DISCUSSION

The salient findings of this investigation which provide a better understanding of the physiology of rooting are discussed.

Time and locale of root primordia initiation

The results of experiment 6 demonstrate that root primordia on hypocotyl cuttings in water and IAA arise near the base of the cutting. This is in accord with the findings of Taylor (1926) and Mahlstedt and Watson (1952) and is probably because of the polar transport of nutritional factors and auxin. Haissig (1970b, 1971a, 1972) suggested that the initiation and development of primordia depends in part upon an auxin such as IAA.

Root initials appear in water and IAA during 24-48 hr as seen anatomically in experiment 6, indicating that all biochemical changes necessary for the initiation of root
primordia take place during the first 24 hours and after 48 hr occurs mainly the development of these primordia into roots so that these emerge out of the cutting at 72 hr. While Cycloheximide delayed the initiation and emergence of root primordia to 96 and 120 hr, respectively, FUdR and Actinomycin-D suppressed it completely implying the necessity of biosynthesis of nucleic acids and proteins in the process of root initiation. The rounded shape of the root primordia in Cycloheximide against the primordia with pointed tips in water and IAA is probably because cell elongation is not able to keep pace with cell division so that the roots take longer to emerge out of the cutting. Cycloheximide, thus, checks both the initiation as well as the development of roots.

It is also interesting to note that roots on cuttings pretreated with FUdR, Actinomycin-D, FU and Cycloheximide appeared first on the region immediately above the dipping part and later on the dipping part as well. This is in contrast to the basal position of the roots on cuttings cultured in water and IAA and is probably due to the break of basal dominance and partly due to the washing out of the inhibitory effect of these antimetabolites with time. The break of basal dominance by 2-thiouracil and 5-BUdR in *Phaseolus mungo* cuttings and by morphactin in *Salix tetrasperma* cuttings has been reported by Ansel *et al.* (1971) and Nanda *et al.* (1971).
Role of cotyledons and apex in rooting

The much higher rooting response of cuttings with then without cotyledons and apex (experiments 1, 7 and 9) clearly demonstrates the beneficial effects of the two organs in rooting. This lends support to the findings of other workers (Bouillenne and Went, 1933; Van Overbeek, 1946; Bachelard and Stowe, 1963; Nanda et al., 1971b). The fact that the number of roots on cuttings with excised cotyledons and apex and cultured in 2% glucose (experiment 1) reached almost that on cuttings with intact cotyledons and apex cultured in water, implies that the leaves serve as source of photosynthates. These results lend support to earlier findings by other workers (Went and Thimann, 1937; Pearse, 1943; Negishi and Satoo, 1956; Sen and Bose, 1958; Katsumi et al., 1969).

Effectiveness of auxin varies with nature of the cutting, concentration of auxin and endogenous level of nutrition.

The stimulatory effect of IAA was more pronounced on cuttings with intact cotyledons and apex than on those from which these had been excised, in which glucose was relatively more effective suggesting that while the former type of cuttings had adequate level of nutrition, the latter type were richer in auxin content (experiments 1, 7 and 9).

The effectiveness of auxin varies also with its concentration. Thus, the stimulatory effect of IAA increased with the increase in concentration up to 1.0 mg/l but decreased with 10.0 mg/l. However, while 10.0 mg/l IAA was less...
effective than 0.1 or 1.0 mg/1 when used alone, it was more effective than these when used together with glucose (experiment 1). In etiolated cuttings also (experiment 3) while rooting of cuttings cultured in water was stimulated by IAA treatment for 8-24 hr, of these cultured in glucose was stimulated by treatment even as long as 8-48 hr. These results show that the effectiveness of auxin varies with the endogenous level of nutrition as well. In fact, 0.5% glucose + 10.0 mg/1 IAA was the most effective combination in cuttings with intact cotyledons and apex and 1.0% glucose + 1.0 mg/1 IAA in those from which these had been excised (experiment 1). An interaction, thus, exists between auxin and nutrition and it is the balance between the two that determines the rate and magnitude of root initiation and development. These findings lend support to those by Nanda and Jain (1971) in etiolated stem segments of Salix tetrasperma.

