AN ABSTRACT OF THE THESIS OF

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Title: STUDIES ON STEROL:UDP-GLUCOSE GLUCOSYLTRANSFERASE IN GERMINATING PEA SEEDLING

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Abstract approved: Derek J. Baisted

Steryl glycoside is synthesized from mevalonate [2-^{14}C] in both maturing pea seeds and etiolated pea seedlings. The maturing seed appeared more active than the seedlings for this synthesis. The sugar moiety was identified predominantly as glucose by acid hydrolysis and paper chromatography of the aqueous extract of the hydrolysate. The lipid moiety extracted from the hydrolysate was found to be mainly sterol by thin-layer chromatography and radiochromatographic scanning. Etiolated shoot-root axes were used for the isolation of the enzyme preparation because of the convenience of obtaining large amounts of material.

UDP-glucose labelled with ^{14}C on the glucose moiety and exogenous sitosterol were used as substrates for the study of sterol:UDP-glucose glucosyltransferase activity. The highest specific activity for this enzyme was found in the shoot, root and shoot-root axial tissues of seven-day old seedlings. Fractionation of the seedling homogenate by centrifugation showed that the 13,000-25,000 g fraction has the highest specific activity in tissue homogenate.

The fraction appeared to be rich in Golgi membranes by the presence of the marker enzyme, IDPase. The glucosyltransferase in this membrane-rich fraction was stimulated best by Ca^{++} and by Mg^{++}. Marked inhibition was observed in the presence of Zn^{++}. The presence of ATP has different effects on the enzyme activity and depends on the kind and quantity of metal ion used in the reaction mixture. In addition, the enzyme activity was enhanced further in the presence of a small amount of methanol and low buffer concentration. Under the assay conditions employed, the enzyme has an apparent Km of 3.33 mM and a V_{max} of 480 nmoles/hr for UDP-glucose. It is inhibited by concentrations of UDP-glucose greater than 4 mM. The enzymatic reaction proceeds preferentially with endogenous sterol as the glucose acceptor and UDP-glucose as the glucose donor.

Glucosyltransferase activity suffers a sharp decline during the course of the assay. The activity could be partially maintained by repeated addition of ATP during the assay. The role of ATP and the possible relationship between ATPase, protein kinase and the glucosyltransferase still needs to be explored.

Attempts to purify this enzyme from the 13,000-25,000 g fraction have been made, but failed. Transferase activity was inhibited by treatments with phospholipases A, C, and D. The lost activity can be fully recovered by adding phosphatidyl serine, or phosphatidyl choline and even

further enhanced by adding phosphatidyl ethanolamine. A transferase with high specific activity of the membrane-rich fraction was separated by sucrose density gradient centrifugation. This method may provide an avenue for further characterization of sterol:UDP-glucose glucosyltransferases in plant tissues.

