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The Biosynthesis of Ethyl-β-glucoside in Extracts of Pea Seedlings[1]

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ABSTRACT

An enzyme, which lacks cellobiase activity, responsible for the synthesis of ethyl-β-glucoside has been found in the extracts of pea hooks (1-centimeter length of the apical portion of epicotyl) and has been partially purified by ammonium sulfate fractionation. The enzyme can transfer the glucosyl moiety from a group of phenolic β-glucosides to ethanol. A specific β-glucosyl donor, isosuccinimide β-glucoside, isolated from the extracts of pea seedlings shows the highest activity. The characteristics of the enzyme which synthesizes ethyl-β-glucoside and the glucosyl donor, isosuccinimide β-glucoside, have been studied. The significance of this system (enzyme and isosuccinimide β-glucoside) has been discussed.

In our studies on ethanol metabolism in etiolated pea seedlings, it was found that ethyl-β-glucoside was one of the major metabolites (11). It is now our purpose to report some observations on the biosynthesis of ethyl-β-glucoside in a cell-free system. The synthesis of glycosides has been reviewed (3, 4). The most likely glucosyl donors (ADPG,[3] CDPG, GDPG, UDPG, glucose-1-P, maltose, sucrose, and cellobiose) for the synthesis of glucosides were tested. However, none of these seemed to be able to donate the glucosyl unit in this system.

In the crude homogenate of pea seedlings, ^{14}C-ethanol could be incorporated into ^{14}C-ethyl-β-glucoside. This system utilized an endogenous glucosyl donor which has been purified and identified as isosuccinimide β-glucoside (Fig. 1) (10).

An enzyme which is able to synthesize ethyl-β-glucoside from ethanol and isosuccinimide β-glucoside was found in pea hooks. Some properties of this enzyme were studied.

MATERIALS AND METHODS

Ethanol-1-^{14}C, 5.55 μc/μmole; isopropanol-2-^{14}C, 2.0 μc/μmole; and uniformly labeled glucose-^{14}C, 0.05 μc/μmole, were purchased from New England Nuclear Corporation. Almond β-glucosidase, ADPG, CDPG, GDPG, UDPG, and cellobiose were obtained from CalBiochem. Glucose-1-P was obtained from Sigma. Maltose was obtained from Nutritional Biochemicals Corporation. Phenyl-β-glucoside, dhurrin, helicin, and *o*-coumarylglucoside were kindly provided by Dr. E. E. Conn and Dr. T. Kosuge.

Pea seeds (*Pisum sativum* L. var. Radio, purchased from Atlee Burpee Corporation) were soaked in water for 6 hr and germinated for 7 days in moist vermiculite in the dark at 23 C. Pea hooks (1-cm length of the apical portion of epicotyl) were used for enzyme source, while the whole shoots were used as the source of ISG.

Quantitative determination of glucose was carried out as previously described (11). Proteins were determined by the method of Lowry *et al.* (12) with bovine serum albumin as standard.

ISG was isolated from etiolated pea seedlings by hot water extraction, ion exchange and paper chromatography, and crystallization from ethanol (10).

Preparation of Enzyme. Five grams of pea hooks were ground in a porcelain mortar and pestle in liquid nitrogen. Then, 12.5 ml of cold 0.1 M potassium phosphate buffer, pH 7, were added. The crude extract was filtered through cheesecloth, and the cell debris was further removed by centrifuging at 600*g* for 10 min. The enzyme was fractionated with $(NH_4)_2SO_4$ or simply precipitated with $(NH_4)_2SO_4$ to 80% saturation in order to remove the endogenous ISG. The enzyme prepared from 10 ml of homogenate was dissolved in 4 ml of 0.1 M potassium phosphate buffer, pH 7. Normally 0.1 ml of this enzyme preparation was used in a 1.2-ml reaction mixture.

In the case of fractionation of organelles, the crude extract was prepared in the way just described except that 0.25 M sucrose was added in order to maintain isotonic conditions. The mitochondrial fraction was obtained by centrifuging at 10,000*g* for 30 min. The microsomal fraction was obtained by centrifuging at 105,000*g* for 90 min. Both mitochondrial and microsomal fractions were washed once with 0.1 M potassium phosphate buffer, pH 7, containing 0.25 M sucrose. The soluble proteins were precipitated from the 105,000*g* supernatant by the addition of 4 volumes of saturated $(NH_4)_2SO_4$. For the enzyme assay, each fraction was suspended in 4 ml of 0.1 M potassium phosphate buffer, pH 7, containing 0.25 M sucrose.