The decrease in rooting by higher concentrations of IAA is probably due to the competition amongst IAA molecules for the active sites on the enzyme and instead of the two-point attachment that is necessary for activity, various molecules of IAA are able to attach to the active site by only one point because they are far in excess of the number of sites available and are rendered inactive. That 2-point attachment is necessary for IAA activity has been put forth by McRae and Bonner (1953). This is apparent from the fact that in experiment 1, 10.0 mg/1 IAA enhanced the production of
Utilization of sugars as source of carbon in rooting

The dependence of auxin action on nutritional factors supplied to the cutting either by cotyledons or by culturing them in a solution containing glucose/IAA+glucose (experiments 1, 7 and 9) is due to the fact that a carbon source is a prerequisite for fresh synthesis of nucleic acids and proteins. Utilization of sugars and starch as carbon sources in etiolated stem segments of *Populus nigra* has been reported by Nanda and Jain (1972a). Yatazawa et al. (1967) and Nickell and Maretzki (1970) also showed that soluble starch can be utilized as a source of energy by maize endosperm, juniper tissue, rice tissue and sugar cane cells.

Variations in temperature affect rooting of cuttings

The results presented in experiment 4 demonstrate that a relationship exists between the nutritional and auxin level and the rooting ability of cuttings, and that this relationship is greatly influenced by the prevailing temperature conditions. That higher temperature decreases the photosynthetic efficiency is shown by the lower content of chlorophyll of cotyledons on cuttings at 31°C than at 25°C resulting in decreased synthesis of carbohydrates as is also evident from these results. Concurrently, higher temperature checked the polar transport of auxin as is indicated by the
high level of auxin in the cotyledons and lower in the hypocotyls during the first 24 hours. Thus, the poor rooting response of cuttings at higher temperatures may be ascribed to the limited resources of auxin and nutrition resulting in decreased synthesis of m-RNA and thus proteins necessary for the initiation and development of roots. This is further supported by the fact that the actual levels of RNA, proteins and amino acids were very low both in the cotyledons as well as in the hypocotyls at 31°C. Jain and Nanda (1972) ascribed seasonal changes in the rooting response of stem cuttings (Nanda and Anand, 1970; Vieitez and Pena, 1968) to changes in the endogenous levels of nutrition and auxin caused by changes in the temperature and light conditions prevailing at different times during the years.

**Period of effectiveness of auxin during rooting**

The results of experiments 2 and 3 demonstrate that the stimulatory effect of IAA increased with the duration of pretreatment indicating that a continuous supply of auxin is necessary for optimal rooting. This is further supported by the fact that pretreatment with water decreased rooting of cuttings cultured in IAA and the decrease increased with the duration of water treatment. It is, however, very interesting to note that the stimulatory effect of short-term treatment with IAA was most pronounced with treatment
during the first twelve hours (experiments 2, 11, 12 and 13).

Effectiveness of antimetabolites varies with the endogenous levels of nutrition and auxin

A significant point that emerges from the results of experiment 9 is that the effectiveness of antimetabolites as inhibitors of root formation is markedly influenced by the endogenous level of nutrition and auxin in the cuttings. Thus, the antimetabolite-caused inhibition was more marked in cuttings without cotyledons and apex than in those with these intact, and was also more marked in water, IAA and glucose than in IAA+glucose. It would, thus, appear that auxin acts as a triggering agent for increased biosynthesis of nucleic acids and proteins and nutrition as a source of carbon and energy required for root production.

Mechanisms of IAA- and antimetabolite-caused inhibition of hypocotyl elongation are different

It may be of interest here to mention that while root formation was stimulated by IAA and inhibited by antimetabolites, hypocotyl elongation was inhibited by both (experiments 5, 11 and 12). Nanda and Jain (1972b) also reported that IAA inhibits internodal elongation but promotes the production of roots on epiphyllous buds of Bryophyllum tubiflorum. It appears that the mechanism of inhibition involved in the two cases is different. IAA checks hypocotyl elongation by channelizing the available food material to the sink, at the base of the cutting, created as a result of increased cell divisional activity. Antimetabolites,
on the other hand, do so by disrupting the metabolic processes and hence the biosynthesis of nucleic acids and proteins.

Mechanism of auxin action in rooting

The results of this investigation show that auxin plays a multifarious role in the rooting of hypocotyl cuttings. These will be discussed under separate heads.

