Acta Bot. Neerl. 30(1/2), February 1981, p. 131–138.

GLUCOSE AND ALCOHOL METABOLISM IN PISUM SATIVUM L.

H. WINTER and P. K. WIERSEMA

Centraal Isotopen-Laboratorium, Biologisch Centrum, Universiteit Groningen, postbus 14, 9750 AA Haren (Gn)

SUMMARY

Glucose absorbed by etiolated pea stem segments was partly metabolized into a number of glucose-derivatives. Lower aliphatic alcohols when applied to solutions in which the stem segments were incubated, strongly stimulated the formation of these compounds. In addition it was found that the label of absorbed ethanol 1-^{14}C was mainly present in one of these sugar-derivatives, which suggested a glycosylation of the ethanol into ethyl-glucoside. Further evidence of the glycosylation of absorbed lower aliphatic alcohols could be obtained with the aid of enzymatical, chromatographical, microchemical and mass-spectrometrical methods.

It is concluded that in etiolated pea stem tissue, glucose will be partly conjugated to lower aliphatic alcohols. If sufficient ethanol is present in the cells, ethyl-β-glucoside becomes the main glucose metabolite.

1. INTRODUCTION

WINTER (1967) reported the formation of an unidentified glucose-derivative synthesized in etiolated stem segments of peas from absorbed glucose. This synthesis could be stimulated by indole-3-acetic acid (IAA). The present publication reports its identification and also the effect of lower aliphatic alcohols on the synthesis of related compounds.

2. MATERIAL AND METHODS

Peas (*Pisum sativum* L. cv. Alaska 7) were grown in trays containing water soaked autoclaved vermiculite. The trays were placed in a dark room at 25 °C and 80% relative humidity. After 7–8 days when the plants had reached an average length of 10 cm, 5–10 mm stem segments were cut from the uppermost portion of the third internode. These segments were floated in 10 ml aerated 20 mM phosphate buffer (pH 6.0) with or without the addition of other substances. After 24 hrs samples of at least 25 stem segments were harvested. The stem segments were successively rinsed in deionized water, frozen in liquid nitrogen and ground in a mortar.

D-Glucose (U–^{14}C), ethanol (1–^{14}C) and methyl (α)-D-glucoside (U–^{14}C) were purchased from Amersham (England). ^{14}C contents were measured with a liquid scintillation counter.

Two directional chromatograms from the ethanol extracts of the ground stem segments were prepared on Schleicher and Schüll 2043 b MgL with butylacetate: acetic acid: water (3:3:1) and pyridine: ammonium-hydroxide: isobutanol

(4:2:1) as solvents. Autoradiograms of the chromatograms were prepared on Kodak No-Sceen X-ray film.

300 g of stem segments were floated in 0.4% ethanol solutions for 24 hrs and then homogenized for 1 min in a Virtis homogenizer at 20000 rpm in cold ethanol 80%. After extraction with hot ethanol for 30 min, the suspension was centrifuged for 15 min at 12000 g. The supernatant was evaporated in a rotavapor at 50°C to a small volume. For radioactivity detection purposes, part of an extract intended for subjection to enzymatic hydrolysis was added to the supernatant. Ionic components were removed on Dowex 50 × 8 and Dowex 1 × 8 (100–200 mesh) columns. Further fractionation was performed on a Bio-Gel P_2 400 mesh column (0.23–1.93 m) and by one-dimensional thin layer chromatography on silicagel with butylacetate: acetic acid: water (3:3:1). The silicagel impurities were removed by shaking the fraction by a mixture of Dowex 50 and Dowex 1. The purity was determined with anthrone reagent after a modification by Fales (1951).

The glucose-derivative was labelled by incubating the stem segments in ethanol -1-^{14}C solutions. After two-dimensional thin layer chromatography, it was dissolved in water (pH 6.0) and subjected to enzymatic hydrolysis by β-glucosidase (EC 3.2.1.21) with a specific activity of 40 U/mg at pH 6.0 (Boehringer Mannheim). The glucose-containing compound and β-glucosidase were mixed (1:1v/v) and incubated at 30°C (pH 6.0). After evaporation to dryness the decrease in activity was measured.

Elementary analysis was carried out with a C-H-N Perkin analyser model 240 with katarometer detection system.

