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Incorporation of Radioactive Label into Nucleic Acids of Compatible and Incompatible Pollen Tubes of *Lilium longiflorum* Thunb.*

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Summary. Stylar canal cells of *Lilium longiflorum* were labeled before pollination with 5-^{3}H-uridine or 5-^{3}H-orotic acid dissolved in water, or the stylar canal was filled 6 hr after pollination with stigmatic exudate supplemented with 5-^{3}H-uridine, 2-^{14}C-uridine or $H_3{}^{32}PO_4$. Nucleic acids extracted from compatible and incompatible pollen tubes grown in these styles were separated by methylated albumin kieselguhr column chromatography. Label occurred in all portions of the RNA profile, but no label appeared in the DNA peak. Using 5-^{3}H-uridine, pollen tubes of different genotype had different labeling patterns.

Introduction

Pollen tubes grown in artificial media synthesize RNA (Dashek and Rosen 1966; Linskens et al. 1971; Mascarenhas 1971b; Mascarenhas and Bell 1970; Mascarenhas and Goralnick 1971; Rosen 1968; Steffensen 1966; Tano and Takahashi 1964). Part of this synthesis occurs both in the generative cell and vegetative nucleus (LaFountain and Mascarenhas 1972; Mascarenhas 1966; Rosen 1968; Stanley and Young 1962). This new RNA probably is not needed for germination or early pollen tube growth but is thought to be required for later pollen tube elongation (Mascarenhas 1966). Most likely, this RNA is not t-RNA or r-RNA, but is m-RNA (Mascarenhas 1971a; Mascarenhas and Bell 1970; Mascarenhas and Goralnick 1971; Steffensen 1966, 1971; Tano and Takahashi 1964).

Injection of the RNA synthesis inhibitor, 6-methylpurine, dissolved in stigmatic exudate into the stylar canal of *Lilium longiflorum* resulted in compatbile pollen tube lengths typical of incompatible pollen tubes, but did not affect incompatible pollen tubes (Ascher and Drewlow 1970). Self incompatibility, a common mechanism enforcing outbreeding in angiosperms, is expressed in the Easter lily as a restriction of incompatible pollen tube length to half that of compatible pollen tubes in the amount of time it takes compatible tubes to traverse the 100 mm long lily style. Apparently, new RNA is needed for compatible pollen tube growth. However, even without this RNA synthesis, lily pollen tubes grow half the length of the style, at least 5 times farther than they grow *in vitro*.

Germinating and growing pollen in artificial media is the usual method for studying pollen tube metabolism (Linskens 1964; Rosen 1968). This procedure has been justified under the assumption that pollen tube metabolism *in vitro* is wholly that of the pollen tube, free of stigma and stylar influences. Perhaps, because of the greater ease of procedure and the relative certainty that *in vivo* results would not be different, pollen germination and initial pollen tube growth is best studied *in vitro*. But, biochemical studies of later stages of *in vitro* pollen tube growth are studied of a greatly impaired metabolism since the length of pollen tubes *in vitro* is usually only a fraction of normal pollen tube length.

Another justification for using *in vitro* systems is that the pollen tubes grown *in vivo* often can not be easily isolated from stylar tissue. Many plant species have loosely packed parenchymatous cells in the center of the style among which pollen tubes grow. This transmitting tissue with embedded pollen tubes has been isolated and analyzed biochemically (Linskens 1958, 1959). While this study pointed out differences in pollen tube-stylar metabolisms between compatible and incompatible pollinations in *Petunia*, it did not separate pollen tube metabolism from stylar metabolism. However, other plant species such as those in the Liliaceae have large, hollow styles lined with specialized cells on which pollen tubes grow. These pollen tubes can be easily removed from the style by bisecting the style and lifting the pollen tubes out of the canal.

The purpose of this research was to study the incorporation of radioactivity into nucleic acids of *in vivo* grown pollen tubes of *Lilium longiflorum*, the radioactivity coming from stylar canal cells previously labeled

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with radioactive nucleic acid precursors or from stigmatic exudate supplemented with label and injected into the stylar canal 6 hr after pollination. A secondary purpose was to see whether unique incorporation patterns were associated with pollen tubes of different genotype or with compatible and incompatible pollen tubes.