Radioactive Assay for Enzyme and Isosuccinimide β-Glucoside. The enzyme assay was dependent upon the formation of ^{14}C-ethyl-β-glucoside from ^{14}C-ethanol. The enzyme and isosuccinimide β-glucoside were incubated in a conical centrifuge tube containing 2.5 μc of ethanol-1-^{14}C and 50 μmoles of sodium acetate buffer, pH 5.5, in a total volume of 1.2 ml. After incubation at 25 C for 1 hr, the reaction vessel was placed in boiling water for 5 min and cooled to room temperature, and the solution was saturated with solid K_2CO_3. The ^{14}C-ethyl-β-glucoside was then extracted 3 times from aqueous phase with an equal volume of 95% ethanol (11). The ethanol extracts were combined and evaporated under a stream of nitrogen. The residue was redissolved in 0.5 ml of water and evaporated to dryness again. The

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[3] Abbreviations: ADPG, CDPG, GDPG, UDPG: adenosine, cytidine, guanosine, and uridine diphosphoglucose; ISG: isosuccinimide β-glucoside.

residue was then dissolved in 1.0 ml of water. An aliquot of 0.5 ml of this preparation was counted in the scintillation counter. The ^{14}C-ethyl-β-glucoside so prepared was chromatographically identical to known ^{14}C-ethyl-β-glucoside, using the solvent systems described previously (11).

The extent of the reaction was represented by the amount (counts per minute) of ^{14}C-ethyl-β-glucoside thus obtained. The specific activity of the enzyme was expressed as cpm/mg protein · hr.

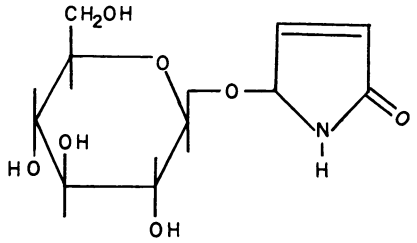
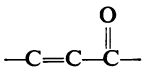


FIG. 1. Isosuccinimide β-glucoside.

RESULTS

Characteristics of Isosuccinimide β-Glucoside. Some of the chemical properties of ISG have been described elsewhere (10). The structure postulated on the basis of these properties is shown in Figure 1. The infrared and ultraviolet spectra of this compound are shown in Figures 2 and 3, respectively. The infrared spectrum shows characteristic bands at 1695 cm^{-1} (C=O), 1600 cm^{-1} (C=C), and two bands between 1600 cm^{-1} and 1695 cm^{-1} which are possibly due to conjugation. The ultraviolet spectrum (Fig. 3) with a pronounced shoulder at 230 mμ likewise supports a conjugated structure of the type



