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The structural characteristics of a diazotization polymer known as DALM (diazoluminomelanin) have been investigated by several methods including chromatographic and electrophoretic separations, nmr spectroscopy, mass spectroscopy and similar approaches. DALM is prepared by the action of sodium nitrite on aqueous solutions containing 3-amino-L-tyrosine (3-AT) and luminol, and a simpler but closely related polymer (poly-3-AT) is prepared by the action of nitrite ion on 3-AT alone. Both substances are complex since they consist of more than one type of polymeric product, and the polymer formation reaction involves not only the aromatic ring of 3-AT but also a partial sidechain scission. Existing evidence suggests that a polyphenylene linkage is the dominant catenation mode in both DALM and poly-3-AT. Diazotized luminol (i.e., luminol diazonium) has been shown to be an excellent sonochemiluminescent agent, and DALM'S luminescent properties, which include a thermochemiluninescent response to dissolved carbon dioxide, probably derive from diazotized luminol and its products. Grant: AFOSR #F49620-95-1-0316.

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FINAL REPORT

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ABSTRACT

The structural characteristics of a diazotization polymer known as DALM (diazoluminomelanin) have been investigated by several methods including chromatographic and electrophoretic separations, nmr spectroscopy, mass spectroscopy and similar approaches. DALM is prepared by the action of sodium nitrite on aqueous solutions containing 3-amino-L-tyrosine (3-AT) and luminol, and a simpler but closely related polymer (poly-3-AT) is prepared by the action of nitrite ion on 3-AT alone. Both substances are complex since they consist of more than one type of polymeric product, and the polymer formation reaction involves not only the aromatic ring of 3-AT but also a partial sidechain scission. Existing evidence suggests that a polyphenylene linkage is the dominant catenation mode in both DALM and poly-3-AT. Diazotized luminol (i.e., luminol diazonium) has been shown to be an excellent sonochemiluminescent agent, and DALM's luminescent properties, which include a thermochemiluminescent response to dissolved carbon dioxide, probably derive from diazotized luminol and its products.

1. OBJECTIVES

The objectives presented here are those stated in the original proposal, The specific objectives of the proposed work were defined during telephone conversations in 1994 between the principal investigator and Dr. Kiel at Brooks AFB. The main objective of the research is a structural characterization of the substance called DALM A structural characterization is expected to provide insight into the mechanisms which cause aqueous solutions of DALM to emit visible light when they are subjected to CW or pulsed microwave fluences. Specific objectives which bear upon the overall purpose are as follows:

A. The identification of a polymer fragmentation method should not overshadow other approaches to structural characterizations. One aspect which should be pursued is a comparative study of substances closely related to 3-amino-L-tyrosine to see how such differences might affect the polymerization. For example, would the presence of tyrosine in the mixture of diazotized products diminish the degree of polymerization? Another approach will be to conduct the polymerization in concentrated aqueous solutions to see if such conditions favor the formation of small oligomers, which should be more amenable to nmr characterizations. It is probably possible to isolate small oligomers using the methods cited here.

B. We now have access to a new HPLC instrument, and it is expected that the chromatographic studies in progress will lead to a more rational choice of columns and conditions. DALM preparations generally ruin expensive HPLC columns (communications with Dr. Kiel at Brooks AFB), and this is likely due to unreacted diazonium groups on the polymer.

C. A further study of various anions which might enhance the sonochemiluminescent properties of alkaline luminol and DALM solutions will be conducted, especially organic species. Anions probably act to convert the more reactive initial unpaired spins (e.g., hydroxyl) to charge-stabilized intermediates (such as carbonate or phosphate radicals) which persist longer and therefore increase the probably of activating luminol's chemiluminescence. There may be an optimum trade-off of radical delocalization versus reactivity with luminol which would maximize the sonochemiluminescent effect, and a number of candidate ions will be tested to see if correlations with spin delocalization and charge are possible. It was found that anion-enhanced sonochemiluminescence of alkaline solutions of luminol show maximum contrast if H_20_2 is omitted from the reacting mixture since the steady chemiluminescence of the unirradiated solution is weak to begin with. The effect of peroxide concentration on this system will also be examined. These objectives are longstanding.

2. STATUS OF THE EFFORT: ACCOMPLISHMENTS AND NEW FINDINGS

2.1. Introduction

This report is the most recent of several in an ongoing investigation of a polymeric substance known to us as DALM (diazoluminomelanin, which contains the chemiluminescent substance luminol), and a summary of the earlier research findings is included as Appendix 1. The central issue of this work, as stated in the objectives, has been a structural characterization of DALM and polymers which are related to it. DALM's structural characterization has not been a straightforward matter, in part because it is not a single polymeric substance and also because workable separation and purification methods have been hard to develop. A secondary issue under investigation has been a characterization of the chemiluminescent properties of DALM and simpler chemiluminescent systems which behave comparably, especially those which either have a large temperature coefficient of chemiluminescence or which respond to sonic irradiation.

The original reason for investigating DALM (1), a complex, polymeric substance formed by diazotizing a mixture of luminol and 3-amino-L-tyrosine, is that it has been shown to interact with (absorb) microwave radiation, producing a chemiluminescence that increases with progressive heating. In high power pulsed microwave irradiations DALM solutions also respond with a flash of visible light. One of the Brooks AFB scientists (Dr. Johnathan Kiel) observed that these solutions produced flashes of visible light when they were irradiated with 6 microsecond pulsed microwave fluences from a one megaWatt, 1.2

GHz transmitter (moderately dilute aqueous solutions have been observed to produce an acoustic wave when irradiated with microsecond pulses of microwave energy at instantaneous power levels of > 100 W/mL; see 2). Dielectric measurements revealed that the solutions of DALM exhibited an anomalous dielectric loss component of permittivity (3), attributable to a conductive or highly dipolar polymer, and such solutions also exhibit an enhanced microwave acoustic effect in high power pulsed irradiations.

This observation led us to consider the possibility that the polymer (which we have found be based on 3-amino-L-tyrosine, hereafter referred to as 3-AT, rather than luminol) was creating an acoustic wave and a diazotized derivative of luminol was responding with sonochemiluminescence. We thus pursued structural characterizations of the polymer of 3-AT alone (poly-3-AT) and separately examined diazotized luminol solutions for sonochemiluminescent tendencies. This proved to be a productive approach because diazotized luminol was found to be an excellent luminescent responder to acoustic energy (4), and chemical and physical characterizations of the polymer of 3-AT have shown that while the material is complex, at least part of it appears to possess DALM's polymer linkage, including an extended pi-electron system needed to explain conductive properties. There are other reasons for characterizing this polymer system, e.g., a derivative of DALM has been found to be thermoluminescent (unpublished, J. Kiel and E. Holwitt, Brooks AFB).

At the current stage of the investigation, a major need area is still the development of analytical methods appropriate for DALM and poly-3-AT, and our department's analytical chemist, Dr. Joel T. Smith (who has expertise in analytical and mass spectrometric applications), was advocated as a collaborative investigator by Dr. Wright and is now fulfilling that need. Dr. Smith applied for and received AFOSR funding, which started October 1, 1997. His grant included much needed HPLC and capillary electrophoresis (CE) equipment.. Also, an award from AFOSR through Dr. Wright's recent grant (along with matching funds from Southeastern State University and the State of Oklahoma) has allowed the addition of a mass spectrometric capability. The instrument has been installed and is in use at this writing.

Another major area of need is a characterization of the polymerization tendencies of analogs of 3-AT. Such studies would resolve structural questions and possibly lead to novel, interesting materials. If funding for further studies is secured, we will pursue the question of 3-AT analogs and their polymerization qualities, with emphasis on structural implications. The proposed work would also examine the physical properties of synthesized materials and especially focus on interactions of polymers with diazotized luminol in an attempt to understand the unique properties of DALM. It was clear from the beginning that DALM-forming mixtures possessed chemical components which were not seen in either diazotized luminol or diazotized 3-AT alone (Fig. 1).

Addressing the outcome of the defined objectives (see above) it is clear that while we gained a better picture of DALM's structural qualities, its complexities have also emerged! The specific objectives were followed with these results: In pursuing objective A it was found that the amino acids (phenylalanine and tyrosine) did not truncate the polymerization of poly-3-AT. Also, when tried alone with the diazotizing reagent, they did not lead to DALM-like pigmented polymers, evidence that the aromatic ring is essential for the reaction. Truncations were effected by KI (not considered originally), though not to the extent of very small oligomers. Good progress was realized for objective B. The low pressure, size exclusion Toyopearl medium did not bind DALM or

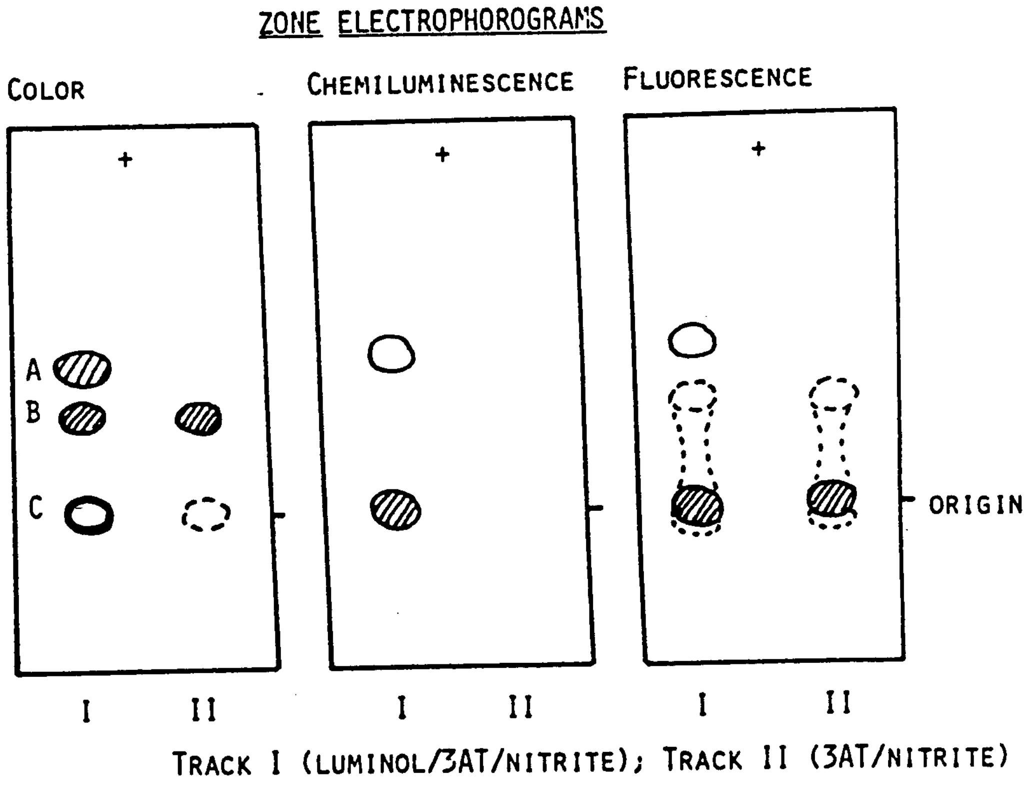


Figure 1. Electrophoresis on cellulose acetate strips showing components that are unique to the diazotized luminol/3-aminotyrosine mixture. Component A was not found in diazotized luminol. Component C is very dark brown; it overlaps a component of diazotized luminol, but since the latter component is maroon colored, C is an entirely different substance.

poly-3-AT irreversibly, the first such case in our experience. The applicability of capillary electrophoresis to DALM and poly-3-AT was also demonstrated. The development of appropriate separation methods is crucial to these investigations.

Objective C had the most successful outcome. The sonochemiluminescent component of DALM has been shown to be luminol diazonium, and this substance might be attached at available carboxylate or amine groups of the poly-3-AT polymer, i.e., it could enter reversible binding equilibria of the following types:

$$\text{R-N}_2^{+} + \text{DALM-COO}^{-} \rightleftharpoons \text{R-N=N-OOC-DALM}$$

or:

$$\text{R-N}_2^{+} + \text{DALM-NH}_2 \rightleftharpoons \text{R-N=N-NH-DALM} + \text{H}^{+}$$

Since diazotized luminol's thermochemiluminescence is markedly enhanced by dissolved carbon dioxide, it could be the reason for DALM's comparable property.

2.2. Significance of the Work

The research has explored the chemistry of a diazotization polymer of luminol and 3-amino-L-tyrosine (DALM). Aqueous solutions of this polymer emit visible light when they are exposed to intense pulse fluences of microwave energy. DALM has analytical applications in studies of microwave absorption in target materials and target phantoms. A DALM-like substance has also been produced in genetically modified bacteria. Aqueous solutions of these polymers enhance the conversion of microwave energy into an acoustic wave. Also, derivatives of DALM are thermoluminescent and have potential applications in sensors.

Perhaps the most significant aspect of this chemical system is its apparent ability to couple microwave energy, with photonic energies less than kT, with chemical reactions, where the bond breaking energies are substantially greater than kT. This is presumably a coupling based the microwave acoustic phenomenon and not quantum mechanical fusion (i.e., not by an anti-Stokes Effect). The system is thus relevant to the whole question of microwaves and chemical effects (including biochemical effects), and if DALM is only one substance deviating from a thousand others which are unable to show such properties it is a system worthy of study. The cited effects are happening at fairly high incident power levels, particularly in pulsed RF fields, and we are not suggesting that weak RF fields could introduce direct chemical effects.

It would seem that the biological properties of high powered microwave (HPM) devices are more a consequence of a generated acoustic wave, i.e., a literal shock wave, than outright heating of the target, and appropriate analytical tools of the type which we are developing will enhance future studies oriented to understanding weapons effects and the safe limits of pulsed microwave fields. The physical property of thermoluminescence observed for DALM derivatives has obvious applications in sensors and alone is sufficient to establish the significance of the proposed work

3. EXPERIMENTAL METHODS

3.1. Materials

Materials used in this work, such as 3-amino-L-tyrosine and luminol, were obtained from Sigma/Aldrich Chemicals and used as received. Sodium nitrite containing 99 atom percent ^{15}N was obtained from Icon, Inc.

3.2. Synthetic Methods (includes labeling)

The methods for preparing various forms of DALM and poly-3-AT have been described in the reports from earlier investigations (see Appendix 1).

3.3. Sonochemiluminescence Measurements

Sonochemiluminescence measurements were obtained using a Turner Model 20D luminometer. This instrument was optically interfaced by means of a fiber optic link to a sonic irradiation cell contained in a light tight box. The acoustic irradiation source was a Branson Sonifier 250, which supplies up to 250 Watts of acoustic power at 20 kHz.