Materials and Methods

Flowers of *Lilium longiflorum* Thunb. cultivars Ace and Nellie White (NW) were cut early the day of anthesis and placed in jars of water on the laboratory bench at room temperature for 1 day. Stigma-styles were removed from the flowers by cutting through the ovary with a triangular needle, and the remaining portion of the ovary was snapped off.

Radioactively labeled nucleic acid precursors diluted with water or stigmatic exudate were injected through the stigma until a drop appeared at the ovarian end of the style. 5-^{3}H-uridine[1] diluted with water to concentrations of 50 to 500 microcuries per experiment (uc/exp) was injected 12 hr before pollination into sets of 32 'Ace' or 'NW' styles, and incubated at 23 °C until pollination. 5-^{3}H-uridine is a specific precursor of RNA synthesis and is not used in DNA synthesis (Hayhoe and Quaglino 1965). At first, the styles were flushed with 10 drops of water just before pollination to remove any free label left in the stylar canal. Since flushing removed only 0.1% of the label injected suggesting that the stylar tissue had absorbed the rest, flushing was discontinued for the remaining experiments. For another set of experiments, 5-^{3}H-uridine or 5-^{3}H-orotic acid was diluted with 9 volumes of water and injected just before pollination into 12 'Ace' or 'NW' styles. 2-^{14}C-uridine, $H_3{}^{32}PO_4$ and 5-^{3}H-uridine diluted with stigmatic exudate collected from 'Ace' flowers to concentrations of 1 to 5 uc/exp for 2-^{14}C-uridine, 300 uc/exp for $H_3{}^{32}PO_4$ and 100 uc/exp for 5-^{3}H-uridine were injected 6 hr after pollination into 32 'Ace' or 'NW' styles. ^{32}P was used to detect DNA synthesis in pollen tubes.

The Easter lily cultivars are clones and are intraclonally (self) incompatible and interclonally (cross) compatible when pollinated. The detached styles were self- or cross-pollinated, placed in petri plates on moistened filter paper and incubated at 23 °C for 24 or 48 hr. After incubation, the styles were cooled to 2-4 °C, bisected longitudinally with a razor blade and the stigma was cut off. The pollen tubes in each stylar half were removed with a fine-pointed forceps by grabbing hold of the pollen tubes at the top of the style and pulling them out of the stylar canal. The pollen tubes were immediately suspended in 2 ml 1xSSC buffer, pH 7.7[2], then added to 30 ml ice-cold 1xSSC buffer; 30 ml buffer-saturated phenol; 1% sodium lauryl sulfate and 5-6 gr fresh-weight nonradioactive lily styles. All was ground in a Virtis Macro "45" Homogenizer at top speed (45,000 rpm) for 3 min, decanted into an extraction flask, shaken at room temperature for 15 min and then centrifuged in a Sorvall RC2B in an S-34 head at 15,000 rpm for 10 min at 0 °C. The supernatant was re-extracted against 40 ml buffer-saturated phenol at 0 °C, recentrifuged and the supernatant combined with 2 volumes of 2-ethoxyethanol overnight at -20 °C. The precipitate was collected by centrifugation at 15,000 rpm for 20 min, washed twice with room temperature 95% ethanol and stored upside down to drain at -20 °C until chromatographed.

Nucleic acids in the samples were chromatographically separated on methylated albumin kieselguhr (MAK) columns, made in 3 layers of 1 gr cellulose powder, 12 gr MAK and finally 1 gr kieselguhr as the sample layer. Each layer was individually suspended in 0.1 M NaCl-$PO_4^=$ buffer, pH 7.6[3] and packed in turn at 4 lb/in^2 air pressure into a 2 × 40 cm glass column. Before packing, the kieselguhr suspensions were boiled to remove the air and 4 ml methylated albumin (MA) was stirred into the 12 gr kieselguhr suspension to produce the MAK. Each column was washed with 100 ml 0.3 M NaCl-$PO_4^=$ buffer, pH 7.6.