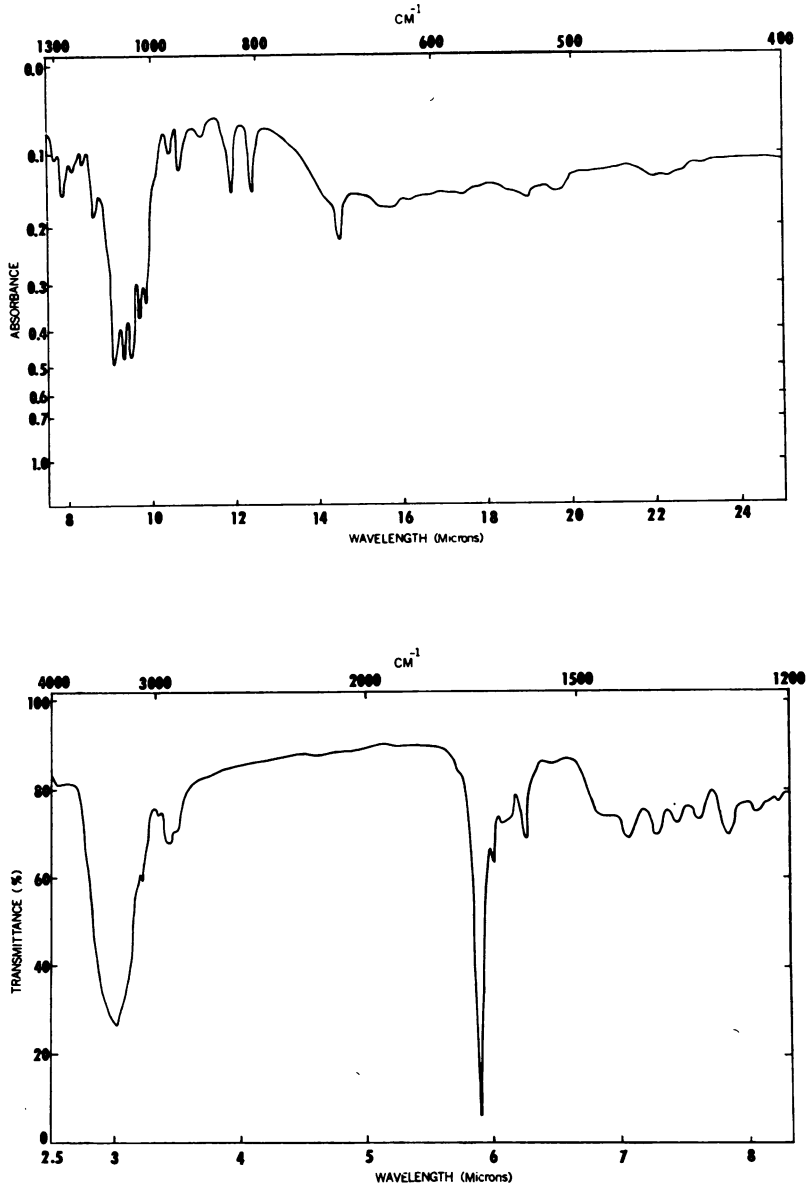


FIG. 2. Infrared spectrum of isosuccinimide β-glucoside. Solvent: 13-mm KBr microdisc. Reference: air.

The spectral shift in 0.1 M alkali is correlated with the irreversible breakdown of the compound.

Intracellular Localization of Enzyme. Ethyl-β-glucoside synthetase, the enzyme which could transfer glucosyl moiety from isosuccinimide β-glucoside to ethanol, was found exclusively in the soluble fraction (Table I). Neither mitochondrial nor microsomal fraction could stimulate or inhibit the enzymatic activity in the soluble fraction.

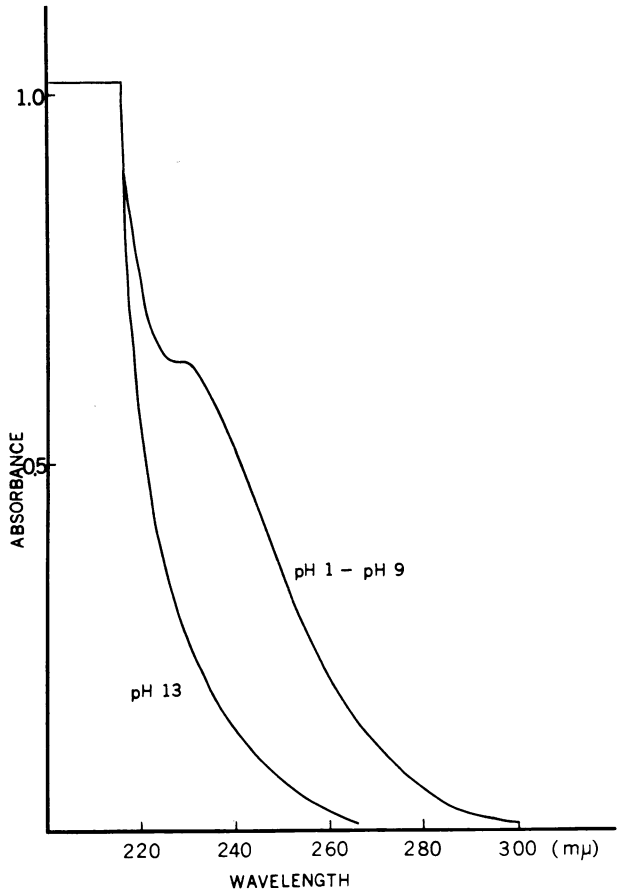


FIG. 3. Ultraviolet spectrum of isosuccinimide β-glucoside. The following buffers (0.1 N) were used: HCl (pH 1), sodium formate (pH 3), sodium acetate (pH 4 and 5), potassium phosphate (pH 6, 7 and 8), ammonium chloride (pH 9), and NaOH (pH 13).

