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PARTIAL PURIFICATION OF THE AGGREGATION PHEROMONE NIPPOLURE FROM FEMALE *Nippostrongylus brasiliensis* (NEMATODA)

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Abstract—Partial purification of a pheromone of the zooparasitic nematode *Nippostrongylus brasiliensis* was achieved. The polypeptide pheromone is degraded by Pronase while increased recovery is obtained after treatment with the protease inhibitors phenylmethylsulfonyl fluoride or L-1-tosylamide-2-phenylethyl chloromethyl ketone. The pheromone has a net negative charge, based on retention on DEAE cellulose and an approximate isoelectric point of 7.3. A combination of double treatments with phenylmethylsulfonyl fluoride and gel filtration enables storage up to nine days which yields biological activity similar to fresh, untreated chromatographic fractions.

Key Words—nematode sex pheromones, Nematoda, pheromone characterization, pheromone purification.

INTRODUCTION

Although knowledge of various biological aspects of nematode sex pheromones is expanding gradually, few advances are evident concerning the physical and chemical properties of these compounds. Chemical studies on the pheromones of zooparasitic worms are scant since only three reports have dealt with this group.

Anya (1976) stated that the male- and female-produced pheromones of

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Aspicularis tetraptera differed in heat lability, while Roberts and Thorson (1977) reported that female *Nippostrongylus brasiliensis* were attracted to male- or female-produced substances which moved with cholesterol and β-sitosterol on thin-layer chromatography. Bone et al. (1979) employed gel filtration chromatography of macerated worms to isolate two elution fractions from female *Nippostrongylus* with pheromonal activity. One fraction at K_{av} 1.0 (<500 mol wt) was found in only females and elicited a male locomotor response only. A second fraction at K_{av} 0.64 (>500 mol wt) was found in both sexes of *Nippostrongylus* and caused a dosage-dependent response by both sexes. These two fractions appeared similar in elution characteristics of those of the free-living nematode *Panagrellus* as reported by Balakanich and Samoiloff (1974).

This study sought to extend our investigations on the elution fraction at K_{av} 0.64, colloquially termed nippolure, of female *Nippostrongylus brasiliensis* which was previously selected as a model organism, based on an extensive literature background. Purification and/or stabilization of this compound(s) would greatly enhance additional studies that are directed toward identification of the pheromonal components. Sufficient progress in these studies may eventually provide a new means of nematode control with medical or agricultural impact.

METHODS AND MATERIALS

Laboratory maintenance of mouse-adapted *Nippostrongylus brasiliensis* was conducted as previously reported (Bone et al., 1977). Additionally, bioassay procedures with Tyrode's solution (MacInnis and Voge, 1970) as a medium were similar to those described earlier with pheromone dosages taken from the aqueous extract of female helminths after various chemical procedures (Bone et al., 1979). Briefly, the pheromone solution is placed behind a filter paper barrier in a Plexiglas chamber (2 × 6 cm). After a 2-hr diffusion period, a single male responder is placed in the middle of the chamber, and after a 2-hr response period the mean distance traveled toward or away from the pheromone source is determined. All pheromone-source and bioassay-responder nematodes were taken from the host at 6 days postinfection.

Female nippolure (K_{av} 0.64) was obtained by Sephadex G-25-80 (Sigma) gel filtration chromatography according to previously reported methods (Bone et al., 1979). This fraction was subjected to several pre- and postchromatographic treatments for characterization, purification, and/or stabilization prior to bioassay of the male locomotor response.

The helminths were macerated routinely in cold (<4° C) glucose-free Tyrode's solution (GFTS), but some stabilization studies employed macer-

ation in hot GFTS at 50 and 60° C to inhibit enzymatic degradation of nippolure prior to gel filtration. Alternatively, the enzyme inhibitors diisopropyl phosphorofluoridate (DPF) (Sigma), phenylmethylsulfonyl fluoride (PMSF) (Sigma) in ethanol, L-1-tosylamide-2-phenylethyl chloromethyl ketone (Sigma) in acetonitrile (TPCK), and *N*-α-*p*-tosyl-L-lysine chloromethyl ketone HCl (Sigma) in acetonitrile (TLCK), were utilized at 10^{-4} M in GFTS to reduce enzymatic degradation of pheromonal activity during processing. PMSF and TPCK were combined also at a final concentration of 10^{-8} M for each inhibitor. Controls consisted of similar concentrations of inhibitors alone. Twenty replicates were obtained for each of five dosage levels for the treatments and control.

