DISCUSSION

Role of segment size, leaves and apex in rooting

The results presented in experiments 1 and 2 clearly demonstrate that the rooting response depends on the size of the etiolated stem segments and also on whether the leaves and apex are left intact or are excised. More profuse rooting on longer segments is in conformity with the findings of other workers (Deuber and Ferrar, 1940; Edgerton, 1944; Ching, 1963; Marin, 1965 and Rubtov, 1964) and is probably because of the presence in such segments adequate levels of all the factors that are necessary for the initiation and development of roots. One or more of these may be deficient in 2.5 cm long segments which may thereby act as (a) limiting factor(s) in the initiation of roots. The production of roots on stem segments with intact
leaves even in water and the considerable increase in the number on such segments in other test solutions, support the findings of Went et al. (1938), who considered that materials beneficial for the initiation and growth of roots were produced in the leaves. Pears (1943) considered that leaves supplied the cuttings with vitamins and nitrogenous substances. In our system leaves may even help in the transport of exogenously applied auxins along the transpiration stream besides supplying photosynthates and may, therefore, be considered to act as chief donors of some specific and non-specific factors that are necessary not only for the production of adventitious roots but also for their development. The apex, of course, seems to be the source of auxin necessary for the differentiation of cambial derivatives into root primordia, as is evident from the fact that segments with intact apices, rooted better than those with excised apices.

The failure of 2.5 cm long segments with excised leaves and apex to root in water or in IAA alone but their ability to root in glucose, suggests that leaves act to supply the nutritional factors necessary for rooting. A decrease in the number of roots with higher concentrations of glucose may be ascribed to high osmotic pressure causing plasmolytic changes. The beneficial effect of carbohydrates on rooting has also been reported by other workers (Went and Thimann, 1937;
Auxin-nutrition balance in rooting

An interesting point that emerges from this investigation is that the effectiveness of auxins in the production of adventitious roots on stem segments is dependent upon nutritional factors. Thus, in experiment 2, 1.0 mg/l IAA inhibited rooting when added to 0.01% glucose, was ineffective with 0.1% and stimulated rooting with 1.0% glucose which was inhibitory when used alone.

Another interesting point that emerges from this investigation is that the concentration of auxin that balances with a given level of nutrition to cause optimal rooting, varies with its nature. Thus in experiments 4 to 5, roots were produced on a few segments with 1.0% glucose. The number and length of roots increased with the addition of auxin, the stimulation increasing with concentration and was stronger with IBA than with IAA. 5.0% glucose used alone inhibited rooting completely but roots were formed when auxins were added to it, IBA being more effective than IAA. In fact 1.0 mg/l IBA produced roots on segments even with as high a concentration of glucose as 10.0%, which was completely inhibitory when used alone. Again in experiment 7, with 0.1% glucose rooting of etiolated stem segments of
Salix tetrasperma was maximum with 0.1 mg/l IAA or IBA, it decreased when the concentration of auxin was increased to 1.0 mg/l. With 1.0% glucose the most effective concentration of auxin was 1.0 mg/l, 5.0 mg/l IBA being inhibitory. The effectiveness of auxin in rooting segments was lower with 2.0 or 5.0% than with 1.0% glucose except 5.0 mg/l IAA in which the number of roots increased with the concentration of glucose. These results, thus, demonstrate that the ability of stem segments to root is determined by a proper balance between the nutritional factors and regulatory substances and that rooting may not occur even when the concentration of one of these is very high.

The rooting of a few segments even with auxin alone in this experiment, may be ascribed to its effect in increasing the activity of hydrolysing enzymes making some of the reserve food materials available for the division of cambial cells and their differentiation into root primordia (e.g. Nanda and Anand, 1970 and Bala et al. 1970).

**Auxin-nutrition balance and callus formation**

An imbalance between nutrition and auxin causes an imbalance between the rates of divisional and differentiation activities of the cambial cells. A relatively higher level of nutrition with lower concentration of auxin increases the
rate of division of cambial cells. The rate of differentiation of these derivatives into root primordia is not able to keep pace with the divisional activity. The cambial derivatives, therefore, increase in size and result in the formation of callus. It is, however, interesting that even the magnitude and distribution of callus on the segments is determined by its own balance with nutrition and auxin which is different from the one that is necessary for the production of adventitious roots. It also varies with the nature of the auxin.