Table I. *Intracellular Localization of Ethyl-β-glucoside Synthetase*

The incubation mixtures contained in a 1.2-ml total volume: 0.1 ml of each enzyme preparation, 0.4 μmole of isosuccinimide β-glucoside, 2.5 μc of ethanol-1-^{14}C (5.55 μc/μmole), 50 μmoles of sodium acetate buffer (pH 5.5), and 300 μmoles of sucrose. Incubations were carried out at 25 C for 60 min.

Enzyme Preparation	Radioactivity in ^{14}C-Ethyl-β-glucoside
	cpm
Mitochondria	210
Microsomes	200
Soluble	6990
Mitochondria + microsomes	230
Mitochondria + soluble	6900
Microsomes + soluble	6700
Mitochondria + microsomes + soluble	6800

A control incubation without sucrose showed that sucrose did not influence the transglucosylation reaction in this case.

Ammonium Sulfate Fractionation. The potassium phosphate extract (prepared in 0.1 M potassium phosphate, pH 7) was fractionated at different concentrations of $(NH_4)_2SO_4$. Table II shows that the enzyme was precipitated mainly between 50 and 80% saturated $(NH_4)_2SO_4$. The precipitate between 70 and 80% saturated $(NH_4)_2SO_4$ had the highest specific activity. The protein yield between these limits was small; therefore, the precipitate between 55 and 80% saturated $(NH_4)_2SO_4$ was used for enzyme assay in many cases.

pH Optimum. The enzyme was precipitated from the crude

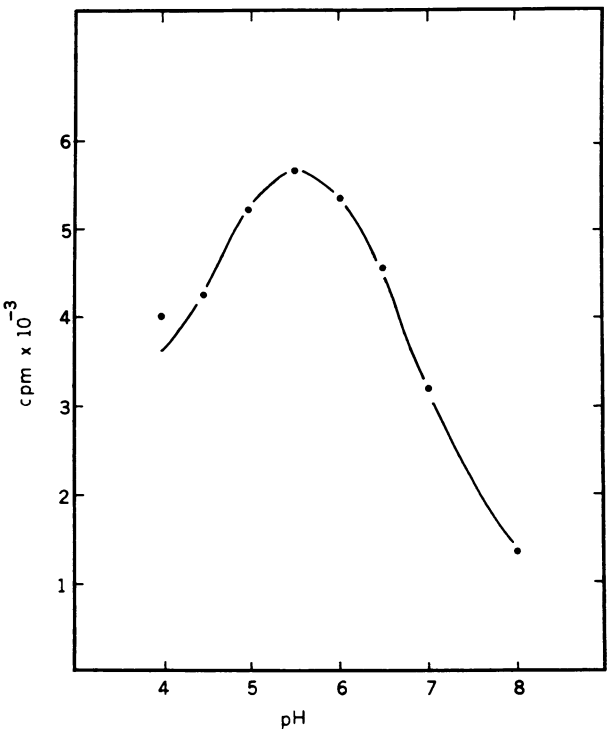


FIG. 4. Reaction velocity as a function of pH. The enzyme was precipitated by 80% saturated $(NH_4)_2SO_4$ from the crude extract. The incubation mixtures contained in a 1.2-ml total volume: 0.1 ml of enzyme preparation; 0.5 μmole of ethanol-1-^{14}C (5.55 μc/μmole); 0.4 μmole of isosuccinimide β-glucoside; and 50 μmoles of each of the following buffers: sodium acetate, pH 4.0, 4.5, 5.0, and 5.5; and potassium phosphate, pH 6.0, 6.5, 7.0, 7.5, and 8.0. Incubations were carried out at 25° for 60 minutes.

