Massive Synthesis of Ribonucleic Acid and Cellulase in the Pea Epicotyl in Response to Indoleacetic Acid, With and Without Concurrent Cell Division[1]

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Summary. Measurements were made over a 4-day period of the effect of added indoleacetic acid (IAA), puromycin, actinomycin D and 5-fluorodeoxyuridine (FUdR) on growth and the levels of total DNA, RNA, protein and cellulase in segments of tissue at the apex of decapitated etiolated epicotyls of *Pisum sativum*, L. var. Alaska.

The hormone induced swelling of parenchyma cells and cell division. By 3 days after IAA application, the amounts of DNA and protein were approximately double, RNA triple and cellulase 12 to 16 times the levels in controls. All of these changes were prevented by both puromycin and actinomycin D. FUdR prevented DNA synthesis and cell division but not swelling or synthesis of RNA, protein and cellulase.

It is concluded that IAA-induced RNA synthesis is required for cellulase synthesis and lateral cell expansion, whether or not cell division takes place.

When high concentrations of indoleacetic acid (IAA) in lanolin paste are applied to the decapitated apex of etiolated pea epicotyls, in the first 10 mm of tissue below the apex there is a marked increase in amount and specific activity of the enzyme cellulase (β-1,4 glucan 4-glucanohydrolase, EC 3.2.1.4.) (3). The increase can be inhibited by including in the lanolin any of a number of substances which interfere with protein synthesis. Accordingly, it appears that IAA selectively brings into operation the coding system(s) needed for eventual biosynthesis of cellulase.

Theoretically, a hormone could regulate the synthesis of a specific enzyme by direct interaction with macromolecules which are important in the processes of information transcription (2) or translation (22). In the case of cellulase, however, it is possible that synthesis occurs specifically in cells which are about to divide and in newly-formed cells as an indirect consequence of IAA-induced mitosis. In the pea epicotyl, cellulase is concentrated in the plumule and hook (16) where the endogenous auxin level is high (21) and where cell divisions are most common. When plumule and hook are replaced by IAA, the most rapid increase in epicotyl cellulase begins at about 18 hours (3) which is the time when the first divisions that lead to formation of root primordia become visible (20). Clearly the ability of an epicotyl cell to form cellulase could depend merely on the time elapsed since DNA replication and not on the continued presence of hormone.

A major question, then, in understanding how cellulase synthesis is induced is whether the action of IAA or the division of cells is the more immediate precursor event. This study examines the problem by measuring the degree to which IAA brings about cellulase synthesis in the epicotyl segment when the formation of DNA, RNA and/or protein is inhibited by 5-fluorodeoxyuridine (FUdR), actinomycin D or puromycin. In epicotyl tissue, both puromycin and actinomycin D inhibit growth and protein synthesis (3, 18, 23, 24) and the latter interferes with RNA synthesis (17, 19, 23). Effects of FUdR on pea tissues have not been reported but this substance prevents thymidylate synthesis (4) and mitosis (11) in a variety of other plant tissues.

Materials and Methods

Etiolated pea seedlings with third internodes 3 to 5 cm long were used throughout this study. Under dim green light the plumule was cut off just below the hook and a point 10 mm below the cut apex was marked with ink to delineate a segment of tissue. The apex was painted with about 2.5 mg of lanolin paste (70 % w/w water) containing IAA (0.5 % w/w) plus or minus FUdR (0.1 % w/w), actinomycin D (0.02 % w/w) or puromycin (0.02 % w/w). At daily intervals 30 to 100 segments from each condition were removed, washed with dilute NaOCl solution and blotted dry. The fresh weight and lengths were recorded. Some of the segments were then used for measurement of cellulase activity and soluble protein in enzyme extracts and others for total protein and nucleic acid determinations. Two experiments are reported here: one (A) using puromycin and actinomycin D and the other (B) using FUdR.