3.4. Elemental Analyses

Purified specimens were submitted to Galbraith Labs for percent assays of C, H, N, O and Na (and S in some cases). Most were sent in duplicate or triplicate (from different preparations) to assess variability.

3.5. Spectroscopic Characterizations

Solid state ^{13}C- and ^{15}N-nmr spectra were obtained using the Oklahoma State University CP/MAS instrument, which resonates ^{13}C-nuclei at 75.7 MHz. High resolution liquid nmr spectra were obtained locally using a Varian/Chemagnetics A-200 spectrometer. In 1997 we gained access to a new 600 MHz nmr spectrometer at Oklahoma State University.

Mass spectra were obtained by means of a Hewlett-Packard model 1100 MSD mass spectrometer with electrospray, chemical ionization and APCI sample introduction capabilities.

A Bomem FTIR instrument was used to acquire infrared spectra.

3.6. Capacitance Measurements

Capacitance measurements were accomplished with a Hewlett Packard 4274A multifrequency LCR meter. The special capacitor contained aqueous poly-3-AT or DALM solutions.

4. RESULTS AND DISCUSSION

Progress during the past three year period is evaluated as follows: In addition to characterizing interesting and potentially useful physicochemical properties of the polymer system in question, continuing efforts to determine the structural features of the polymer have employed most of the methods known to polymer science: chemical degradations, truncation of the polymerization (to obtain smaller polymers), spectroscopic characterizations, isotope labeling methods, chromatographic and electrophoretic separations, elemental analyses, and polymer fractionations by molecular weight. The available evidence obtained by these methods indicates a plurality of linkage modes. While a diazo-linked component of the polymer mixture isn't entirely ruled out, the bulk of the polymer appears to be based on a polyphenylene backbone, probably formed by an aryl S_N1 process (e.g., involving an aryl cation intermediate), which would help to explain the pigmentation and other properties of the material. A non-conjugated (non-pigmented) component of the polymer is not ruled out, and Dr. Smith's HPLC and CE detectors have shown that some of the polymeric components do not absorb significantly at visible wavelengths.

We also discovered that a portion of the 3-AT sidechains come off during the polymerization, which could be the result of a cationic process. Nuclear magnetic resonance spectra of the polymer fraction with a molecular weight range below 5,000 g/mol is predominantly aliphatic while the polymer above 10,000 g/mol has an elevated aryl content, a consistent result. There is evidence that side-chain scission results in a polymerization of both aryl and aliphatic fragments. A structural characterization of poly-3-AT thus continues to be the central focus of the investigation.

The work also established that diazotized luminol is an excellent chemiluminescent responder to accoustic radiation. This finding fulfilled one of the stated goals of the original proposal. We now know that strobes of light from pulsed microwave irradiated solutions of DALM *could* originate through an enhanced microwave acoustic effect since the luminol component is clearly disposed to sonochemiluminescence. There is a question about the intensity range of pulsed microwave fluences appropriate to produce only sonochemiluminescent effects since powerful pulses produce sparking, which also results in strobes of light and sound. Weak pulses produce no effect; the phenomenon is very non-linear.

4.1. Sonochemiluminescence of diazotized luminol

As used here, the term sonoluminescence refers to visible radiation originating from cavitation sites in a liquid subjected to intense acoustic irradiation, while sonochemiluminescence means activation of a chemiluminescent substance such as luminol by free radicals originating from cavitation sites. The latter mode is inherently able to produce a much greater light output. We examined diazotized preparations of luminol (alone), to see if it would be possible to induce sonochemiluminescence from the solutions in combination with hydrogen peroxide (since that was the composition of the above cited microwave irradiation specimens (2)). These experiments were based on measurements of the luminescent output of of diazotized luminol solutions before irradiation with acoustic energy (Io) and during acoustic irradiation (I). It was found that under optimum conditions, i.e., in the absence of dissolved carbon dioxide, neutral diazotized luminol solutions containing hydrogen peroxide produce an I/Io value exceeding 150, and this is increased to more than 200 on adding formate ion to the preparation. Diazotized luminol solutions clearly have high selectivity for acoustic energy and this finding is the basis of a potentially useful sonic energy dosimeter (4).

As shown in Fig. 2, the diazotized luminol acoustic response is pH dependent and has an optimum value between pH 7.0 and 7.5. The significant interactions between dosimeter components are collected in Table I, and are based on 10 replications of measurements of I and Io. These sonochemiluminescent effects of diazotized luminol are well above the sonoluminescence of deionized water, as shown in Fig. 3, which presents intensity ratios relative to those of deionized water, i.e., the ratio of the acoustically activated luminescence of a defined mixture to the sonoluminescence of pure water. The sonoluminescence of deionized water is comparable to aqueous solutions containing just one of the solutes, e.g., hydrogen peroxide, sodium formate or even diazotized luminol, and aqueous solutions containing both hydrogen peroxide and sodium formate also yield a comparable level of sonoluminescence. The aqueous solution containing both sodium formate and diazotized luminol yielded a modest but significant (about 3-fold) enhancement in acoustically induced luminescence over that of pure water. In contrast, a marked increase accredited to sonochemiluminescence was observed for aqueous solution of hydrogen peroxide and diazotized luminol, and the I/Io ratio exceeded 200 when formate ion was added to the latter mixture. Individual measurements of Io are not highly reproducible (Table I) because the intrinsically low values of Io are barely above the

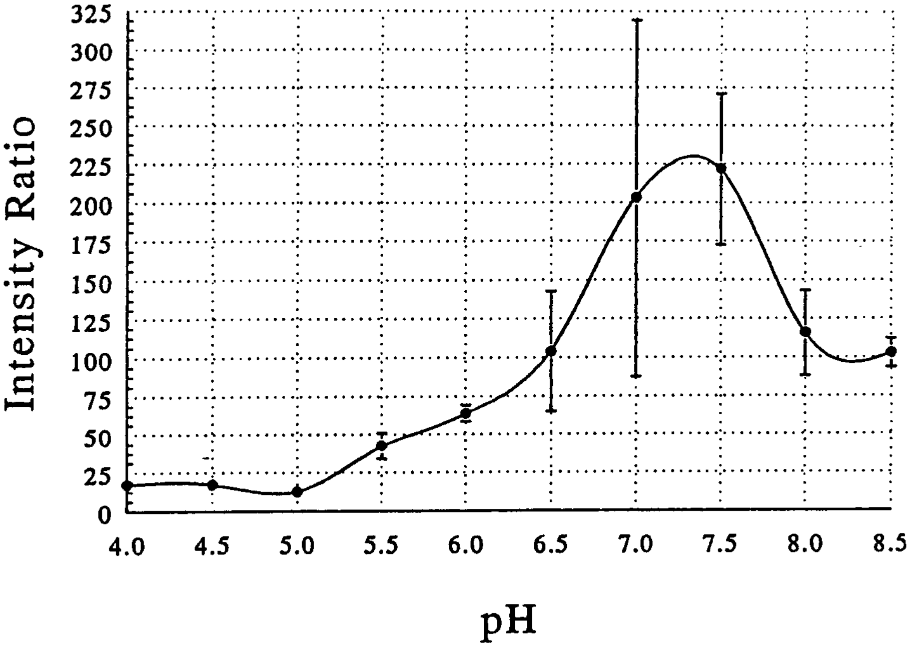


Figure 2. The pH Dependence of the diazotized luminol dosimeter. The response curve of measured I/Io values shows a maximum between pH 7 and 7.5.

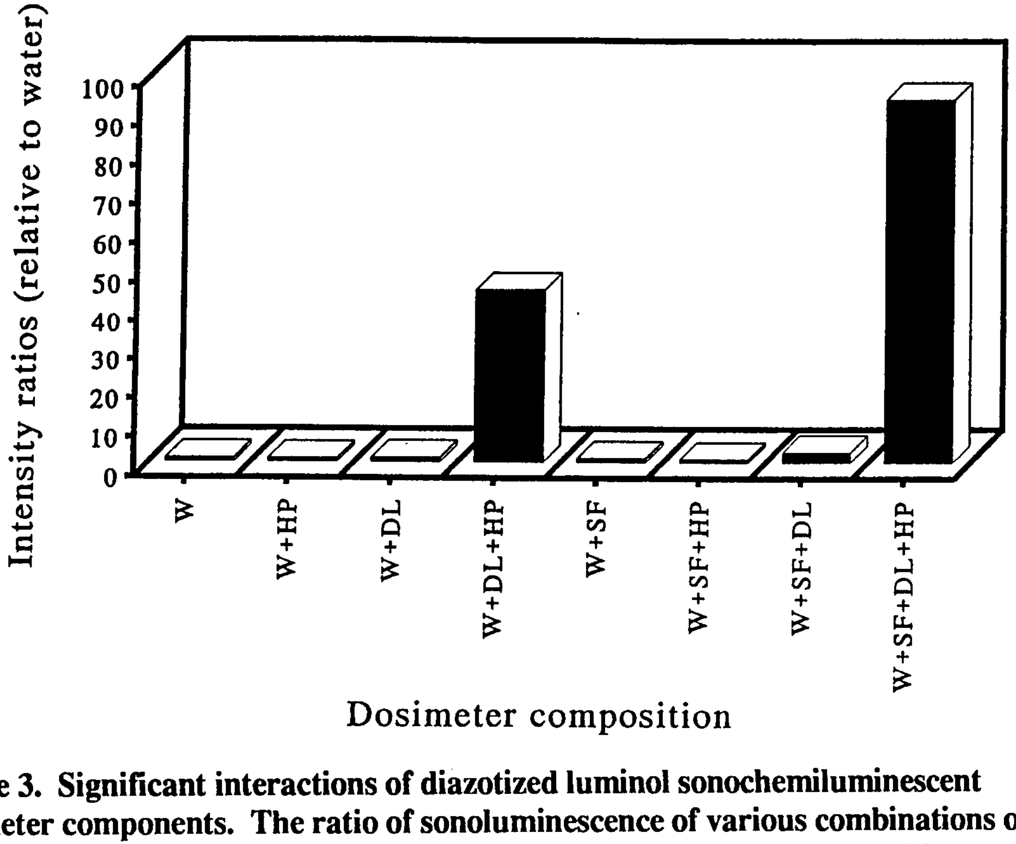


Figure 3. Significant interactions of diazotized luminol sonochemiluminescent dosimeter components. The ratio of sonoluminescence of various combinations of the dosimeter's components to pure water's sonoluminescence. By definition the value for W ("H_2O only", the left-hand column) is unity. Abbreviations: DL - diazotized luminol; HP hydrogen peroxide; SF - sodium formate; W - water.

instrument noise level. Diazotized luminol solutions are truly sensitive to sonic energy, with minimal thermal chemiluminescence in the quiescent state. Dissolved carbon dioxide acts to increase the value of Io while I is essentially unchanged.

This property of diazotized luminol easily explains one of the observed characteristics of DALM solutions used in the microwave experiments, namely that its level of thermal chemiluminescence is markedly increased on exposure to carbon dioxide or to a lesser extent, to dissolved bicarbonate ion. Merely exhaling one's breath over the solution or adding an aliquot of carbonated water raises the Io value and thus diminishes I/Io.

It is known that various anions such as bicarbonate, carbonate, phosphate, etc., enhance luminol's chemiluminescence (5), probably through the formation of charged radicals, which are able to initiate the chemiluminescent reaction and which persist longer through charge stabilization (which slows radical recombination), but the cited species also increase thermochemiluminescence. The reason for the enhancement due to formate ion is not yet clear. At pH 7.5, formate was found to be markedly more effective than other anions which were tried with diazotized luminol, i.e., nitrite, nitrate, bicarbonate, oxalate, glyoxylate and phosphate. Formate affects primarily the irradiated intensity (I).

In further control experiments the omission of luminol from the solution resulted in negligible sonochemiluminescence. From ten repetitions of the dosimeter prepared in this manner, the mean value of Io was found to be -0.0108 (S.D. = 0.0223) and the mean value of I was 0.1245 (S.D. - 0.0277), pointing to the luminol derivative as the source of sonochemiluminescence and ruling out nitrite or nitrate ion as the active agent.

The initial product of diazotization should be luminol diazonium, and the proton-nmr spectrum of the product mixture is consistent with the expectation (4). Capillary electrophoresis shows evidence for low oligomers (up to tetramer), but the the diazotization of luminol does not yield high polymers. However, in DALM most of the luminol present is associated with high molecular weight material, revealed by nmr line broadening even in alkaline solutions, and the luminol is probably present as a pendant group. Luminol diazonium is not an unknown substance since it has been used to prepare labeled antibodies for chemiluminescent immunoassays (6). Some diazonium species are more stable than others, but most are explosive and sensitive to mechanical shock. The acoustically sensitive properties of the diazotized luminol solutions described here might derive from the decomposition of an unstable diazoperoxide intermediate:

$$\text{R-N=N-O-O-H} \;\; \text{->} \;\; \text{R (luminol aryl radical)} + N_2 + \text{00H (hydroperoxyl)}$$

It would not be surprising if this type of free radical producing reaction were sensitive to an acoustic field, and free radicals are known to initiate the chemiluminescence of luminol and luminol derivatives (7). Consistent with the possibility, alkaline solutions of hydrogen peroxide (pH 12) react immediately with added diazotized luminol, producing a bright blue burst of chemiluminescence (8).

TABLE I - Measured Values of Io and I for Various Combinations of the Dosimeter Components: Evaluation of Component Interactions. The values shown here are relative intensities taken directly from the luminometer since the intent is to measure intensity ratios.

Components Present	Io	S.D.	I	S.D.	I/Io**	S.D.**
W	-0.0073	0.0119	0.1065	0.0121	*	*
W + HP	-0.0040	0.0117	0.0938	0.0136	*	*
W + DL	-0.0055	0.0130	0.1164	0.0180	*	*
W + DL + HP	0.0321	0.0124	4.7350	0.4452	179.4	97.3
W + SF	0.0084	0.0159	0.1060	0.0132	*	*
W + SF + HP	-0.0198	0.0145	0.0829	0.0112	*	*
W + SF + DL	-0.0020	0.0159	0.2724	0.0332	*	*
W + SF + DL + HP	0.0482	0.0135	9.9590	0.7625	221.2	64.3

* Io values are near zero or negative.
** Based on I/Io ratios from each of the ten individual experiments.
Legend: W - deionized water; HP - hydrogen peroxide; DL - diazotized luminol; SF - sodium formate. Where present, the amount of the component is as described in the experimental part, and the pH is 7.5.