Females were macerated in Pronase (Calbiochem) at 2.6 units/ml GFTS to assess the effect of this broad-spectrum proteolytic enzyme on nippolure. The Pronase-treated solution was held at room temperature (20–21° C) for 5 min prior to gel filtration for isolation of the nippolure fraction. Twenty replicates were done for each of five dosages.

Ion-exchange purification was conducted in a 1-cm ID glass column that was filled to a 5-cm depth with DEAE cellulose (medium mesh, 0.85 mEq/g) (Sigma). Cellulose was pretreated with 0.5 N hydrochloric acid and 0.5 N sodium hydroxide rinses for activation prior to washing with distilled water and equilibrating with 10% GFTS in distilled water. The nippolure fraction from females was taken from gel filtration with GFTS as an elutant ($I = 0.225$) and diluted with 9 parts distilled water ($I = 0.0225$) prior to ion exchange chromatography. After rinsing the column with three column volumes of GFTS (10.5 ml, $I = 0.0225$), the DEAE cellulose matrix was eluted with GFTS ($I = 0.225$) at pH 8.5, 8.0, 7.8, 7.6, 7.4, and 7.2, after which these fractions were adjusted to pH 7.2 with 0.1 N HCl for bioassay. After this preliminary determination of the conditions that were needed to elute nippolure from the DEAE column, the interaction of ionic strength and pH on elution of nippolure was examined with GFTS elution at pH 7.2, 7.0, 6.8, 6.4, and 6.0 at an ionic strength of 0.056 (25% GFTS) prior to adjustment to pH 7.2 or at pH 7.2 with ionic strength of 0.05, 0.09, 0.15, 0.18, and 0.225 (25, 40, 65, 80, and 100% GFTS, respectively).

Twenty replicates were done for each five dosages on the preliminary determination of binding and exclusion. Twenty replicates were also performed for each pH and ionic strength variable at a constant dosage of 100 female-equivalents.

Isoelectric point determinations were conducted at 4° C with precast, wide-range (pH 3.5–9.5) polyacrylamine gels (LKB) on an LKB 2117 Multiphor with an LKB 2103 power supply. An initial power of 7 W/quarter gel was used and gels were focused for 30 min after reaching minimal resistance and maximal voltage (1400V). The helminth heme bands served as

internal indicators while bovine heme, egg albumin, and catalase served as external markers of known isoelectric point. Some gels were stained with Coomassie blue according to Winter et al. (1977).

After applying the centrifugation supernatant from maceration of varying numbers of females in distilled water to the gel surface and focusing for the previously described period, nine pH bands were taken by longitudinal slicing of the gel slab. Each isolated pH band was then subdivided into 15 pieces that were placed in bioassay chambers for determination of the male response after a 2-hr diffusion period. Fifteen replicates were done for each of five dosages. Controls consisted of similar focusing treatment and assay of a distilled water blank.

These individual purification steps were combined to evaluate the possibility of achieving highly purified female nippolure for eventual identification. This scheme was initiated with gel filtration according to earlier procedures with distilled water as an elutant. The peak nippolure fraction (600–650 μl) from maceration of females in PMSF at 10^{-4} M was then focused on wide-range polyacrylamide plates. The pheromonally active gel region was next mixed with 1.5 ml of 10% GFTS and centrifuged. After three extractions, the pooled supernatants were passed through a DEAE cellulose column and bioassayed dosage-wise after elution with pH 7.2 GFTS at 0.225 I. Total protein, using bovine serum albumin as a standard, was determined at each step by UV absorbance at 280 nm or Lowrey's and by fluorometric assay with fluorescamine (Stein et al., 1973). Twenty replicates of five dosages were obtained for this procedure and each treatment in the following study.