The nucleic acid sample was dissolved in 20 ml 0.1M NaCl-$PO_4^=$ at 0 °C, loaded onto the column and washed with 100 ml 0.3 M NaCl-$PO_4^=$. A 600 ml gradient containing 300 ml 0.4M NaCl-$PO_4^=$ in the mixing chamber and 300 ml 1.2M NaCl-$PO_4^=$ in the reservoir was attached to the column and 3 lb/in^2 air pressure applied to the gradient. After the salt gradient, the column was washed with 100 ml of cold 0.74 M NH_4OH. Fractions of 5.4 or 7.6 ml were collected.

The absorbance at 260 nm (A_{260}) for each of the 100-140 fractions was obtained, the test tubes and their contents cooled to 0 °C and 1 ml of a 1% albumin[4] solution added to each test tube. Enough 40% trichloroacetic acid (TCA) was added to each test tube to make the solutions 5% in TCA and the test tube contents were mixed vigorously. The precipitate was allowed to form for at least 6 hr and then collected by filtering the solutions through 2.4 cm Whatman GF/A fiberglass filter disks, rinsing the test tubes twice with 5 ml cold 5% TCA. The disks were dried in scintillation vials and 10 ml of a scintillator solution[5] added. Counts per min (CPM) data were obtained for each vial, twice, by counting for 20 min or until 10,000 counts accumulated. Counting was done in a Beckman Scintillation Spectrophotometer or a Packard 3375 Automatic TriCarb Liquid Scintillation Spectrophotometer at 8 °C, with the gain set to give an automatic external standard (AES) ratio of 1.0 for the unquenced background standard found in each machine.

Quench curves were obtained for each machine with the formula of $y = ax^b$, plotting efficiency of counting the isotope versus AES ratio. These quench curves were encoded in a computer program for a Wang 2000 desk computer, and the average disintegrations per min (DPM) per fraction was calculated utilizing the raw CPM data and the AES ratio from the 2 replicates of counting. Efficiency of counting on the 2 spectrophotometers was close to 100% for ^{14}C and ^{32}P, and approximately 45% for ^{3}H.

The planned comparisons were 'Ace' × 'Ace', 'NW' × 'NW', 'Ace' × 'NW' and 'NW' × 'Ace' pollen tubes using a 5-^{3}H-uridine stylar labeling. While all of these comparisons were done several times, many individual experiments were discarded before critical analysis (including all the 'Ace' × 'NW') because the absorbancy profile of stylar nucleic acids indicated poor nucleic acid extraction or a column problem, or because the radiactivity profile had very low counts and was erratic or there was signs of micro-organism con-

[1] 5-^{3}H-uridine, New England Nuclear (NEN), Specific Activity (SA) 29.3 curies (c)/mM, 1 mc/ml; 5-^{3}H-orotic acid, NEN, SA 12.2 c/mM, 1 mc/ml; 2-^{14}C-uridine, Schwarz BioResearch, SA 49.6 mc/mM, 10 uc/ml; $H_3{}^{32}PO_4$, carrier-free, NEN, 1 mc/ml.

[2] 0.01M tris (hydroxymethyl) aminomethane, 0.15 M sodium chloride, 0.015 M sodium citrate, 0.01 M 2-mercaptoethanol.

[3] 0.1 M, 0.3 M, 0.4 M and 1.2 M NaCl in 0.05 M Na phosphate buffer, pH7.6.

[4] Bovine serum albumin, Fraction V, Sigma.

[5] 4 gr 2,5-diphenyloxazole (PPO), 0.1 gr dimethyl-POPOP, 1000 ml toluene.

tamination. For final comparison, there were 2 of 6 'Ace' × 'Ace', 2 of 8 'NW' × 'NW' and 2 of 5 'NW' × 'Ace' experiments retained using 5-^{3}H-uridine. There were originally 5 'Ace' × 'NW' experiments. Other experiments retained were 'NW' × 'Ace' and 'NW' × 'NW' using 5-^{3}H-orotic acid and 'NW' × 'NW' using 5-^{3}H-uridine suspended in stigmatic exudate. Overall, of 39 separate MAK column runs, 9 were useable.