The methods used for buffer-extraction of soluble protein and cellulase were described in previous papers (3, 16). Cellulase activity in the enzyme ex-

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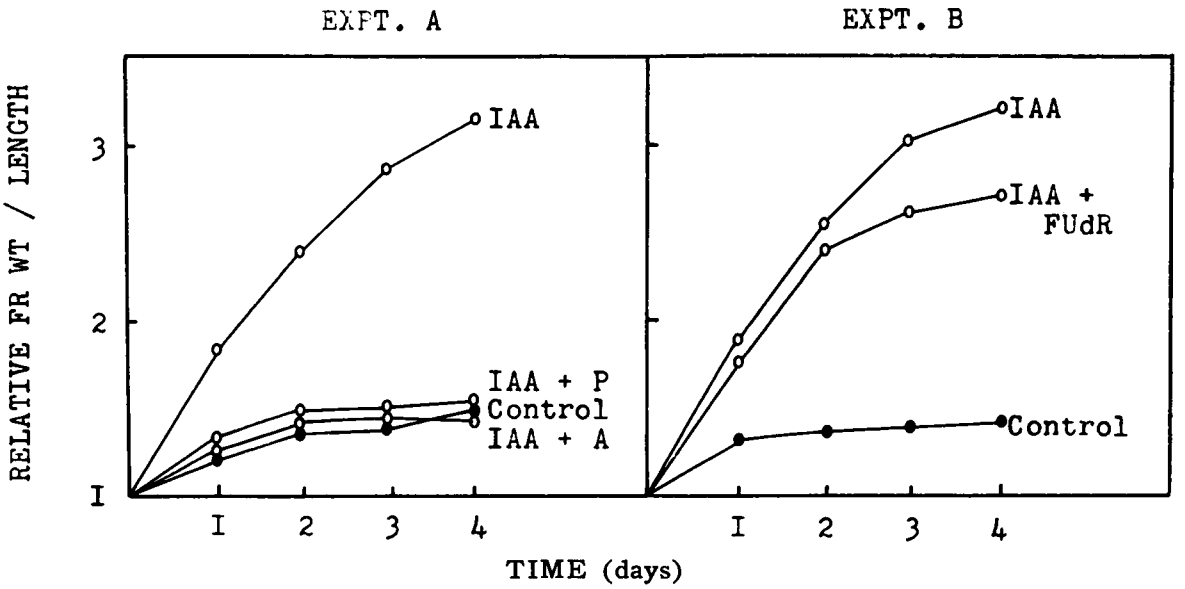

FIG. 1. Swelling of segments treated with IAA in combination with puromycin (P), actinomycin D (A) or fluorodeoxyuridine (FUdR). Expts. A and B were carried out on separate occasions. Swelling is indicated by an increase in the fr wt/unit length (see table I for original data for expt A).

tract was measured by the initial rate of loss of viscosity in solutions of carboxymethylcellulose (3). Soluble protein was determined with the biuret reagent standardised against bovine serum albumin (5). Total protein, RNA and DNA determinations were carried out on homogenized 80 % ethanol- and ether-insoluble fractions from the segments. The methods (15) included extraction of total nucleic acid into warm 0.5 N perchloric acid, its estimation by ultraviolet absorption and DNA determination with diphenylamine (RNA = total nucleic acid minus DNA). Total protein was determined in the perchloric acid-insoluble residue after Kjehldahl digestion by nesslerization. The yield of buffer-soluble protein accounted for 90+ % of total segment protein.