Studies on Sterol:UDP-Glucose Glucosyltransferase in Germinating Pea Seedling

by

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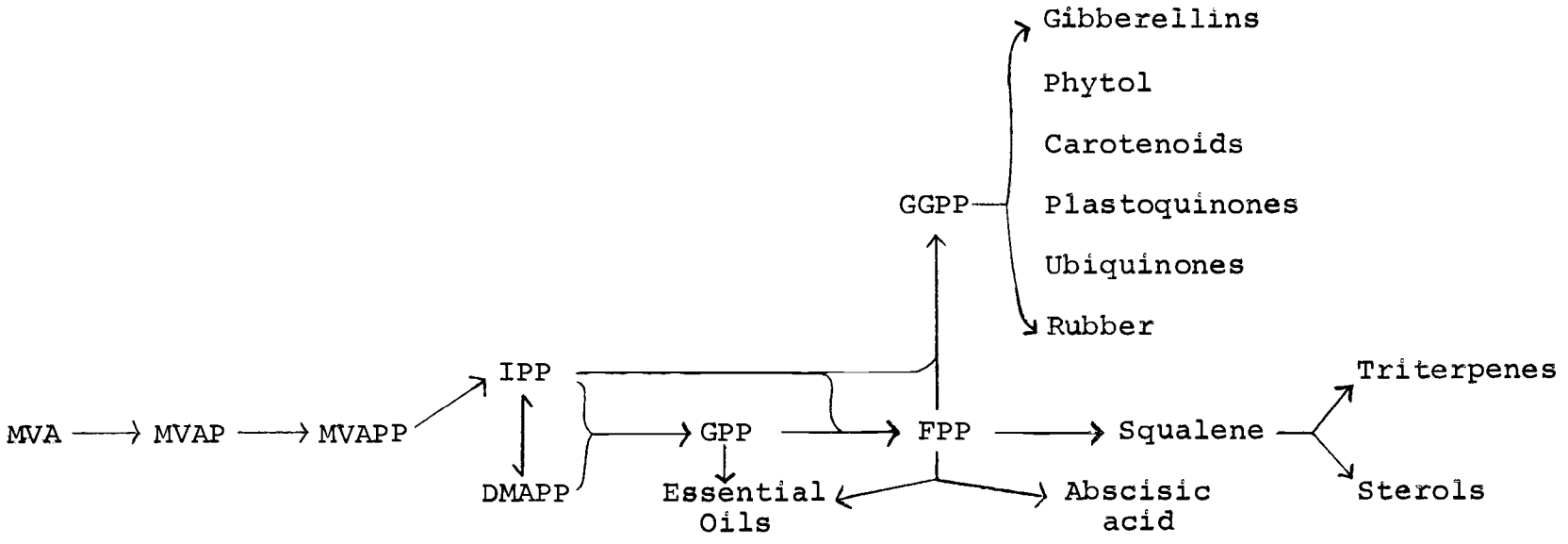
STUDIES ON STEROL:UDP-GLUCOSE GLUCOSYLTRANSFERASE IN GERMINATING PEA SEEDLING

INTRODUCTION

The biosynthetic pathway leading to the isoprenoid, cholesterol in animals has been elucidated (8). This pathway is essentially the same as that leading to the higher isoprenoids of plants. Differences between the two pathways occur as a result of the greater diversity of plant isoprenoids compared with animal isoprenoids, thereby giving rise to reactions such as cyclizations and rearrangements of the pyrophosphate intermediates of the pathway. Scheme 1 shows the highly branched pathway to various plant isoprenoids from mevalonic acid (MVA). Among the end products, sterols have drawn considerable interest in recent years. Specifically, their undefined function in plants is particularly intriguing. In addition, the regulation of sterol synthesis in plants is still a mystery to biochemists even though some aspects of the regulation of cholesterol synthesis in mammals appears to be understood. In animal systems, sterols have been found to be further metabolized into bile acids, hormones, and to conjugated forms such as esters, glucuronides and glycosides. Many biological functions of these metabolites of sterols have been clearly demonstrated and, as a result, the functions of sterols in animals are considered to be: 1) as hormones; 2) as membrane components; and 3) as

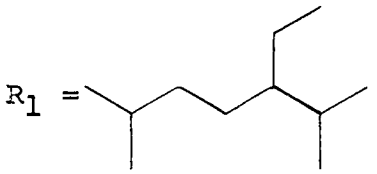


Scheme 1. Biosynthetic pathway in plants of higher isoprenoids from MVA.

precursors of other steroids (35). However, the function of many sterol derivatives is still unknown.

