Studies on Phytoalexins

THE RELATIONSHIP BETWEEN ACTINOMYCIN D AND RIBONUCLEIC ACID SYNTHESIS DURING THE INDUCTION OF PHASEOLLIN IN THE FRENCH BEAN (PHASEOLUS VULGARIS L.)

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Received for publication March 10, 1972

ABSTRACT

Actinomycin D stimulated phaseollin production in endocarp tissues of the French bean (Phaseolus vulgaris L.), maximum production being obtained with 25 to 30 micrograms per milliliter of antibiotic. Under these conditions, net incorporation of 3H-uridine into total cell ribonucleic acid was inhibited by more than 80% over a 6-hour induction period. If allowance was made for a 2-hour lag in the action of actinomycin D, inhibition of incorporation was greater than 95%. Contrary to other reports, no evidence was obtained of an increased formation of any specific ribonucleic acid fraction. Actinomycin D applied in the cold (4 C) was not found to be effective in stimulating phaseollin production. When applied in this way, actinomycin D did not affect induction of phaseollin by a fungal peptide, Monilicolin A, although ribonucleic acid synthesis was inhibited by more than 95%. It is suggested that the induced formation of phytoalexins may not be dependent on increased ribonucleic acid synthesis as has previously been claimed.

Other experiments indicated that the apparent effects of actinomycin D on ribonucleic acid synthesis could be influenced by the choice of precursor used to label ribonucleic acid and by the order of addition of precursor and antibiotic to the plant tissue. These effects were only observed in the period immediately following application of actinomycin D. It is suggested that such effects could critically influence the results obtained in short term experiments and may explain some differences in reported action of actinomycin D from different laboratories.

Formation of the phytoalexins pisatin and phaseollin may be induced in tissues of the pea (Pisum sativum L.) and the bean (Phaseolus vulgaris L.) respectively by a number of agents including germinating fungal spores (4, 7), extracts of fungal mycelium (6), and a number of compounds normally considered as antimetabolites, including heavy metal ions, metabolic inhibitors, and antibiotic inhibitors of nucleic acid and protein synthesis (7, 12, 19, 23, 24). Recently it has been reported that increases in RNA and protein synthesis were observed in excised pea pods during the early stages of pisatin induction by low concentrations of actinomycin D (10 µg/ml) (24). It was also reported that induction of pisatin by low levels of actinomycin D could be inhibited by applying considerably higher levels of the same antibiotic (450 µg/ml) early in the induction period and that the inhibitory effects of these high levels became less pronounced if applied later in the induction period. From these studies it has been inferred that pisatin production is dependent on de novo formation of RNA, and that the induction mechanism involves the activation of specific host genes in a manner similar to the Jacob-Monod model of gene activation in Escherichia coli (9, 24).

In this laboratory we are primarily interested in investigating the mode of action of Monilicolin A, a peptide of molecular weight 8000 derived from the mycelium of Monilinia fructicola (6). At low concentrations (ED50 = 8 nM), this peptide induces phaseollin production in bean endocarp tissues, but does not appear to be active against other legumes including the pea (6). Recently it has been suggested that the gene activation mechanism described for pea tissues also applies to phaseollin induction in beans (11, 13). Since it has been reported that actinomycin D is capable of stimulating phaseollin production in bean tissues (7, 12), the present study was initiated to examine more closely the postulated requirement for RNA synthesis in phaseollin induction. This paper reports that contrary to previous findings on pisatin induction, no evidence was obtained of an increase in RNA synthesis during the induction of phaseollin in bean endocarp tissues. Rather, results were obtained which are not readily interpreted in terms of a gene activation mechanism.

MATERIALS AND METHODS

French beans (Phaseolus vulgaris L. var. Red Kidney) were grown in a glasshouse as previously described (7). Detached pods (chiefly class II, Table 2 of ref. [7]) were harvested and half seed-cavity sections prepared under aseptic conditions some 15 to 24 hr prior to inoculation to allow the cut surface surrounding the seed cavity to dry. This procedure does not appreciably affect phaseollin production (7). Provided that suitable precautions are taken in maintaining aseptic conditions during plant preparation and in subsequent inoculation steps, the bacterial population is maintained at a low level. In typical experiments the bacterial density in the inoculating solutions harvested after 6 hr on the bean surfaces at 20 C was usually below 10⁷/ml, with an occasional sample reaching 10⁸ cells/ml.