Results

Growth. Table I records the growth in segment length and fresh weight; figure 1 shows the changes in relative fresh weight per unit length, i.e., a measure of the extent of segment swelling. In untreated segments (control), both length and fresh weight increased mainly during the first 2 days. In IAA-treated segments, elongation was slightly inhibited but the fresh weight increment was much greater than in controls with the result that segments showed marked swelling (3-fold increase in fr wt/length in 4 days). When puromycin or actinomycin D was included with IAA, elongation was greater than that observed after any other treatment given in these

Table I. *Effect of Added IAA in Combination with Puromycin or Actinomycin D on the Growth of Decapitated Pea Epicotyl Tissue Segments*

Time (days)	Untreated (control)	+ IAA	+ IAA + puromycin	+ IAA +actinomycin D
		Length (mm/segment)		
0	10.0	10.0	10.0	10.0
1	12.0	12.2	13.1	13.2
2	13.6	12.5	16.4	18.6
3	14.1	14.0	18.6	22.0
4	15.0	14.3	24.0	24.5
		Fr wt (mg/segment)		
0	23.0	23.0	23.0	23.0
1	34.2	53.4	38.8	39.2
2	43.2	69.7	56.8	62.6
3	45.5	92.7	68.5	82.4
4	54.8	106.0	89.0	89.5

tests. The fresh weight kept pace with elongation so that a major effect of these antibiotics was the complete prevention of IAA-induced swelling. In contrast, FUdR had no effect on elongation and it interfered only slightly with swelling.

Figure 2 illustrates the effects of IAA and FUdR on the anatomical structure of segments at 1 and 3 days. In untreated segments some swelling of parenchyma cells but no cell division occurred. Similar results (not shown here) were observed in segments treated with IAA plus puromycin or actinomycin D. In segments treated with IAA alone, marked swelling and some cell division were visible at 1 day. By 2 days many swollen parenchyma cells throughout the cortex had disintegrated to leave lacunae filled with cell and wall debris (see 20). By 3 days recognisable root primordia had been generated in vascular regions. This did not result in much further swelling of the whole epicotyl (cf. fig 1) because the masses of new cells merely occupied the spaces left by collapsed and disoriented cortical cells. In segments treated with IAA plus FUdR, considerable parenchyma swelling but no visible cell division or wall disintegration took place.

Changes in DNA, RNA and Protein. Figure 3 shows effects of IAA and inhibitors on total DNA, RNA and protein levels. Table II records the ratios of the weight of total RNA/DNA and protein/RNA. In untreated segments, the total and relative amounts of these components showed little change up to 4 days. With IAA treatment, the amounts of each macromolecule increased linearly during the whole experiment. The approximate times necessary for initial levels to double were 2.2 days for DNA, 1.2 days for RNA and 3.4 days for protein. Accordingly, the RNA/DNA ratio steadily increased in the presence of IAA and the protein/RNA ratio decreased. Only the RNA level increased at a rate comparable to the growth rate, i.e., RNA concentration per unit fresh weight was maintained during swelling whereas DNA and protein concentrations decreased (cf. table I and fig 3).

Puromycin and actinomycin D prevented the IAA-induced increases in DNA, RNA and protein (fig 3). FUdR completely inhibited the increase in DNA which accounts for its effectiveness against cell division (fig 2). However, FUdR did not interfere seriously with the increase in RNA and protein until after about 2 days incubation. There was therefore a marked increase in the RNA/DNA ratio up to that time (table II). Evidently this RNA and protein synthesis, as well as most of the lateral segment expansion (fig 1), was due to action of IAA in cells which were present in the segment at zero time (pre-existing cells) and was relatively independent of DNA synthesis or cell division.

Cellulase Activity. Figure 4 shows the changes observed in cellulase activity. By 3 days, the amount of enzyme per segment reached levels 12 to 16 times the level in controls. This increase was much greater than those in fresh weight (table I), total protein, RNA or DNA (fig 3), i.e., the cellulase level followed a typical sigmoid induction curve regardless of the basis on which it was calculated.

In the presence of IAA plus FUdR, cellulase activity increased rapidly for 2 days, i.e., for as long as rapid synthesis of total RNA and protein continued, and then began to decrease slowly as the total protein level declined (cf. figs 3 and 4). Since there was no DNA synthesis or cell division in the presence of this inhibitor, the extra cellulase must have formed as a result of direct IAA action in pre-existing cells. The maximum cellulase level reached in these cells (4.5 × zero time) was less than the level reached in segments treated with IAA alone, and the discrepancy between the 2 increased after the first day as cell division proceeded. Evidently newly-formed cells also synthesized cellulase.