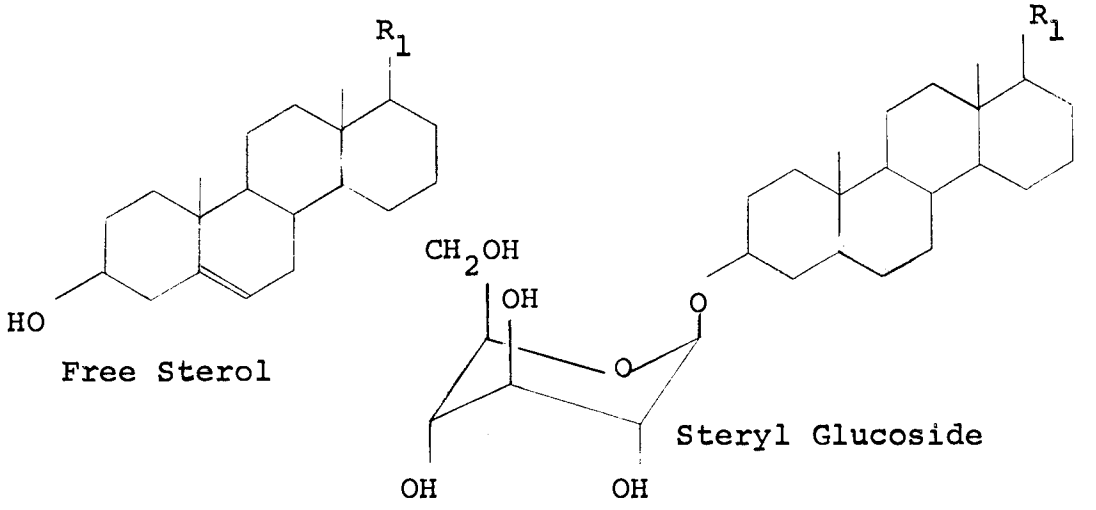
The total sterol content of plants usually refers to the combined content of free, esterified, glycosylated and acylated glycosidic sterol of a hot acetone extract of the plant tissue (12). All four kinds of sterol have been found to occur in both green and etiolated barley shoots (12), soybean seeds (44) and germinating tobacco seeds (13). Sterols and sterol esters of green and etiolated bean leaves have been isolated and their sterol compositions have been compared (9). The physiological roles of sterols in plants have been suggested to be an inert stockpile of precursors that are readily convertible to biologically active compounds, or to exert hormonal activity (35), or to be the structural components of cell membranes (33, 35, 43). Recently, it has been postulated that sterols and their conjugates may also play an important role in membrane permeability (32, 34, 79).

Naturally occurring steryl glycosides were first characterized in 1913 by Power and Salway. They were named sterolins (60) or cholane saponins (6). The most well known within this group is digitonin, which is widely used to precipitate sterols quantitatively (83).



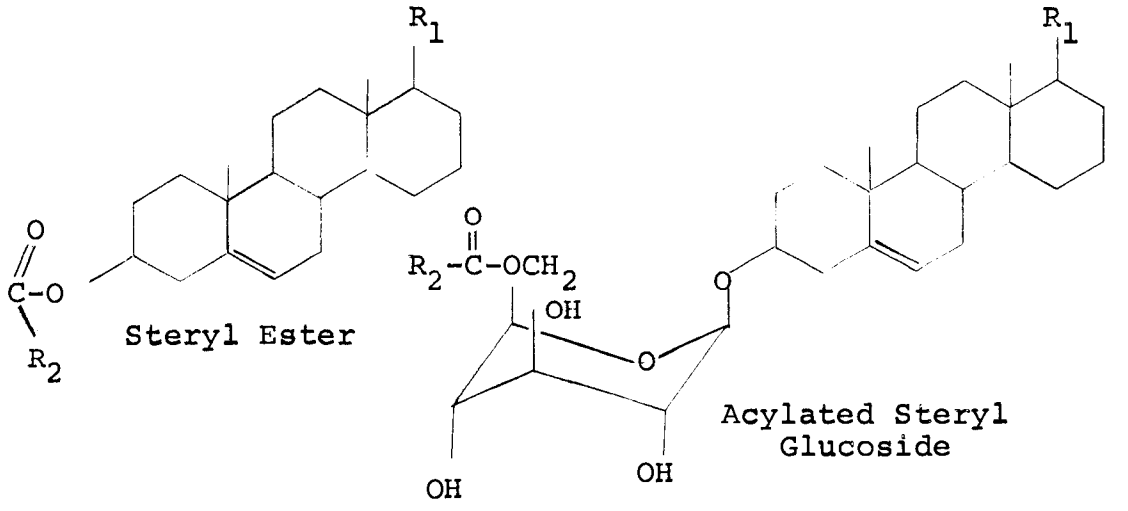
for sitosterol

R_2 = long chain fatty acid



Free Sterol

Steryl Glucoside



Steryl Ester

Acylated Steryl Glucoside

In vivo Biosynthesis of Steryl Glycosides

Biosynthesis of steryl glycosides in plant tissues and intact plants has been demonstrated (14, 23, 75). Labelled steryl glucoside was found by incubating UDP-glucose-^{14}C or UDP-galactose-^{14}C with lettuce leaf discs (23). The same workers showed that green plant tissue such as leaf discs of yellowwood, bean, maple and spinach were also able to synthesize steryl glycosides from UDP-galactose-^{14}C. Conjugation of exogenous steroids such as dehydroisoandrosterone and isoandrosterone with endogenous glucose has been demonstrated with surviving potato slices, green bean and pineapple slices in alkaline pH buffer solutions (75). Cholesteryl glucoside was also formed from exogenous cholesterol by pleuropneumonia-like organisms (70).