Seed cavity segments were set out in Perspex incubation chambers, and solutions were applied topically at a level of 5 to 6 ml per 40 to 50 segments. Treated tissue was incubated in the dark at 20 C for the times indicated. At the end of the incubation period, solutions were removed by suction and discarded, and the bean sections were chilled immediately in an ice/water mixture and then stored under ice. Since phytoalexin
induction appears to be a localized event (5), only the endocarp tissues in the immediate vicinity of the site of inoculation were taken for examination to minimize dilution by tissue not actively involved in phytoalexin production. The following procedure was routinely employed for preparing endocarp tissues for analysis: the pod sections were chilled in ice after removal of inoculum, washed quickly in 2 x 1 liter changes of cold tap water, and then rechilled in ice water. Batches of 15 to 20 sections were removed from the ice water and blotted dry. Discs were cut (5 mm diameter, one per cavity section) from the area of inoculation with a steel punch and transferred immediately to liquid nitrogen. Total exposure of any one section to room temperature under this regime did not exceed 5 min, and in most cases the time was considerably less. While the discs were still frozen, endocarp slices (0.3-0.5 mm thick) were removed with a sharp scalpel and either transferred to liquid nitrogen when RNA was to be extracted with phenol, or for the Schmidt-Thannhauser procedure, transferred to tared vials containing redistilled ethanol (2 ml).

Total RNA was extracted in 150- to 200-mg samples of endocarp tissue (fresh weight) by the Schmidt-Thannhauser procedure as described by Smillie and Krotkov (25), except that, (a) prior to extraction with trichloroacetic acid the ground tissue was extracted five times at 70 C with 60% ethanol (v/v) containing 0.2 mg/ml of unlabeled RNA precursor as described by Click and Hackett (2), (b) the final digestion time was reduced to 1 hr since it was found that maximum release of nucleotides had occurred in this period, and, (c) the Dowex-1 chromatography step (25) was omitted since almost all (94%) of ultraviolet-absorbing material behaved in the manner described for nucleotides. The final supernatant after neutralization was taken to dryness on a rotary evaporator and redissolved in distilled water (2 ml). The RNA content of the tissue was determined spectrophotometrically from the absorption at 260 nm of an aliquot of the aqueous extract in 1% (v/v) HClO4, and by reference to a sample of yeast RNA (P.L. Biochemicals U.S.A.) hydrolyzed under identical conditions. For radioactivity determinations 1 ml of the final aqueous solution was combined with 10 ml of a dioxane-based scintillation fluid (1) and counted in a Packard scintillation spectrometer. Under these conditions efficiency of counting was about 15% for 3H and about 50% for 14C as determined by reference to 14C- and 3H-hexadecane standards (Radiochemical Centre, Amersham).

To examine individual RNA species, RNA was extracted from frozen endocarp tissues (about 2 g fresh weight representing 400-500 endocarp slices) with phenol-sodium dodecyl sulfate as described by Spencer and Whitfield (27). The final pellet from the ethanol precipitation was redissolved in tris-HCl (10 mM) MgCl2 (10 mM), pH 7.8, at 0 to 4 C containing 1 mg/ml of unlabeled uridine and reprecipitated with ethanol at —20 C. This procedure was repeated three times. The RNA was further purified by precipitation as a cetyltrimethyl ammonium salt, washed, and reconverted to the sodium salt as described by Ralph and Bellamy (20). The final pellet was dried under a stream of argon or nitrogen and taken up in a minimum volume of distilled water (0.3-0.4 ml). Throughout this procedure, only acid-washed or diethylpyrocarbonate-treated (Baycovin, Bayer, Germany) apparatus was used, and all solutions were prepared from distilled water which had been treated overnight with diethylpyrocarbonate (2 ml/liter) and boiled to inactivate ribonucleases.