(a) Auxin, carbohydrate metabolism and hydrolysing enzymes

The results of experiments 1, 7, 9 and 13 demonstrate that auxin acts to hydrolyze the reserve food materials and mobilize them to the site of root primordia initiation. This is evident from the drifts in carbohydrate metabolism in experiment 13 where the carbohydrate content of the cotyledons was lower but that of the hypocotyls higher in IAA than in water. Concurrently, the activity of amylase was much higher in the cotyledons than in the hypocotyls and in IAA than in water. That auxins are able to mobilize reserve food materials by enhancing the activity of hydrolysing enzymes has been reported earlier (Wort and Cowie, 1953) Nanda and Anand, 1970). This assumption is further supported by the fact that Actinomycin-D decreased the amylase activity of the cotyledons as well as the hypocotyls implicating the induction of de novo synthesis of the enzyme by IAA. Varner and Chandra (1964) and Chrispeels and Varner (1967) have suggested the de novo induction of α-amylase by GA₃ in
barley endosperm. Auxin is also reported to cause the synthesis of some new enzymes (Morris, 1966; Patterson and Trewavas, 1967).

The decrease in the carbohydrate content of the hypocotyls in FUdR and Cycloheximide in spite of the high level in the cotyledons may be ascribed to the blockage of transport of carbohydrates from the cotyledons to the hypocotyl because of the disruption of metabolic activity in the hypocotyls by these inhibitors.

(b) Involvement of nucleic acids and proteins in auxin effects on rooting

From the foregoing discussion, it appears that auxin is most effective during the first 12 hours and that it channelizes the metabolites to the site of root initiation.

An attempt was made to investigate the site of auxin action in the chain of biochemical reactions that take place prior to organization of root initials with the help of metabolic inhibitors of nucleic acids and proteins.

The complete inhibition of rooting by 10.0 mg/l FUdR and Actinomycin-D and decrease in rooting by 100.0 mg/l FU and Cycloheximide in experiments 5,6,11,12 and 13 suggests the involvement of protein synthesis in the production of adventitious roots and its mediation through multiplication of DNA, production of m-RNA and r-RNA. Thus, auxin probably acts by derepressing genes and, thereby, inducing synthesis of new proteins necessary for increased production
of roots. This is supported also by the fact that the inhibitory effect of short-term treatment with FUDR and Actinomycin-D was most pronounced when given during the first 6-12 hours and FU during 0-18 hours and this period of inhibition by antimetabolites coincides with the period during which the stimulatory effect of IAA is most pronounced (experiments 11, 12 and 13). The role of nucleic acids and proteins in other manifestations of growth such as auxin-induced cell elongation and wall loosening etc have been demonstrated by other workers (Morre, 1965; Key and Ingle, 1964; Masuda et al., 1967; Nooden and Thimann, 1966; Evans and Ray, 1969; Barkley and Evans, 1970) with the help of antimetabolites.

The involvement of nucleic acids and protein synthesis in auxin-induced rooting is further evident from the studies of the effect of metabolic inhibitors on the metabolic drifts during rooting. Thus, higher concentrations of FUDR, Actinomycin-D and Cycloheximide all decreased the DNA-, RNA-, and protein contents of the cotyledons as well as of the hypocotyls (experiments 10 and 13) whether these were given as a pre- or continual treatment. The results of experiments 7, 8, 10 and 13 which demonstrate an antagonism between IAA and metabolic inhibitors go further to prove that IAA acts at the transcriptional and translational levels. Thus, IAA was able to alleviate completely the inhibitory effect of 0.1 and 1.0 mg/l FUDR in cuttings with intact cotyledons.
and apex regardless of whether FUdR was given as a pre- or continual treatment. It was also able to overcome the inhibitory effect of 0.1 mg/l Actinomycin-D and the delaying effect of 1.0 and 10.0 mg/l FU.

(c) Auxin and the oxidative enzymes

An interesting point that emerges from this investigation is the significance of oxidation products of IAA in the formation of roots. In experiment 13, high peroxidase and IAA-oxidase activities in IAA-treated cuttings correspond with profuse rooting and low activities in cuttings treated with FUdR and Actinomycin-D correspond with poor rooting. The activity of these enzymes decreased even in Cycloheximide after 6 hr. The inhibition of peroxidase and IAA-oxidase activity by these antimetabolites clearly suggests that the blockage of the synthesis of these enzymes may be mediated through the inhibition of synthesis of specific m-RNA.

