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THE ROLE OF GLYCOSYLATED AMINO ACIDS OF WALL-BOUND PROTEIN IN CELL EXTENSION OF STEMS OF PISUM SATIVUM L. PLANTS

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SUMMARY

The effect of cell extension regulating plant hormones, GA and IAA were studied in view of differences in protein properties of the primary cell wall. Lamport's hypothesis that wall-bound protein is a polysaccharide cross-linking agent and hence affects wall extensibility was tested. The results show that during cell extension new synthesis of wall-bound protein occurs, though the highest rate coincides with a declining rate of cell extension. Consistent with Lamport's hypothesis, the elongating cells of dwarf variety (Rondo) plants have a higher glycosylated hydroxyproline content than the standard variety (Alaska). The application of GA to the top of the slow growing dwarf variety increased the growth rate until comparable with the standard variety, while decreasing the glycosylated part of the hydroxyproline content. Experiments performed with excised elongating stem segments, grown in a culture solution containing phosphate, demonstrated that the glycosylated hydroxyproline content of the cell walls at least doubled during an incubation time of 24 hrs. IAA strongly inhibited this increase while stimulating elongation. However, culture solutions stimulating growth in length do not always cause a reduction in glycosylated hydroxyproline synthesis. Moreover, during the period of rapid cell extension, large differences in growth rate may occur with hardly any difference in glycosylated hydroxyproline synthesis.

It was concluded that an inverse linear relationship between glycosylated hydroxyproline content of primary cell walls and growth rate does not necessarily exist. This conclusion also holds true for the glycosylated wall-bound serine content. No significant change in concentration during the period of rapid cell extension could be detected and therefore the conclusion that wall-bound glycosylated aminoacids play a role as a stiffening agent of the primary cell wall is not justified.

1. INTRODUCTION

Ever since HEYN (1931) obtained evidence of plasticity changes of the plant cell wall caused by a growth hormone, it is believed that cell extension is due to a weakening of the cell wall. A further hypothesis in this concept was that weakening involved a loosening of the interconnected cellulose microfibrils (BONNER 1936).

BENNET-CLARK (1956) introduced a new point of view by suggesting that the pectins of the cell wall might play a role in cell extension. The plastic and elastic extensibility of a polygalacturonic acid was thought to be controlled by the ratio of uronic carboxyl to uronic methyl carboxylate and this ratio to be influenced by IAA. LAMPORT (1965) emphasized, in a review about the protein component of primary cell walls, the importance of hydroxyproline-rich protein for the mechanism of cell extension. This might contribute to the wall tensile strength by forming cross-links between wall polysaccharides. Lamport's hypothesis is primarily based on the relation between the hydroxyproline content of the cell wall

and the ability of the cell to extend.

CLELAND & KARLSNES (1967) showed that hydroxyproline containing proteins increase markedly in the cell wall of Alaska Pea epicotyls during the transition of rapidly growing tissue into non-elongating mature tissue.

WINTER et al. (1971) concluded that growth rate of cells is not always inversely correlated with the amount of hydroxyproline.

ALBERSHEIM et al. (1973) greatly extended our knowledge of the structure of the primary plant cell wall by using specific enzyme cell wall degradation techniques and proposed a model, showing that the primary sycamore cell wall could be regarded as a single molecule.

LAMPORT (1973) reported that preliminary experiments indicated that the number of glycosylated serine residues might be a function of growth. In this paper experiments are described testing the structural role of hydroxyproline-rich protein in cell extension.

2. MATERIAL AND METHODS

Peas (*Pisum sativum* L.) of the varieties Alaska (standard) and Rondo (dwarf) were grown in pans containing water soaked autoclaved vermiculite. The pans were placed in a dark room at 27 °C and high humidity. After germination for about 60 hrs the plants either remained in the pans or were placed in the greenhouse.

