c.c.u./mg.

This product was chromatographed in approximately 2-g portions on a column of macroreticular polystyrene resin (400 g). In a typical run, 2·1 g of toxin (121 000 c.c.u.) was applied in water (50 ml), washed with additional water (2 litres) and eluted with aqueous methanol (1 : 1 v/v, 1·5 litres) and methanol (2 litres), the three solvents being collected separately. After removal of solvent, the residues obtained were 1·5 g, 3925 c.c.u. from water; 250 mg, 7500 c.c.u. from aqueous methanol; and 380 mg, 112 600 c.c.u. from methanol. The methanol eluates from several runs were combined to give an amber gum (2·55 g, 644 000 c.c.u., 250 c.c.u./mg).

This high-activity concentrate was dissolved in a small volume (200 ml) of mixed solvent in order to prepare standard aliquots. To achieve complete solution in this volume it was necessary to use approximately 155 ml tetrahydrofuran, 15 ml ethanol, 25 ml methanol and 5 ml water. On keeping at 0°C for a brief period the solution deposited crystals. Several batches were obtained, in all about 600 mg, specific toxicity 250 c.c.u./mg. The remainder of the bulk concentrate, after removal of the crystals, had a specific toxicity of 210 c.c.u./mg.

### *Some Properties of the Crystalline Toxin*

The crystals effloresced when removed from solvent and lost additional weight on storage. When inserted by direct entry into a mass spectrometer, the spectrum of tetrahydrofuran was obtained. Tetrahydrofuran, at the level present in the crystals, did not affect the bovine kidney cell culture.

The toxin was recrystallized by evaporating a solution in the above mixed solvent to near dryness at room temperature, taking the residue up immediately in a small volume of 25% aqueous tetrahydrofuran at room temperature and allowing it to crystallize. The product consisted of colourless prisms which decomposed without melting when heated above 205°C in an evacuated tube.

When submitted to microanalysis, the crystals lost an additional 2·9% by weight on drying at 60°C *in vacuo*. Found on the dried sample: C, 53·4; H, 6·2; N, 10·3; O, 26·2; S, 0·0%. U.v. absorption, molar extinction coefficients calculated for molecular weight 250: in methanol, $\lambda_{max}$ 209 ($\log\varepsilon$ 4·20), 228 (sh) ($\log\varepsilon$ 3·83), 289 ($\log\varepsilon$ 3·70) and 302 (sh) nm ($\log\varepsilon$ 3·54); in water, $\lambda_{max}$ 202 ($\log\varepsilon$ 4·26), 227 ($\log\varepsilon$ 3·9) and 288 nm ($\log\varepsilon$ 3·65); in 1 M NaOH, $\lambda_{max}$ 206 ($\log\varepsilon$ 4·69), 254 ($\log\varepsilon$ 3·74) and 306 nm ($\log\varepsilon$ 3·40). I.r. absorption in KBr disc: $\nu_{max}$ 3510 (sh), 3400 (sh), 3360 (OH), 1685, 1670, 1640, 1615 (possibly amide CO), 1480, 1460, 1370, 1280, 1200, 1120, 880, 860 $cm^{-1}$. N.m.r. spectrum measured at 60 Hz in $CD_3OD$–$D_2O$ containing $K_2CO_3$: multiplets at $\delta$, 7·40, 6·83, 6·27, 5·88, *c.* 4·70, 3·1, 2·1, 1·4–1·6, 0·8–1. Interpretation was made difficult by the lack of recognizable line patterns and by an obviously non-integral relationship of H-atom numbers corresponding to the peaks.

Paper electrophoresis in borate buffer (pH 9·2) led to the separation of two anionic compounds which were visible as dark absorbing spots in u.v. light, one more intense than the other. These spots corresponded with mobilities of 5·7 and 4·5 $cm\,h^{-1}\,kV^{-1}$ for the major and the minor components respectively. They were also detected by spraying the paper with the potassium permanganate–chromium trioxide–sulphuric acid reagent when they appeared immediately as yellow spots on a pink background changing to white spots on a brown background.