The sonochemiluminescence of diazotized luminol solutions containing added hydrogen peroxide is easily visible to the dark adapted eye, and while sonoluminescence is brightest near the tip of the transducer, a glow immediately appears throughout the solution on application of sonic irradiation, either with- or without vigorous stirring. When sonic power is removed, the luminescence fades with a latency of approximately 0.5 s, an effect that was also observed in the above described pulsed microwave irradiations. The value of Io is minimized by chilling the solution in an ice bath, an Arrhenius effect. This also slows the further reactions of the diazonium species. The contrast ratio is thus enhanced since I is fairly insensitive to temperature variations.

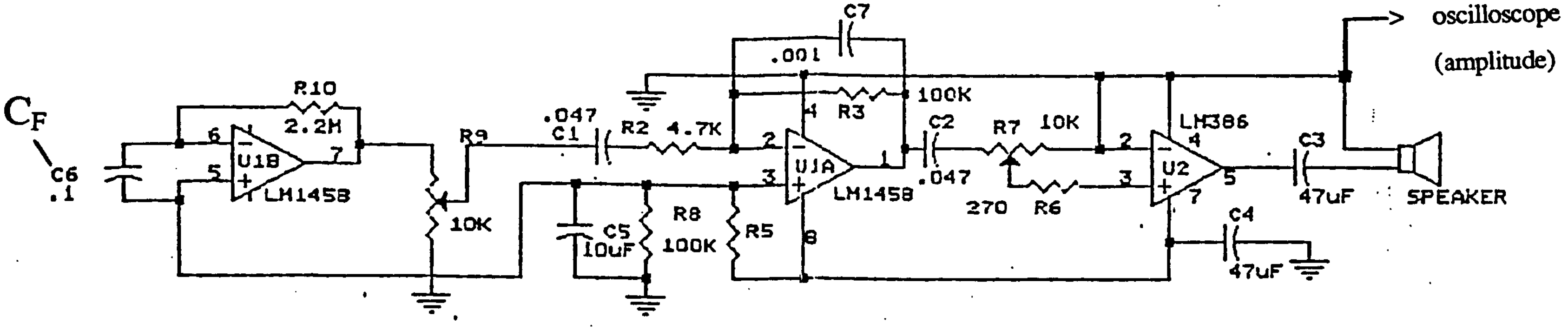
4.2. Findings concerning the poly-3-aminotyrosine diazotization polymer and their relationship to DALM

4.2.1. Physical Properties

We have hypothesized that pulsed microwave induced emission of visible light might be at least in part a coupling of the microwave acoustic effect (9) with a sonochemically active component of the polymer mixture, but induced sparking near the irradiated material can cause similar strobes of light and sound at high pulse power levels. The cited sparking effects have also been observed in high power, pulsed microwave irradiations (10). It is difficult to quantify the relative contributions of the two phenomena.

The anomalous microwave absorption effects observed by Kiel et al. (3), which involve a large complex component of permittivity (i.e., the loss factor, e", due to dissipation of applied power as heat), point to a conductive or strongly dipolar polymer as the source of the absorption and (presumably) the acoustic wave effects. During the reporting period we obtained measurements of capacitance properties using concentrated aqueous solutions of poly-3-AT as the dielectric of a capacitor. These measurements, accomplished with a Hewlett Packard 4274A multifrequency LCR meter, produced no detectable effects of dielectric loss beyond that of pure water at frequencies below 100 kHz (which should be observed as a reduction of the capacitor's Q-value), although it needs to be pointed out that the special liquid dielectric capacitor's value (~ 5 pF) is near the instrument's lower capacitance limit; thus, the anomalous loss factor seen at microwave frequencies appears to go through a maximum at a point below the range of frequencies of the cited earlier study (3). The reason behind the anomalous loss factor is still puzzling (and a reason for pursuing this investigation). Based on the very preliminary studies reported here, a characterization of the dielectric properties of aqueous solutions of DALM and poly-3-AT as well as combinations of poly-3-AT and diazotized luminol over a wide frequency range is identified as a high priority need.

Concentrated poly-3-AT solutions did not enhance the sensitivity of a circuit (Fig. 4) known to detect pulsed microwave radiation but without the usual high speed solid state electronic components. The circuit is essentially a regenerative (narrow bandwidth), high gain audio frequency amplifier in which the frequency determining capacitor (C_F, containing the concentrated polymer solution as its dielectric) and lead wires form a resonant microwave antenna. This solid state detector is not very sensitive but is prone to microphonics. Because of the latter property it was thought that the circuit might be detecting pulsed microwave energy through a microwave acoustic effect acting in capacitor C_F (Fig. 4). Measurements of the detector's output amplitude were obtained at 1 GHz and pulse powers under 0.1 W (in an anechoic enclosure) and were thus within the frequency range of the earlier permittivity measurements (3). The microwave acoustic effect is nonlinear and much less pronounced at low instantaneous pulse powers, and it may simply be that the circuit in question works entirely on the basis of rectification at a PN junction, though inefficiently. However, when the voltage rating (but not the dielectric composition) of capacitor C_F was increased, which increases the dielectric volume but not the capacitance C_F, it was found that the circuit became more sensitive to applied pulses, a result not inconsistent with an acoustic effect because the larger dielectric volume should cause larger deviations in the capacitor's dimensions. C_F amounts to a



R3, 5	100K RESISTOR(BRN BLK YEL)	C3 C4	47uF ELECT CAP
R8	100K RESISTOR (BRN BLK YEL)	C5	10uF ELECT CAP
R6	270 OHM RES (RED VIO BRN)	C6	.1 uF MONO CAP
R7, 9	10K POT PC MOUNT VERTICAL	C7	.001 uF DISC CAP
R10	2.2 MEG RES (RED RED GRN)	U1	LM1458 IC
R2	4.7K RESISTOR (YEL VIO RED)	U2	LM386N-1 I C
C1 C2	.047uF DISC OR MONO CAP	P C BOARD	080-00219
		MYLAR SPEAKER	

Figure 4 - Microwave sensitive circuit that might detect induced acoustic effects. The circuit shown here is the schematic supplied with the kit ("radar detector", purchased from Rainbow Electronics). Although it is not a high sensitivity design, it readily detected the pulse recurrence frequency and horizontal scan rate of an airport surveillance radar from two miles. The roll-off frequency of the 1458 IC is in the low MHz range, definitely not a microwave device!

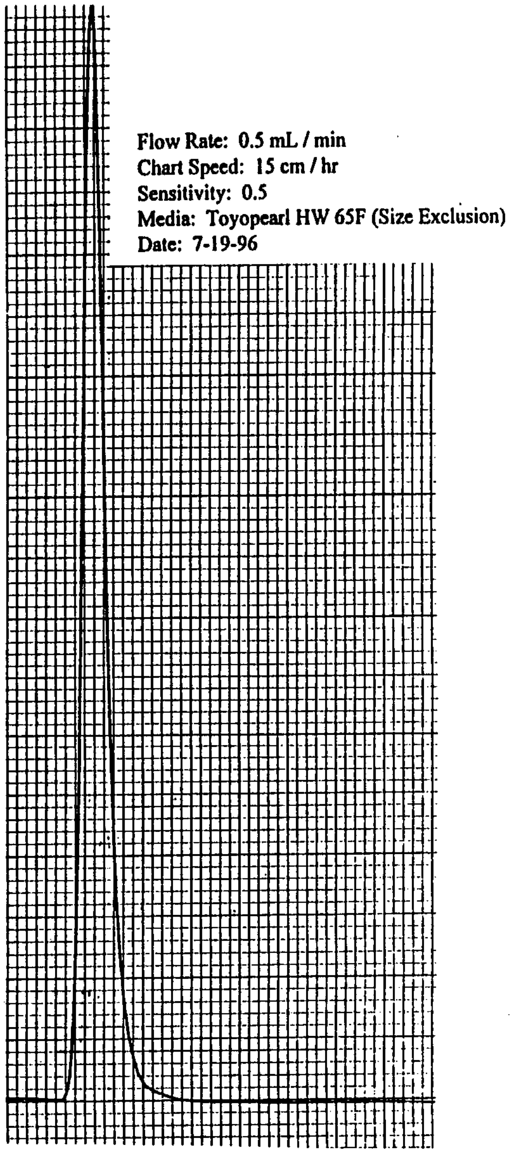
capacitance microphone in this circuit. The results with the poly-3-AT containing capacitor are not conclusive because we were unable to construct capacitors larger than about 20 pF, and values in the 0.01 - 0.1 microFarad range needed.

4.2.2. Structural Characterizations

The structure assigned to a chemical substance must account for all of its spectroscopic properties, as well as its chemical and physical characteristics. The new findings presented here have been examined in relation to our previously reported results (see Appendix 1).

The polymer of 3-AT is prepared by mixing 4.14 g of $NaNO_2$ in 20 mL water with 3.23 g 3-AT in 20 mL water and stirring at 26 C for 30 minutes. The reaction product mixture is then stirred into 1800 mL acetone in a large beaker and allowed to stand for 24 hours. Acetone precipitates the reacting material, and the polymerization reaction appears to continue after the precipitation step. Because of this continuing reaction, polymer preparations were allowed to "cure" for a month before further use. The dried polymer may be dissolved in a few mL of water for further procedures.

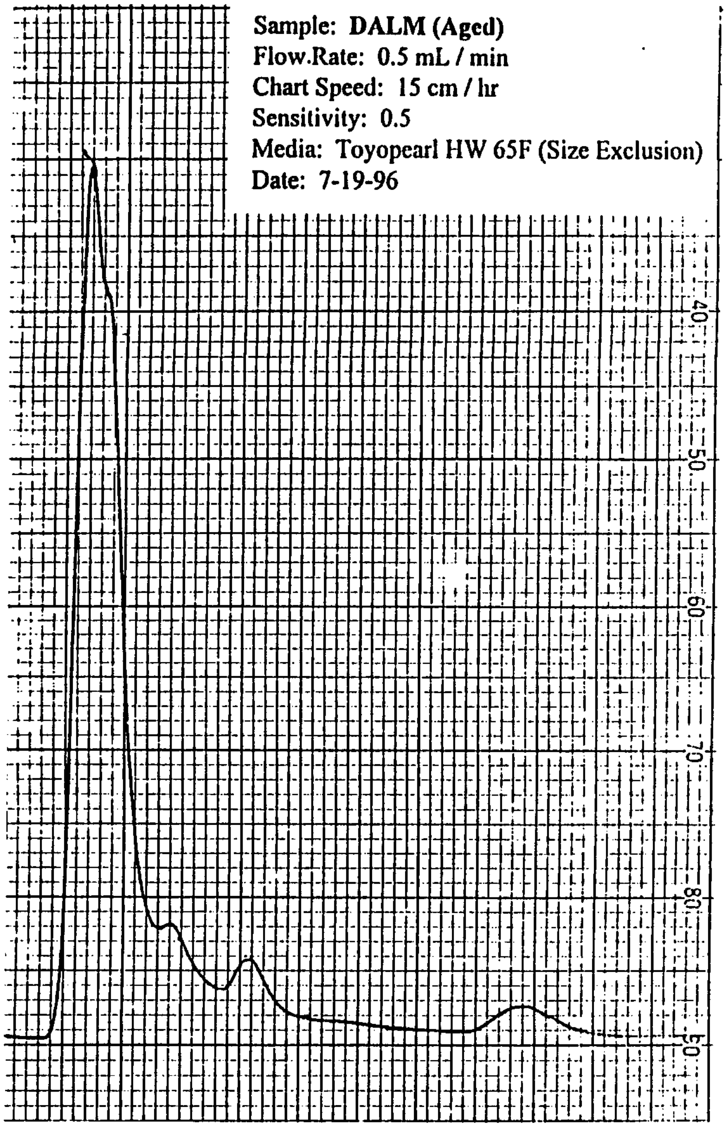
For analytical purposes, the Toyopearl HW 65F size exclusion chromatographic medium was found to be superior to all others tried so far in separating the components of DALM and related polymers; all pigmented components elute from columns of HW 65F, but other solid phases tried retained a substantial amount of pigmented material, which could not be removed by any means. Fig. 5 shows Toyopearl separations of DALM, poly-3-AT and a biologically produced DALM from the group at Brooks AFB. Although HW 65F functions as a molecular permeation/size discrimination medium in most of its



Flow Rate: 0.5 mL / min
Chart Speed: 15 cm / hr
Sensitivity: 0.5
Media: Toyopearl HW 65F (Size Exclusion)
Date: 7-19-96

Poly-3-AT on Toyopearl. The peak corresponds to the void volume.

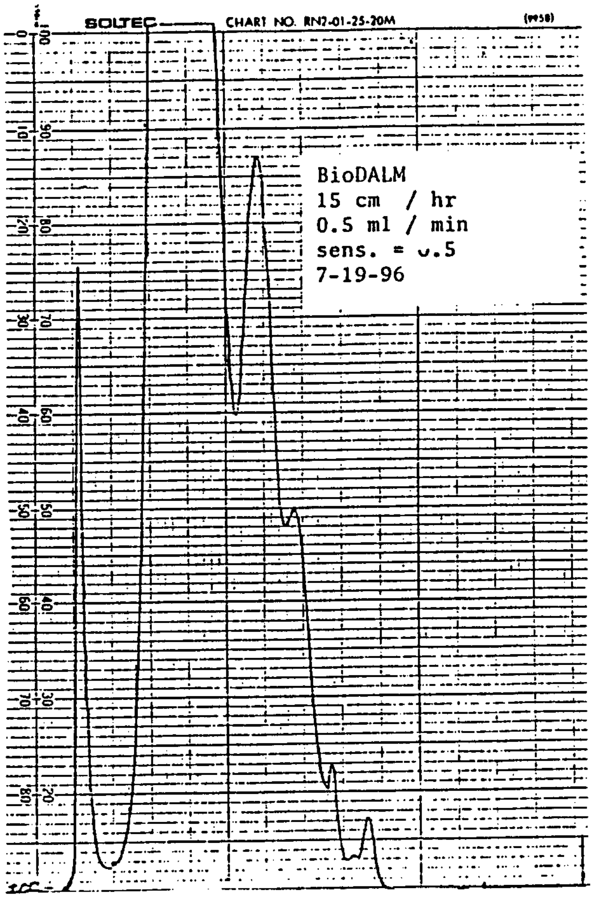
Figure 5 - Toyopearl separations of poly-3-AT, DALM and a biological DALM.



Sample: DALM (Aged)
Flow.Rate: 0.5 mL / min
Chart Speed: 15 cm / hr
Sensitivity: 0.5
Media: Toyopearl HW 65F (Size Exclusion)
Date: 7-19-96

DALM on Toyopearl. The left peak corresponds to the void volume.

Figure 5 - continued



BioDALM
15 cm / hr
0.5 ml / min
sens. = 0.5
7-19-96

Biological DALM on Toyopearl.