Mass spectra were recorded with an A.E.I.-MS 9 instrument. The source temperature was 100°C and the ionizing potential 70 eV. The dried fractions were acetylated in the conventional manner prior to mass-spectrometry. They were solved in acetic anhydride-pyridine (10:1) and heated in a sealed flask for 1 hr at a temperature of 120°C. The solutions were evaporated at 55°C and stored overnight under vacuum above KOH.

3. RESULTS

Table 1 shows the distribution of ^{14}C over the main 80% ethanol soluble metabolites after incubating the stem segments in ^{14}C glucose solutions for 24 hrs. These metabolites are amino acids, malic acid and the glucose-derivative (WINTER 1967). Addition of IAA, but above all, of ethanol to the solutions, stimulates the synthesis of the glucose-derivative. It causes a shift in the conversion pattern of glucose at the expense of the synthesis of amino acids and malic acid.

Besides ethanol also methanol, propanol and butanol significantly ($P < 0.05$) stimulate the synthesis of glucose-derivatives (*table 2*). The longer the chain length, the less stimulation was involved. The Rf-values of the spots on the radioautographs suggested the synthesis of four glucose-derivatives, their com-

Table 1. The effect of IAA and ethanol on glucose absorption and conversion.

Medium solution	Glucose absorbed/25 segments	Glucose not metabolized	Glucose-derivative	Aminoacids	Malic acid
0.005% ^{14}C-glucose	42 µg	12%	18%	40%	16%
+ 10 µg/ml IAA	33	6	27	36	10
+ 0.03% ethanol	56	14	52	28	5
0.1% ^{14}C-glucose	820	52	18	15	6
+ 10 µg/ml IAA	780	41	25	11	9
+ 0.03% ethanol	980	41	46	10	3

position corresponding to the alcohol applied. These results intimated the biosynthesis of alcohol-glucosides.

To test this, ^{14}C-ethanol was applied to the stem segments (*fig. 1*). After two directional chromatography the label of the absorbed ethanol was mainly present in a compound with Rf-values 0.66–0.85. The percentage metabolized depended on the ethanol concentration applied and varied from 90% at low to 60% at high ethanol concentrations. The Rf-values were identical with the Rf-values of the main glucose-derivative formed when ^{14}C-glucose and ethanol were applied to the stem segments (*table 2*). This reversed labelling experiment strongly suggests the synthesis of ethyl-glucoside. Glucose addition to the medium hardly effected its synthesis up to an ethanol concentration of 0.4%.

Adding methyl (α)-D-glucoside (U-^{14}C) to a 80% ethanol extract of the stem segments and subsequent chromatography and autoradiography resulted in Rf-values identical with the Rf-values of the glucose-derivative synthesized when methanol was applied to the stem segments.

Table 2. The effect of lower aliphatic alcohols on the synthesis of glucose-derivatives.

Medium solution	Glucose absorbed/25 stem segments	Glucose-derivatives	Rf-values	Relative amounts %
0.1% ^{14}C-glucose	775 µg	17%	0.55–0.80	55
			0.66–0.85	45
+ 0.05% methanol	1050	64	0.55–0.80	100
+ 0.05% ethanol	925	40	0.55–0.80	14
			0.66–0.85	86
+ 0.05% propanol-1	900	34	0.55–0.80	15
			0.74–0.87	85
+ 0.05% butanol-1	910	31	0.66–0.85	55
			0.94–0.93	45
+ 0.05% pentanol-1	825	19	0.55–0.80	35
			0.66–0.85	65
+ 0.05% hexanol-1	750	21	0.55–0.80	52
			0.66–0.85	48

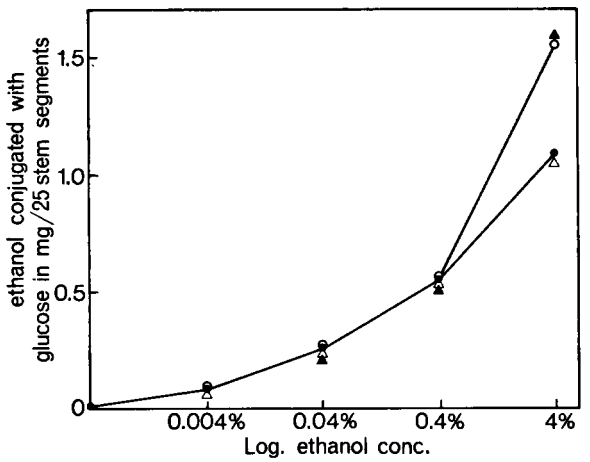

Fig. 1. Ethanol metabolite, isolated from pea stem segments after incubation for 24 hrs in medium solutions containing various concentrations of ^{14}C-ethanol and glucose. ▲——▲ 1% glucose. o—— o 0.5% glucose. ●——● 0.1% glucose. △——△ 0.01% glucose.