To standardize A_{260} profiles and the radioactivity profiles 1 experiment to the other (see Fig.2), the RNA portions of the MAK profile were replotted with each test tube's A_{260} and DPM designated as a % of the total A_{260} units and DPMs present in the total RNA of MAK chromatographic profile for each individual experiment (see Fig.3). According to standard methods of describing the profile of plant nucleic acids separated on MAK columns, the 2 major RNA portions of the profile were divided into 5 separate areas (Fig.1). The light-RNA was divided into 4S-RNA and 5S-RNA, and the heavy-RNA was divided into 18S-RNA and 28S-RNA. The 28S-RNA was subdivided into peak-RNA (from 18S- to 2 fractions past the highest A_{260} reading in the 28S-RNA portion) and rapidly-labeled-RNA (the shoulder of the 28S-RNA portion). There was no attempt in this study to determine whether the nucleic acid species designated as being present in the various portions of the MAK profiles were actually present. The A_{260} units or radioactivity within the 5 portions of the profile were summed and the 5 sectional totals were expressed as a % of the total A_{260} units or radioactivity present in RNA. Subsequently, the ratio $R_{D/A}$ was calculated using this formula,

$$R_{D,A} = \frac{\% \text{ DPM}_X}{\% \text{ A}_X},$$

where X was either any 1 of the 5 portions of the RNA profile or was any summed part of the RNA profile. Also, ratios were calculated for the 2 major areas of the RNA profile, the light-RNA and heavy-RNA (see Figs. 3,4,5,7).

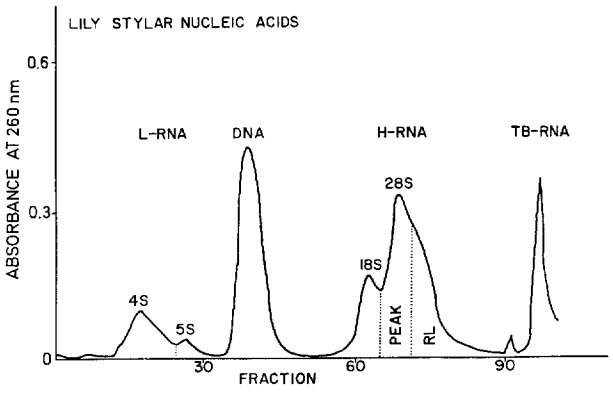

Fig.1. Typical absorbancy profile of *Lilium longiflorum* stylar nucleic acids chromatographed on a MAK column: light-RNA (1-RNA) = 4s- + 5S-RNA; heavy-RNA (h-RNA) = 18S- + 28S-RNA; peak- + rapidly-labeled-(rl) RNA = 28S-RNA; tenaciously bound-(tb) RNA is removed by NH_4OH

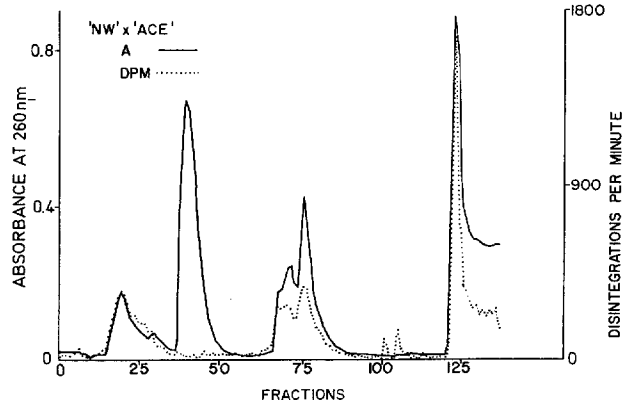

Fig.2. Absorbancy profile of nucleic acids from non-radioactive styles of *Lilium longiflorum* cultivar Nellie White (NW). Radioactivity profile was contributed by 'Ace' pollen tubes which had been grown 48 hr at 23 °C in 8 'NW' styles. The 'NW' styles had been labeled just before pollination with an injection of 267 ul 5-^{3}H-uridine