Bush and Grunwald (14) have shown acylated steryl glycosides to be synthesized more rapidly than steryl glycosides by exposing intact tobacco seedlings to cholesterol-^{14}C and sitosterol-^{14}C in phosphate buffer. They concluded that steryl glycoside is not a precusor to the acylated derivative in this tissue. This is in contrast to the findings of Hou *et al.* (36) and Ongun and Mudd (55), who in time-course studies with cell-free extracts demonstrated a precursor-product relationship between steryl glycoside and the acylated derivative. Conceivably the same relationship holds in the intact seedlings used by Bush and Grunwald but

the organization of the glycosylating and acylating enzymes is such that release of steryl glycoside is slow compared with its acylation by the adjacent acylating enzyme. As the acylated product accumulates and acyl CoA becomes rate-limiting, steryl glycoside accumulates.

In vitro Synthesis of Steryl Glycosides

Enzymatic studies on the biosynthesis of steryl glycosides in plants did not begin until recent years. Hou et al. (36) were the first to use a particulate enzyme preparation from immature soybean seeds. The preparation transferred glucose from UDP-glucose to sterol (Equation 1).

$$\begin{array}{l} \text{Sterol} + \text{UDP - glucose} \\ \qquad \Big\downarrow\Big\uparrow \text{ sterol:UDP-glucose glucosyltransferase} \\ \text{Steryl glucoside} + \text{UDP} \end{array} \qquad (1)$$

Since that time, this enzyme has been studied by some other groups with pea root mitochondria, spinach chloroplast, cauliflower mitochondria and avocado mitochondria (55), with particulate fractions of mung bean shoot (39, 47), tobacco seedling (14) and germinating wheat root (57).

Although enzymatic activity was found in different particulate fractions, the preparations were crude. Generally speaking, they can be divided into two categories: (1) high speed spin particulate fractions (36, 57); and (2) low speed spin particulate fractions (14, 39, 47, 55).

Hou et al. (36) showed that endogenous sterols present in the 10,000-105,000 g fraction obtained in 0.1 M Tris·HCl (pH 8) buffer were available for the enzymatic reaction. Although only sitosterol was added as an acceptor of glucose from UDP-glucose-^{14}C, the isolated sterol glucosides after hydrolysis yielded campesterol and stigmasterol in addition to sitosterol.

With a 1,000-34,000 g particulate enzyme preparation made with 0.05 M potassium phosphate buffer (pH 7.3) from etiolated mung bean shoots, Kauss (39) was able to incorporate glucose-^{14}C from UDP-glucose-^{14}C into steryl glucosides. The products were identified as sitosteryl glucoside and stigmasteryl glucoside with some contamination by acylated steryl glucosides. In addition, this enzyme preparation could also form cellulose from UDP-glucose. It was demonstrated, however, that steryl glucoside is not an intermediate in cellulose synthesis. Experiments which were terminated by digestion of the unused UDP-glucose by phosphodiesterase demonstrated no further turnover of synthesized steryl glucosides. Exogenous sterols did not act as glucose acceptors with their enzyme preparation.

Ongun and Mudd (55) used a 1,000-18,000 g particulate fraction of pea roots made with 0.01 M phosphate buffer (pH 7.4) containing 0.5 M sucrose which they identified as the mitochondrial fraction. Both UDP-glucose-^{14}C and UDP-galactose-^{14}C were used as the sugar donor and no exogenous

sterol was added into the reaction mixture. It was noticed that in the case of the pea root mitochondria preparation, steryl glucosides were found even when UDP-galactose was used as the hexose donor indicating the presence of a 4-epimerase in the preparation. Steryl glucosides were also formed efficiently from UDP-galactose with cauliflower mitochondria and avocado mitochondria preparations.