Thus, 0.1 and 1.0 mg/l IAA produced the maximum amount of callus with 2.0% but 5.0 mg/l IAA with 5.0% glucose. In contrast to this 0.1 mg/l IBA produced maximum callus with 1.0% glucose, 1.0 mg/l IBA with 2.0% and 5.0 mg/l with 5.0% glucose. Again while with IAA callus was confined to the basal end, it was distributed all over the length of the segments with IBA. Secondary roots were also produced with IBA but not with IAA.

The difference in the effectiveness of the two auxins in the production of adventitious roots as well as in callus formation may be partly due to the differential rates of polar transport and partly to the oxidation of IAA but not of IBA, caused by endogenous IAA oxidase. IBA, thus, persists within the system and therefore is stronger in its effect than IAA. The relative effectiveness of different auxins on rooting has also been reported by other workers (Pearse, 1939;
Light effects on auxin-nutrition balance and rooting

The more profuse rooting of segments in the dark than in the light and the substitution of the effect of darkness, at least to some extent, either by an exogenous supply of auxins or by increasing the size of the segments, is suggestive that the effect of light is probably mediated through its effect on photo-oxidation of endogenous auxin through increased activity of IAA oxidase (e.g. Galston and Baker, 1953), although Guttenburg et al. (1956) considered that light decreased the auxin level in cuttings by increasing the rate of polar transport and diffusion into the medium causing thereby a decrease in the production of roots. The determinative role of light in rooting stem cuttings of *Populus nigra* has also been reported by Shapiro (1957) and Nanda (1970).

Temperature effects on auxin-nutrition balance and rooting

The results presented in experiment 6 demonstrate that the balance of nutrition and auxin necessary for root initiation as is discussed in a previous paragraph, is markedly influenced by the prevailing temperature conditions. 5.0 mg/l IAA, thus, balanced with 1.0% glucose at 30°C for
optimum production of roots but with as low a concentration as 0.1% glucose at 15°C. The seasonal changes in the rooting response of stem cuttings reported earlier (Nanda and Anand, 1970; Vieitez and Pena, 1968 and Hartmann and Loreti, 1965) may, therefore, be ascribed to changes in the level of nutrition and endogenous auxin that are caused by changes in the temperature, light and other environmental conditions at different times during the year.

**Carbon source and rooting**

The differences in rooting response of segments with the type of sugar reported in experiment 8, may be ascribed to differences in the amount of energy released in oxidation which will depend, not only on the nature of the sugar supplied exogenously but also on the prevailing conditions and the nature of the end products, that together with determine the extent to which a given sugar can be oxidised. Ball (1953) found fructose to be the best amongst sugars for the growth of plant tissues of *Sequoia*, while Yatazawa et al (1967) found glucose best for rice tissue and Nickell and Maretzki (1970) raffinose best for sugarcane tissue. Gautheret (1955) reported that sucrose was the most suitable carbon source for the growth of most plant tissues.

**Starch as a carbon source in rooting**

However, the most interesting point that emerges from
this investigation is that stem segments can even use starch as carbon source in spite of the fact that it is osmotically inactive and cannot permeate through plasma membrane and that hydrolysis of starch into sugars to be utilized for rooting is brought about by the enzymes that leach out of the segments. That soluble starch can be utilized as a source of energy by maize endosperm, Juniper tissues, rice tissues and sugarcane cells, has also been demonstrated by other workers (Straus and La Rue, 1954; Constable, 1961, 1963; Yatazawa et al., 1967 and Nickell and Maretzki, 1970). Nickell and Maretzki (1970) also considered that utilization of starch by sugarcane cells was due to secretion of \( \beta \)-amylase. The secretion of \( \beta \)-amylase by Rumex virus tumors in vitro and also of an enzyme from intact cells of \( R. \) acetosella, were also reported earlier by Brakke and Nickell (1951, 1952, 1955).