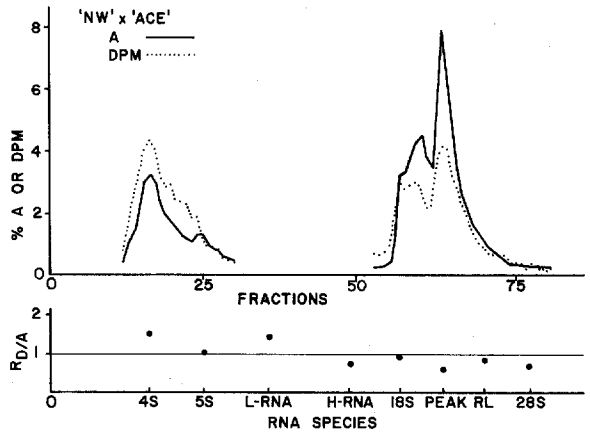

Fig.3. RNA species of the MAK profile in Fig.2 have been replotted with the A_{260} units or DPM in each column fraction expressed as a percentage of the total A_{260} units (A) or DPMs present in the column RNA. $R_{D/A}$ is the ratio of the % radioactivity (%D) to % nucleic acids (%A) contained within 6 separate portions of the RNA profile and within the 2 major parts of the profile, the 1- and h-RNA

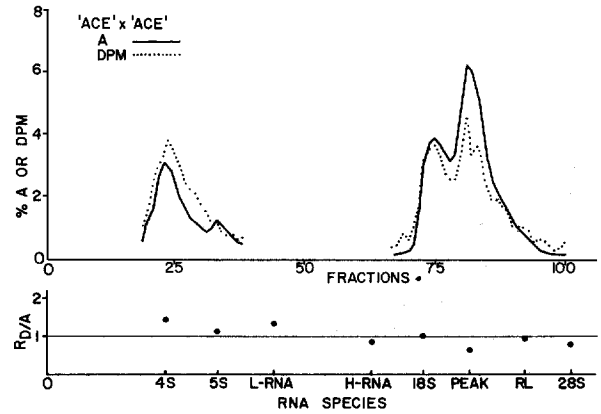

Fig.4. Adjusted absorbancy profile of the RNA from non-radioactive 'NW' styles. Adjusted radioactivity profile was contributed by 'Ace' pollen tubes which had been grown 48 hr at 23 °C in 8 'Ace' styles. The 'Ace' styles had been labeled just before pollination with an injection of 267 ul of 5-^{3}H-uridine

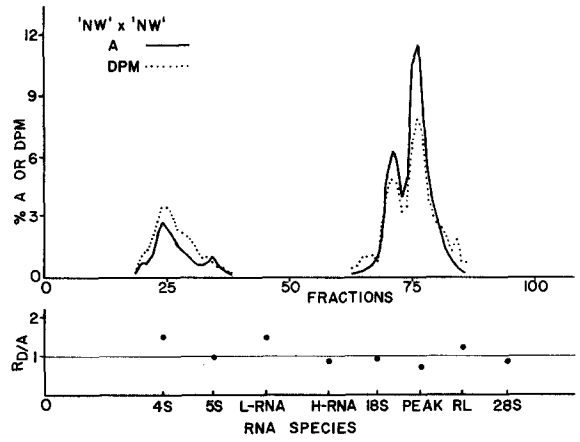

Fig.5. Adjusted absorbancy profile of the RNA from non-radioactive 'NW' styles. Adjusted radioactivity profile was contributed by 'NW' pollen tubes which had been grown 48 hr at 23 °C in 32 'NW' styles. The 'NW' styles had been labeled with a 24 hr pre-pollination injection of 43.2 ul 5-^{3}H-uridine