The RNA thus prepared was fractionated on linear 5 to 20% (w/v) sucrose gradients containing 0.1 M tris-HCl, 0.1 M NaCl, 1 mM MgCl2, pH 7.8, at 0 to 4 C. Aliquots (50 μl containing 120-140 μg RNA) were layered carefully on top of the gradients and centrifuged at 35,000 rpm in the Spinco SW 39 rotor for 6 hr. The sucrose solutions were treated with diethylpyrocarbonate to inactivate ribonucleases following the procedure described by Solymosy et al. (26) except that double-strength sucrose only was treated, and was then diluted with double strength buffer which had been prepared in diethylpyrocarbonate-treated water. To avoid changes in pH buffer solutions were not treated. After the run, the bottom of the tube was pierced and 10-drop fractions (0.13 ml) collected. Each fraction was diluted to 1.0 ml with water and the ultraviolet absorbance measured at 260 nm in a Shimadzu Model Double 40 S spectrophotometer. Each fraction was then mixed with 10 ml of Bray’s solution (1), and the radioactivity was determined in a Packard scintillation spectrometer.

Phaseollin was extracted from drop diffusate solutions with light petroleum, and estimated spectrophotometrically as previously described (7). Recent experiments have indicated that such preparations also contain small amounts of a closely related compound, phaseolidin, induced together with phaseollin, but that the proportions of these compounds remained constant under the experimental conditions described in this paper.

Actinomycin D was a gift from Merck, Sharpe and Dohme, Rahway, New Jersey. Uniformly labeled uridine-3H (2.5 c/mumole), orotic acid-6-3H (60.8 mc/mumole) and uridine-5'-H5'-triphosphate (1.0 c/mumole) were purchased from the Radiochemical Centre, Amersham, U.K.

**RESULTS**

Stimulation of RNA Formation not Observed During Phaseollin Induction by Actinomycin D and other Compounds. Although it has been reported that stimulation of RNA formation was observed during actinomycin D-induced formation of pisatin in pea tissues (9, 24), this laboratory has not been able to confirm these observations for bean tissues. In numerous experiments conducted over the past 2 years phaseollin induction by this antibiotic has always been accompanied by a reduced incorporation of radioactive precursors into RNA. In no case has an increase been observed. Rather, data have been obtained which suggest that phaseollin induction may be more closely related to inhibition than to stimulation of RNA synthesis. Figure 1 shows the concentration dependence curves of

![Fig. 1. Concentration dependence curves of phaseollin-inducing and RNA inhibiting activities of actinomycin D. To measure effects on RNA synthesis, sets of 50 pod cavities were inoculated with 6 ml of solution containing 3 μc of 14C-orotic acid (60.8 mc/mumole) with and without actinomycin D at the concentrations indicated. RNA synthesis was measured over 6 hr, phaseollin production over 24 hr. Phaseollin production is from reference 7: a: Phaseollin production; b: inhibition (%) of incorporation into RNA.](https://www.plantphysiol.org/doi/fig/10.1104/pp.50.1.661)
Table 1. Effect of a Number of Inducers of Phaseollin Production, other than Actinomycin D, on the Incorporation of \(^{14}\)C-Orotic Acid into RNA

Beans were treated as described in the text. For incorporation studies, 1 \(\mu\)g of \(^{14}\)C-oarcic acid (60.8 mc/mmole) was added per 5 ml of inoculating solution.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Specific Radioactivity of RNA at 6 Hr</th>
<th>Phaseollin in Diffusate After 24 Hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>cpm per mg RNA</td>
<td>(\mu)g/ml</td>
</tr>
<tr>
<td>Cycloheximide (0.36 (\mu)M)</td>
<td>6,470</td>
<td>0.5</td>
</tr>
<tr>
<td>Monilicolin A (60 nm)</td>
<td>2,740</td>
<td>8.9</td>
</tr>
<tr>
<td>HgCl(_2) (10 (\mu)M)</td>
<td>3,280</td>
<td>17.4</td>
</tr>
<tr>
<td></td>
<td>1,420</td>
<td>8.7</td>
</tr>
</tbody>
</table>

The consistent failure to observe an increased incorporation of labeled precursors into RNA during phaseollin induction by actinomycin D and other compounds has led us to query the concept that induction is dependent on RNA synthesis and to investigate more fully the role of actinomycin D in the induction mechanism.