This is supported by the fact that these inhibitors, especially Actinomycin-D decreased the contents of DNA, RNA and proteins in the cotyledons as well as in the hypocotyls. Changes in peroxidase activity and isoenzyme pattern concomitant with tissue differentiation have been reported by many workers (Siegel and Galston, 1967; Galston and Davies, 1969; Gorden and Allridge, 1971). Melnar and LaCroix (1972a) showed that peroxidase was the first enzyme whose activity increased with root initiation as well as
with root development in Hydrangea cuttings. IAA has been reported to enhance peroxidase activity by Abeles (1966), Sakai and Imaseki (1971). Gurumurti et al. (1974) have shown that the physiological effects of IAA are probably caused by the active oxidation products which catalyse the action of IAA.

The results of experiments 14 and 15 demonstrate that besides the increase in the activities of peroxidase and IAA-oxidase during auxin-induced rooting of cuttings, new isoenzymes of these enzymes are induced which are associated with the initiation and development of roots. Thus, in experiment 14, isoenzyme F was specific to IAA-treated hypocotyls. In etiolated cuttings also in experiment 15, six new isoenzymes A-F developed in water during the course of root initiation and another two G and H were specific to IAA+glucose. These results are in accord with earlier findings of some workers that the isozyme patterns may vary during root primordium initiation and development (Chandra et al., 1971).

The results also show that the delay/inhibition of induction of new isoenzymes by higher concentrations of Cycloheximide corresponds with the inhibition of rooting of these cuttings. In experiment 15, no new band of peroxidase developed in IAA+glucose+10.0 mg/l Cycloheximide and the appearance of some IAA-oxidases was delayed (B and D) or inhibited (A). The inhibition of isoenzymes by Cycloheximide leads us to assume that the induction of new isoenzymes is
mediated through the fresh biosynthesis of proteins specific to rooting. Chandra *et al.* (1971) also reported that new peroxidases developed in cuttings that produced roots but not in those that were treated with streptomycin that suppressed rooting. Nanda *et al.* (1973b) have suggested that changes in cytoplasmic eosoperoxidases can be used as an analytical criterion for root initiation.

The possible significance of these isoenzymes lies possibly in the regulation of the level of endogenous auxin to balance with the available nutritional supply. It would also appear that some of the peroxidases act as IAA-oxidases. This is apparent from a comparison of the elution profiles of IAA-oxidase and peroxidases (experiment 14) which show that IAA-oxidase activity of these peroxidases in general was very high. Endo (1968), Yoneda and Endo (1970), Nanda *et al.* (1973b) and Gurumurti and Nanda (1974) also reported that some peroxidases act as IAA-oxidases. Hoyle (1972) reported that IAA-oxidase and peroxidase activities are dual catalytic functions of the single enzyme. Retig and Rudich (1972) found that the active sites of both peroxidase and IAA-oxidase on the gels were similar.

It is probable that these IAA-oxidase type peroxidases may cause the oxidation of available auxin into active constituents which result in the stimulation of rooting. Fox *et al.* (1965) and Hinman and Lang (1965) have shown that oxidation of IAA by peroxidase enzymes catalyses the formation of free radicals isomerise to form two tautomeric
species B and C. Meudt (1965), Meudt and Galston (1962) suggested that B forms an active complex essential for the catalytic activity of IAA on growth. Meudt (1971), therefore, considered that oxidative transformation of IAA was essential for its activity as a growth hormone.

**Paradoxical effects of antimetabolites**

Most of the workers hitherto have speculated that root primordium initiation and development is blocked by substances which interfere with or modify DNA, RNA or protein syntheses (Fellenberg, 1965, 1966; Guillot, 1965; Jain and Nanda, 1972; Kaminek, 1967; Melichar, 1964; Mitsuhashi et al., 1969). FUdR, Actinomycin-D and Cycloheximide have been used in a large number of studies in vivo to answer: Is de novo synthesis of nucleic acids and proteins required for some biological process? Cycloheximide is used also to answer: Is a particular protein made on ribosomes of the cytoplasm or on those of an organelle? Accurate answers to these questions depend upon the assumption that these metabolic inhibitors are specific to the reactions in question. The results of this investigation demonstrate that FUdR, Actinomycin-D, FU and Cycloheximide can inhibit growth at higher concentrations only, which of course will vary with the system. Thus these results lend support to the findings of other workers as far as the higher concentrations are concerned.