Figure 5 - continued

applications, some of the DALM pigments are retained beyond the salt permeation volume, indicating that affinity is also a factor in these separations.

Removal of low molecular weight products from polymerized 3-AT may be accomplished (preparatively) by simple dialysis but is best done by ultrafiltration on a Millipore 10,000 nmwl regenerated cellulose membrane in a pressurized cell. Polymer samples used in osmometric and nmr measurements were prepared in this manner. Further fractionations according to molecular weight are straightforward, e.g., by means of Millipore 1,000 and 3,000 nmwl membranes. The observed deep pigmentation of the 3-AT polymer obtained this way is due to an absorption beginning in the ultraviolet and trailing into the near infrared. The observed absorption is most likely due to extended pi-bond conjugation, a characteristic structural quality of conductive polymers, and thus consistent with the above cited anomalous microwave permittivity properties (3). Polymer fractions below 3,000 Daltons do not show the absorption extending into the near infrared but rather, a definite maximum near 410 nm and stronger absorption in the ultraviolet. These lower molecular weight materials have more of a red color than the brown of dilute higher molecular weight DALM or poly-3-AT solutions in water. Proton nmr spectra of the lower molecular weight fractions show more aliphatic resonance than aromatic resonance. The only possible source of the aliphatic material is from 3-AT; therefore it is now clear that the sidechain group is somehow being removed in a competing reaction. In any case these kinds of reaction products are apparently only part of the total reaction yield.

The number average molecular weight of the polymer of luminol and 3-AT was determined by means of simple membrane osmometry at 100,000 Daltons, and both it and the polymer from 3-AT alone form near-black- saturated aqueous solutions. The two polymers, if dialyzed for four weeks using Spectra/Por 12,000-14,000 Dalton molecular weight cutoff membrane tubing or purified by 15 cycles of 6:1 volume reductions (and reconstitution with pure water) over a Millipore 10,000 nmwl membrane, also yield very similar black solid residues which do not adhere to glass surfaces upon evaporation of the aqueous polymer solutions. When shaken gently, these residues break up into long black ribbons with a metallic sheen. The ribbons of both polymers are remarkably uniform along their length. Lower molecular weight fractions did not produce the ribbon forms. These similarities of DALM and the 3-AT polymer suggest that they have similar structural features, especially polymer linkages.

The CE analysis of a diazotized 3-AT reaction product mixture is typical of polymers. The electropherogram is dominated by a broad distribution envelope rather than an abundance of narrow peaks of individual low molecular weight products. More significantly this separation revealed two sets of low molecular weight oligomers fitting log mass-linear migration velocity relationships, merging into the larger polymer distribution. Thoroughly dialyzed poly-3-AT shows *two* distribution envelopes (11), and we note that our liquid state proton nmr spectra of poly-3-AT also show evidence of two components (Fig. 6a; compare with the spectrum of the material before dialysis, Fig. 6b; Fig 6c shows DALM before dialysis). This raises the likelihood of two different linkage modes in polymeric 3-AT. The 3-AT polymer is stable and decomposes (i.e., is fragmented) slowly even in strong reagents such as aqua regia.

As stated above, the UV/visible spectrum is suggestive of extended conjugation and band structure. Absorbance is maximum at 200 nm and falls away steadily with

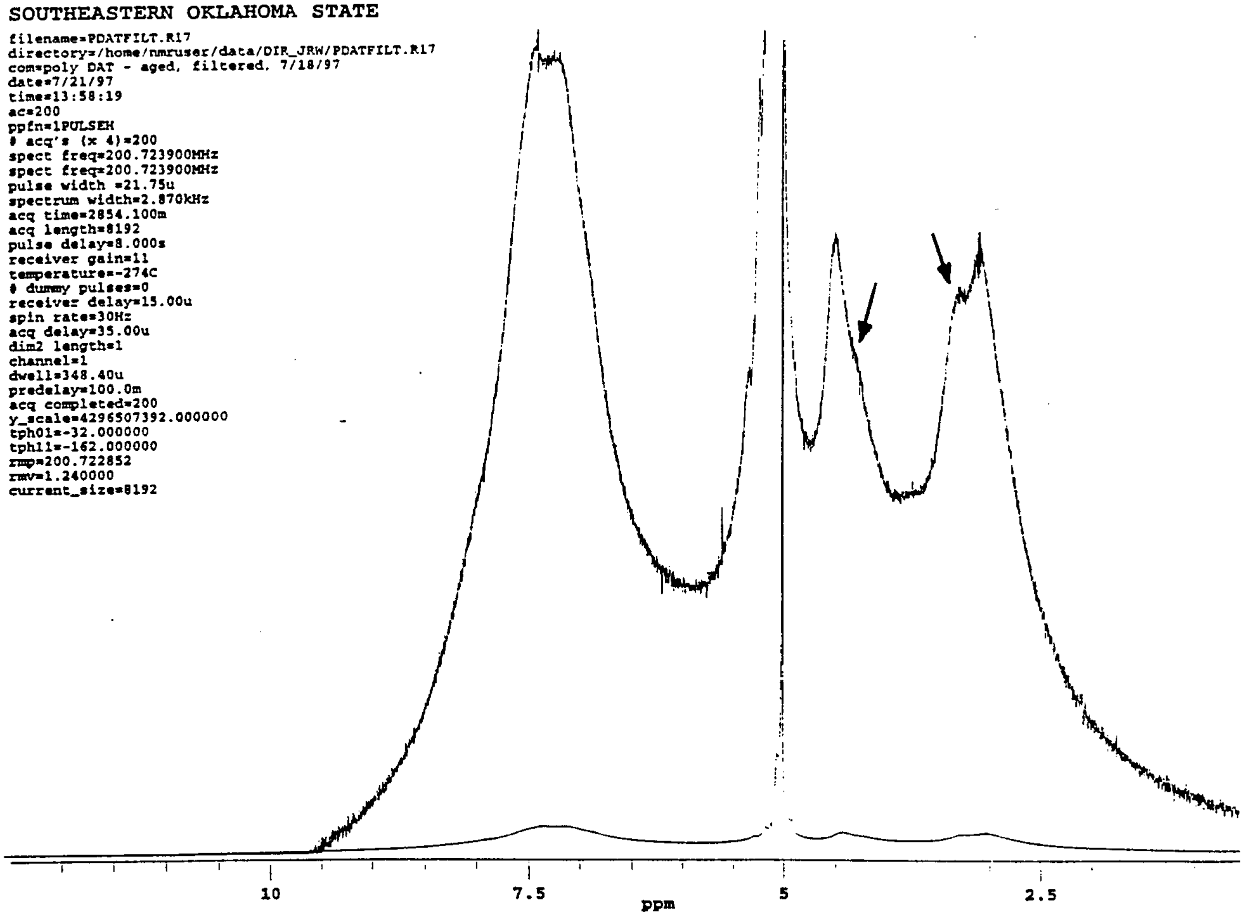


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Figure 6a. Proton nmr spectrum of thoroughly dialyzed poly-3-AT in deuterium oxide. Arrows mark two shoulders corresponding to alpha and beta hydrogens that may represent a second type of polymer; the spectrum is consistent with two CE envelopes observed for this material (11). Relative areas at 4.4 ppm (alpha hydrogen) and 3.1 ppm (beta hydrogens) are consistent with 1 and 2 hydrogens, respectively; the area associated with the broad resonance at 7.3 ppm is consistent with 3 hydrogens rather than the 2 required by the polyphenylene structure of Figure 7. This integration is a better fit with the alpha amino/aryl linkage, a structure that is also consistent with the elemental analysis but unable to account for the strong pigmentation. With compelling evidence for at least two polymers, perhaps one is conjugated while the other is not. The excess of aryl resonance is more easily explained by loss of a portion of the aliphatic sidechains (see below). Although poly-3-AT is simpler than DALM, it possesses a degree of complexity.

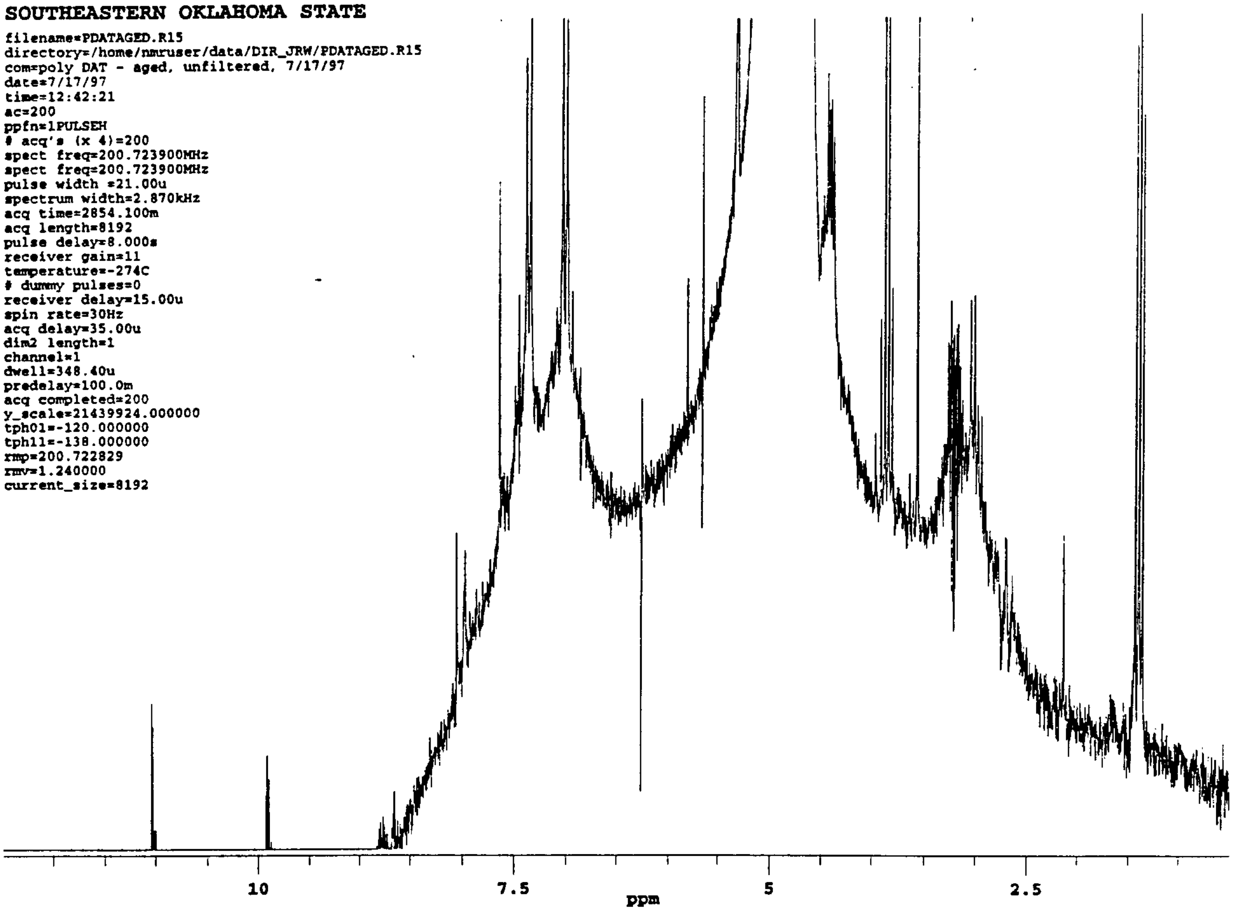

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Figure 6b. Proton nmr of poly-3-AT without dialysis.

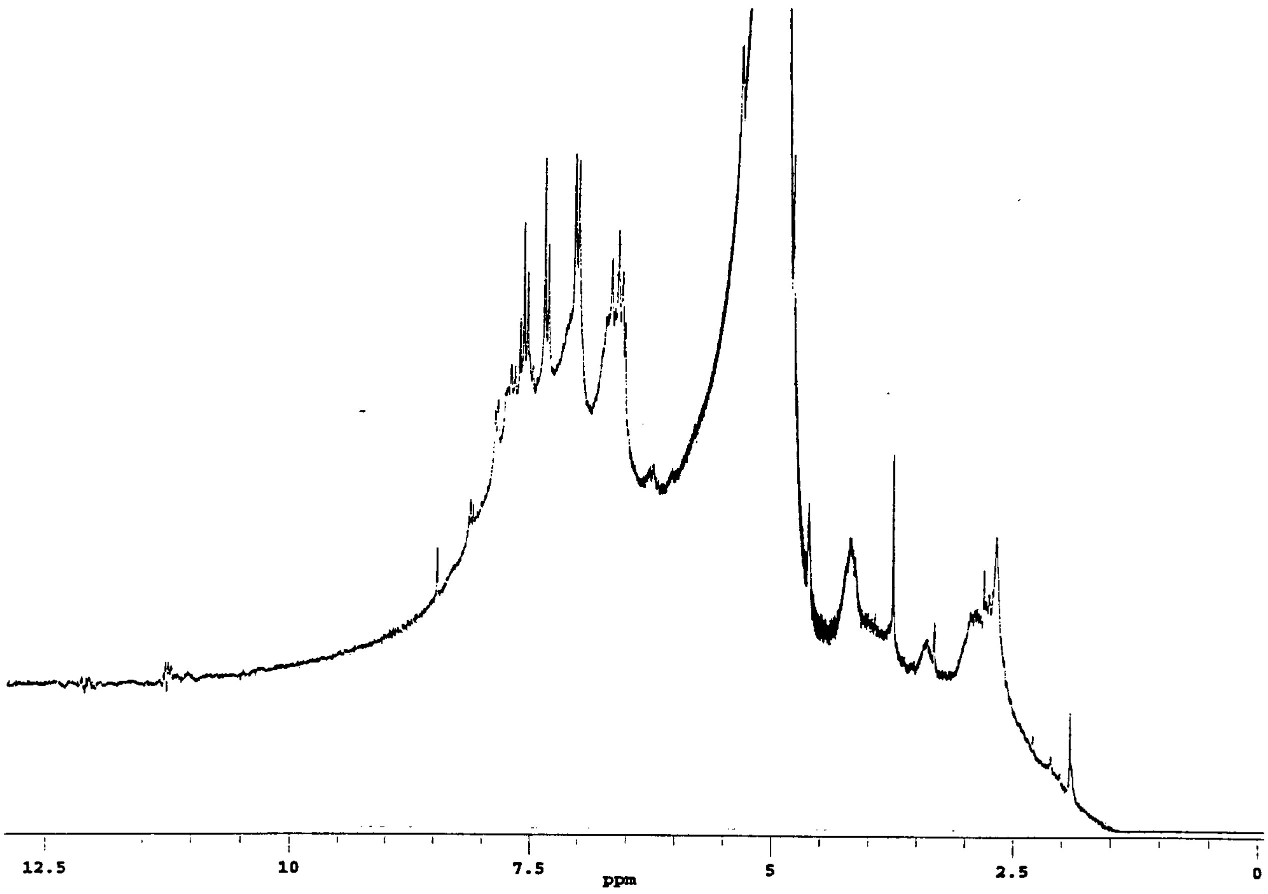

Figure 6c. Proton nmr of DALM without dialysis.

increasing wavelength, extending measurably into the near infrared to about 900 nm. Fluorescence spectra obtained by Dr. Tom Cooper at Wright-Patterson AFB are indicative of extended conjugation, a finding that cannot be ignored. A longstanding question about the pigmentation DALM and poly-3-AT still open: is the bulk of the polymer a pigment or is the pigment just one of two or more components of a mixed polymer?