Stabilization of the aggregation pheromone was studied by storage of the female fractions at 4° C for one day after individual or various combined purification procedures. Initially, the highest dosages on the dose–response lines for each treatment were examined. Storage solutions revealing any or some activity were explored further through dose–response lines at several dosages to determine the level of remaining activity. Storage purification steps attempted were gel filtration only, PMSF treatment at 10^{-3} M and 10^{-4} M prior to gel filtration, gel filtration with ion exchange, PMSF treatment at 10^{-4} M prior to gel filtration and ion exchange, and isoelectric focusing only. Additionally, PMSF treatment at 10^{-4} M or 10^{-3} M was performed prior to gel filtration after which PMSF (10^{-4} M or 10^{-3} M) was reintroduced into the nippolure fraction for storage. The second PMSF treatment was then removed by a second gel filtration prior to nippolure bioassay. TPCK (10^{-4} M) and TPCK-PMSF (each at 10^{-8} M) were also used for stabilization effects with storage.

Data were analyzed by linear regression and analysis of variance or covariance. The 0.05 probability level was considered statistically significant.

RESULTS

Enzymatic Degradation and Inhibition. Maceration of female helminths in Pronase resulted in a reduction in the attractancy of the aggregation pheromone (Figure 1). No tested dosage was significantly different from zero according to the mean standard error. Thus, female nippolure retains little activity after exposure to Pronase.

Figure 1 shows also the effect of PMSF (10^{-4}) pretreatment of female worm macerates. The pheromone fraction that was recovered from gel filtration after PMSF pretreatment revealed almost a threefold increase in activity when compared to that obtained from gel filtration only. The male response to PMSF-pretreated nippolure was significantly dosage dependent but did not differ in slope from pheromone that was derived from gel filtration only ($F^4_{99} = 4.42$). However, the intercepts of the regression lines of the male response to the PMSF-treated and untreated gel filtration pheromone indicate greater activity in the pretreated fractions. Similar results were obtained with TPCK pretreatment at 10^{-4} M and TPCK-PMSF at 10^{-8} M for both compounds. The PMSF (10^{-4} M) or TPCK (10^{-4} M) controls revealed no attractancy, although posttreatment gel filtration should separate the pheromone and enzyme inhibitor without the need of a control for this procedure.

Attempts to block enzymatic degradation of female nippolure with DPF or TLCK at 10^{-4} M revealed no increase in pheromonal activity. Maceration of females in 50 or 60° C GFTS to denature degradative enzymes failed also to increase activity and resulted in an elimination of attractancy probably as a result of the thermal lability of the pheromone.

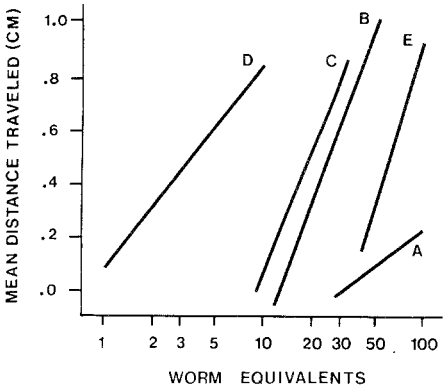

FIG. 1. Male locomotor response of *N. brasiliensis* to female pheromone dosages that were treated with Pronase (A), PMSF (B), TPCK (C), or PMSF-TPCK (D) prior to gel filtration, or untreated gel filtration pheromone (E).

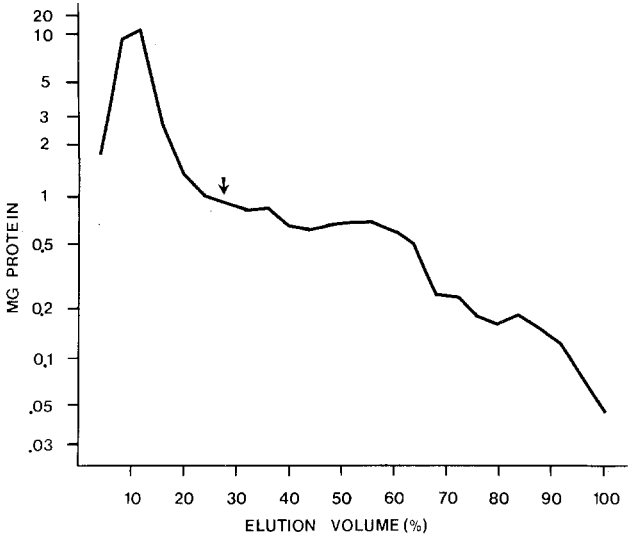

FIG. 2. Elution of female *N. brasiliensis* total UV absorbance from a Sephadex G-25 column. The arrow indicates the female pheromone peak. Void volume (V_o) = 5.1 ml; inclusion volume (V_t) = 10.3 ml.