Laine and Elbein (47) synthesized steryl glycosides with the material sedimenting between 1,000 and 30,000 g from mung bean shoot homogenate. However, a description of the homogenizing medium was not reported. Radioactive UDP-glucose and endogenous sterols were used as substrates. By combined gas-liquid chromatography-mass spectrometry, they were able to identify the glucosides of sitosterol and stigmasterol and tentatively stigmastatrienyl glucoside.

Peaud-Lenoel and Axelos (57) found sterol:UDP-glucose-glycosyltransferase activity in a crude particulate preparation, 2,500-130,000 g, of germinating wheat root obtained in 0.1 M Tris·HCl (pH 7.5) containing 0.7 M sucrose. An active preparation was separated from this by sucrose density centrifugation. The active fraction was believed to be fragments of plasmalamella from endoplasmic reticulum. An acetone powder of this partially purified enzyme has been made and found to require lipid substrate in order to have the reaction proceed. Endogenous lipid acceptor was no better than exogenous sterols at this stage. The acetone

powder was relatively stable in TME (0.1 M Tris·HCl buffer containing 5 mM β-mercaptoethanol) suspension for several days at -25°C, but was labile in Triton X-100 solution. This work also claims to support Kauss' conclusion (39) that steryl glucoside was not an intermediate of β-glucansynthesis by demonstrating that steryl glucoside was metabolically active in the synthesis of acylated steryl glucoside, as they concluded. This does not exclude the possibility of steryl glucoside being a carrier for glucan synthesis as well as being a precursor in acylated steryl glucoside.

Bush and Grunwald (14) used a 500-20,000 g particulate fraction of tobacco seedlings prepared in 10 mM Na^+-K^+ phosphate buffer (pH 7.4) for the study of sterol:UDP-glucose glucosyltransferase. ^{14}C-Labelled cholesterol was used as the glucose acceptor. The enzyme transferred glucose to endogenous sterol acceptors as well as to the exogenous ^{14}C-cholesterol. However, they demonstrated chromatographically that for prolonged incubation (40 minutes or more), added cholesterol was more readily available to the enzyme preparation than the endogenous free sterols. This could be reasoned that after prolonged incubation, the endogenous sterol pool has been depleted and the exogenous cholesterol became the only available sterol acceptor.

Reaction Conditions for the Assay

Optimal conditions used for the enzyme activity vary from experiment to experiment. The optimum pH for the transfer of glucose from UDP-glucose to sterol varies from 6.3 to 6.6 in cacodylate buffer (39) to 7.5 to 8.0 in Tris·HCl buffer (36, 55, 57). Hou et al. (36) observed maximum steryl glucoside synthesis at 30° to 35°C, but Ongun and Mudd (55) concluded in their study that the synthesis was inhibited at temperatures higher than 30°C.

There have been few studies to determine the cofactor requirements of the enzyme. ATP was found to be stimulatory to the transferase activity by Hou et al. (36) and Bush and Grunwald (14). It was suggested that it functions by preventing the hydrolysis of UDP-glucose by contaminating phosphatase (36). No divalent metal ion was required for steryl glucoside synthesis in the preparation of Kauss (39). Bush and Grunwald (14) have demonstrated that high concentrations of UDP-glucose inhibit synthesis of steryl glycoside.

Acetone powders of the particulate enzyme preparations have been prepared (55, 57). Sterols were required for steryl glucoside synthesis at this stage. However, addition of sterol to the original particulate enzyme has also been found to stimulate the reaction (36).

UDP-glucose:sterol glycosyltransferase activity was observed in the crude extracts prepared by sonicating cells

of the yeast Candida bogoriensis (24). The lipid acceptor for glucose was identified to be ergosterol, but authentic cholesterol was also able to serve the same purpose. Even after 1-butanol-acetone extraction, the remaining powder still contained a small amount of endogenous acceptor. It was also found that 7-15 percent (by volume) of ethanol in the reaction mixture not only helped to disperse the exogenous lipid acceptors but also to stimulate the enzyme activity.