To the eye the polymer is an indicator and is brown at neutral pH but changes to a red-brown above approximately pH 8.5 (the color is similar to that of the low molecular weight fractions at neutral pH). In contrast a plot of pH versus titration equivalents added shows a change near pH 6.5. The color changes are associated with a broad shoulder centered near 500 nm. If the chromophore is based on catenation of 3-AT's aromatic ring and the phenolic -OH groups are retained in the polymer, the latter could be the basis of the polymer's indicator properties; but the data do not establish this. The titration could just as well involve a carboxylate group. The indicator properties could also be due to a lesser component of the product mixture.

From the anticipated reaction chemistry, the polymer backbone might be diazo-linked; however, there is no spectroscopic evidence for such a structure. Neither Raman nor infrared detected an aryl linkage involving the -N=N- group. Also, one polymer batch was prepared from ^{15}N-enriched sodium nitrite and subjected to ^{15}N-CP/MAS nmr spectroscopy, with the result that no resonance was found at the expected aryl diazo chemical shift. The spectrum was very weak and attributable to residual nitrate and nitrite impurities (but not $C=N^+=N^-$ because the strong 2030 cm^{-1} infrared absorption is absent in unlabeled poly-3-AT preparations). Although $SnCl_2$ appeared to cleave the polymer in one attempt, the experiment did not prove to be reproducible.

The polymer's elemental analysis is also inconsistent with a diazo linked polymer, which (counting the alpha amino group of 3-AT) should have three nitrogens per monomeric unit, i.e., a C/N molar ratio of 9/3, or 3.00. As shown in Table II, the proportion of nitrogen in the polymer is substantially lower. The mean value of measured C/N molar ratios is 6.82. The full analysis of batch 1 is 44.48% C, 4.34% H, 29.91% O, 7.36% N and 8.39% Na.

Table II. Carbon and Nitrogen Analyses of Different Poly-3-AT Batches

Batch	Carbon	Nitrogen	C/N Molar Ratio
1	44.48%	7.36%	7.05
2	39.69%	7.06%	6.56
3	49.62%	8.44%	6.85

The structure could be predominantly of a polyphenylene type, formed through an electrophilic aromatic substitution involving an aryl cation derived from the expected diazonium species (S_N1 process with N_2 the leaving group). It is noted that HMO calculations for the 3-diazonium derivative of tyrosine (3-DT) indicate that the electron density at position 5 is not diminished from that of 3-AT, and a proton nmr spectrum which we obtained for 3-DT shows no downfield shifting of the position 5 hydrogen. A possible linkage resulting from cationic attack at ring position 5 is shown in Fig. 7, but until more mass spectroscopic evidence is secured (see below), the structure is regarded as conjecture. At this writing the Hewlett-Packard mass spectrometer has been installed, and

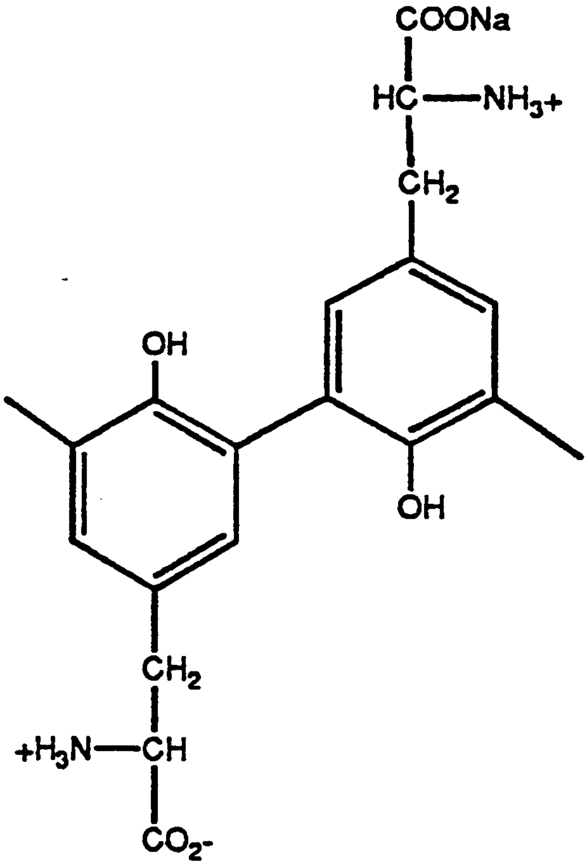

Figure 7. A postulated polyphenylene-linked polymer of 3-amino-L-tyrosine.

Dr. Smith hopes to realize some progress into mass spectrometric characterizations by mid-summer of 1998.

An alternative structure which also approximates the observed elemental analysis, an aryl amine linkage involving the alpha-amino nitrogen of 3-AT (also by an aromatic S_N1 process, only with the alpha amino group replacing the diazonium), does not lead to extended conjugation and is thus hard to reconcile with the properties of poly-3-AT and DALM; however, it could be one of the two components of the polymer if the other has extended conjugation to explain the observed pigmentation and dielectric properties of the material. There is real concern that both the aryl amine and phenolic -OH groups of 3-AT (and also the sidechain nitrogen) might be somehow involved directly in the polymerization, and if so, the reaction would be of an uncommon variety. That the reactivity depends on the aromatic portion of the molecule is shown clearly by the fact that tyrosine does not react with nitrite to form a pigmented polymer like DALM or poly-3-AT under similar reaction conditions.

The C/N ratio expected for the hypothetical structure of Fig. 7 is 9/1. Considering the CE evidence for two polymer types, the measured C/N ratio (6.82) could find an explanation in a mixture, with one component containing more nitrogen.

The ^{13}C-CP/MAS TOSS spectrum of the polymer (Fig. 8a) approximates a line broadened carbon spectrum of 3-AT itself, with the aryl and carbonyl resonances between 110 and 180 ppm and two aliphatic resonances at 38 and 72 ppm, showing that the alpha and beta carbons of 3-AT are retained as sp^3 centers in the polymer, as in the structure of Fig. 7 (and also expected for the alpha amino/arylamine linkage). Fig. 8a does not show clear evidence for two polymers types; the downfield

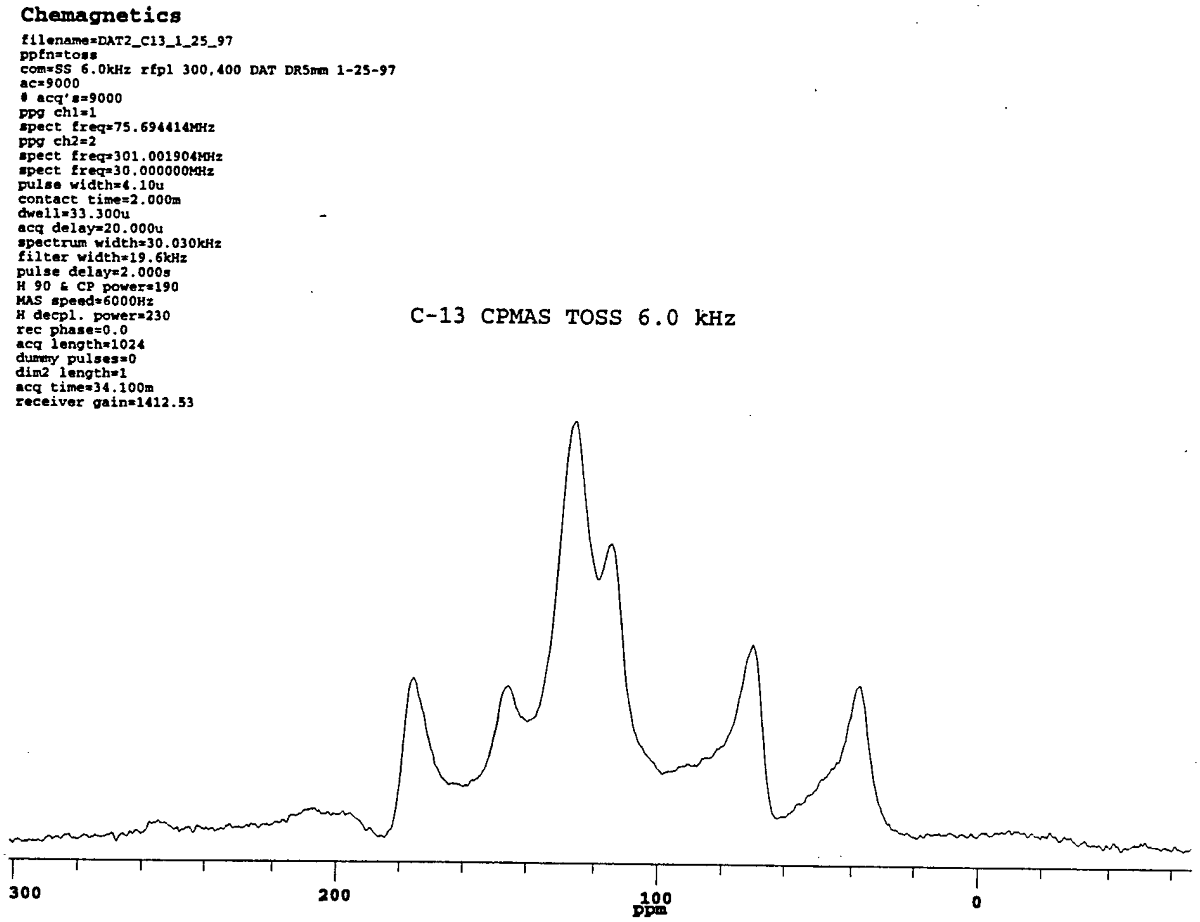

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Figure 8a. Carbon CP/MAS spectrum of solid poly-3-AT.

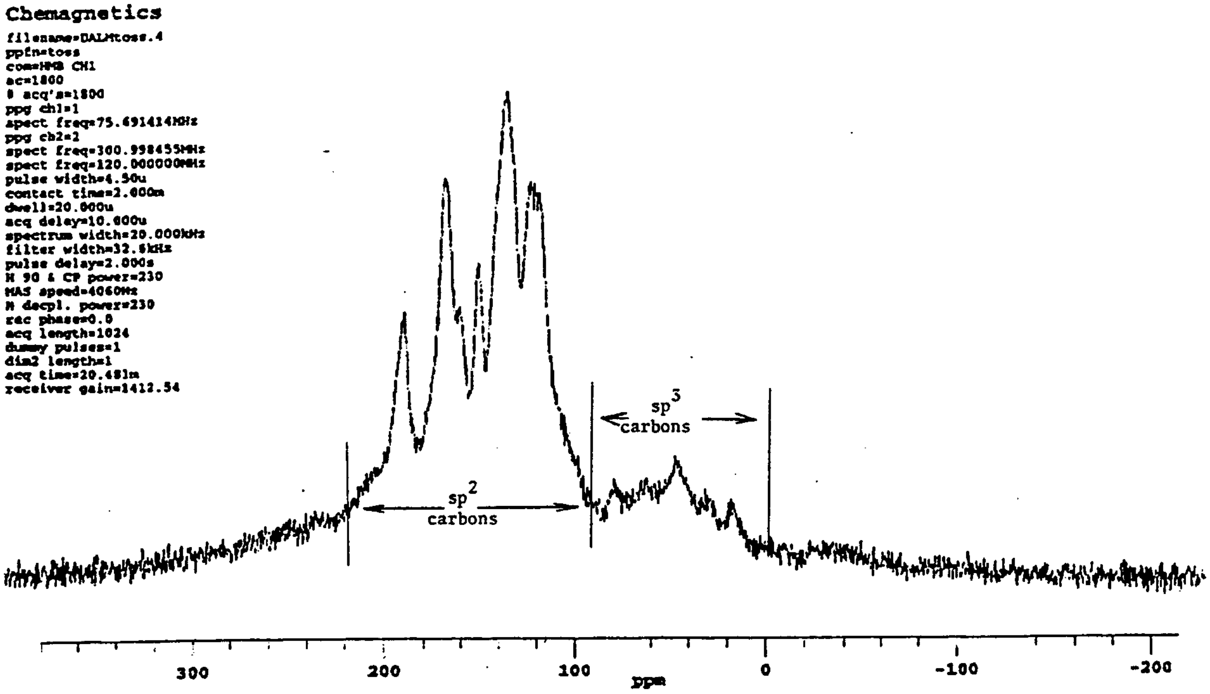

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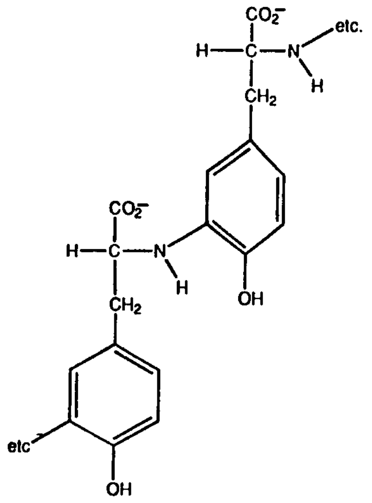
Figure 8b Carbon CP/MAS of solid DALM

A



B

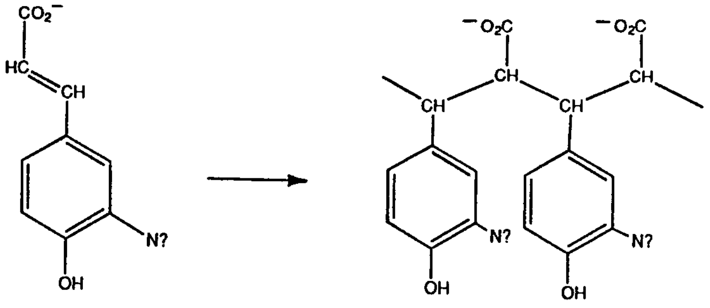


Figure 8 - continued. The aliphatic resonances of both poly-3-AT and DALM are consistent with sidechain groups in the polymer. However, in view of evidence of a degree of sidechain scission and sidechain polymerization in these reactions, it is possible that the aliphatic resonance could be due to a separate polymer. Otherwise, the structure of Fig. 7 is consistent with these carbon nmr spectra, the infrared spectra, the strong absorption of light across the visible and part of the near infrared spectrum and the elemental analysis (granting some impurities), but it does not fully explain the anionic charge, which is a worrisome point. An aryl amine structure (A) satisfies both the proton and carbon nmr spectra, the anionic charge and the elemental analyses but not the observed pigmentation since conjugation is limited to isolated aromatic rings. Deamination to a *trans*-cinammic acid derivative and an acrylic acid-type polymerization (B) leads to a product with properties similar to the aryl amine (A), and for the same reason does not explain the pigmentation or the strong evidence (and expectation) that the aryl amine is involved in the reaction. The underlying problem is that both DALM and poly-3-AT are polymer mixtures; we have not yet isolated a pure component.

shoulders on the two sp^3 carbons may be phase errors. The ^{13}C-CP/MAS spectrum of DALM (Fig. 8b) is considerably more complex. DALM is a more random polymer, as is evidenced by broader nmr lines and more diverse chemical shifts. The water solubility of both DALM and poly-3-AT is assumed to be a result of ionophoric sidechain groups. Also, luminol is a part of the DALM polymer (which results in a relatively larger amount of sp^2 resonance compared with that in Fig. 8a) and not just luminol or a luminol derivative mixed with the 3-AT polymer.