Gel Filtration of Total Protein. Protein elution patterns from 3500 macerated female *N. brasiliensis* are given in Figure 2 based on a bovine serum albumin standard as determined by UV absorbance at 280 nm. Most 280-nm absorbing material (73.1%) is eluted from the Sephadex matrix in the exclusion volume or first 20% of the column inclusion volume while the nippolure fraction consists of only 2–3% of the total UV absorbance in the elution volume. The nippolure fraction from gel filtration exhibited a peak absorbance at 260 nm and a steep rise in absorbance towards 210 nm when scanned from 350 to 200 nm. In this representative determination the pheromone fraction contained about 880 μg total protein while the total elution volume represented about 35.08 mg of total protein according to UV absorbance. Lowrey's determination gave comparable results. Thus, gel filtration alone yields a relatively high overall purification. An additional reduction in total protein (>150-fold) without loss of pheromonal activity was obtained from the double PMSF-gel filtration procedures that are presented with the pheromone storage data.

Ion-Exchange Chromatography. The dose–response line for male locomotion to nippolure female equivalents from gel filtration followed by ion exchange is shown in Figure 3. The male's response was dosage dependent ($F^4_{99} = 3.78$), but did not differ significantly in slope or intercept from the male's response to gel filtration alone female equivalents. However, some

pheromone apparently was lost quantitatively or qualitatively based on nonsignificant differences in the regression lines. This loss can probably be accounted for by failure to bind to the matrix or a trailing effect upon dissociation. Nippolure was bound to the DEAE cellulose matrix at pH 7.2 (0.0225 I) and released with an increase in ionic strength to 0.09 without pH alterations. A constant ionic strength of 0.056 I retained the pheromone at pH 7.2 or 7.0, but the pheromone dissociated from the matrix upon elution at pH 6.8.

Isoelectric Focusing. Bioassay of female equivalents from PAG isoelectric pH bands revealed only one region that elicited a dosage-dependent male response (Figure 3). This region had a median pH of 7.3 and was demarked by two worm heme bands (Figure 4).

The regression line ($F^4_{74} = 2.79$) for the male response did not differ significantly from that response to gel filtration or ion exchange in slope or intercept. However, as in ion exchange, the regression line suggests a slight, but consistent, loss of qualitative or quantitative attractancy that may result from retention of the pheromone within the gel, thus reducing the effective diffusion gradient.

Figure 4 shows the protein isofocusing patterns of 1000 male or female helminths. Most stainable protein bands were found to have a pI higher than 5.4 according to the catalase reference. The approximate 7.3 pI of nippolure agrees with the location of the bovine hemoglobin reference at pI 6.8. Bioassay of the individual regions of the active band that exhibited stained protein

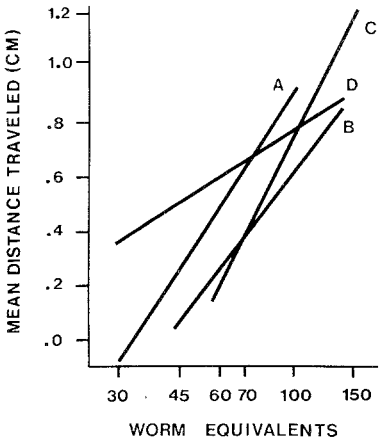

FIG. 3. Male locomotor response of *N. brasiliensis* to female pheromone dosages from gel filtration (A), ion exchange (B), isoelectric focusing (C), or these procedures combined (D).