In the experiments either intact internodes or 5 mm stem segments cut out of the growth zone were harvested and analysed. After harvesting, samples of the stem segments were frozen in liquid nitrogen, ground in a mortar, suspended in icewater and centrifuged at 1000 g at 2 °C. The pellet was resuspended and extracted in a chloroform-methanol mixture (1–1 v/v 0 °C) and homogenized to remove lipids. The cell wall was separated from the soluble fraction by filtration on a Büchner funnel and subsequently washed three times with a cold chloroform-methanol mixture and three times with acetone. (KARR & ALBERSHEIM 1970). For determining its dry weight, the residue (cell wall fraction) was dried at room temperature.

2.1 Determination of the ratio glycosylated and non-glycosylated hydroxyproline

Samples of this dried cell wall fraction were hydrolyzed using 0.22 M Ba $(OH)_2$ at 105 °C for 6 hrs. (1 ml $Ba(OH)_2$/10 mg cell wall). The hydrolysate was neutralized with sulfuric acid, centrifuged at 2000 g for 15 min and subsequently lyophilized. The precipitate was dissolved in 2 ml 0.1 N acetic acid and filtrated on a blue-band filter. The resulting solution was fractionated on a Sephadex D25 super fine column and tested for the relative amounts of 'free' hydroxyproline and sugar attached hydroxyproline according to a method derived from Hutterer and Singer (1960).

2.2 Acid hydrolysis

Samples of the dried cell wall fraction were hydrolyzed with 6 N HCl in a glycerine

bath at 110 °C for 14 hrs. After filtration on a white-band filter and neutralisation with 7.5 N NaOH, the hydrolysate was tested for the amount of hydroxyproline.

2.3 Determination of glycosidically bound serine

Glycosidically bound serine was determined by the difference between the amount obtained after hydrolysis with 6 N HCl at 110 °C and the amount obtained after hydrolysis with hydrazine 1.5% (w/v) according to a method used by HEATH & NORTHCOTE (1971). For the assay an amino acid analyser was used.

2.4 Gibberellic acid treatment

Gibberellic acid 0.01% was applied to the vegetative apex of the plants as a very small droplet attached to the apex with the aid of a microsyringe.

3. RESULTS

In the first experiment the growth in length of the first three internodes of Alaska Peas was measured with time, concurrently with the wall-bound hydroxyproline content. The results are shown in *fig. 1* and *fig. 2*. *Fig. 1* shows the large difference in final length of the internodes and the time needed to obtain this. Whereas the growth in length of the first internode is already completed after one day and the

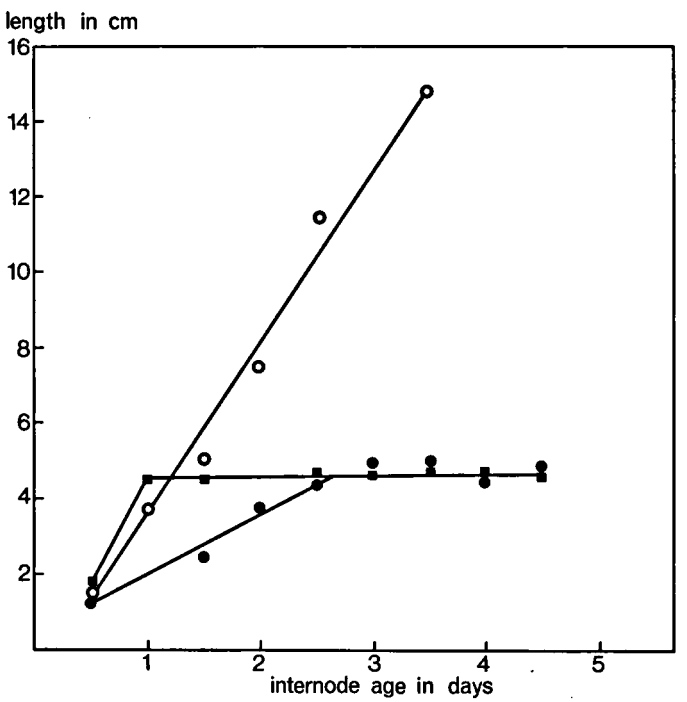

Fig. 1. Increase in length of pea internodes (Alaska) in time. ■——■ First internode, ●——● second internode, ○——○ third internode.