Proton nmr spectra of crude DALM solutions at high pH (i.e., no dialysis and conditions which solubilize luminol and luminol derivatives) retain the very large low-field broadline component with small amounts of free, unreacted luminol showing as narrow lines on top of the broad polymer resonance (Fig. 6c). Nearly half of the aromatic resonance of DALM is due to a luminol component associated with the polymer. Free luminol and luminol derivatives produce narrow line spectra, not the broad lines seen in DALM.

As noted above, tyrosine and phenylalanine do not form DALM-like polymers and are relatively unreactive under diazotization conditions, even considering the low solubility of phenylalanine. As noted above, these substances were also tried in attempts to truncate the polymerization of DALM and poly-3-AT, with no success. Such results show clearly the need for the aryl amine group and are further support for a structure like that of Fig. 7.

A structure of the type in Fig. 7 is further supported by preliminary mass spectroscopic evidence. Hewlett-Packard carried out demonstration separations of low molecular weight poly-3-AT fractions using an HPLC system and a mass spectrometer of the type that we were purchasing from them. One of the components yielded a primary mass peak at 376 (M+H), consistent with a dimer of the type shown in Fig. 7; also, the M+1 and M+2 peak intensities are in agreement with the formula. More surprising was the fragmentation pattern of another component (the first component from the HPLC column), which shows clear evidence of being a *nitrogen containing* polymer with a mass repeat interval of 82. This mass interval is too small to account for the aromatic nucleus of 3-AT and its substituent atoms.

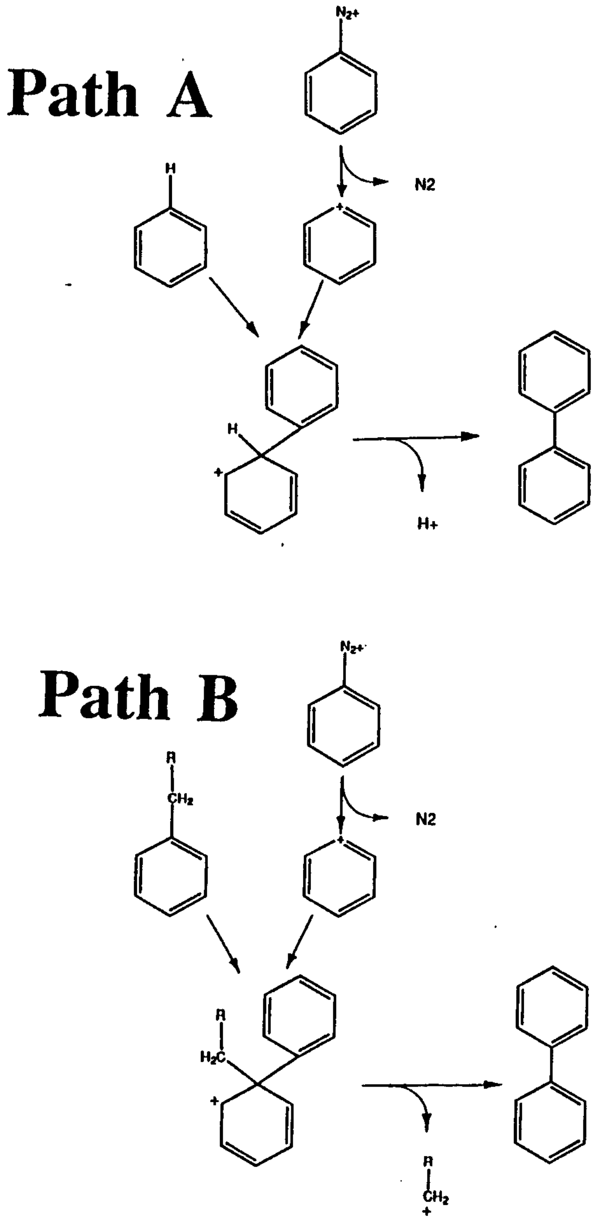
That the latter substance could be derived from the aliphatic sidechain is supported by recent nmr findings. Using ultrafilters, poly-3-AT was fractionated into several molecular weight ranges, e.g., below 500; 500-1,000; 1,000-3,000; 3,000-5,000; 5,000-10,000 and above 10,000 (g/mol). Using ^{1}H-nmr spectroscopy, an aliphatic envelope (with some resolution of narrow lines) was observed in the 500-1,000 g/mol fraction but almost no aryl nmr absorption was detected in this specimen. In agreement with this result, an integration of aryl and aliphatic resonances in poly-3-AT above 10,000 g/mol finds too much aryl absorption to satisfy the structure of Fig. 7, consistent with the loss of part of the sidechain. The high-field ^{13}C liquid state spectrum of this material (OSU 600 MHz nmr; one of Dr. Smith's preparations) shows mostly a broad *aryl* resonance.

Significant quantities of material are found in the lower molecular weight fractions of poly-3-AT. In one peparation the amount of polymer above 10,000 g/mol (0.292 g) was only slightly more than the content of the 3,000 - 10,000 g/mol band (0.272 g). The 1,000 - 3,000 g/mol band from this batch contained 0.043 g of material.

If a polyphenylene polymer is formed by way of an aryl S_N1 reaction involving the reactive phenyl cation (12) the sidechain (rather than a proton) could conceivably be displaced to a nucleophilic receptor (perhaps a growing sidechain polymer?), as suggested in Fig. 9. This scheme is admittedly speculative, but the aliphatic material appearing in the reaction products has only 3-AT as its source. An explanation is needed. These are important findings, and they justify earlier concerns that an unusual reaction might be taking place.

Dr. Smith is currently considering the possibility that a diazotization at the aliphatic (alpha) amino group might lead to deamination with the formation of a cinnamic acid derivative, i.e., one with an olefinic sidechain that could enter a polymerization analogous to that of acrylic acid. Although this type of polymerization would not lead directly to extended conjugation, it would explain the anionic nature of DALM and poly-3-AT. In earlier work we looked for but did not find the infrared absorption of an aliphatic diazo group in these polymers (i.e., the C=N=N group, which ought to be strong at 2030 cm^{-1}; even residual material might be detectable). Perhaps we did not catch the reaction at the right stage. The 2122 cm^{-1} infrared absorption observed in thoroughly dialyzed poly-3-AT was thought to be due to a zwitterionic pair (the alpha amine and carboxylate groups), a feature that is characteristic of amino acids. It is possible that the assignment is incorrect, and since we now have evidence that sidechain modifications are taking place, the possibility of a deamination needs to be explored.

When this period of funding began, we were encouraged to be starting it with a working polymer fragmentation method that yielded large amounts of low molecular weight products. It had been found that aqua regia (a source of nitrosyl chloride) converted the broadline resonances of DALM into a complex of narrow line, superimposed spectra (Fig. 10). Partial chromatographic purifications of these products were monitored by proton nmr spectroscopy, and this permitted a recognition of the



Not free but to a nucleophile.

Figure 9. Possible cationic aromatic substitution modes. Loss of molecular nitrogen from a diazonium compound generates aryl cations in what has been called the "aryl S$_N$1 reaction". Electrophilic aromatic substitution through cationic attack according to path A is completed by loss of a proton, which is the generally envisioned mechanism, but in path B the leaving group is a carbocation. The primary cation is not expected to occur as a free entity but as its equivalent by nucleophilic capture. This tentative model is offered to explain the sidechain scission that is actually observed.

spectroscopic signatures of definite compounds in the mixture; these were labeled X, Y, Z and A, as shown in Fig. 10. The X product was isolated, purified (by recrystallization) and characterized by elemental analysis, melting point and spectroscopic methods as 3-chlorophthalic acid, obviously a derivative of luminol. But this fragment is completely disconnected from whatever it was attached to in DALM and thus provides no connectivity evidence other than to rule out the three protonated ring positions as attachment sites. The aqua regia fragments are acidic, titrating with a pKa of 3.25.

Product Y is hygroscopic and our attempts to purify it have failed. It is definitely derived from the 3-AT component of DALM. Poly-3-AT, prepared by the method for DALM but omitting luminol, may be digested by the HCl/HNO_3 reagent, and the reaction product contains mostly Y, an electrolyte mixed with other contaminating electrolytes. Considering the simplicity of Y's nmr spectrum, it is almost certainly an isolated small molecule, possibly a symmetric chlorinated/nitrated cinnamic acid derivative, and might not provide unequivocal connectivity information, although it could suggest ring substitution positions. Similar arguments apply to components Z and A, which appear to derive from luminol. The available evidence from the fragmentation of DALM by aqua regia has not contributed significantly toward a structural characterization of the polymer.

$SnCl_2$, a reagent which reductively cleaves -N=N- linkages (forming $-NH_2$ groups) appeared to act on poly-3-AT in one attempt, producing a low molecular wright product which we designated BSn. Several subsequent attempts to reproduce a fragmentation with the reagent have failed. This reagent should completely fragment an azo-linked polymer. The reagent $Na_2S_2O_4$ (which also cleaves azo bonds) did not fragment a type of poly-3-AT prepared in solution (rather than from acetone precipitated material; see the

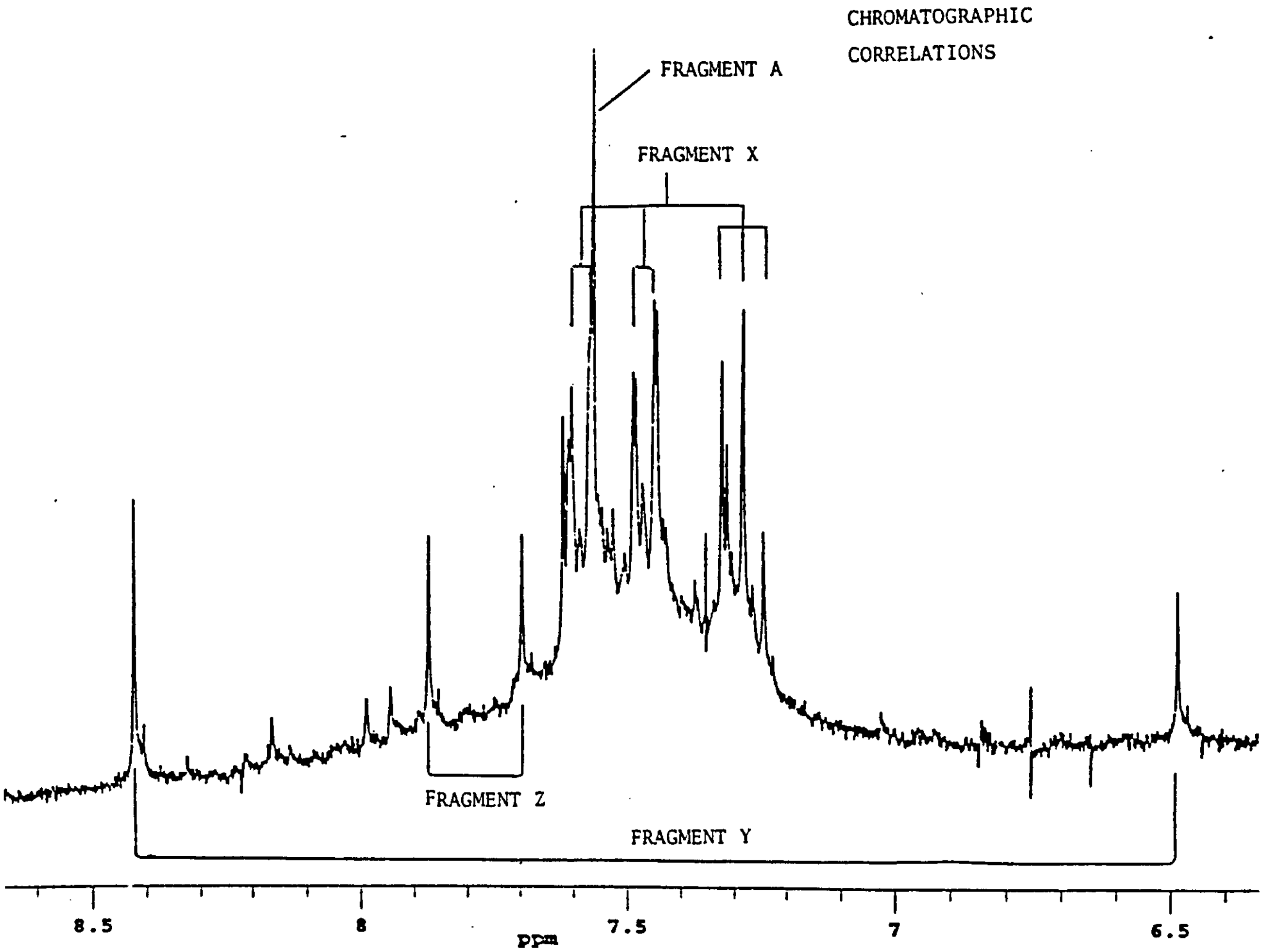


Figure 10. Proton nmr of Aqua Regia Fragments from DALM. Partial chromatographic separations of the components labeled X, Y, Z and A were achieved on silical gel columns using various mixtures of dioxane and acetone as the eluting medium. The eluted fractions were monitored by proton nmr spectroscopy, which found the distinct components shown here. Ion exchange chromatography was also tried, with disappointing results.

report of F49620-89-0530). The poly-3-AT which forms in standing solutions of diazotized 3-AT may have an entirely different structure than the polymer which forms in the acetone precipitate of diazotized 3-AT, although the elemental analyses of these substances are not very divergent. The solution polymer is much less pigmented than the one which forms in acetone precipitates. As noted above, elemental analyses of poly-3-AT find a C/N ratio much too large to account for a predominantly azo-linked polymer.