Results

Compatible and incompatible pollen tubes of *Lilium longiflorum* grown down stylar tissue previously labeled with 5-^{3}H-uridine acquired radioactivity in all RNA species separated by MAK column chromatography, but no label appeared in the DNA peak (Fig.2,6). Close examination of the radioactivity profile revealed consistent relationships for all types of pollen tubes. The light-RNA area had a greater percent of label in relation to the A_{260} profile than the heavy-RNA area (Fig.2-7), a fact evident both from the profiles and from the $R_{D/A}$ values which were greater than 1.0 for light-RNA and less than 1.0 for heavy-RNA. Within the light-RNA, 4S-RNA was consistently more highly labeled than the 5S-RNA (Fig. 2-7). Within the heavy-RNA, peak-RNA had the lowest $R_{D/A}$ of any portion of the profile (Fig.3-5,7). Also, the $R_{D/A}$ for 28S-RNA (peak-RNA plus the rapidly-labeled-RNA) was both less than the $R_{D/A}$ for the 18S-RNA and less than 1.0 (Fig.3-5,7).

The labeling pattern of 'NW' and 'Ace' pollen tubes differed so that, in 'NW' pollen tubes, the rapidly-labeled-RNA had an $R_{D/A}$ greater than that of 18S-RNA (Fig.5,7) but, in 'Ace' pollen tubes, a reversal of the 'NW' pattern occurred with the $R_{D/A}$ of the 18S-RNA being greater than that of rapidly-labeled-RNA (Fig.3,4).

Labeling pattern of incompatible and compatible 'Ace' pollen tubes differed in that the compatible pollen tubes had a lower $R_{D/A}$ for the heavy-RNA (and therefore a higher $R_{D/A}$ for the light-RNA), with no portion of the heavy-RNA having an $R_{D/A}$ greater than 1.0 (Fig. 3,4). Comparison of the labeling pattern of incompatible and compatible 'NW' pollen tubes could not be used to verify this conclusion because of the lack of compatible 'NW' pollen tube data.

The labeling pattern of pollen tubes grown in styles previously labeled with 5-^{3}H-orotic acid closely resembled the labeling pattern derived from 5-^{3}H-uridine except that 'Ace' pollen tubes did not differ from 'NW' pollen tubes. In both, the $R_{D/A}$ of rapidly-labeled-RNA was greater than that of 18S-RNA. Compatible and incompatible pollen tube differences could not be determined because of insufficient data.

Labeling of pollen tube nucleic acids derived from 2-^{14}C-uridine and $H_3{}^{32}PO_4$ suspended in stigmatic exudate was too low (^{14}C) or so erratic (^{32}P) that no consistent pattern could be recognized. However, the radioactivity profile followed the profile of absorbancy closely enough to say that ^{14}C and ^{32}P was found in all portions of the RNA profile, but not in the DNA portion.

5-^{3}H-uridine dissolved in stigmatic exudate and injected into the style 6 hr after pollination gave a pattern of labeling in 'NW' pollen tubes identical to that caused in 'NW' pollen tubes by a pre-pollination stylar injection of 5-^{3}H-uridine (Fig.6.7).

The amount of label appearing in the total nucleic acids off the MAK column was approximately 5.9×10^{-3}% of the amount used to pre-label stylar tissue and 3.7×10^{-3}% of the label suspended in exudate.

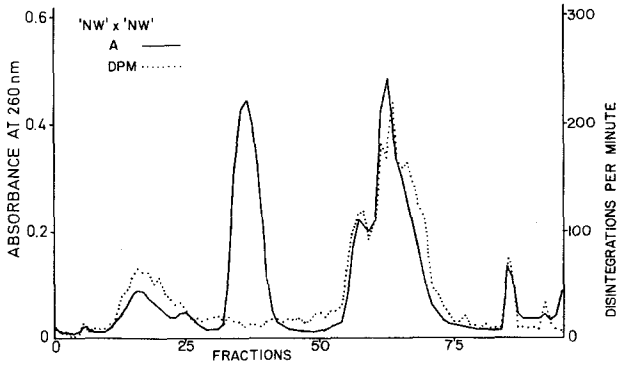

Fig.6. Absorbancy profile of the nucleic acids from non-radioactive 'NW' styles. Radioactivity profile was contributed by 'NW' pollen tubes which had been grown 48 hr at 23 °C in 28 'NW' styles. The 'NW' styles had been injected 6 hr after pollination with 43.2 ul of 5-^{3}H-uridine suspended in stigmatic exudate