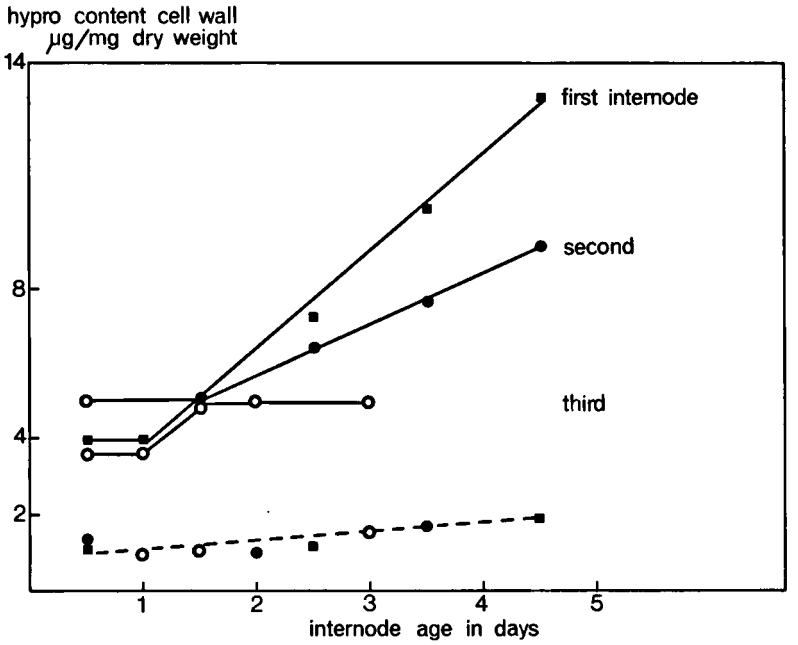

Fig. 2. Increase in hydroxyproline content of pea internodes (Alaska) with time. ——— glycosylated, - - - - - - non-glycosylated.

second after two days, the growth in length of the third internode is not yet completed after three days and a half. In the latter case this results in a final length which is many times the length of the other two internodes.

The glycosylated hydroxyproline content of the cell wall increases during this ageing process especially in the first and second internode (*fig. 2*). The non-glycosylated hydroxyproline content is low as compared to the glycosylated part and increases somewhat on ageing. Furthermore *fig. 2* shows that the three internodes do not differ significantly with respect to the non-glycosylated hydroxyproline content.

In the next experiment the amount of wall-bound total and glycosylated hydroxyproline in elongating third and matured second internode cells of 7 days old Alaska Peas were compared with those of Rondo Peas of the same age. The length of the two varieties differs at that age but physiologically they were in the same phase. The results are represented in *fig. 3*. It shows that the dwarf variety cells (Ro) whether matured or elongating contain much more hydroxyproline than the standard cells (A1). This is in agreement with earlier results (WINTER et al. 1971). However, it now appears that this conclusion also holds for the glycosylated part. Next gibberellic acid 0.01% was added to the apices of part of the dwarf variety plants on the fourth day after germination and during a number of days the second internode was analysed for wall-bound hydroxyproline content. *Fig. 4* shows that