**Effect of Actinomycin D on the Incorporation of \(^{3}\)H-Uridine into Specific Classes of RNA.** Since measurements of total RNA do not differentiate between the various types of RNA within the cell, the possibility could not be excluded that in the previous experiment there could be an increased formation of a minor fraction of RNA following actinomycin D treatment, which may be masked by quantitatively greater changes in the bulk of the cell RNA. To examine this possibility, therefore, it was necessary to fractionate total cell RNA and examine the extent of incorporation into specific classes. Bean pods were, therefore, treated with \(^{3}\)H-uridine in the presence and absence of actinomycin D, the RNA isolated from the endocarp tissues with phenol-sodium dodecyl sulfate and fractionated by sucrose gradient centrifugation. The results are shown in Figure 2. At 1, 2, and 4 hr, total incorporation into RNA in actinomycin D-treated beans was reduced by some 25, 60, and 80% of water control values respectively, indicating a lag in the action of this antibiotic. It is apparent however that actinomycin D has effectively inhibited the incorporation of labeled uridine into all classes of RNA: in water controls the bulk of the incorporation was accounted for by the 19S and 28S ribosomal RNA peaks and the SS transfer and ribosomal RNA peak. Following application of actinomycin D, only the SS transfer and ribosomal RNA peak was clearly defined; some incorporation into the remainder of cell RNA was evident but was strongly repressed. Expressing the results as specific radioactivities (cpm per mg RNA) did not alter the picture: RNA extracted from beans treated with actinomycin D showed a lower level of incorporation at all points than did RNA from

![Fig. 2. Incorporation of \(^{3}\)H-uridine into specific classes of RNA. Pod sections (400–500) were inoculated with 50 ml of solution containing 500 \(\mu\)c of \(^{3}\)H-uridine and where appropriate actinomycin D at 30 \(\mu\)g/ml. At the times indicated, the bean pods were treated, the RNA extracted with phenol-sodium dodecyl sulfate, and fractionated by sucrose gradient centrifugation as detailed in "Materials and Methods." Note the change in scale between the 2-hr and 4-hr samples.](image-url)
Table II. Effect of Applying Actinomycin D and Monilicolin A at Low Temperature on Subsequent Phaseollin Production

Beans were treated as described in the text. The results shown represent the means of duplicates except in the water control of Monilicolin A.

<table>
<thead>
<tr>
<th>Solutions Applied</th>
<th>Mean Phaseollin Conc in Diffuse Solutions after 24 Hr at 20 C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No pretreatment</td>
</tr>
<tr>
<td></td>
<td>µg/ml</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td></td>
</tr>
<tr>
<td>25 µg/ml</td>
<td>6.6</td>
</tr>
<tr>
<td>10 µg/ml</td>
<td>3.7</td>
</tr>
<tr>
<td>Water control</td>
<td>1.5</td>
</tr>
<tr>
<td>Monilicolin A</td>
<td></td>
</tr>
<tr>
<td>(60 nm)</td>
<td>15.8</td>
</tr>
<tr>
<td>Water control</td>
<td>2.8</td>
</tr>
</tbody>
</table>

1 One of the duplicates showed an abnormal spectrum and was not included in the calculations.

Water controls. Thus there did not appear to be an increased formation of any fraction of RNA as reported for pea tissues (23, 24), and the changes in total RNA observed in the previous experiment reflect changes in individual classes of RNA.

Lost of Phaseollin-inducing Activity of Actinomycin D on Infiltration into Tissue at Low Temperatures. The aim of the following experiments was to determine whether phaseollin production could be induced after RNA synthesis had been inhibited. Presumably if the induction mechanism involves RNA synthesis, then one would not expect phaseollin induction under these conditions. In approaching this question a major difficulty had to be overcome in that all inhibitors of RNA synthesis so far tested had been found capable of stimulating phaseollin production. However, during the course of the present experiments it became apparent that actinomycin D, if infiltrated into tissue at 4 C,2 is not effective in stimulating phaseollin formation, as shown in Table II. When applied to bean endocarp surfaces at 20 C, actinomycin D at 25 and 10 µg/ml respectively induced the formation of 6.6 and 3.7 µg of phaseollin per ml of diffuse solution in 24 hr. After cold pretreatment, these values fell to 1.8 and 1.6 µg/ml respectively, which are comparable to water control values (1.5 µg/ml). Since Monilicolin A (6) induced the production of comparable quantities of phaseollin under both conditions it would appear that the induction mechanism itself has not been inactivated by incubation at low temperatures. Thus the possibility arises that induction by actinomycin D may be due to transient effects which are somehow circumvented under conditions of low temperature infiltration.