IAA failed to raise cellulase levels in the presence of puromycin or actinomycin D (fig 4). This cannot have been due to inhibition by these antibiotics of IAA-induced DNA synthesis and cell division since the results with FUdR show that these events were not required for cellulase synthesis. Rather, it may be presumed to result from their effectiveness in preventing RNA and protein synthesis in pre-existing cells.

Discussion

It is clear from the effects of inhibitors (figs 1–4) that IAA can promote the synthesis of protein in general and cellulase in particular in cells which are in the process of expanding. It is legitimate, therefore, to speculate on the question of where the hormone must act in the sequence of events leading to protein synthesis. Recently it has been suggested

Table II. *Effects of IAA and FUdR on the Weight Ratios of RNA/DNA and Protein/RNA*

Time (days)	RNA/DNA			Protein/RNA		
	Untreated	+ IAA	+ IAA + FUdR	Untreated	+ IAA	+ IAA + FUdR
0	6.5	6.5	6.5	4.9	4.9	4.9
1	7.3	7.8	10.5	3.6	3.5	3.0
2	9.1	8.7	18.5	2.7	3.5	2.7
3	7.1	9.6	16.1	3.5	2.9	2.3
4	6.5	12.0	20.3	4.0	2.2	2.0

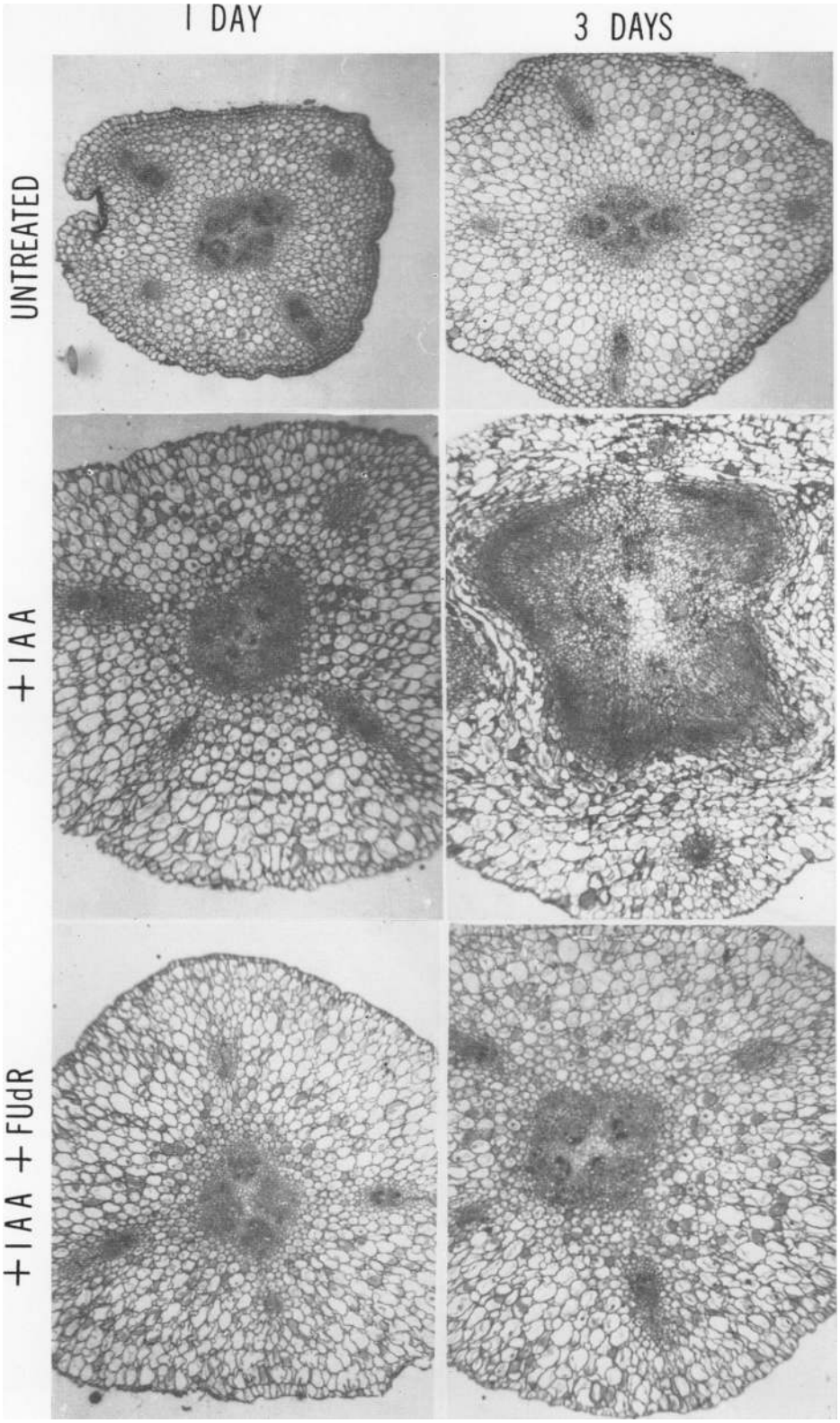

FIG. 2. Anatomical structure of cross sections of segments at 1 and 3 days after treatment with IAA or IAA plus FUdR. Sections (10 μ) were removed from the most swollen regions of tissue after it had been dehydrated in *t*-butanol and embedded in paraffin wax. Magnification: × 29; stain: saffranin. Note swelling and formation of root primordia in tissue treated with IAA; FUdR prevented cell division.

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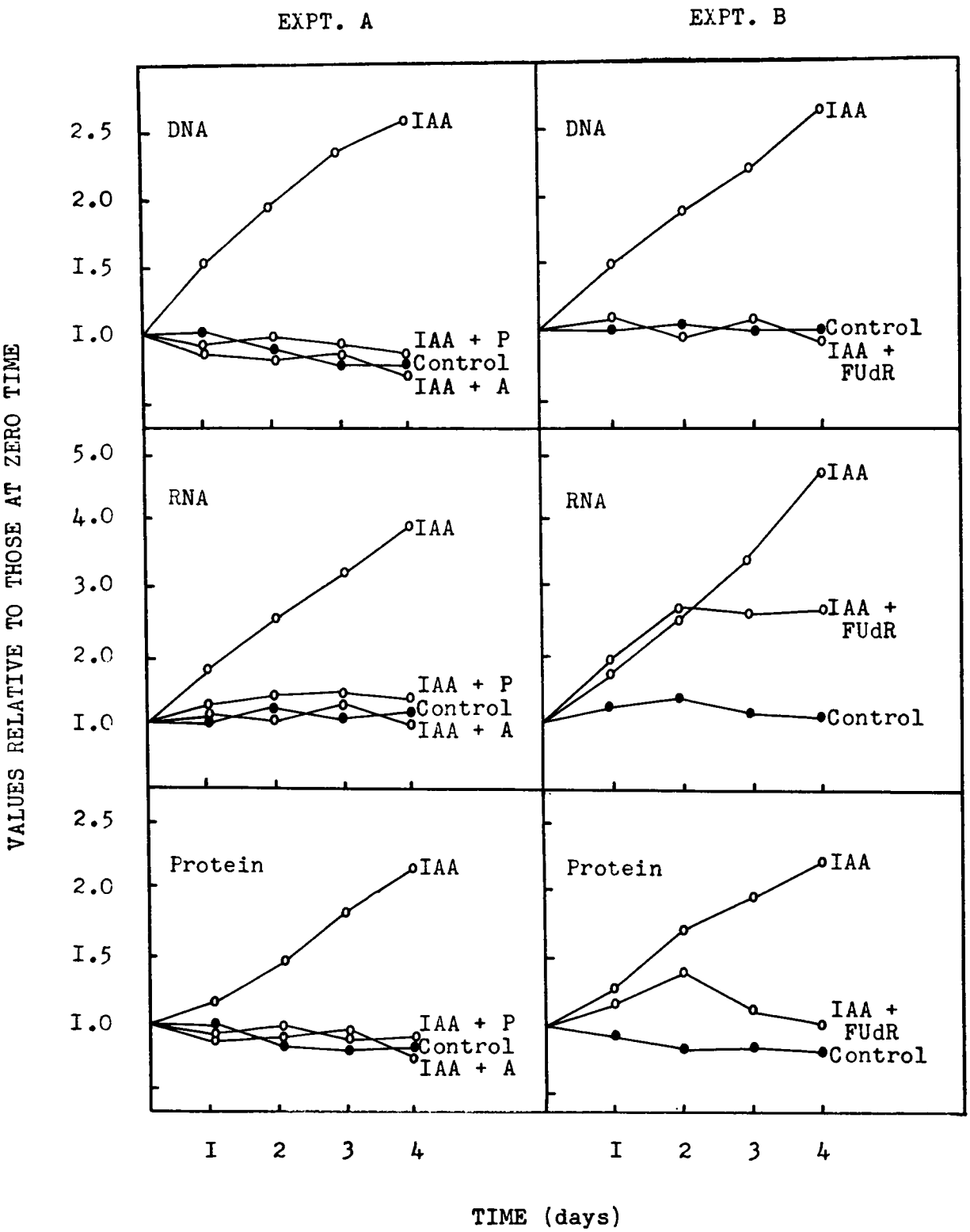