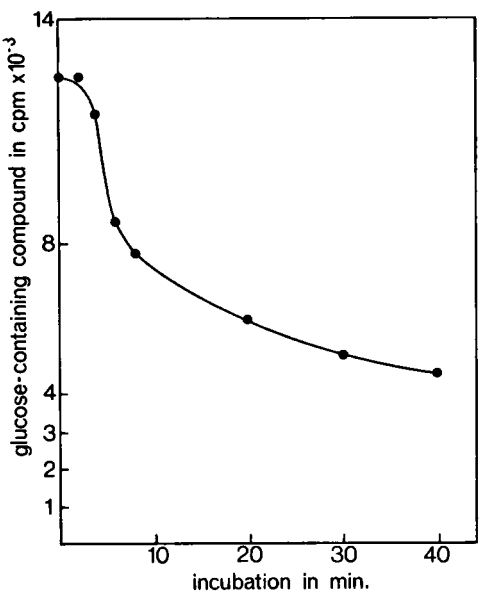

Fig. 2. Enzymatic hydrolysis by β-glucosidase (pH 6.0) of the ^{14}C-glucose derivative obtained from pea stem segments after incubation in a ^{14}C-ethanol solution.

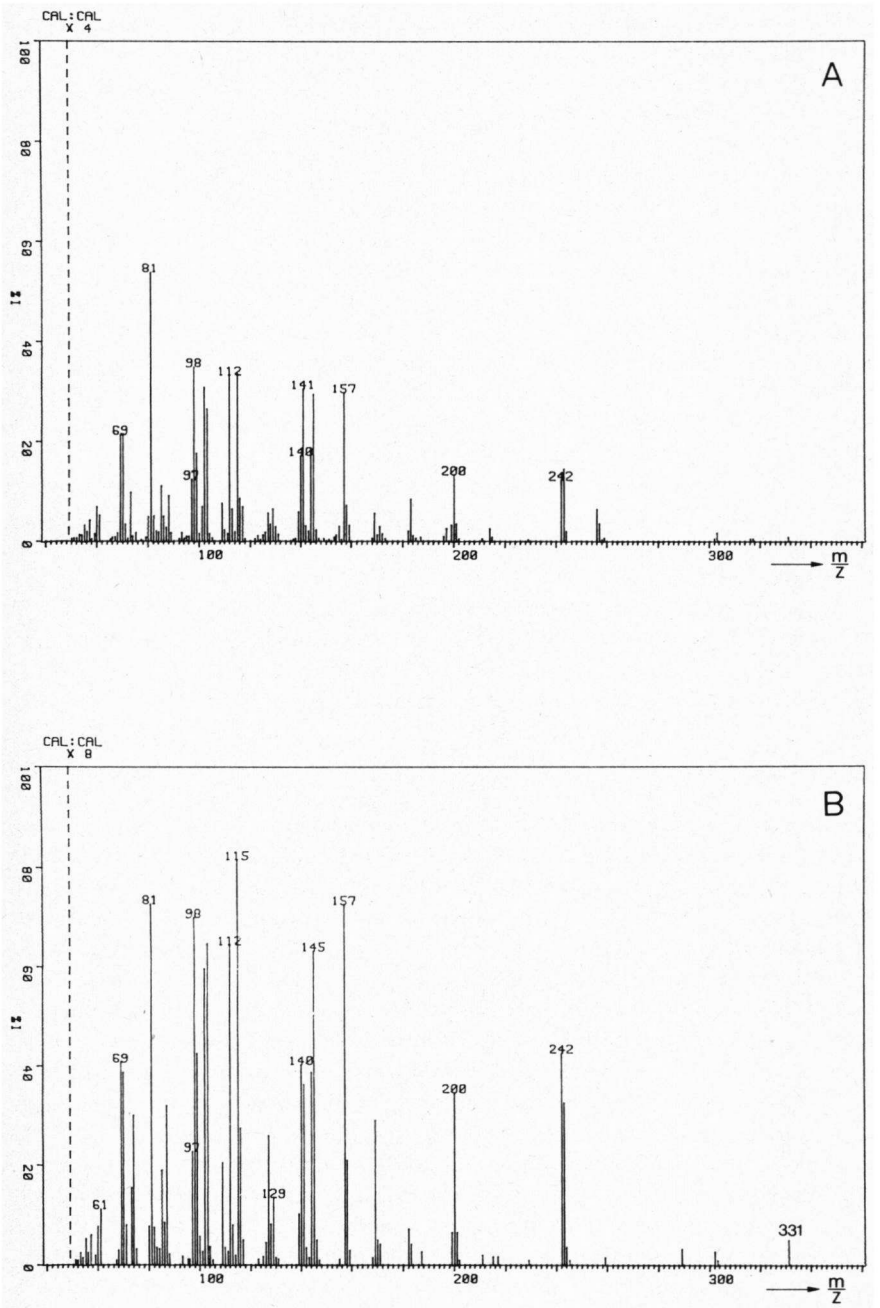

Fig. 3. Mass-spectra of the glucose-derivatives synthesized in pea stem segments during incubation in A: 0.5% ethanol, B: 0.4% methanol.

The labelled glucose-derivative, prepared by incubating the stem segments in ^{14}C-ethanol solutions, was subjected to enzymatic hydrolysis by β-glucosidase. *Fig. 2* shows that the greater part is broken down. Micro-analysis of this glucose derivative yielded a hydrogen/carbon ratio of 2, which is in agreement with ethyl-glucoside.

Highly purified compounds extracted from the stem segments, were analysed by electron impact mass-spectrometry. The stem segments were incubated in solutions containing either methanol or ethanol. The results are shown in *fig. 3*. The main fragment was CH_3CO^+ which was present in the spectra of all peracetylated sugars and gave a base peak with an intensity of about 10 times the highest peaks of the other fragments. Typical of peracetylated sugars with 3 acetyl groups in succession were the fragments at m/z 103, m/z 145 and m/z 157 (BIEMANN et al. 1963). The molecular ion M^+ of the peracetylated 'ethyl-glucoside' was small but nevertheless clearly present on the original record. It corresponded to a molecular weight of 376. The molecular ion M^+ of the peracetate of 'methyl-glucose' could not be detected, which is quite usual for sugar-derivatives upon ionization by means of electron impact (HEYNS & SCHARMANN 1963). However the fragment at m/z 331 was present and very likely originated from the molecular ion with m/z 362 by a