Table II. *Ammonium Sulfate Fractionation of Ethyl-β-glucoside Synthetase*

Each enzyme preparation was incubated under the same conditions as that of Table I, but without sucrose.

Enzyme Preparation	Radioactivity in ^{14}C-Ethyl-β-glucoside	Protein	Specific Activity of Enzyme
	cpm	*mg/1.2 ml*	*cpm/mg protein·hr*
Crude extract	9,724	5.72	1,700
0–40% saturated $(NH_4)_2SO_4$	738	1.59	464
40–50% saturated $(NH_4)_2SO_4$	1,460	1.71	854
50–60% saturated $(NH_4)_2SO_4$	2,946	0.597	4,935
60–70% saturated $(NH_4)_2SO_4$	2,979	0.266	11,199
70–80% saturated $(NH_4)_2SO_4$	2,244	0.109	20,587

extract by 80% $(NH_4)_2SO_4$. The enzyme shows the highest activity at pH 5.5 (Fig. 4); therefore, the routine assay was carried out at this pH.

Effect of Substrate Concentration on Velocity. For the study of the effect of ethanol concentration on velocity, the concentration of ISG was kept constant at 0.4 μmole/1.2 ml of incubation mixture. For the study of the concentration of ISG, the concentration of ethanol was kept constant at 0.5 μmole/1.2 ml of incubation mixture.

The enzymatic activity as a function of ethanol concentration is illustrated in Figure 5. The Michaelis constant (K*m*) for ethanol is 0.5 M.

The enzymatic activity as a function of ISG concentration is illustrated in Figure 6. The Michaelis constant (K*m*) for ISG is 0.48 mM.

Specificity of the Substrate. ADPG, CDPG, UDPG, glucose-1-P, sucrose, maltose, and cellobiose could not donate glucose for the synthesis of ethyl-β-glucoside. However, the crude enzyme is capable of transferring the glucosyl moiety from a variety of phenolic β-glucosides (Table III). ISG is more active than any of the substrates tested.

Besides ethanol, isopropanol could also accept the glucosyl moiety from ISG to give isopropyl-β-glucoside (Table IV). However, ^{14}C-disaccharides were not formed when ^{14}C-glucose was incubated with ISG.

Incubation of ^{14}C-ethyl-β-glucoside with the crude enzyme did not cause appreciable hydrolysis (Table V). Therefore, the glucosyl transfer from ISG to ethanol is practically irreversible, and radioactive ethyl glucoside cannot be formed by an exchange reaction between endogenous ethyl glucoside and ^{14}C-ethanol.

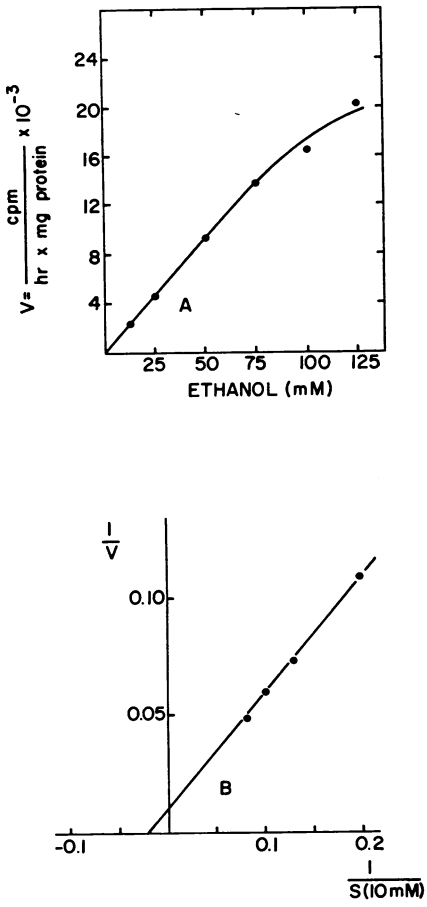


FIG. 5. A: reaction velocity as a function of ethanol concentration; B: Lineweaver-Burk plot of the effect of ethanol on the reaction velocity. The incubation mixtures contained in a 1.2-ml total volume: 0.1 ml of enzyme prepared as Table III; 0.4 μmole of isosuccinimide β-glucoside; 50 μmoles of sodium acetate buffer (pH 5.5); and ethanol-1-^{14}C (0.002 μc/μmole) as indicated. Incubations were carried out at 25 C for 60 min.