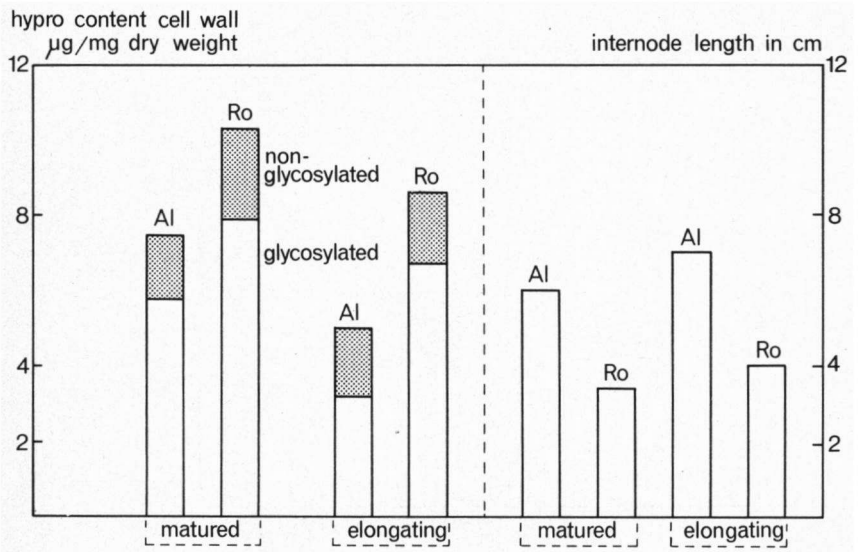

Fig. 3. Wall-bound hydroxyproline content of elongating third and matured second internode cells of 7 days old pea's. Rondo (Ro) and Alaska (Al).

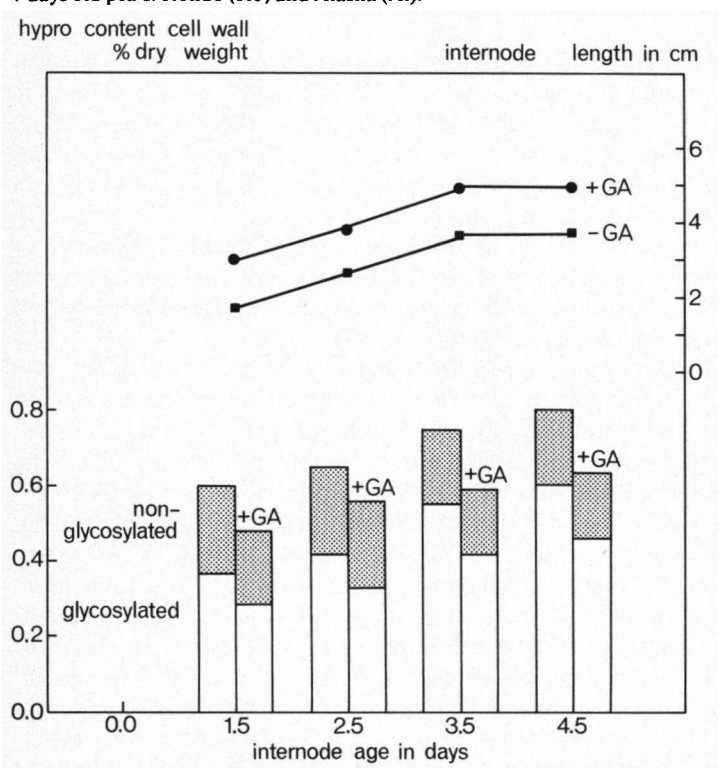

Fig. 4. The effect of gibberellic acid on the growth in length of second internodes of pea (Rondo) plants and wall-bound hydroxyproline content.

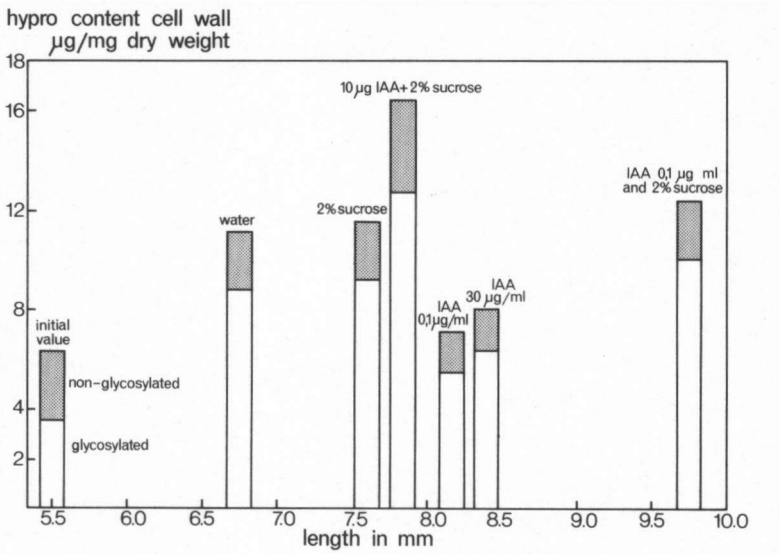

Fig. 5. Hydroxyproline content of cell walls and growth in length (arbitrary units) of pea stem segments (Alaska) after incubation in various culture solutions for 24 hrs.

the GA treated Rondo plants have less hydroxyproline, total and glycosylated, in the cell walls of the elongating cells than the controls ($P < 0{,}05$). At the same time the growth in length is stimulated up to 35% by GA. In fact the difference in final second internode length between the standard and dwarf plants disappears completely by the GA treatment. However, this does not quite hold for the difference in glycosylated hydroxyproline content (*fig. 3*).