The enhanced rooting in starch in combination with auxin is due to enhanced activity of hydrolysing enzymes which mobilize starch into soluble sugars to a level that balanced correctly with the concentration of exogenously applied auxins. The role of auxins, therefore, is two-fold: first to mobilize reserve food materials; and secondly to enhance cell division, elongation and differentiation. That auxins are able to mobilize reserve food materials by enhancing the activity of hydrolysing enzymes has been demonstrated earlier (Wort and Cowie, 1953 and Nanda and Anand, 1970).
Nitrogenous compounds and rooting

The increased production of adventitious roots on stem segments supplied with serine and tryptophane particularly in the presence of glucose shown in experiment 10, lends support to the findings of other workers (Went and Thimann, 1937; Doak, 1941; van Overbeek and Gregory, 1945 and Trione and Avellenda, 1963) and demonstrate that nitrogenous compounds are other nutritional factors that should be provided in adequate quantities together with carbohydrates to satisfy energy and tissue building requirements for the initiation and development of roots.

Mode of action of IAA and GA\textsubscript{3} in rooting

The inhibitory effect of GA\textsubscript{3} on auxin induced root formation on etiolated stem segment reported in experiment 11 is in accord with the findings of other workers (Brian et al., 1960; Bachelard and Stowe, 1963; Mitsuhashi et al., 1968 and Nanda et al., 1968). Varner (1964), Varner and Chandra (1964) and Chrispeels and Varner (1967) have suggested that GA\textsubscript{3} acts at transcriptional or translational level to produce some new enzyme(s). Auxin is also reported to cause the synthesis of some new enzymes (Morris, 1966 and Patterson and Trewavas, 1967). It may, therefore, be assumed that the antagonistic effect of GA\textsubscript{3} on auxin induced rooting may be caused by interference in the production of enzymes or in
the products of enzymatic reactions. This postulate is supported by the results presented in experiments 12-14 which show that while GA₃ and darkness stimulate internodal growth but inhibit the production of roots, IAA inhibits internodal elongation but promotes the production of roots on epiphyllous buds of *Bryophyllum tubiflorum*. The greater elongation of the first internode of epiphyllous buds in the dark than in the light is in accord with the findings of Lockhart (1956, 1959, 1961) who considers that light inhibition of elongation of *Pisum sativum* and *Phaseolus vulgaris* is due to the fact that light interferes with gibberellin metabolism within the plant. Brian and Hemming (1958) and Cleland (1964) consider that stem elongation is controlled by both auxin and gibberellin and that the response to GA₃ depends upon the availability and level of auxin in the tissue. The inhibitory effect of IAA on internodal elongation of *Bryophyllum tubiflorum* in experiment 12, thus increase with concentration both in the light and in the dark (Fig. 19) but decreased when the epiphyllous buds were pre-treated with IAA for 4-8 hrs and subsequently transferred to water (Fig. 21). Again, internodal elongation was stimulated in the light but inhibited in the dark when GA₃ was added to IAA (Fig. 20). There are three hypotheses that have been put forth to explain this control, namely (i) gibberellins act by promoting the synthesis of auxin (Kuraishi and Muir, 1964), (ii) gibberellins may
protect the auxin from inactivation (Pillet, 1957 and Brian and Hemming, 1958) and (iii) gibberellins may be concerned in the synthesis of RNA and consequently of proteins (Kaufman et al., 1968) and the primary action of auxin may be dependent upon these proteins (Ockerse and Galston, 1967).