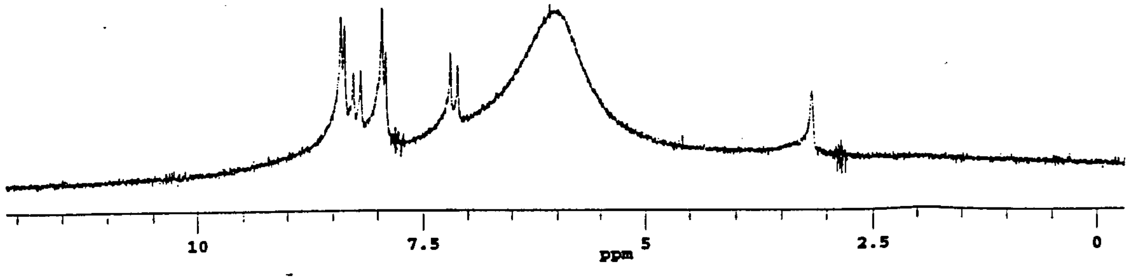
The molecular weight of poly-3-AT is decreased in the presence of potassium iodide, which replaces the diazonium group with an iodine atom, thus apparently competing with the polymerization process (i.e., the diazonium group is removed before it can react with another monomer). This approach did not lead to polymer fragments which were small enough for preparative isolations and characterizations. An option which has not been fully explored is the Sandmeyer reaction, which uses a copper catalyst to decompose diazonium groups.

4.2.3. Additional findings

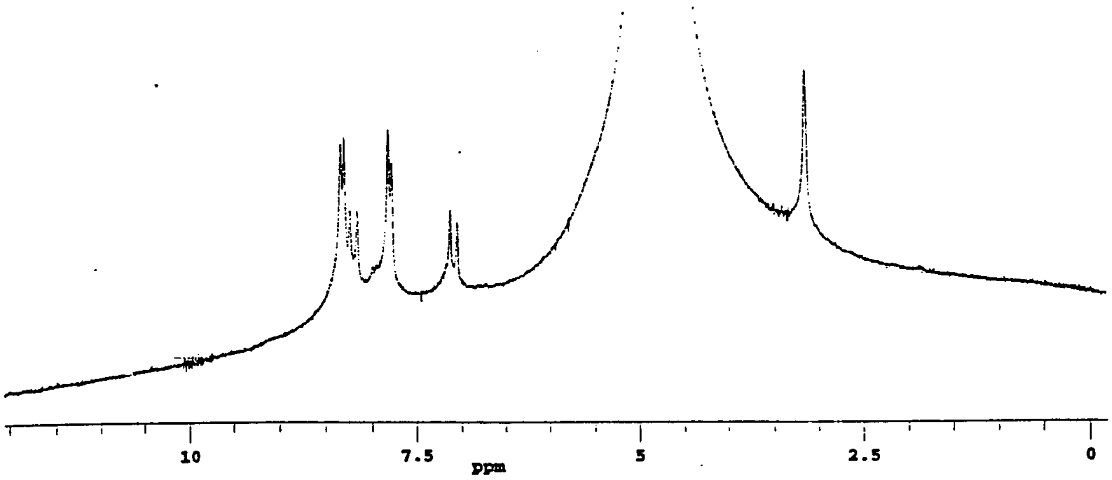
During the last six months of the project, the technician (Brenda Russo, a microbiologist) established a culture of the yeast *Rhodosporidium toruloides*, which produces an L-phenylalanine deaminase (18). This was done with an intent to deaminate 3-AT at the alpha position, i.e., to obtain 3-amino-p-coumaric acid. This compound would have allowed us to evaluate possible DALM-like polymerizations with a modified sidechain; this is one approach to the question of sidecain involvement in the polymerization reaction, and in hindsight it is also a way to test Dr. Smith's very recent postulate that sidechain deamination (by aliphatic diazotization) leads to an unsaturated sidechain derivative (a p-coumaric acid derivative) and a subsequent acrylic acid polymerization.

Brenda had a measure of success in this attempt since her yeast enzyme apparently acted upon 3-AT. Fig. 11a shows a proton nmr spectrum of the reference compound p-aminocinnamic acid, and Fig. 11b presents the spectrum of a substance obtained by means of a yeast broth containing 3-AT (as the nitrogen source) in place of sodium nitrate. The aryl/olefin signatures of these two substances are very similar, and Fig. 11b is like 11a in having a doublet grouping typical of a symmetrical, p-substituted aromatic ring, i.e., it is more like the spectrum of p-coumaric acid than the desired 3-amino-4-hydroxy-cinnamic acid, which would be the result if the yeast also removed nitrogen at the 3-position of the aromatic ring! It is noted that the spectrum of Fig. 11b came from a reddish brown film that formed at the interface between the aqueous and ether phases in the separatory funnel during the extraction process. This zone may be removed with a long Pasteur pipet.

An additional finding surfaced during the writing of this report (late June, 1998). Electrospray mass spectrometric measurements for anionic species obtained from solution polymerized 3-AT (see the report of F49620-89-0530/89-NL-258, Appendix 1) has shown a polymer repeat structure with a mass increment of 136 (Fig. 12). This mass is smaller than the monomer's and can account for an aryl ring and only part of the substituent groups. The HPLC component yielding the 136 mass repeat unit did not absorb at wavelengths beyond about 300 nm (thus does not have extended conjugation), and it is a fairly polar material. Formulas which fit a 136 mass structural unit are limited, and the candidates which fit the (M + 1)/M ratio are being evaluated at this writing. In



p-Aminocinnamic acid, proton nmr spectrum.



Product with 3-amino-L-tyrosine in *Rhodosporidium toruloides* broth, proton nmr spectrum.

Figure 11. Nmr spectra of p-aminocinnamic acid and a derivative of 3-amino-L-tyrosine prepared enzymatically.

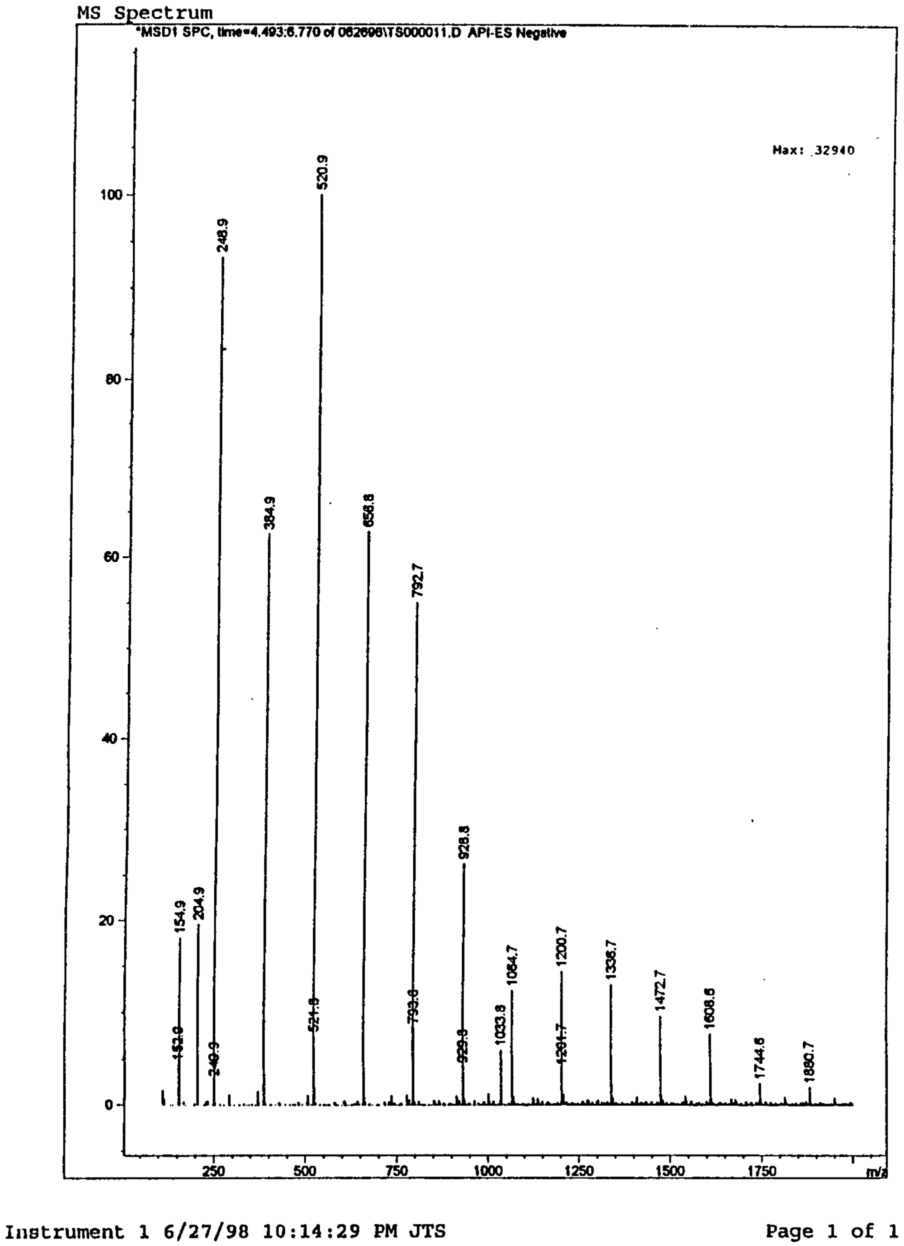


Figure 12. Mass spectrum from 3-AT polymerized in solution showing a 136 mass repeat unit. Although the products formed in acetone precipitates are different, this component might be a lesser product in the latter situation.

contrast, dialyzed preparations of the polymer which forms in acetone precipitates of diazotized 3-AT did not show this repeat pattern.

As noted above, the nitrogen-containing polymer with a repeat mass of 82 is so small that it would have to originate from the sidechain part of 3-AT. Here again is evidence that some kind of sidechain scission is taking place in these polymerizations. Work underway by Dr. Smith's group should decide if these polymer modes represent the bulk of the polymer or if they are lesser components. The findings certainly support the earlier stated view that poly-3-AT and DALM are mixtures of different polymers. Mass spectroscopy is proving to be effective in resolving questions about DALM and poly-3-AT.

5. CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE INVESTIGATIONS

Earlier investigators may have overlooked diazotization polymerizations of compounds such as 3-AT and its structural analogs because of the required conditions, i.e., formation of the diazonium species in solution, followed by removal of the solvent. The polymerization takes place in the condensed material, and we have good evidence that the solution and condensed state polymerization products are not identical. Some of the characteristics of DALM and poly-3-AT obtained from condensed material are summarized in Table III.

Given the available evidence, the working hypothesis is that both DALM and the simpler polymer obtained by diazotization of 3-AT alone have predominantly the same backbone linkage, and further, it is thought that the linkage might be mostly of a polyphenylene type, though not exclusively a polyphenylene structure. It is further hypothesized that the microwave interactive properties of DALM and possibly the thermoluminescence of DALM derivatives are consequences of the conductive properties of a polyphenylene polymer, or at least some kind of conjugated, conductive polymer present in the material. Accompanying polymeric component or components may not be conjugated. Since available evidence rules out large polymers based on luminol alone it is postulated that luminol interacts with the 3-AT polymer at peripheral functional groups, specifically at the carboxylate and amine positions on the aliphatic sidechain.

These working hypotheses are in principal subject to testing by means of spectroscopic and similar characterizations of the polymers, many of which have already been carried out but with less than definitive results. Methods which have not been thoroughly explored and should be the basis of a strategy for further investigation are as follows:

A. Comparative studies of structural relatives of 3-amino-L-tyrosine should reveal the molecular features needed to obtain DALM-like polymers. This would involve diazotization polymerization attempts with variants of the 3-AT structure, i.e., with or without specific functional groups and also with groups relocated to different positions. If these synthetic approaches lead to novel DALM-like substances the latter could be subjected to spectroscopic and chemical characterizations and other measurements.

B. The strategy should also focus on attempts to establish (or rule out) the postulate that luminol attaches to the core polymer at peripheral groups. In this approach purified poly-

3AT/diazotized luminol combinations would be tested to determine if they mimic the properties of DALM .

C. In both A and B above, the characterization methods (spectroscopic and chemical) should also be supplemented with measurements of polymer microwave absorptive properties, both continuous and pulsed.

D. The pigmented component is thought to be a structure with extended conjugation. If it is conductive the possibility of a quantum wire-like phenomenon needs to be considered in explaining the microwave interactive effects (19).

Table III - Summary of dialyzed poly-3-AT and DALM characterizations:

Method	Results
^{1}H-nmr	Shows only broad polymer envelopes with both aryl and aliphatic resonances. The aryl/aliphatic ratio is larger than the value predicted from the structure of Fig. 7 but is consistent with the finding of an excess of aliphatic material in lower molecular weight fractions and partial sidechain scission. The two aliphatic resonances have shoulders and could be the spectra of at least two different polymers superimposed.
^{13}C-CP/MAS	The spectrum from solid poly-3-AT clearly shows the two aliphatic sidechain carbons, consistent with the two aliphatic hydrogen environments in spectra. The ^{13}C-CP/MAS spectrum of poly-3-AT is much simpler than the ^{13}C-CP/MAS spectrum of DALM. The former is comparable to a linebroadened spectrum of the monomer while the latter has relatively more aryl absorption due to its content of a luminol derivative.
^{15}N-CP/MAS	This was a labeling experiment with ^{15}N -enriched sodium nitrite, and the spectrum was weak and devoid of a resonance attributable to the -N=N- group.
Infrared	No evidence for an aliphatic diazo group ($C=N^{+}=N^{-}$), although it could have been a transient; there is some evidence for amino acid-like zwitterionic groups on the sidechain.
Raman	No evidence for -N=N-
Elemental analysis	The C/N ratio is too large to support -N=N- linkages.
Electrophoresis (CE)	Evidence for more than one type of polymer.
Mass Spectra	Evidence for more than one type of polymer. One mass and its (M+1)/M ratio was consistent with a dimer like that of Fig. 7. Mass spectroscopy studies are just starting.
UV/Visible spectra	At least one component of poly-3-AT has extended conjugation. An absorption trailing into the near infrared suggests band structure.