In order to study the possible correlation between hydroxyproline content of cell walls of elongating cells and IAA affected cell extension, it was necessary to cut stem segments out of the growth zone of the stem internodes (5 mm length) and grow them for 24 hrs in various culture solutions (*fig. 5*).

Fig. 5 shows that during the period in water (phosphate buffer added) the glycosylated hydroxyproline content of the cell walls increased considerably with hardly any change in the amount of non-glycosylated hydroxyproline. The addition of IAA to the phosphate-buffered water solutions strongly stimulated the growth in length whereas the increase in hydroxyproline content is inhibited.

This result agrees well with Lamport's former hypothesis. However, stem segments floated in a solution containing 2% sucrose and IAA in a low concentration (0.1 µg/ml) almost doubled their length in 24 hrs without any reduction in total and glycosylated hydroxyproline as compared to the stem segments floated in water. It is known (WINTER et al. 1971) that the growth response for isolated epicotyls is almost completed during the first 12–18 hrs of the experiment. Therefore the difference in growth rate might be related to differences in glycosylated hydroxyproline synthesis during the first 12–18 hrs of the experiment. Hence a time course experiment on hydroxyproline synthesis during 24 hrs was carried out.

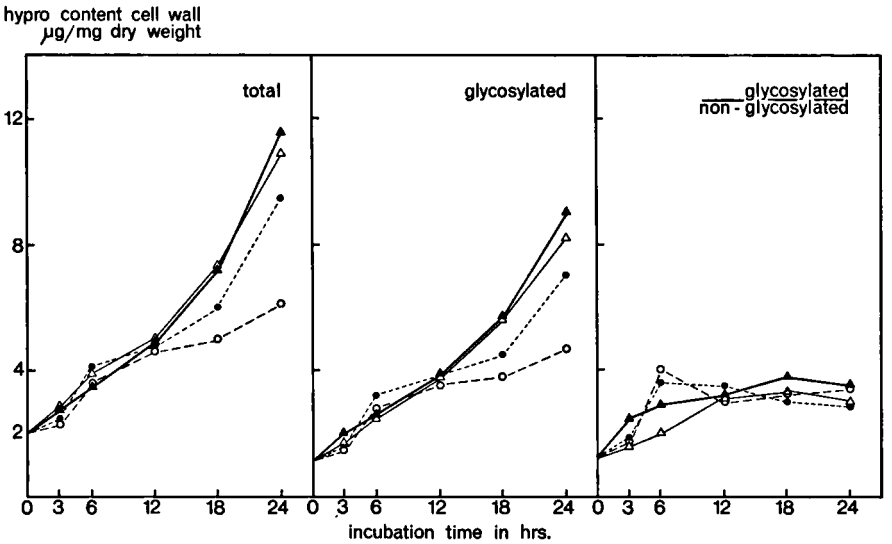

Fig. 6. Increase with time of hydroxyproline content of cell walls of stem segments of pea internodes (Alaska).
▲——▲ IAA 0.1 µg/ml and 2% sucrose. ○——○ IAA 0.1 µg/ml. △——△ water. ●——● 2% sucrose.

Fig. 6 shows that the synthesis of wall-bound hydroxyproline does not occur exclusively in the last 6–12 hrs of the experiment but probably starts right from the beginning. This is especially true for the glycosylated part of the hydroxyproline content. The possibility that the synthesis of hydroxyproline-rich protein in stem segments starts after the completion of the growth in length can therefore be ruled out. Neither is there an inverse relationship between growth rate and increase in glycosylated hydroxyproline-rich protein during the period of cell extension.