Dithiothreitol has been used widely in enzymic preparations as an antioxidant and enzyme stabilizer (18, 49). Storm and Hassid have demonstrated just recently with a particulate fraction of mung bean and pea shoots (81) that dithiothreitol can conjugate with glucose enzymatically as well as with other hexose donors. Therefore, substantial errors may occur when assaying sterol:UDP-glucose glucosyltransferase in the presence of dithiothreitol since DTT-glucoside is soluble in $CHCl_3$-MeOH and MeOH (81). These same workers have found a soluble glycosyl transferase in mung bean seeds (82) in which glucose is most effectively transferred to phenol and n-butyl alcohol among the aromatic and aliphatic acceptors examined. Methanol proved to be a very poor acceptor of glucose. The rate of incorporation of glucose or cholesterol into steryl glycosides for preparations from a number of different sources ranged from 0.1 (55) to 8.7 (14) nmoles per hour per mg of protein,

which might be considered to be low in terms of enzyme activity.

So far, it seems that sterol:UDP-glucose glucosyltransferase from plant sources belongs to one family of glucosyltransferase. It has broad substrate specificity in terms of sterol, the glucose acceptor. Its properties differ somewhat, depending on the source of plant material.

Glycosyltransferase in Mammalian Systems

In vitro studies of sterol:UDP-glucose glucosyltransferase in animal tissue preparations have not been so extensive. In general the enzyme displays considerable specificity for the substrate and it has also been difficult to purify. A steroid:UDP-glucose glucosyltransferase has been found and partially purified from rabbit liver microsomes (20). The enzyme has a specific requirement for UDP-glucose for the transfer of glucose to the phenolic 3-hydroxyl group of estrone and 17α(β)-estradiol and their 3-glucuronosides. A purification has been accomplished by means of solubilization of the microsomal preparation with 0.1 percent Triton X-100 solution. Further attempts to purify this enzyme by $(NH_4)_2SO_4$ precipitation were unsuccessful. This enzyme is widely distributed in the rabbit. It has been found in liver, kidney, large intestine and small intestine of rabbit, but the richest source is in liver microsomes. The results also showed that the steroid glucosyltransferase

activity was closely associated with two other microsomal steroid glycosyl transferase activities, namely N-acetyl glucosaminyl transferase and glucuronyl transferase, which have been investigated previously in more detail (19).

In partially purified rabbit liver the UDP-glucuronic acid:steroid glucuronyl transferase has been recognized to consist of a group of enzymes with similar physical properties and different substrate specificites (63). Similar characteristics have been found for UDP-N-acetyl glucosyamine:steroid N-acetylglucosaminyl transferase. The enzyme has a strict specificity for the 17 α-hydroxy group of phenolic steroids which carry an acidic conjugating group on C-3 (19). But, another transferase was specific for the 15 α-hydroxy group on phenolic steroids (15). In contrast, the UDP-glucose:steroid glucoside glucosyl transferase discussed above did not have such strict substrate specificity.

In partially purified rabbit liver microsomal preparations, glucuronyl-, glucosyl- and N-acetyl glucosaminyltransferase have been identified (20). Triton X-100 precipitation and $(NH_4)_2SO_4$ fractionation have shown that the glucosyltransferase was not purified as effectively as the other two enzymes. No other purification method has been tried on the UDP-glucose:steroid glucosyltransferase of animal origin.

Other glycosyl transferases isolated from various sources have been studied more extensively. Among them, the glucose acceptors include proteins (51, 72), polyisoprenols (7, 37, 69), ceramide (77), and phenols (30, 59, 88). Although different enzymes catalyze the synthesis of glycosides some generalizations can be made about them: they are usually particulate and, most likely, membrane-bound (19, 37, 51, 59, 72, 77, 92, 93); their activity is low (68, 72, 77); detergents such as Triton X-100 and digitonin enhance the enzyme activity (59, 72, 88, 92); divalent ions, Ca^{++}, Mg^{++} and Mn^{++} are stimulatory (7, 72, 77, 92); and finally, they are generally unstable during purification processes (20, 46).

Sterol and Membrane Permeability

One particularly important aspect of the study of this enzyme is the fact that sterol is known to be a modifier of membrane properties (16). Rottem et al. (71) have recently obtained information with Mycoplasma mycoides var. capri cells grown with low cholesterol concentrations and have concluded that cholesterol functions as a regulator of membrane fluidity. Studies of Papahadjopoulas et al. (56) using model membranes of mixtures of phospholipids and enzymes strongly indicated that cholesterol could regulate the permeability of biological membranes by affecting the internal viscosity and molecular motion of the lipid within the membrane. The degree of cohesion between the lipid molecules appears to determine the motional freedom and localization of membrane enzymes.