Another interesting point that emerges from this investigation is that cycloheximide, which is considered to be a potent inhibitor of protein synthesis, inhibited both internodal elongation as well as root initiation, demonstrating thereby the dependence of both these manifestations of growth on the biosynthesis of proteins. Even the pre-treatment of buds with cycloheximide for as short a period as 4 hrs inhibited internodal elongation as well as rooting. In contrast to this, pre-treatment with IAA or GA₃ even for as long as 8 hrs did not alleviate the inhibitory effect of cycloheximide suggesting thereby, that there is a lag period between the application of these regulators and the biosynthesis of proteins. The difference in the effects of IAA and GA₃ on shoot and root growth, is considered to be due to the induction of specific types of proteins which may favour cell division in the root meristem in the former, but that in the shoot meristem in the latter. This is supported by the findings of Patterson and Trewavas (1967) and Morris

**Mechanism of auxin action in rooting**

From the foregoing discussion it would appear that the mode of auxin action in the production of adventitious roots as well as in internodal elongation essentially involves the biosynthesis of some specific enzyme protein(s). The question that arises is: What is/are the site(s) of action of auxin in the process of regeneration? Studies on the action of auxins in simple growth processes like cell elongation or expansion have provided strong evidence to suggest that auxin effects growth through enhanced production of RNAs and consequently proteins (e.g. Noodén and Thimann, 1963, 1965, 1966; Key, 1964; Key and Ingle, 1964; Key and Shannon, 1964; Bonner, 1965; Masuda, 1966 and Masuda et al., 1967). According to Noodén and Thimann (1966) the primary action of auxin may be dependent upon the presence of a particular protein not directly involved in the growth process. It is suggested that auxin might act directly to promote the synthesis of an enzyme protein (or the RNA which code for it) and since such protein synthesis should be controlled by a relatively unstable messenger RNA, inhibitors
of syntheses of RNAs and proteins would be expected to inhibit growth. An attempt was made in experiments 15 to 22 to elucidate the possible mechanism of auxin action in the production of roots with the help of metabolic inhibitors. The inhibition of rooting by FUDR, FU, actinomycin-D and cycloheximide suggested the involvement of the syntheses of both nucleic acids and proteins in the production of adventitious roots. It would also appear that the synthesis of proteins may be mediated through either the multiplication of DNA or the production of messenger, ribosomal or soluble RNAs or both. However, the ineffectiveness of FU in this regard observed in experiment 16 on etiolated stem segments of Salix tetrasperma may probably be due to the presence of adequate amount of soluble and ribosomal RNAs obviating the necessity of their fresh synthesis for the biosynthesis of proteins. This is in contrast to the inhibition of rooting by FU on stem segments of Populus nigra (experiment 19) which means that even ribosomal and soluble RNAs were not adequate in this system and their fresh biosynthesis was, therefore, essential for the production of proteins necessary for the formation of adventitious roots.

Effectiveness of antimetabolites and nutrition and auxin

An interesting point that emerges from these experiments is that the effectiveness of antimetabolites in inhibiting the production of roots is markedly influenced by
nutrition and auxin. Thus, the inhibition of rooting by these antimetabolites was more marked in glucose alone than in glucose + IAA/ or IBA. It would appear that auxin acts as a triggering agent and nutrition as a carbon source and the dependence of auxin action on nutritional factors is due to the fact that a carbon source is a pre-requisite for the fresh biosynthesis of nucleic acids and proteins.

**Rooting dependent on size of protein pool**

The increasing inhibition of rooting with the increasing concentration of these antimetabolites observed in experiments 15-20 indicates that the magnitude of rooting is determined by the size of the protein pool that is available in the tissue at the time of root initiation. This postulate is supported by the fact that the inhibitory effect on the production of adventitious roots decreased with the delay in the application of cycloheximide in experiment 20, as the size of the protein pool had already increased above the minimal level required prior to cessation of fresh biosynthesis caused by antimetabolites.

**Metabolic drifts in rooting**

A study of the changes in the water soluble and acid hydrolysable fractions made in experiment 21 showed that the general level of both fractions of carbohydrates decreased with time in segments cultured in water. In marked contrast
to this, the content of water soluble fraction of carbohydrates remained high up to 48 hrs to decrease rapidly by 72 hrs in segments cultured in glucose + IBA. On the other hand, the content of water soluble sugars did not change much in segments cultured in cycloheximide and rather increased in those cultured in glucose + IBA + cycloheximide. As has been seen, profuse rooting occurred only in glucose + IBA in this system. It is likely that these sugars were actively used in the division of cambial cells and in the differentiation of cambial derivatives into root primordia in segments cultured in glucose + IBA but remained almost unchanged in segments cultured in cycloheximide alone or even together with glucose + IBA probably due to inhibition in the activity of enzymes caused by cycloheximide.