In the light of the new results obtained about cell wall structure (KEEGSTRA et al. 1973, BAUER et al. 1973, TALMADGE et al. 1973 and LAMPORT 1973), Lamport pointed out: "it is considered most likely that the major polysaccharide attachment to extensin is by way of the serine hydroxyl gropus while the hydroxyproline arabinosides could conceivably function to stabilize the hydroxyproline-rich extension polypeptide backbone as a rigid rod".

For this reason second internode stem segments of Alaska and Rondo plants of various age were analysed for their serine content. The results are shown in *fig. 7*. They indicate that neither a significant increase nor a decrease in serine concentration of primary cell walls of the second internode sets in during the period of attaining a length of 3 cm. This is the period which synchronizes with a high cell extension rate. However, there is a rather sharp decrease for Alaska just as for Rondo in total serine concentration during the increase in internode length from 3 to 4 cm.

It is also striking that this falls in with an increase in the degree of glycosylation.

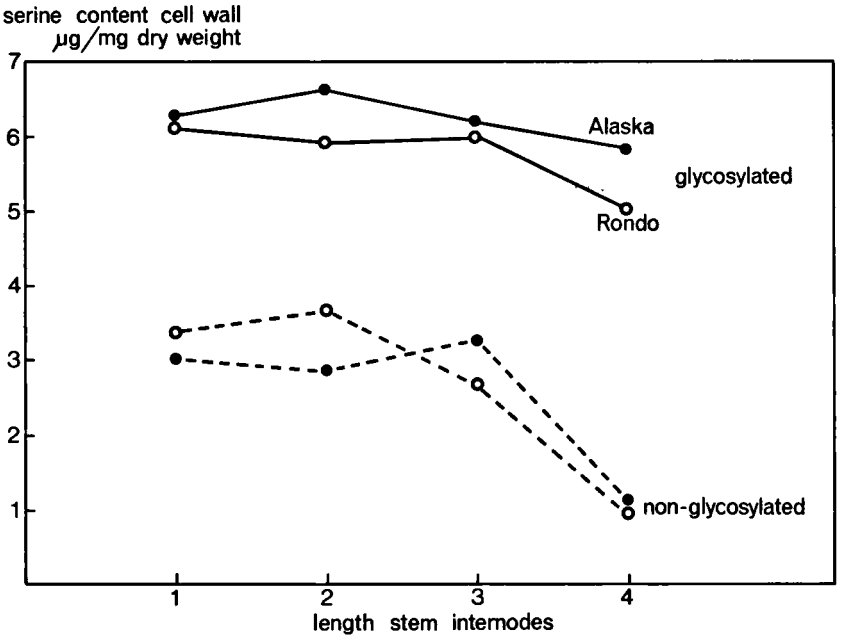

Fig. 7. Serine content of cell walls of second internodes of Alaska and Rondo peas of various length (age).

This might coincide, especially for the Rondo plants with a final internode length of about 4 cm (*fig. 3*), with a decreasing cell extension rate.

The general conclusion which could be drawn from the results is that there does not necessarily exist an inverse linear relationship between glycosylated hydroxyproline resp. serine concentration of primary cell walls of peas and growth rate.

4. DISCUSSION

WINTER et al. (1971) concluded that cell extension and hydroxyproline content of cell walls of pea epicotyls are not necessarily inversely correlated. LAMPORT (1965) suggested the hypothesis of wall-bound protein as a cross-linking agent regulating wall extensibility. However, cross-linking cellulosic microfibrills in this theory, demands glycosylation of aminoacids of the wall-bound protein and the degree of glycosylation might therefore be an important factor in determining cell extensibility.

The dramatic increase in glycosylated hydroxyproline content of intact stem internodes during ageing, especially after the cessation of growth (*fig. 2*), is in agreement with the results of CLELAND & KARLSNES (1967) and SADAVA et al. (1973).