Induced Formation of Phaseollin following Inhibition of RNA Synthesis by Actinomycin D. This technique, by eliminating or markedly diminishing the phaseollin-inducing activity of actinomycin D but without at the same time affecting its ability to inhibit RNA synthesis has allowed a direct approach to the question of the involvement of RNA synthesis in phytoalexin induction. Figure 3 shows a typical experiment in which an inducer, Monilicolin A, was applied to bean tissue after cold infiltration of actinomycin D. It is evident that subsequent production of phaseollin was not affected despite the inhibition of RNA synthesis. In the presence of Monilicolin A, the phaseollin content of drop diffuse solutions reached some 9 µg/ml, irrespective of treatment with actinomycin D, although incorporation into total RNA had been inhibited by some 70% prior to addition of Monilicolin A, and was blocked almost entirely (95%) in the 6-hr period afterwards. In the presence of actinomycin D alone, phaseollin was marginally above water controls, and comparable to other water control values obtained previously (Table II). Monilicolin A alone did not appear to stimulate the incorporation of 3H-uridine into total RNA, indeed incorporation was slightly lower in beans treated with this peptide than in water controls, despite the large differences in phaseollin induction under these conditions.

Effect of Varying the RNA Precursor and the Timing of Addition of Precursor and Antibiotic on the Apparent Action of Actinomycin D on the RNA Synthesizing System of Bean Endocarp Tissue. The present results are consistent with other reports of inhibition of RNA synthesis by actinomycin D in bean tissues (22, 29), but do not confirm previous observations of an increase in RNA synthesis during pisatin production in pea tissues (24). It would seem unlikely that this lack of agreement is due to differences in reaction of pea and bean tissues with actinomycin D, since in a number of other studies on pea tissues (3, 15, 17) inhibitory effects of this antibiotic on RNA synthesis were recorded. In comparing these various reports it became evident that different experimental conditions were employed in different laboratories, notably with respect to time of incubation. RNA precursor used, and timing of addition of

2 In all experiments involving cold infiltration, care was taken not to permit the bean sections to reach room temperature during the experiment. The beans were chilled overnight (4 C) before cutting, and all subsequent manipulations were performed in a cold room. All solutions were chilled in ice before application, and the bean pod sections were placed in trays which had also been stored overnight in a cold room (4 C) before use. Six hours after application of solutions to the bean surfaces, the trays containing the bean sections were transferred to a constant temperature room at 20 C.

Fig. 3. Effect of low temperature infiltration of actinomycin D (25 µg/ml) on incorporation of 3H-uridine into RNA, and subsequent induction of phaseollin formation by Monilicolin A. Fifty bean pod sections were treated with 50 µc of 3H-uridine (6 ml) in the presence and absence of actinomycin D for 6 hr at 4 C, then transferred to 20 C. Samples were taken for RNA analyses (after 6 hours). At the time shown (arrow), solutions were removed from all samples (including controls) and replaced with fresh solutions identical to those applied previously but containing Monilicolin A (0.5 µg/ml, 63 µm) where indicated. The phaseollin content of diffuse solutions was measured 24 hr after the change indicated by the arrow.
percuro and actinomycin D to tissue. Experiments were therefore devised to assess the effects of some of these parameters on the type of results obtained.

Figure 4 shows the effect of actinomycin D on the incorporation of orotic acid, uridine, and UTP into RNA. These compounds are all precursors of the pyrimidine moieties of RNA. It is apparent that in the period immediately after inoculation, actinomycin D has effect the incorporation of each of these compounds differently. With orotic acid as precursor, no inhibition of incorporation was observed by 30 min and some 33% by 1 hr. Incorporation of UTP on the other hand was inhibited by some 70% in the first 30 min and by a similar amount at 1 hr. Uridine gave results intermediate between these two extremes. By 2 hr these differences were no longer evident and subsequent incorporation of all three compounds into RNA was inhibited by more than 95%. Thus it appears that within the 1st hr after inoculation, the apparent action of actinomycin D on RNA synthesis can vary greatly depending on the precursor used. In longer term experiments (>2 hr) in bean tissues, these effects do not appear to be important.