Chapman and Wallach (16) have proposed a dual role for cholesterol in membranes through hydrophobic interactions of the large, planar cholesterol molecule with the fatty acid chains of the phospholipid component of membranes. This in turn can restrict the motion of certain fatty acid chains in liquid crystalline regions and prevent the formation of crystalline gel of other fatty acid chains in the membrane. Thus, the hydrocarbon chains are in an "intermediate fluid" condition.

Purpose of Study

As the enzyme sterol:UDP-glucose glycosyltransferase is suggested to be membrane bound (14, 19, 36, 51, 55, 77, 92), this enzyme activity has a potential means of altering the level of free sterol in a membrane. Such alterations could lead to changes in membrane properties in the vicinity of the enzyme and influence the activities of other membrane-bound enzymes. Clearly, if the enzyme has this property, then an investigation of factors regulating it would be important.

Although the enzyme has been observed in different particulate fractions from several plant sources, its dependence upon a lipid environment and the effects of different metal ions and of ATP have not been investigated. In this study, the enzyme is characterized in more detail with respect to these three factors.

MATERIALS AND GENERAL METHODS

Materials

Pea seeds, Pisum sativum L. var. Alaska, were purchased from W. Atlee Burpee Co., Riverside, California. Vermiculite used to germinate the seeds was Terra Lite from W. R. Grace and Company.

Radioactive materials were from different sources. UDP-glucose-^{14}C was from ICN (International Chemical and Nuclear). It was diluted to the desired specific activity by addition of unlabelled UDP-glucose. ATP-γ-^{32}P tetrasodium salt was also from ICN. DL-MVA-5-^{3}H was obtained as the dibenzylethylenediamine salt from New England Nuclear. D, L-MVA-2-^{14}C lactone was obtained from Amersham/Searle. Both preparations of MVA were converted to the sodium salt before use.

The unlabelled UDP-glucose and ATP were purchased from Calbiochem and Sigma Chemical Co., respectively. Other chemicals purchased from Sigma were sitosterol, practical grade, which was recrystallized from $CHCl_3$-MeOH before use; ADP, as the sodium salt; and phospholipase A, from bee venom. Phospholipase C and D were from Clostridium perfringens and cabbage, respectively; both were obtained from Miles Laboratories. Phospholipids were obtained from Sigma and Supelco.

Ficoll, molecular weight 400,000, was from Pharmacia, Uppsala. Amberlite XAD-4 was obtained from Rohm and Hass.

The counting fluor Aquasol was obtained from New England Nuclear.

Thin layer chromatogram sheets coated with a 100μ layer of silica gel were purchased from Eastman Kodak Company.

Miracloth for filtration of the blended plant tissue was obtained from Calbiochem, LaJolla, California.

Authentic cholesteryl glucoside was a generous gift from Dr. J. J. Schneider, Department of Medicine, Jefferson Medical College, Philadelphia, Pennsylvania.

General Methods

Seed Germination and Maturation

Seeds were washed and presoaked with several changes of water for 24 hours before germination on vermiculite. For the first four days of germination (including the day of presoaking), the seeds were covered with moistened paper towels and maintained wet. The last three days of growth the paper towels were removed. Except for the first day of presoaking, the rest of the germination was carried out in the dark. The temperature for growth was between 24-26°C. The seedlings were thoroughly rinsed in running tap water and the cotyledons removed before homogenization.

Pea plants were grown in the greenhouse with a photoperiod of 16 hours and a day-night temperature cycle of 24°C and 18°C. The light intensity at the bench top was 9700 Lux.

Determination of Radioactivity

Lipid-soluble materials were evaporated to dryness and counted in non-aqueous fluor.* Water-soluble materials were counted in Aquasol.

Liquid scintillation counting was carried out on a Packard Tricarb, Model 574 scintillation counter. All data are corrected so as to express radioactivity in disintegrations per minute (dpm). Counting efficiencies were determined using ^{14}C and ^{3}H standards. Samples of ^{32}P-labelled materials were counted and expressed in counts per minute (cpm).