KLIS (1976) failed to find an increase after the cessation of growth and assumed that the different results might be due to different experimental methods. Our

results were obtained on the basis of hydroxyproline content of the wall expressed as amount per mg of dry wall material. Klis used the same method, so the conflicting results cannot be explained by differences in method. Though our results with intact stem internodes do not rule out the possibility of a certain increase during the process of cell elongation, it is evident that this increase is less conspicuous than after the period of cell extension. The sharp increase in glycosylated hydroxyproline in addition to the relatively low amount of non-glycosylated hydroxyproline, which does not decrease but increases, is proof of a new synthesis of hydroxyproline-rich protein.

Because of the low increase in non-glycosylated hydroxyproline as compared with the glycosylated part, this protein necessarily has a higher degree of hydroxyproline glycosylation. The evidence for the existence of a synthesis of glycosylated hydroxyproline which is less pronounced during the period of extension growth, the inverse correlation between glycosylated hydroxyproline content and the capacity of cells to extend, wether due to variety (*fig. 3*) or internode number, are all in favour of Lamport's hypothesis. This is supported by the fact that dwarf plants (Ro) of which cell extension is stimulated by GA, do have a lower total and glycosylated hydroxyproline content than the control plant (*fig. 4*). However, the absence of a linear relationship conflicts with the existence of a close connection. Equally the results on glycosylated hydroxyproline formation obtained from excised elongating cells (*fig. 5*) do not point to a close relationship between hydroxyproline content and the ability of cells to extend. Stimulation of hydroxyproline synthesis was found to be correlated with a stimulation in cell extension.

It could also be shown (*fig. 6*) that hydroxyproline formation in excised elongating stem segments started at least within three hours from the beginning and therefore there existed no lag period during the first 12–18 hrs which is after all the period of greatest cell elongation. Besides, in this period of large differences in growth rate (WINTER et al. 1971), due to the various experimental conditions, no significant difference in glycosylated hydroxyproline synthesis could be detected.

Nevertheless, except in the event of inhibition by IAA, it appears that the increase in amounts of hydroxyproline is lowest during the period of cell extension. Therefore the possibility of inhibition of cell extension by the hydroxyproline content of the cell wall cannot be completely ruled out, though only in the case when the hydroxyproline content of the cell wall exceeds a certain level. Below this level the cell extension rate should then be largely dependent on other factors than the hydroxyproline-rich protein of the primary cell wall.

The amount of non-glycosylated hydroxyproline does not change much during the first 12 hrs of the experiment. This result strengthens the conclusion drawn from the experiments with intact stem internodes (*fig. 2*) that synthesis of hydroxyproline-rich protein with a much higher degree of glycosylation must be involved.

The reduction in hydroxyproline formation by IAA could be explained by assuming a competition between IAA stimulated cellulose synthesis (WINTER 1966, RAY 1967) and protein formation. Both processes need sugar and in the case

of wall-bound protein both for the protein backbone and for the galacto-araban moiety of the glycoprotein complex. Without the addition of externally applied sugar, it could be shown (WINTER 1967) that within 14 hrs the amount of reducing sugar became rate limiting for the synthesis of cellulose.

The results obtained from the experiments about the serine concentration of the primary cell wall (*fig. 7*) as well show that a close relationship between aminoacid content, whether glycosylated or not, and the ability of cells to extend was absent. The sharp decrease of the serine concentration during the increase in length from 3 to 4 might be explained by assuming that in this period secondary cell wall formation takes place, at least in the oldest part of the internode. This must result in a decrease in the serine concentration. This explanation is supported by the fact that the total wall-bound protein concentration decreases as well. This is especially true for the Rondo plants.

The increase in the degree of glycosylation is not accompanied with a net increase in serine concentration as observed for the hydroxyproline content. If the serine and hydroxyproline measured in our experiments are part of the same glycoprotein, this result is not to be expected. Therefore it looks as though different glycoproteins are involved.

The obvious conclusion to be drawn from the experiments on hydroxyproline and serine synthesis with reference to cell extension is that no close relationship exists between glycosylated aminoacid content of the primary cell wall and growth rate. Therefore the data thus far obtained do not support the hypothesis of a stiffening of the wall by glycosylation of either hydroxyproline or serine.

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