The high level of amino acids in segments cultured in glucose + IBA in experiment 21 is indicative of their enhanced synthesis as a prelude to enhanced synthesis of cellular proteins. It is rather interesting that the content of amino acids as well as of proteins are enhanced during 48 to 72 hrs (Tables 15 and 16). The enhanced protein synthesis in the segments during this period may therefore be considered to be related to the synthesis of some new enzyme protein that may be concerned in the production of adventitious roots. This assumption is supported by the results presented in experiments
21 and 22 which demonstrate that protein synthesis decreases appreciably in the presence of cycloheximide in the medium (Table 16 and Fig. 26C). Of course, the content of proteins determined at any time reflects the net amount left unused in the tissue at any stage. These results, thus, demonstrate that the size of the protein pool necessary for the initiation of roots is reached only in segments cultured in glucose + IAA/or IBA and remained lower in other cases.

Again, the contents of both RNA and DNA were quite high in segments cultured in glucose + IAA where rooting occurred as compared to those cultured in water or in glucose + IAA + cycloheximide/or + actinomycin-D where rooting did not take place (refer Fig. 26). It means that the enhanced protein synthesis is mediated through the multiplication of DNA as well as enhanced production of RNAs. However, it may be noted that even DNA content of segments cultured in glucose + IAA + actinomycin-D/or + cycloheximide and RNA content of those cultured in glucose + IAA + cycloheximide, remained lower than that of the controls and much lower than that of the segments cultured in glucose + IAA and this happens in spite of the fact that cycloheximide is an inhibitor of the synthesis of proteins and actinomycin-D of DNA-dependent RNA (refer Fig. 26). This may be due to the fact that the proteins whose synthesis is inhibited by cycloheximide may also include the enzymes concerned in the
syntheses of DNA and RNA. The involvement of nucleic acids and proteins in the production of adventitious roots on stem cuttings has also been shown by other workers (Fellenberg, 1969a, 1969b, 1970; Hassig, 1970a, 1970b; Jalouzot, 1971; Roychouchary, 1971; Nanda, 1970; Nanda and Jain, 1972b; Nanda et al., 1973 and Jain and Nanda, 1972).

**Physiology of root initiation**

Based on the foregoing discussion of results it may be suggested that the mechanism of hormone action involves an interaction with the genetic apparatus of the cell. A repression-derepression mechanism in controlling protein synthesis may be considered to be operative in a regenerating system, the sequence of events taking place being as follows: When a shoot is detached and made into cuttings, the endogenous auxin accumulates by polar transport at the morphological base. It acts as a derepressor (inducer) and initiates the system that brings about the synthesis of specific enzyme protein(s) that are responsible for regeneration. If the level of endogenous auxin is low, an exogenous supply of auxin at the base of the segment would bring about the same effect provided the amount of nutrition is adequate to satisfy the energy and tissue building requirements of the segment. From the physiological standpoint, other conditions
such as light and temperature remaining favourable, the endogenous auxin-nutrition balance would, therefore, be a crucial factor determining the regeneration of roots on stem segments. It is also proposed that auxin acts as a triggering agent for the synthesis of specific enzyme protein(s) that are required for the initiation of root primordia at transcriptional level and the magnitude of rooting is determined by the size of the protein pool that is available in the tissue at that time. However, the possibility of auxin acting at translational level in this system can not be completely ruled out as some workers considered that while some hormonal responses requiring the involvement of DNA-dependent RNA synthesis may be caused at transcriptional level, others at the level of translation and still others probably are mediated by mechanisms other than that involve the synthesis of RNA or proteins. It may however be pointed out that no attempt has been made in this thesis to provide a clue to the function of specific enzyme protein(s) in the formation of adventitious roots on stem segments. Work to elucidate this is in progress in this laboratory.