However, the interesting observation during the course
of experiments 7, 8, 9, 10, 13, 14 and 15 was that low concentrations of these antimetabolites or even higher concentrations given as short-term pretreatment stimulated rooting of hypocotyl cuttings and this stimulatory effect was more pronounced when IAA was used together with the antimetabolite or was given subsequently to cuttings pretreated with these. Some reports of this kind have appeared earlier. de Ropp (1949) obtained stimulatory effect with penicillin in tissue cultures but attributed these to contamination of the antibiotic with indole acetic acid. Camus and Lane (1955) obtained consistent stimulation of artichoke tuber tissue cultures with apparently pure penicillin G; however the fact that they obtained stimulation only with auxin-requiring tissues and the antibiotic had no effect in the presence of IAA led them to assume that either penicillin replaces IAA, which seemed unlikely or the sample was contaminated with traces of auxin. Acceleration of seedling development following treatment of soil with penicillin and oxytetracycline has been reported by many workers (Nickell, 1952, 1953; Cortesi and Girard, 1953a,b, 1954a,b; Harris, 1953) who attributed the stimulatory effect of these antibiotics to their antibacterial property. Iyengar and Starkey (1953) however, speculated a possibility that some antibiotics at least could accelerate growth by interfering with auxin metabolism.

The stimulation of root primordia initiation and
development by DNP (Krul, 1968), 2TU and 5BUDR (Anzai et al., 1971), 5 BU (Guillot, 1971; Melichar, 1964; Fellenberg, 1965; 1966) has also been reported. At least it appears that the influence of IAA on root primordia initiation and development is manifested through quantitative or qualitative changes in protein synthesis during an early part of the regeneration period (Fellenberg, 1965; Kaminker, 1967) after an initial lag (Mitsuhashi et al., 1969; Moore and Lovell, 1972; Nanda and Jain, 1972 b).

The results of these experiments also show that though low concentration-or pretreatment with-antimetabolites stimulated the number of roots, these delayed the appearance of roots, and the roots produced were spread all over the surface of the cutting. The stimulatory effect of these antimetabolites may, therefore, be due to lengthening of the lag phase and partly due to break of basal dominance as reported earlier by Anzai et al. (1971). Another possibility is the existence of endogenous inhibitor(s) or negative modulator(s) exercising a control on rooting process. The lower concentrations of antimetabolites may directly or indirectly decrease the content of these negative modulators resulting in increased rooting, provided the available auxin and/or nutrition is adequate. Such modulators may be protein(s) inhibiting the action of some enzyme(s) implicated in the differentiation process or controlling their biosynthesis at transcriptional or
translational level. The existence of such an inhibitor(s) of IAA-oxidase in *Ipomoea fistulosa* affecting rooting has been reported by Chibbar *et al.* (1974). Stimulation of GA\textsubscript{3}-induced differentiation of floral buds and leaves in *Impatiens balsamina* and promotion of flower formation and fruit set in citrus by other antimetabolites of protein and nucleic acid synthesis have been reported (Nanda *et al.*, 1973a; Goren and Moncelise, 1969).

The results of experiments 14 and 15 demonstrate the induction of new isoenzymes of peroxidase and IAA-oxidase by low concentrations of Cycloheximide corresponding with the increase in the rooting of cuttings. Similarly, Parish (1968) and Novacky and Wheeler (1971) also reported that low concentrations of Actinomycin-D caused the development of isoperoxidases. That Cycloheximide cannot be considered as a general inhibitor of all proteins has been shown by other workers (Macdonald and Ellis, 1969; Ellis and Macdonald, 1970; Lee, 1971; Gurumurti and Nanda, 1974; McMeekan, 1975). It was also noticed that the stimulatory effect of Cycloheximide manifests itself more in cuttings transferred to IAA than water (experiment 14). Moreover, a comparison of the elution profiles of peroxidases and IAA-oxidases shows that the peaks of IAA-oxidase activity coincide with the peaks of peroxidase activity corresponding to the new isoperoxidase bands A, B, C, D and G induced by Cycloheximide treatment.
These results lend support to findings of Endo, 1968; Yoneda and Endo, 1970; Hoyle, 1972; Retig and Rudich, 1972; as mentioned earlier. Thus Cycloheximide may cause stimulation of rooting by increasing the oxidation of available auxin into active constituents which are essential, as already discussed earlier, for the activity of IAA as a growth hormone.

However, at present there is insufficient experimental evidence to warrant further speculation concerning the mechanism of these stimulations by antimetabolites.