FIG. 3. Changes in DNA, RNA and protein levels in segments treated with IAA plus puromycin, actinomycin D or FUdR. Values are calculated relative to those at zero time, namely, for expt. A and B respectively: DNA = 8.5 and 7.5 μg; RNA = 58.0 and 48.5 μg; total protein = 278 and 238 μg/segment.

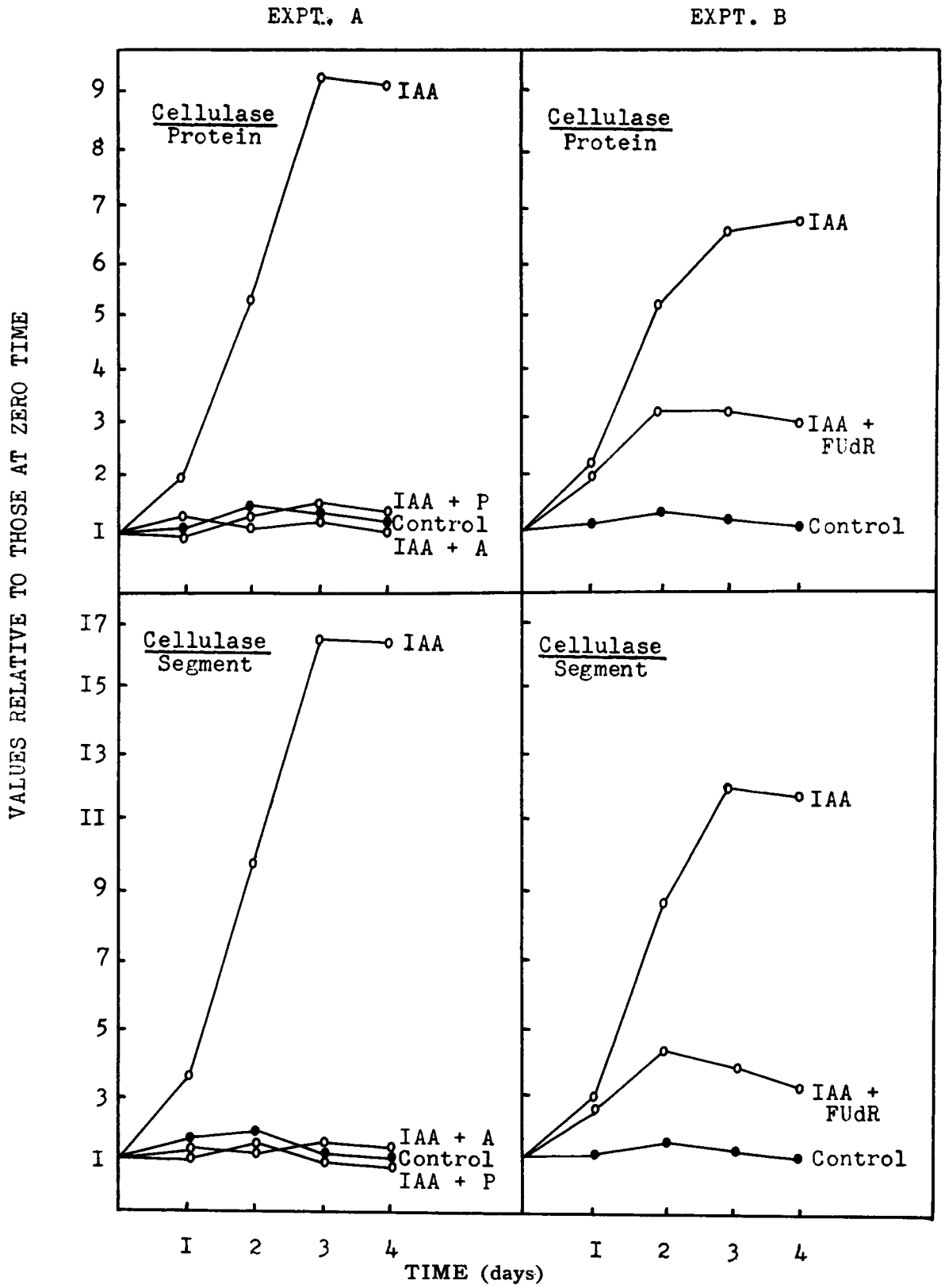

FIG. 4. Changes in cellulase activity per unit soluble protein and per segment. Values are relative to those at zero time, namely, for expt. A and B respectively; cellulase activities = 0.60 % and 0.65 % loss in viscosity/2 hours/segment; soluble protein = 253 and 228 μg/segment.

that plant hormones promote synthesis of specific enzymes by unmasking the appropriate preformed messenger RNA (22, 26) or by charging a particular variety of transfer RNA needed to initiate synthesis (1). Such effects on the process of translation would not necessarily require RNA synthesis. Alternatively, hormones may selectively de-repress part of the genome of maturing cells so that certain species of messenger RNA and eventually the proteins for which they code are synthesized (2, 8, 23). Such control over the transcription of DNA to RNA appears to be the best explanation for nearly all the effects of IAA observed in this study.

IAA induced very great increases in total RNA in the epicotyl segment (fig 3), not only in time as an eventual result of cell division but from the beginning by direct action in pre-existing cells (cf. RNA/DNA ratios, table II). Similarly pronounced and differential effects on RNA synthesis have been reported to follow 2,4-D-treatment of intact soybean hypocotyls and in this tissue most of the newly-formed RNA was ribosomal (9, 10). In pea seedling tissues, nearly all of the total cell RNA is in microsomes (12) associated with cytoplasm rather than the nucleus (14, 19). Thus, much of the IAA-induced RNA is probably also ribosomal, especially in view of its apparent stability (fig 3, Expt B). In this event, merely as consequence of an increased number of sites where synthesis can proceed, a non-specific increase would be expected in the production of total cell protein. Certainly preferential action of auxin on transcription rather than translation is implied by the fact that auxin causes protein/RNA ratios to decrease in both the pea epicotyl (table II) and the soybean hypocotyl (10).