Figure 5 shows the apparent action of actinomycin D in bean tissues may also be influenced by the order in which precursor and antibiotic are applied to the plant tissue. When H-uridine was added 30 min prior to actinomycin D, subsequent incorporation of RNA was less sensitive to inhibition than when precursor and antibiotic were added simultaneously. At 30, 60, and 90 min after addition of actinomycin D, incorporation of uridine into RNA was inhibited by some 8, 25, and 50% of water control values respectively in the former treatment compared to about 40, 50, and 60% in a corresponding period when antibiotic and precursor were added simultaneously. At the concentration used in these experiments (30 µg/ml), the reverse order of addition, actinomycin D followed by H-uridine (not shown) did not substantially affect the results compared to simultaneous addition. This may be due to saturation effects, however, since at lower concentrations (10 µg/ml) actinomycin D applied prior to precursor effected a greater degree of inhibition (85%) than when added simultaneously (60%).

**DISCUSSION**

The concept that RNA synthesis is required for phytoalexin induction is based largely on observations (a) of an increased incorporation of precursors into RNA following treatment of pea pod tissues with low concentrations of actinomycin D (10 µg/ml) (23, 24); and (b) of inhibition of pisatin production by high concentrations of actinomycin D and other antimetabolites (10, 24). The increases in RNA synthesis have been reported variously to involve certain fractions of rapidly labeled RNA (23), while apparently in short term experiments all types of RNA showed increased incorporation (24). These observations are not consistent with other studies on the same variety of peas (Pisum sativum cv. Alaska) in which only inhibitory effects of actinomycin D on RNA synthesis were reported (3, 15, 17). In the present studies no evidence was obtained of a stimulation of RNA synthesis during phaeollin induction by actinomycin D in bean tissues comparable to the changes reported to occur in pea tissues during pisatin induction. In attempting to correlate these various results it is of interest to consider the implications of some of the experiments described in this paper regarding experimental parameters involved in the study of RNA synthesis. As shown in Figure 5, the timing of addition of reagents was found to influence the apparent ef-

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**Fig. 4.** Effect of actinomycin D on the incorporation of 3H-orotic acid, 3H-uridine, and 3H-UTP into RNA of bean endocarp tissue. Fifty cavities were inoculated with either 3, of the 3H-orotic acid or 30 µc of 3H-uridine or 3H-UTP in 6 ml, in the presence and absence of actinomycin D (30 µg/ml). Tissue sampling and analysis of RNA are detailed in "Materials and Methods." Timing was taken from commencement of inoculation. O: Actinomycin D treatment; □: water controls.

**Fig. 5.** Effect of altering the timing of addition of actinomycin D and 3H-uridine on the apparent action of actinomycin D on RNA synthesis. I: Water control; II: actinomycin D and 3H-uridine added simultaneously; III: actinomycin D added 30 min after 3H-uridine. Solutions were applied at 6 ml containing 16 µc of 3H-uridine per 50 pod sections. When applying actinomycin D to III, the solutions on the bean surfaces were removed and replaced with identical solutions but containing 30 µg/ml of actinomycin D. The hatched bars indicate the degree of variation in duplicates.
ACTINOMYCIN D AND PHASEOLIN INDUCTION

The inhibitory effect of actinomycin D on RNA synthesis in bean endocarp tissue. The reported stimulation of RNA synthesis in pea pod tissue was observed under conditions where a single precursor, orotic acid, was applied to pod tissue 10 min prior to actinomycin D (10 μg/ml). The total duration of the experiment was 45 min (24). Johri and Varner (15) on the other hand, using a comparable concentration of actinomycin D (12 μg/ml) reported some 40% inhibition of incorporation of labeled precursors into all types of RNA in a comparable time period (1 hr).

In their experiments however, the actinomycin D was applied to pea tissue prior to precursor. In other reported cases of inhibition of RNA synthesis in pea tissues by actinomycin D, it is noteworthy that actinomycin D was also applied to the tissues prior to precursor (3, 17). Thus it appears that in pea as in bean tissues the timing of addition of reagents can influence the type of results obtained.