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7. PERSONNEL SUPPORTED

Southeastern Oklahoma State University is a designated minority institution (Native American), and the AFOSR participants have reflected the ethnic composition of the institution, as we believe they should. Although the project is concerned with purely scientific questions, it has also served to pipeline students and technicians into pure or applied science careers. The various individuals involved have gained experience with chemical methods and concepts and acquired critical thinking skills. Other than a requirement that technicians have at least a baccalaureate degree, there is little distinction between technicians and students because most of our research participants go on to graduate programs. This is illustrated by a follow-up summary of the activities of individuals involved in the AFOSR project during the reporting period:

7.1. Technicians

Gene McDaniel is now a second year medical student in Florida and has told us that he is doing well in his courses.

Kenneth Roberts is currently working toward his Ph.D. degree in chemistry at Iowa State University (second year), doing very well.

Joe Studer is in a chemistry Ph.D. degree program at Oklahoma State University. He told us that he thought physical chemistry would wipe him out, but I note that he survived the ordeal and is now going into his second year.

Kris Wood graduated Summa (GPA = 4.0) and became the technician for two semesters before taking a forensic chemistry position with Oklahoma State Bureau of Investigation (OSBI). He states that he wants to pursue a forensic science Ph.D. degree.

Brenda Russo, the last technician, holds a master's degree in technology and has expertise in biochemical and microbiological methods. She is now an adjunct faculty member at East Central Oklahoma State University.

7.2. Students

Middleton Reeves is a student in a registered nurse program at University of Central Oklahoma.

John Wetzel is a member of an engineering group at Texas Instruments Corporation.

Kin Whittington is a medical student (Doctor of Osteopathy) at Oklahoma College of Osteopathic Medicine and Surgery (OCOMS, Tulsa Oklahoma), and he reports that he is doing well.

Cassie McLemore finished her undergraduate chemistry degree in the fall of 1997 and intends to enter a physician's associate program.

LaChelle Maddox and Keri Campbell are working toward their baccalaureate degrees in chemistry, and both want to pursue pure chemistry careers

8. PUBLICATIONS

8.1. Published or In Press:

L. Maddox, M. Reeves, K. Wood, K. Roberts, J. Studer, J. Wetzel, J. T. Smith, K. Whittington, J. L. Alls, J. E. Parker, E. Holwitt, J. Kiel and J. R. Wright, **1998**, "Acoustic Wave Dosimetry Based on Diazotized Luminol Solutions", *Microchemical Journal*, *58*, pp209-217. (AFOSR funded research)

John R. Wright, Johnathan Kiel, Eric Holwitt, Joel T. Smith, Kenny Roberts, Joe Studer, Cassie McLemore, Keri Campbell, Brenda Russo and Kris Wood, **1998**, "Preliminary

Characterization of a Polymer Prepared by Diazotization of 3-Amino-L-tyrosine", *Polymer Preprints (ACS), 39(1),* pp365-366. (AFOSR funded research)

V. M. Russo, J. Williamson, K. Roberts, J. R. Wright and M Maness, **1998,** "Carbon Nuclear Magnetic Resonance Spectroscopy to Monitor Sugars in Pith of Internodes of Shrunken2 Corn at Developmental Stages", *HortScience* (*in press*; SEOSU collaboration with the USDA Lab at Lane Oklahoma)

8.2. Other manuscript submitted: (non-AFOSR work)

S. Hertzler Musgrove, R. Brown, M. Wilkett, R. Boyd, M. Ellexson, C. Chambers, C.Plunkett and J. R. Wright, **1998**, "A Colorimetric Immunoaffinity Assay Based on Poly-DL-Lysine and a Pigmented Copper Cluster", *Bioconjugate Chemistry (ACS)* (NIH/NIGMS funded research)

9. INTERACTIONS AND TRANSITIONS:

9.1. Meeting Presentations:

Presentations of AFOSR research results during approximately the past three years:

J. R. Wright, J. Kiel, G. McDaniel, K. Roberts, K. Wood, L. Maddox, C. Dillworth, M. Zulkey, D. Powell and K Whittington, "Properties of a Polymer Formed by Diazotizing Mixtures of Luminol and 3-Amino-L-tyrosine", Joint Southeast-Southwest Regional ACS

Meeting, Hotel Peabody, Memphis TN, November 29-December 1,. 1995.

J. R. Wright, "Chemical Structure of DALM Based on NMR", AFOSR Workshop on DALM (Diazoluminomelanin), North Redington Beach Hilton Hotel, North Redington Beach, FL, March 3-5, 1996.

M. Reeves, L. Maddox, K. Wood and J. R. Wright, "Acoustic Wave Dosimetry Based on Diazotized Luminol Solutions", 42nd Oklahoma ACS Pentasectional Meeting, Cameron University, Lawton OK, March 1, 1997.

J. R. Wright, K. Roberts, J. Studer, T. Smith and J. R. Wright, "The Diazotization Polymer of 3-Amino-L-tyrosine, Which Has Interesting Properties", 53rd Southwest Regional ACS Meeting, Tulsa OK, September 1, 1997.

J. R. Wright, J. Kiel, E. Holwitt, J. T. Smith, K. Roberts, J. Studer, C. McLemore, K. Campbell, B. Russo and K. Wood, "Preliminary Characterization of a Polymer Prepared by Diazotization of 3-Amino-L-tyrosine", 215th National ACS meeting, Hyatt Regency Hotel and Dallas Convention Center, Dallas TX, March 29-April 2, 1998.

9.2. Consultation:

(none)

9.3. Transitions:

(none)

10. DISCOVERIES, INVENTIONS AND PATENT DISCLOSURES:

A sensitive sonochemiluminescent dosimeter was discovered during this work. There were no inventions or patent disclosures. The Air Force funded this work and is entitled to pursue any desired patent rights.

11. HONORS AND AWARDS:

One of the student research participants, Kris Wood, graduated *summa cum laude,* with a 4.0 GPA.

12. OTHER PERTINENT DEVELOPMENTS

The most significant improvement to our facility has been the acquisition of a Mass spectrometric capability. The instrument is now installed and in use (at this writing). Also, the inclusion of a second P.I. (Dr. Tim Smith) brought in analytical expertise and much needed capillary electrophoresis equipment, which is now solely committed to the DALM polymer structural question. Dr. Smith's efforts should assure progress into the structural characterization of DALM. Dr. Smith is taking on the difficult problem which has been the main impediment to DALM studies - analytical methods.

13. APPENDIX 1

(A SUMMARY OF PREVIOUS FINAL REPORTS)

During the past decade the structural and physical properties of DALM have been investigated in an effort that has been discontinuous, but the sum of the funded research periods was equivalent to approximately 7.4 years of continuous effort. A number of pertinent findings which cannot be ignored came out of the earlier investigations, and they need to be considered along with the new evidence presented in this final report. The following is thus a summary of the earlier final reports. Commentaries on relationships to more recent findings are in square brackets.

Report of F49620-87-R-0004 (Summer Faculty Research Participant Program at Brooks AFB):

Zone electrophoresis (cellulose acetate) and Sephadex (gel permeation) chromatography were employed to study diazotizations of 3-Amino-L-tyrosine (3-AT) or luminol, either alone or in combination (DALM). Both of the reactants have aryl amine groups and yield distinct diazotization products; however, if 3-AT and luminol are diazotized together, two additional, dark-pigmented products are observed, one anionic and the other neutral (Fig. 1). As a result, the product mixture is quantifiably darker with both reactants present (i.e., by colorimetry) than with one or the other alone. These results show that DALM is not just a mixture of 3-AT and luminol diazotization products; unique substances are involved. [These DALM-associated compounds undergo further chemical changes on a time scale of about a month. A deviation from the usual diazotization procedure, namely that of carrying out the reaction at room temperature (25-30 C) rather than chilled in an ice bath has been used in the DALM synthesis procedure from the beginning. The effect of lowering the temperature is something that should have been investigated but hasn't been attempted to our knowledge. DALM is simply a polymer that forms under the stated conditions.]

The work also began a study of metal oxide colloid/luminol systems that show a positive temperature coefficient of chemiluminescence and thus mimic a property of DALM.

Report of F49620-88-C-0053/SB5881-0378 (AFOSR Minigrant)

During this period various metal oxide colloid/luminol systems were examined for temperature sensitivity. Of the metal oxide systems tested, cupric oxide was found to have the most desirable temperature coefficient and is suitable for microwave dosimetric applications. Numerical integrations of the Arrhenius equation in which temperature is treated as a variable are in agreement with observed light curves of progressively heated metal oxide colloid dosimeters. Integrations for an analytical expression were attempted, but a simplification of the the log/exponential integral expression involved has not been found. [We did find an empirical equation that approximates the luminosity function.]

A cobalt/luminol system was found to be sensitive to both heating and dissolved carbon dioxide, i.e., its chemiluminescence output increases in the presence of dissolved carbon dioxide, a property shared with DALM. The work also found that an aqueous scandium (III) oxide/luminol system was sensitive to acoustic energy, i.e., sonic irradiation increased its light output. [As in the case of the relatively simpler, metal ion-catalyzed chemiluminescent reactions, DALM is also a type of catalyst; apparently it acts to generate free radicals, to start the chemiluminescence of luminol or a luminol derivative.]

Report of USAF-UT89 Subcontract (Lab Director Initiated Research)

The immediate reaction products obtained by diazotization of luminol and 3-AT, thought to be diazonium ions, were characterized by spectroscopic and other methods. Infrared spectra of the luminol product presented an absorption band consistent with the diazonium functional group. Similarly, the proton nmr spectrum shows a reversal, relative to luminol, in the chemical shift order of the three aryl hydrogens (two doublets and a triplet), also consistent with a diazonium structure. The luminol product was recovered from solution, and differential scanning calorimetry revealed an exotherm close to 150 C, which is typical of the thermal decomposition of a diazonium compound. The elemental analysis of the solid product was in agreement with luminol diazonium chloride. The half time of its formation reaction in the reaction medium is approximately 3 minutes. Although all diazonium compounds are unstable and reactive, luminol diazonium is more stable than most.

The diazotization of 3-AT proceeds faster, with a half time of about one minute, and the product is fairly reactive. The immediate proton nmr spectrum of this material shows distinct changes in the aromatic ring, but the aliphatic resonances (alpha and beta protons) are not greatly changed. The spectrum is consistent with a diazonium ion [this evidence does not rule out the possibility that further diazotization at the aliphatic nitrogen might occur subsequently, i.e., perhaps after polymerization]. Gases produced by the luminol/3-AT system include nitrogen, carbon dioxide and either methane or carbon monoxide.

The findings of this investigation supported the reasonable expectation that the polymerizing reactants are diazonium compounds and that the diazonium of 3-AT is the more reactive material.

Report of F49620-89-?-0530/89-NL-258 (AFOSR Grant)

HMO calculations of luminol and 3-AT and their respective diazonium derivatives were carried out and the charge densities were found to be in agreement with the observed chemical shifts of these substances, supporting the earlier conclusion that reactive diazonium compounds were responsible for the polymerization.

It had been noted that freshly prepared DALM did not have the same properties as the aged product, and in this work the progress of the long-term reaction was followed by means of differential scanning calorimetry. The exotherm near 150 C, due to luminol diazonium, is prominent at first but fades away over an approximate one month period [in light of the evidence of greater instability of 3-AT and later findings, this is probably due to luminol reacting with already formed 3-AT polymers, possibly at sidechain pendant groups]. During the month-long "curing" period the proportion of anionic material increases, and narrow line nmr resonances disappear into the broad polymer envelope. These results left us with the feeling that the early, dark-colored DALM products were somehow reacting and forming the ultimate polymeric material which is called DALM. It was during this work that a distinction was made between reactions in aqueous solutions and those in acetone precipitates; DALM forms under the latter conditions.

During this work the group at Brooks AFB suggested that we consider their finding that 3-AT alone formed a polymer in solutions standing for nine days, and this product was less pigmented than DALM. We reproduced their results The proton nmr spectrum of this product approximated a line broadened spectrum of the 3-AT monomer [This work was done near the end of the funding period, and the equivalent of the DALM reaction, i.e., in precipitated material had to wait for a later funding window. The latter conditions produce a polymer much more like DALM.]. The reagent $Na_2S_2O_4$ did not cleave the polymer which formed in solution, arguing against -N=N- linkage groups, but some bleaching did occur. The idea that DALM and 3-AT polymers might be mixtures of pigmented and non-pigmented polymeric components entered our minds during this work.

The reagent $Na_2S_2O_4$ was only one of several which were tried as possible cleavage reagents, and none led to an effective fragmentation of the polymer. This was a frustrating outcome since one approach to establishing a polymer's linkage mode is through the isolation of small fragments which retain the catenation bonds.

This report also included a tabulation of the known reactive properties of diazonium compounds, which often enter electrophillic aromatic substitutions (leading to azo products) or react by the aryl S_N1 process (nitrogen loss), i.e., through an aryl cation mechanism. A non-conjugated arylamine linkage was more consistent with the reaction possibilities and the evidence available at that time, including the anionic charge of the material [but later data would modify that inference; an explanation of the basis of the anionic charge is still troublesome and has to be consistent with the ultimate structural assignment].

Report of F49620-92-J-0191 (AFOSR Grant)

Structural progress was minimal during this funding period. It was found that metal ions quenched the chemiluminescence (and sonochemiluminescence) of DALM solutions. However, sonochemiluminescence was observed in DALM solutions [in hindsight, DALM solutions from material which had not been aged], and the level of luminescence was increased by added formate ion. [But these results proved hard to reproduce; they were interesting because the polymer component of of DALM might be enhancing the pulsed microwave acoustic effect with the consequence that its sonochemiluminescent properties responding to the generated acoustic wave. [The source of a strong sonochemiluminescent effect (luminol diazonium ion) was identified during the current reporting period, along with the reasons for irreproducibility.]

Report of F49620-94-1-0014 (AFOSR Grant)

Further attempts to develop better polymer separation methods were carried out during this period. The lack of good separation methods has been a hindrance to the progress of DALM's characterization. A method of precipitation of the anionic polymer with Ba^{2+} cation was found during this period. Ion exchange over the resin Chelex-100 (sodium form) restores solubility; unfortunately elemental analyses revealed that some of the barium remained tenaciously bound to the polymer.

The most significant finding during this period was that a mixture of HCl and HNO_3 (essentially aqua regia) decolorized and fragmented the polymer, leading to low molecular weight products (e.g., as revealed by numerous narrow nmr lines. Attempts to separate and purify these reaction products using silica gel columns, TLC and similar methods were only partially successful. Again, the thing that had been holding back progress - separation methods - was still at work!

A sample of the fragmentation product was sent off-campus for capillary electrophoresis. The resulting electrophorogram revealed that while 3-4 components predominated, more than two dozen chemically distinct fragments were present in this mixture.