*Toxicity of the Crystalline Toxin*

In the cell culture and nursling rat assays the following results were obtained for the specific toxicities of the crystals, the original crude toxic extract and the final bulk concentrate after removal of the crystals:

| | Cell culture (c.c.u./mg) | Nursling rat (n.r.u./mg) | Ratio (n.r.u./c.c.u.) |
|---|---|---|---|
| Original crude extract | 13 | 240 | 18·5 |
| Crystals | 240 | 8730 | 36·4 |
| Final extract after removal of crystals | 210 | 2040 | 9·7 |

A 19-kg wether, when dosed intraperitoneally with 1·2 mg of the crystalline mixture in sterile water, showed clinical, biochemical and histological changes characteristic of lupinosis. These included a loss in body weight and jaundice, elevations in plasma ornithine carboamyltransferase and alkaline phosphatase levels. Histologically the liver displayed a uniform fatty vacuolation, moderate individual hepatocyte necrosis and a moderate degree of mitotic activity.

*Electrophoretic Separation of Phomospins A and B and Individual Toxicities*

The crystalline toxin (1·0 mg), run on 3MM paper in ammonium carbonate buffer, gave distinct bands for phomopsins A and B. After excision and extraction with water, the bands yielded residues, 0·90 and 0·20 mg respectively. Each residue

was dissolved in 1 ml water and tested against nursling rats. The results indicated specific toxicities of 5000 and 1100 n.r.u./mg for phomopsins A and B respectively.

*Concentration of Toxin via Sparing Solubility in Water and Alcohols*

The extraction of crude toxin (16 g, 160 000 c.c.u., 10 c.c.u./mg) into n-propanol, water and propanol–ethanol (4 : 1) led to the highest specific activity in the fraction which remained undissolved in water and propanol–ethanol (4 : 1) — 50 mg, 20 400 c.c.u., 409 c.c.u./mg. The low overall yield made this process unattractive but the product was shown by electrophoresis to be similar in composition to the high-activity eluate from the polystyrene resin and to contain phomopsin A. The high value for the specific toxicity implies that one or more components of this fraction has higher activity against bovine kidney cells than do the phomopsins.

*Countercurrent Distribution*

The distribution of a crude extract (2·0 g, 26 000 c.c.u.) between 20-ml volumes of n-butanol and ammonium acetate buffer (pH 4·5) in seven separating funnels gave the following fractions in order, beginning with the first separating funnel: 51·7 mg, 5690 c.c.u.; 64·5 mg, 12 250 c.c.u.; 57·2 mg, 5720 c.c.u.; 97·6 mg, 3220 c.c.u.; 27·1 mg, 255 c.c.u.; 294 mg, 469 c.c.u.; 1443 mg, 0 c.c.u. Specific toxicities for the first three fractions were 100, 190 and 100 c.c.u./mg. Recoveries of both weight and toxicity were close to 100% in this fractionation and most of the toxin was recovered with considerably enhanced specific toxicity.

*Preparative Thin-layer Chromatography*

(i) *Solvent*: *ethanol–water (80 : 20 v/v)*

Application of crude extract (29·3 mg; 293 c.c.u.) and running overnight at 0°C resulted in only faint spots at $R_F$ 0·8 under u.v. light. Fractions obtained at $R_F$ 0–0·11, 0·26–0·53 and 0·68–1·0 contained the main weight of material (12·8, 33·8 and 24·3 mg respectively) and toxicity was confined to the last fraction (212 c.c.u.) and $R_F$ 0·53–0·68 (4·6 mg, 70 c.c.u.). The blanks showed considerable residue weights (3·6–11·3 mg) and zero toxicity.

(ii) *Solvent*: *n-propanol–n-butanol–water–ammonia (sp. gr. 0·880) (65 : 15 : 17 : 3 v/v)*

Using fraction 3 from the countercurrent separation (6·7 mg, 670 c.c.u.) and running overnight at 0°C resulted in fractions showing maximum toxicity and strong u.v. absorbance at $R_F$ 0·21–0·25, but with appreciable toxicity in all other fractions.

*Behaviour of the Toxin in other Fractionating Procedures*

Other methods of purifying the toxin were tried and found to be unsatisfactory. Chromatography on A29 macroreticular resin and Sephadex G25 gave fairly good recoveries but little concentration of the toxin. On silica gel, fairly good separations were achieved but recoveries were usually poor. On activated charcoal, the toxin was strongly adsorbed from aqueous solution and could not be desorbed. On QAE A25 ion-exchange Sephadex with elution by ammonium acetate buffers at pH 8·3,

6·8 and 5·4, the toxin was rapidly eluted without concentration. On LH20 Sephadex, recovery was very low. On Pyrex glass powder moistened with phosphate buffer, pH 7·5, and using ether–tetrahydrofuran mixtures as mobile phase, the toxin eluted at each solvent change without concentration, and stripping of the buffer phase occurred at the higher proportions of tetrahydrofuran.