loss of the OCH_3 group. Conclusive for the structure was the observation that the fragmentation pattern and relative intensities of signals at the various m/z values of this plant-made glucose-derivative were very much the same as those of methyl-β-D-glucopyranose purchased from Baker Chemicals B.V.

4. DISCUSSION

The experiments and analyses show that glucose externally applied to the stem segments is partly used for the conjugation with ethanol. This process is concentration dependent (*table 1*). However, this is not the total amount of ethyl-β-glucoside synthesized. The internal sugar reserves also combine with ethanol (*fig. 1*). Ethanol absorbed by the stem segments in the absence of applied glucose is also converted to ethyl-β-glucoside. Up to an ethanol concentration of 0.4%, externally applied glucose hardly effects the total amount of ethyl-β-glucoside synthesized, though it does compete with the internal glucose reserves.

Evidently the rate at which the internal sugar reserves are mobilized for the synthesis of ethyl-β-glucoside is at least up to 0.4% ethanol not the rate limiting step in the process. Raising the ethanol concentration as high as 4%, which strongly stimulates the absorption of ethanol, it seems that the total amount of available sugar is now the rate limiting step for the synthesis of ethyl-β-glucoside. Under these conditions externally applied sugar (0.5%) stimulates the synthesis. The absence of a significant difference in synthesis at external glucose concentrations of 0.5 and 1% indicates that somewhere in this concentration range the availability of ethanol again is the rate limiting step in the process. In this concentration range the absorption of glucose is still concentration dependent (WINTER et al. 1970).