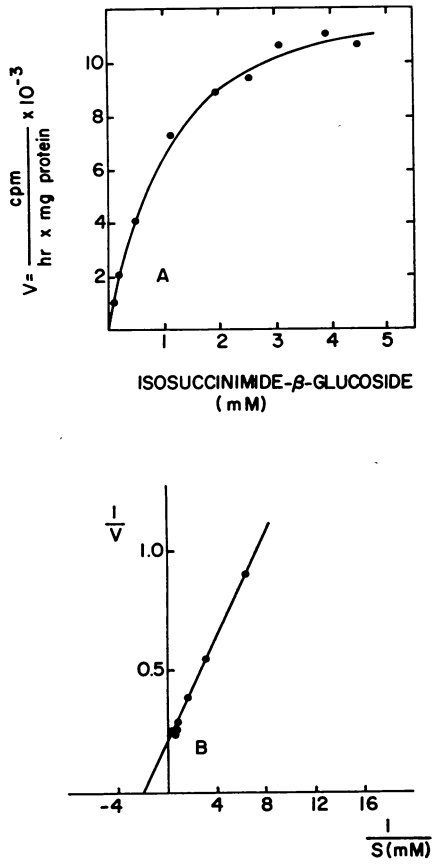


FIG. 6. A: Reaction velocity as a function of isosuccinimide β-glucoside concentration; B: Lineweaver-Burk plot of the effect of isosuccinimide β-glucoside on the reaction velocity. The incubation mixtures contained in a 1.2-ml total volume: 0.1 ml of enzyme prepared as Table III; 2.5 μc of ethanol-1-^{14}C (4.6 μc/μmole); 50 μmoles of sodium acetate buffer (pH 5.5); and isosuccinimide β-glucoside as indicated. Incubations were carried out at 25 C for 60 min.

Table III. *Specificity of the Glucosyl Donor*

The enzyme was prepared by the precipitation of between 55 and 80% saturated $(NH_4)_2SO_4$. The incubation mixtures contained in a 1.2-ml total volume: 0.1 ml of enzyme preparation, 0.4 μmole of each β-glucoside, 2.5 μc of ethanol-1-^{14}C (4.6 μc/mole), 50 μmoles of sodium acetate buffer (pH 5.5). Incubations were carried out at 25 C for 60 min.

β-Glucoside	Radioactivity in ^{14}C-Ethyl-β-glucoside	Relative Activity
	cpm	
Isosuccinimide β-glucoside	9669	1.00
Helicin	7761	0.80
Dhurrin	7782	0.80
Salicin	3813	0.39
o-Coumarylglucoside	2728	0.28
Phenyl-β-glucoside	1836	0.18

Table IV. *Specificity of the Glucosyl Acceptor*

The incubation mixtures contained in a 1.2-ml total volume: 0.1 ml of enzyme prepared as in Table III, 2.5 μc of ethanol-1-^{14}C (4.6 μc/μmole) or 2.5 μc of isopropanol-2-^{14}C (2.0 μc/μmole), 1.6 μmoles of isosuccinimide β-glucoside, 50 μmmoles of sodium acetate buffer (pH 5.5). Incubations were carried out at 25 C for 60 min.

Glucosyl Acceptor	Radioactivity in ^{14}C-Ethyl-β-glucoside	Radioactivity in ^{14}C-Isopropyl-β-glucoside[1]	Relative Activity
	cpm	*cpm*	
Ehthanol-1-^{14}C	32,454	...	1.00
Isopropanol-2-^{14}C	...	17,064	0.53

[1] Isopropyl-β-glucoside was isolated as described for ethyl-β-glucoside.

Table V. *Stability of Ethyl-β-glucoside toward Its Synthetase in Presence of Other Compounds*

The incubation mixtures contained in a 1.2-ml total volume: 0.1 ml of enzyme prepared as in Table III, 6522 cpm of ^{14}C-ethyl-β-glucoside, 50 μmoles of sodium acetate buffer (pH 5.5). Incubations were carried out at 25 C for 60 min.