## Discussion

The crystalline mixture of phomopsins A and B, given intraperitoneally to a sheep, produced typical signs of lupinosis. Phomopsins A and B, after electrophoretic separation, are each highly active in producing mitotic arrest in liver cells of the young rat. There seems little doubt that one or both of these metabolites are the main cause of lupinosis toxicity in lupins infected with *P. leptostromiformis*. The question of whether there are other substances present in the toxic extracts capable of causing lupinosis has not yet been definitely answered. The extract remaining after removal of the crystals from the toxic concentrate still contains a substantial proportion of the toxicity measured by the bovine kidney cell culture assay (about 77%) and by the young rat assay (about 45%) but the nursling rat toxicity, which is regarded as a specific indicator of lupinosis toxicity, is at least largely accounted for by the phomopsins A and B shown to be still present by electrophoresis. This suggests that the residual extract contains constituents which have high specific toxicity in the cell culture assay but little or no toxicity to the young rat.

Biologically active metabolites which have previously been isolated from *Phomopsis* species (Sphaeropsidales : Sphaerioidaceae) appear to be restricted to the cytochalasin group. Two metabolites considered to be closely related to cytochalasin D have been isolated from *P. paspali*, a fungal pathogen of kodo millet (Patwardhan *et al.* 1974). One is apparently identical with a compound isolated from a culture of a *Phomopsis* sp. found in weevil-damaged pecan nuts (Wells *et al.* 1976) and named cytochalasin H, following an X-ray crystallographic determination of structure (Beno and Christoph 1976) which confirmed that proposed by Patwardhan *et al.* (1974) for the *P. paspali* metabolite. Cytochalasins A and B are also produced by *Phoma* spp., which are classified with *Phomopsis* in the family Sphaerioidaceae; for example both cytochalasins A and B have been reported in an unidentified *Phoma* sp. (Rothweiler and Tamm 1970) and cytochalasin B has been reported in *Phoma exigua* (Anon 1976). The cytochalasins, especially cytochalasin B, which has been the main subject of investigation, produce unique biological effects which include extrusion of the nuclei from cells and inhibition of cytoplasmic cleavage without interference with division of the cell nucleus.

Culture extracts of another *Phomopsis* sp. and of *Diaporthe phaseolorum* var. *sojae* isolated from mouldy soybeans have recently been shown to produce grossly fatty livers and sometimes liver necrosis and haemorrhaging in day-old chicks (Kung *et al.* 1976). The lesions differed from those produced by *P. leptostromiformis* strains from the American Type Culture Collection under the same conditions. The hepatotoxic metabolites produced by these species are still unknown.

From the biological effects induced by toxic lupins and the chemical and physical properties evident in the present investigation, phomopsins A and B appear to be unrelated to the cytochalasins. They are distinguished in particular by a high nitrogen

content (of the mixture, at least) and by low solubility in lipid solvents. They appear to be closely related to each other in structure since they have similar mobilities on electrophoresis and show similar absorption of u.v. light and similar response to the potassium permanganate–chromium trioxide–sulphuric acid reagent. They also give similar colours with diazotized p-nitroaniline and diazotized sulphanilic acid. The available data do not yet show the class of compound although electrophoretic results based on comparison with reference compounds (unpublished data) suggest that the main constituent, phomopsin A, is a phenol of equivalent weight approximately 280 and the i.r. spectra indicate strong OH absorption (above 3000 $cm^{-1}$) and probably amide absorption (1600–1700 $cm^{-1}$). The preliminary n.m.r. spectra show resonance in the regions for aliphatic and aromatic hydrogen but are not capable of detailed interpretation. The u.v. absorption indicates little conjugation beyond one aromatic ring or its equivalent but is not definitive for a particular chromophore. Elucidation of the molecular structures of phomopsins A and B must await resolution of the mixture and purification of each component. Further work is being directed to these ends.

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