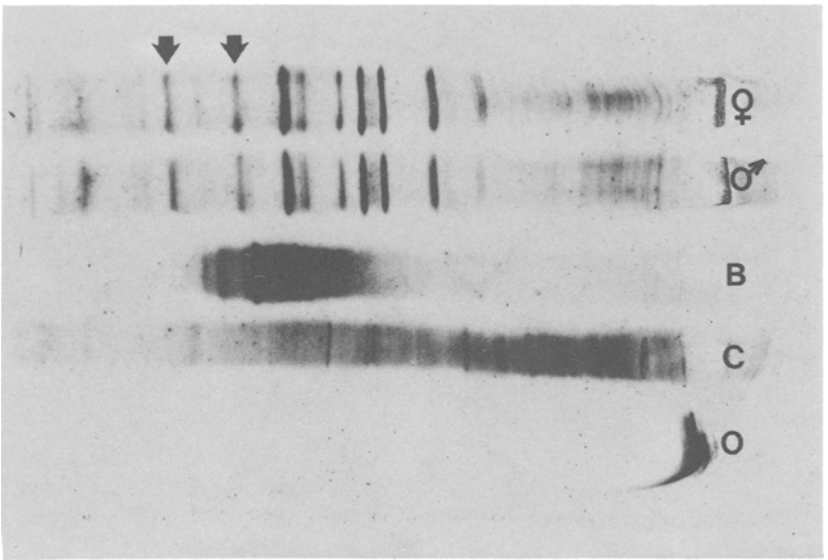

FIG. 4. Isoelectric focusing of 1000 female or male *N. brasiliensis*. Standards are bovine hemoglobin (B), catalase (C), and ovalbumin (O). The region of pheromonal activity is between the two helminth heme bands indicated by arrows.

bands on a reference gel, prepared simultaneously with the gel bands for bioassay, failed to yield activity. Thus, the amount of pheromone at its isoelectric point is probably too quantitatively small for stain visualization. Both stained regions within this pI band represented heme or heme-breakdown products as determined by their visual color. Bioassay of the distilled water control from this gel region revealed no attractancy.

Pheromone Purification. The male response to nippolure after combined purification steps is shown in Figure 3. The female-equivalent dosages on which the dose–response line is based are estimates of activity that consider the gel filtration regression line as a standard and are calculated from the approximate losses incurred from isofocusing and ion-exchange procedures alone and the increase in activity that results from PMSF pretreatment.

The male response to these female-equivalent dosages was dosage dependent ($F^4_{99} = 3.35$). The slope of the regression line was not significantly different from those line for gel filtration, ion exchange, or isofocusing as single procedures. However, the intercept differed at the 0.05 probability level. Our interpretation is that the combined procedures yield more variability in the final recovered activity than any of the individual purification techniques as supported by the shallower regression slope, but decreased intercept.

Total protein yields and purification factors for a representative purification are presented in Table 1. The total purification factor based on total protein and near-quantitative recovery of biological activity (Figure 3) was more than 460-fold with gel filtration, giving greater purification than isofocusing or ion exchange within the sequential order of utilization. Interpretation of the total protein and purification yields suggests that the recovered pheromone represents less than 0.25% of the total female protein.

Rechromatography with gel filtration during the dual PMSF treatments for storage yielded relatively high purity. Based on UV absorbance at 280 nm, the second nippolure fraction exhibited more than a 150-fold decrease in total UV absorbance in comparison to the first chromatographic separation. The peak absorbancy from rechromatography coincided with the initial elution volume of nippolure activity and exhibited little loss of pheromonal activity. However, precise quantification of the obtained purification is difficult since the small amount of total material is below reliable values that use bovine serum albumin as a standard. These results are not unexpected and indicate that the maximum capacity of the Sephadex matrix may be approached during initial chromatography. Similar results in purification would be anticipated from an increase in column size to attain greater linear distribution of filtrates. This high purification from gel filtration indicates that nippolure has an apparent molecular weight quite different from the majority of the UV-absorbing material in the macerates.

Pheromone Storage. Figure 5 shows the bioassay results after storage of pheromone that was subjected to various pre- and post-gel filtration procedures. Storage of nippolure for 24 hr after gel filtration alone yielded no activity. Similar results were obtained for isofocusing only and gel filtration

TABLE 1. PURIFICATION YIELDS FROM 3800 FEMALE *N. brasiliensis* ASSAYED AT AN ESTIMATED 140 FEMALE-EQUIVALENT DOSAGE

Source	Total protein (mg)[a]	Protein decrease (*X*-fold)	Biological activity[b] (total female equivalents)	Specific activity (units/mg protein)
Crude extract	38.81[a,b]	47.8	7600	196
Post-gel filtration	0.812[a,b]	4.93	7410	9,101
Post-isoelectric focusing	0.164[c]	1.96	4468	27,243
Post-ion exchange	0.084[c]		2993	35,630

[a]Protein determinations by Lowrey's (a), absorbance 280 nm (b), or fluorescamine (c).
[b]Pheromonal activity was derived from the approximated female equivalents obtained from dose–response lines for individual procedures alone (Figure 1, 3) with the untreated gel filtration sample as a standard.