Compound Added	Radioactivity Recovered in ^{14}C-Ethyl-β-glucoside
	cpm
None	5904
Ethanol (0.4 μmole)	5928
Isosuccinimide (0.1 ml, about 0.1% solution)	6151
ISG (0.4 μmole)	5955
ISG (0.4 μmole) incubated with boiled enzyme	6048

DISCUSSION

The presence of two substances which give a yellow-orange coloration with the common diphenylamine sugar reagent, was demonstrated in pea seedling extracts by Vitek (13). The ratio of these two substances differs considerably in the different parts of the pea seedling. Both substances were found in large amounts in the epicotyls. Chromatographically, the two substances reported by Vitek (13) agree with ISG and its aglycone. During growth the amount of glucoside remained relatively constant while that of the aglycone increased. The aglycone was detected in the root only occasionally, while in the shoot it was always present. In the cotyledons both the glucoside and its aglycone were practically absent. The level of these two substances was greatly influenced by radiation (8). However, these authors did not identify these two compounds but confined themselves to studying their R_F values and color reactions on paper chromatograms. As the seedling grows, ISG decreases and its aglycone increases. This observation could be explained if ISG is the glucosyl donor for the synthesis of other glucosides, releasing the aglycone during the transglucosylation reaction. In our own studies we found both substances in the etiolated pea seedlings in large amounts; however, we failed to find them in soybean or mung bean seedlings.

Recently Kocourek *et al.* (9) reported the isolation of a new glucoside from pea seedlings which they named "pisatoside." The physical and chemical properties reported by Kocourek *et al.* (9) for this compound are in good agreement with those which we have found for ISG. The structure which has been assigned to "pisatoside" is isomeric to the one we have proposed for ISG (Fig. 1). It is very likely that we are dealing indeed with the same compound and that the discrepancies between the assigned formulas will be resolved by further experimental work.

The enzyme preparation which transfers glucose from ISG to ethanol (precipitated between 55 and 80% saturated $(NH_4)_2SO_4$) was able to hydrolyze ISG without the presence of an added glucosyl acceptor (alcohol). Since this enzyme preparation was rather crude, it is not known whether the hydrolysis and the transglucosylation reactions were carried out by the same enzyme. The enzyme so prepared lacks cellobiase activity since no ^{14}C-ethyl-β-glucoside was obtained when ^{14}C-ethanol and cellobiose were incubated with this enzyme preparation. (The authors were able to obtain significant amounts of ^{14}C-ethyl-β-glucoside by incubating ^{14}C-ethanol and cellobiose with emulsin). Furthermore, when ^{14}C-glucose and ISG were incubated in the presence of this enzyme, ^{14}C-cellobiose was not obtained. Enzymes catalyzing β-D-glucosyl transfer from aryl β-D-glucosides are often distinct from those catalyzing similar transfers from oligosaccharides (4). Enzyme preparations possessing aryl β-glucosidase activity but devoid of cellobiase activity have been prepared from the cellulolytic fungi (1, 2, 5–7).

The crude enzyme preparation which is able to synthesize ethyl-β-glucoside from ISG and ethanol does not catalyze the hydrolysis of ethyl-β-glucoside (Table V). The fact that ISG is readily hydrolyzed by the crude enzyme while ethyl-β-glucoside is not hydrolyzed even in the presence of added aglycone suggests that the glucosyl transfer reaction, *ISG + ethanol → IS + ethanol-G* is practically irreversible. Normally irreversible transglucosylations occur when sugar nucleotides are the glucose donors. Therefore, it would appear that ISG is—like the sugar nucleotides—an energy-rich compound because of the highly reactive nature of the aglycone.

The affinity of ethanol with enzyme is low (Km = 0.5 M); that of ISG is reasonably high (Km = 0.48 mM). This suggests that ethanol might not be a physiological acceptor for this glucosyl transfer while ISG may be a physiological donor. On the other hand, the content of ISG in pea seedling is high (13). Therefore, it appears possible that ISG takes part in the synthesis of other physiologically important β-glucosides such as phenolic glucosides or cell wall substances. The physiological role of this highly reactive glucoside will be the object of further research.

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