A considerable amount of the absorbed glucose is conjugated with ethanol or methanol even if these alcohols are not externally applied (*table 2*). In our opinion there are two possible sources. In the first place bacterial growth starts in the sugar containing medium during the experimental period of 24 hrs. This might result in alcohol synthesis in the incubation medium. In the second place the source might be internal for meristematic cells tend to anaerobic respiration (BETZ 1960). Likewise the relatively small but significant effect of IAA on the alcohol-β-glucoside synthesis (*table 1*) could be explained by assuming that IAA by stimulating the respiration (REINDERS 1938, BONNER 1949, and KELLY & AVERY 1951), indirectly effects fermentation in the rapidly growing cells. Under these conditions it will be even more difficult to maintain a sufficient oxygen supply. LIU & CASTELFRANCO (1970) succeeded in isolating a crude enzyme from etiolated pea seedlings which was able to synthesize ethyl-β-glucoside from ethanol and isosuccinimide-glucose (ISG). They suggested that ISG, detected in extracts of pea seedlings by VITEK (1964), might be an energy-rich compound because, as they demonstrated, the aglycon was of a highly reactive nature. Besides it takes part in the synthesis of other physiologically important β-glucosides. However, the physiological role of ethyl-β-glucoside is not clear. It might be necessary to render ethanol into less toxic substances by conjugation with glucose. LIU et al. (1966) suggested that it might be advantageous to the meristematic cells to maintain a high NAD/NADH ratio without increasing the acetaldehyde concentration to toxic levels. The main conclusion to be drawn from the experiments on glucose metabolism in stem segments of peas is that glucose either present as storage in the cells or absorbed from the medium can be conjugated to lower aliphatic alcohols. In the presence of sufficiently large amounts of alcohol in the tissue, this appears to become the main glucose metabolite.

ACKNOWLEDGEMENTS

The authors wish to thank A. Kiewiet (org. chem. lab. RU Groningen) for this assistance on the mass-spectrometrical analysis and Dr. J. Bakker for the correction of the English text.

REFERENCES

BETZ, A. (1960): Die aerobe Gärung in aktiven Meristemen höherer Pflanzen. *Encyclopedia of Plant Physiology* (Edited by W. RUHLAND). Vol. **XII.2**: 88–113. Springer-Verlag Berlin.

BIEMANN, K., D. C. DE JONGH & H. K. SCHNOES (1963): Application of Mass Spectrometry to Structure Problems. *J. Amer. chem. Soc.* **85**: 1763–1771.

BONNER, J. (1949): Relations of respiration and growth in the Avena Coleoptile. *Amer. J. Bot.* **36**: 429–436.

KELLY, S., & G. S. AVERY (1951): The age of pea tissue and other factors influencing the respiratory response to 2.4 dichlorophenoxy-acetic acid and dinitro compounds. *Amer. J. Bot.* **38**: 1–5.

FALES, F. W. (1951): The assimilation and degradation of carbohydrates by yeast cells. *J. Biol. Chem.* **193**: 113–124.

HEYNS, K. & H. SCHARMANN (1963): Die Massenspektren von Derivaten der Monosaccharide und Aminozucker. *Liebigs Ann. Chem.* **667**: 183–193.

LIU, T. Y., A. OPPENHEIM & P. CASTELFRANCO (1965): Ethyl alcohol metabolism in leguminous Seedlings. *Plant Physiol.* **40**: 1261–1268.

— & P. CASTELFRANCO (1970): The biosynthesis of ethyl-β-glucoside in extracts of Pea Seedlings. *Plant Physiol.* **45**: 424–428.

REINDERS, D. E. (1938): The process of water-intake by discs of potato tuber tissue. *Proc. Kon. Ned. Akad. Wetensch.* **41**: 820–831.

VITEK, V. (1964): Sugar-like diphenylamine-positive metabolites in Pea seedlings. *Biochim. Biophys, Acta* **93**: 429–432.

WINTER, H. (1967): The effect of IAA on sugar metabolism by growing pea stem segments. *Proc. Kon. Ned. Akad. Wetensch.* **C70**: 294–301.

—, S. SPAANDER & P. K. WIERSEMA. (1970): The relation between cellulose synthesis and an unidentified glucose-complex in pea stem segments. *Acta. Bot. Neerl.* **19**: 525–532.