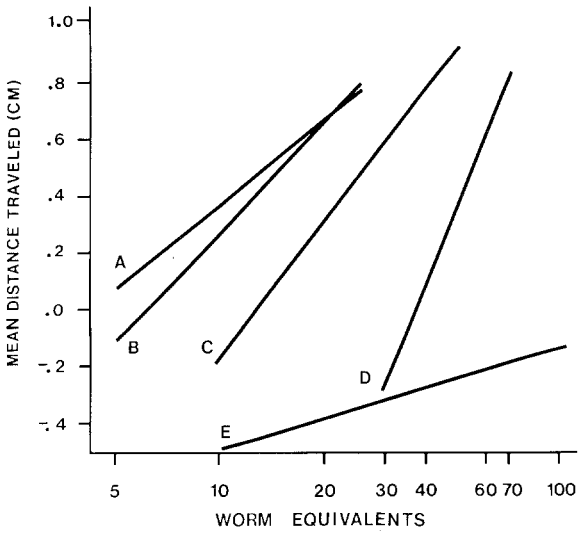

FIG. 5. Male locomotor response of *N. brasiliensis* to female pheromone after treated storage: A = PMSF, 10^{-4} M (2×), 1-day-old; B = PMSF, 10^{-3} M (2×), 3-day-old; C = PMSF, 10^{-3} M (2×), 6-day-old; D = PMSF, 10^{-3} M (2×), 9-day-old; E = PMSF, 10^{-3} M (2×), 11-day old.

followed by ion exchange. However, pretreatment with PMSF enhanced considerably the retention of attractive activity (Figure 5). PMSF at 10^{-4} M pretreatment with gel filtration suggested little activity remained at 24 hr while PMSF at 10^{-3} M also revealed only slight activity after 3 days. However, PMSF pretreatment at maceration followed by gel filtration before storage with PMSF and a second gel filtration prior to bioassay increased the recovery of nippolure activity considerably. Double treatment with PMSF at 10^{-3} M enabled a twofold recovery of activity after 6 days storage at 4° C when compared to fresh untreated pheromone from a single gel filtration. However, even greater activity was found in this procedure after only 3 days, which indicates loss of activity even with this methodology. The male dose–response line to 11-day-old female nippolure showed little, if any, pheromonal activity. Double gel filtration separations failed to give any stabilization without inhibitors. However, these results indicate that storage for up to 9 days with a recovery that exceeds or equals that of fresh pheromone from gel filtration only is feasible. TPCK at 10^{-4} M and PMSF-TPCK, each at 10^{-8} M, were less effective for stabilization based on pheromonal activities that remained after 9 days storage. These results should enable usage of stored pheromone after evaluation of remaining calculated activity for up to 9 days.

DISCUSSION

A somewhat less nebulous image of female nippolure is gradually emerging. Based on elution volume as previously presented, the female pheromone has a molecular weight of 500–1000 (Bone et al., 1979). This weight apparently indicates that five to eight amino acids comprise the pheromone if it is, in fact, a peptide without other residues such as carbohydrates and if an average amino acid molecular weight is assumed. The pheromone is heat and freeze labile, at least in the unpurified and semipurified states from the procedures of this study. However, the heat lability may be an indirect result of increased enzymatic breakdown.

No information is available concerning the constituent amino acids of nippolure due to quantitative difficulties that have yet to be surpassed. However, the approximate pI of 7.3 and the retention on an anion exchanger, such as DEAE cellulose, indicate that nippolure possesses a net negative charge under our experimental conditions.

Nippolure can be maintained for a brief period provided proper storage conditions are met. Additional studies using additional or combined enzyme inhibitors may yield longer periods of retained activity and facilitate the accumulation of sufficient nippolure to approach amino acid composition and peptide sequence studies on relatively purified material. A multitude of various biological aspects of nippolure remain unexplored and may contribute valuable information related to continued chemical investigation.

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