The other main effects of IAA, namely DNA and unilateral cellulase synthesis and growth by cell expansion and cell division, were all closely correlated with the ability of the segments to synthesize RNA. It is difficult to account for these events without supposing that IAA brings about formation of coding varieties of RNA besides ribosomes. In fact, auxins have been shown to stimulate the synthesis of all fractions of RNA including messenger in experiments with attached (10) and detached (9) soybean hypocotyl tissue and with oat coleoptile sections (6). Messenger RNA is the one fraction of total RNA which is still synthesized in detached pea root sections and presumably required in order for growth on solution to proceed (13). It is necessary, of course, to show that only selected messengers are induced in order to explain the specific formation of those enzymes which are needed for growth. To this end, preliminary studies in this laboratory (E. Davies, unpublished) have indicated that polyribosomes isolated from pea epicotyl segments after brief IAA treatment are enriched in bound cellulase and appear to have acquired the capacity to synthesize this particular enzyme.

The above considerations apply to reactions brought about by IAA in pre-existing cells. After 2 days of IAA treatment, most or all of the further increases in RNA, protein and cellulase appear to result from synthesis in newly-formed cells. Even here, however, the continued presence of IAA seems to be needed to promote cellulase synthesis. In these and previous (3) tests, the cellulase level in IAA-treated tissue stopped rising after 3 days although DNA, RNA and protein synthesis continued without abatement. Indeed, when such experiments were carried on for longer time periods, the cellulase level began to decrease while root primordia proliferated and grew through the epidermis. Undoubtedly the concentration of IAA within the segment also decreased with time as a result of translocation, degradation and detoxification reactions, all of which are well known to occur in peas. The implication is that new cells readily form RNA and protein but they lose capacity to form cellulase unless high IAA levels are maintained. Separate tests (unpublished) have confirmed that cellulase activity can be made to increase further after 3 days if fresh 0.5 % IAA in lanolin is applied at that time.

It is possible to compare the relative capacities for cellulase synthesis of new and pre-existing cells by calculating the cellulase levels per unit DNA which were reached in segments treated with IAA plus or minus FUdR. The data are assembled in table III. Values close to 5 times initial values were attained in 2 to 3 days whether or not DNA synthesis or cell division took place. After reaching this peak level, the cellulase activity per unit DNA began to decrease under both treatments. It appears, therefore, that cells in this tissue, regardless of age, were capable of generating cellulase at similar high rates provided the genome was fully de-repressed by IAA.

With respect to growth effects in this system, all of the data are consistent with the view (3) that IAA-induced cellulase activity helps to bring about lateral swelling of parenchyma cells and fragmentation of their walls (fig 2). Special attention should be drawn to the fact that greatly enhanced elongation without swelling occurred in segments treated with IAA plus actinomycin D or puromycin (3, table I). This result was unexpected since these antibiotics have consistently inhibited auxin-induced elongation in detached pea epicotyl sections (17, 18, 24) and in other excised tissues (8, 9, 13, 18). Nevertheless,

Table III. *Effects of IAA on the Formation of Cellulase in New and Pre-existing Cells*

Cell division occurs in IAA-treated segments from the first day unless FUdR is present (fig 2).

Time (days)	Cellulase activity per unit DNA		
	Untreated	+ IAA	+ IAA + FUdR
0	1.0	1.0	1.0
1	0.8	1.8	2.4
2	1.2	4.8	4.8
3	0.8	5.6	4.0
4	0.6	4.8	4.0

several other inhibitors of protein synthesis, e.g. thiouracil, azaguanine, terramycin, etc., have been reported to increase elongation, especially in tissues left attached to the plant (3, 7, 13, 25). Apparently elongation does not require any concurrent increase in the level of RNA, protein or cellulase provided both IAA and some other essential factor derived from elsewhere in the plant are available.

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