Another factor which can influence the type of result obtained in short term experiments is the choice of precursor used to study RNA synthesis (Fig. 4). In the 1st hr following application of actinomycin D to bean pods, incorporation of orotic acid into RNA of the endocarp tissue was affected only by a minor degree, whereas incorporation of UTP was strongly inhibited. The effect on uridine incorporation fell between these two extremes. After this period, the influence of the nature of the precursor diminished, and after 2 hr no differences were detected. Inasmuch as the reported stimulation of incorporation into RNA of pea tissues was observed: (a) in the 1st hr following application of actinomycin D, the period during which the precursor effect was most marked in bean tissues, (b) it was observed with orotic acid as precursor, whose incorporation in bean tissues was least affected during this period; and (c) it was observed under conditions where the orotic acid precursor was applied to tissues prior to actinomycin D, it seems likely that the increased observed may be transient effects occurring as a result of a particular set of experimental conditions. Clearly these aspects require further clarification.

As additional evidence of involvement of RNA synthesis in phytoalexin induction, it has been reported that high levels of actinomycin D (450 μg/ml) or 6-methylpurine (1 mg/ml, about 7 mm) inhibit phytoalexin induction if added simultaneously with lower inducing levels of actinomycin, but become decreasingly effective if application is delayed (10, 24). From such studies it has been inferred that the postulated mRNA is formed in the first 2 to 3 hr after induction and that continued synthesis is not necessary (10). However 6-methylpurine has been observed to inhibit respiration in other plant tissues at considerably lower concentrations (0.5 mm) (8) than used in these studies. In addition, actinomycin D at low concentrations has also been observed to affect systems other than RNA synthesis, including glycolysis and respiration (16). Phospholipid synthesis (18), accelerated breakdown of pre-existing RNA (28), and has had effects on protein synthesis apparently independent of action on RNA synthesis (14, 21). Thus specificity of action by these compounds at high concentrations cannot be assumed. The effects could be on cell processes other than the RNA synthesizing system, a suggestion not inconsistent with published evidence that even with 300 μg/ml of actinomycin D only 10 to 20% inhibition of incorporation of orotic acid into RNA was observed (24). The postulated mRNA would, therefore, be required to be represented in that 10 to 20% of RNA which formation was reportedly blocked. In the present experiments phaseollin induction was apparently unaffected despite 95% inhibition of RNA synthesis. Based on incorporation data and effects of antibiotics, therefore, evidence for involvement of RNA synthesis in phytoalexin induction is not conclusive.

The marked diminution in stimulatory activity of actinomycin D on phaseollin production when the antibiotic is infiltrated into tissues at low temperature is not readily explained on the basis of a gene activation mechanism (9, 24). It does not appear to be due to a change in the concentration dependence curve (Fig. 1) since altering the concentration of antibiotic in a 2-fold geometric progression from 0.12 to 32 μg/ml did not affect the results. Nor did increasing the incubation time from 24 to 48 hr have any substantial effect on inducing activity. Clearly actinomycin D was able to inhibit RNA synthesis after cold treatments, and therefore it is still capable of affecting the transcription systems of bean endocarp. A process of differential gene activation by actinomycin D would not be expected to be affected under these conditions. Since Monilicin A was capable of stimulating phaseollin production after such treatments, it would appear that the induction system also remained functional under these conditions. It would seem that the induction of phaseollin by actinomycin D, but not necessarily by other compounds, could be dependent on an initial step, as yet undefined, which is somehow circumvented under conditions of cold infiltration. It would also appear that conditions may be critical for activating this initial step, since induction by this antibiotic can apparently be bypassed by a superficially simple physical technique of low temperature infiltration, which at the same time does not appear to affect other biochemical processes in bean or other plant tissues (8). At present this technique provides a useful, if somewhat empirical, means of altering the reaction of certain inducing agents with the host tissue. The nature of the changes in the plant tissue during the early stages of the induction process, and whether other inducers are affected by this technique are possible subjects for future investigation.

Acknowledgments—The author is grateful to Dr. I. A. M. Cruickshank and Mrs. Down R. Perrin for helpful advice and critical discussion during the course of these experiments. To Miss M. Mould for technical assistance and to Merck, Sharpe and Dohme for generous donations of actinomycin D.

LITERATURE CITED