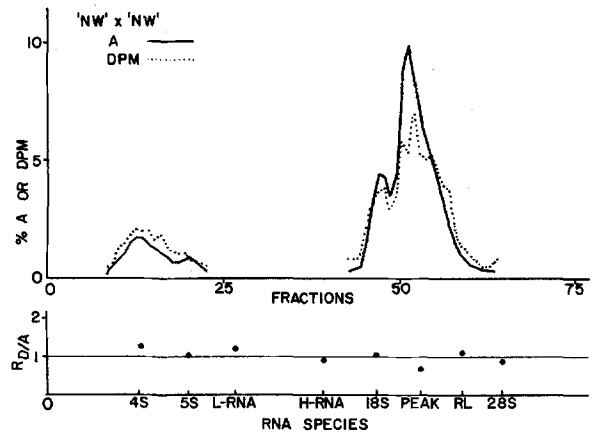

Fig.7. Adjusted MAK profile of the RNA for the experiment in Fig.6

Discussion

Although *in vivo* pollen tube treatment with inhibitors of RNA synthesis indicate that incompatible pollen tubes of *Lilium longiflorum* do not require RNA synthesis for growth but compatible pollen tubes do (Ascher and Drewlow 1970), nucleic acids extracted from both compatible and incompatible pollen tubes contain label derived from radioactive nucleic acid synthesis precursors. The label in the pollen tubes is not that of a few stylar canal cells accidentally removed along with the pollen tubes, for the radioactivity pattern of the canal cell RNA on MAK column chromatography differs in many respects from that of the lily pollen tubes (Campbell and Ascher 1975). Specifically, the radioactivity profile for RNA extracted from canal cells labeled 48 hr with 5-^{3}H-uridine more closely follows the absorbancy profile, with the 28S-RNA portion of the heavy-RNA proportionately more heavily labeled than the 18S-RNA portion and the $R_{D/A}$ value of 5S-RNA less than 1.0.

Even though the total labeling of nucleic acids from pollen tubes was low, more label seemed to occur in pollen tube RNA when the pollen tubes grew in previously labeled styles than when they grew in association with label suspended in stigmatic exudate. Two possible explanations for this are tied to pollen tube derivation of nourishment from stylar canal cells and the inability of stigmatic exudate injected into the stylar canal to equal that stylar nourishment (Ascher 1975). On the one hand, label from the exudate may be absorbed by the canal cells and subsequently secreted into the canal and be taken up by the pollen tubes. Little or no label would go directly from exudate into pollen tube RNA synthesis. Less label would then appear in pollen tubes by this circuitous route compared to pollen tubes growing in previously labeled styles. On the other hand, the label suspended in exudate may be taken into pollen tubes and used for pollen tube RNA synthesis, but in the stylar canal cells might be secreted into the stylar canal in an altered form more readily incorporated by pollen tubes than the label suspended in exudate. This last alternative could explain why both compatible and incompatible pollen tubes contain label, especially if the new material secreted by the canal were an RNA or a nucleic acid and was incorporated into pollen tubes but was not used in pollen tube RNA synthesis. Therefore, the differences in RNA labeling patterns observed between 'NW' and 'Ace' pollen tubes and the possible difference between compatible and incompatible pollen tubes may result from some pollen tube synthesis plus a stylar contribution associated uniquely with the specific physiological or genotypic state of the pollen tube.

The presence of label in all portions of the RNA profile separable by MAK column chromatography does not necessarily mean that each type of RNA from 4S-RNA to rapidly-labeled-RNA specifically contains label. Before statements can be made that specific RNA species contain label, base ratio and further biochemical analysis must be done.

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