The distribution of radioactivity on paper chromatograms and thin layer chromatograms was examined with a Packard Radiochromatogram Scanner, Model 7301. Further quantitation of radioactive areas on the chromatographs was made by scintillation counting of the eluates from the scrapings of radioactive zones on the thin layer chromatograms.

The quenching by $CHCl_3$-MeOH solvent mixtures (74) was minimized by: (i) absorbing the water in the extract with anhydrous sodium sulfate granules; (ii) evaporating the solvent in the scintillation vial under nitrogen before addition of the scintillation counting fluid.

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* Non-aqueous fluor: 4 gm POP (2,5-diphenyloxazole)
30 mg POPOP [1,4-bis-2'-(5'-phenyloxazolyl)-benzene]
Dissolved in 1 liter of toluene.

Chromatography

1. Thin Layer Chromatography: Thin layer chromatography was carried out on Eastman Chromagram sheets precoated with 100μ silica gel. The sheets were activated at 100°C for 20 minutes prior to use. The solvent systems used for the various separations are listed in Table I under section 4. Authentic samples of appropriate compounds were run alongside the biosynthesized compounds.

2. Detection of Compounds on Thin Layer Chromatogram: Triterpenes, sterols and steryl glucosides were detected by exposing the plates to iodine vapor or spraying with 50 percent H_2SO_4 and heating the plate in an oven at 100°C for five minutes. Phospholipids were sprayed with a chromogenic reagent prepared according to Goswami and Frey (27). Metallic copper (0.08 g) was placed into 1 ml of 25 percent ammonium molybdate solution. The mixture was chilled in ice and 1 ml H_2SO_4 was added. This solution was kept at room temperature for two hours with occasional shaking. Distilled water (40 ml) was added and the copper metal was removed. After addition of 3.2 ml H_2SO_4, the solution was light-brown and remained stable at room temperature. Plates with phospholipid samples were sprayed twice with this reagent. After each spray, plates were kept in an oven at 65-70°C for five minutes. A blue color developed after the second spraying and heating.

Detection of radioactive compounds on the plate was made with the radiochromatogram scanner as previously described.

3. Paper Chromatography: Whatman #1 filter paper was used for paper chromatography. Five cm x forty cm strips were used to separate UDP-glucose, UDP and sugars by descending chromatography, and 3 cm x 20 cm strips were used to resolve ATP and P_i in an ascending system. Solvent systems used are listed in Table 1. After development, the paper was dried at room temperature. Authentic samples were run alongside the unknown mixtures and radioactivity was detected with the radiochromatogram scanner.

Sugars were detected with p-anisidine spray. The spray solution was made by dissolving one gram of p-anisidine-HCl in 100 ml n-butanol. Sugar spots became distinguishable after brief heating.

4. Chromatographic Solvent Systems: Numerous solvent systems were used for thin layer chromatography and paper chromatography. To avoid describing the composition of each solvent system repeatedly, they are listed in Table 1 and will be referred to as solvent system A, B, C, ... etc., hereafter.

Table 1. Solvent systems for thin layer and paper chromatography.

Solvent	Composition	Type of Chromatography	Separation Effected*	Ref.
A	12% ethyl acetate in hexane	TLC	Neutral lipids (sterol, triterpene, squalene)	5
B	$CHCl_3$:MeOH:HOAc, 65:25:8 (v/v)	TLC	Phospholipids	54
C	$CHCl_3$:EtOH:H_2O, 90:9:1 (v/v)	TLC	Polar compounds (SG, ASG) and Triterpenoid	--
D	$CHCl_3$:MeOH:HOAc:H_2O, 65:15:10:4 (v/v)	TLC	Polar compounds (SG, ASG)	54
E	$CHCl_3$:MeOH:7N NH_4OH, 65:25:4 (v/v), saturated tank	TLC	Polar compounds (SG, MGG, DGG)	54
F	n-Butanol:HOAc:H_2O, 12:3:5 (v/v)	Paper	Sugars	81
G	100 ml 0.1 M $NaPO_4$ buffer (pH 6.8) + 60 gm $(NH_4)_2SO_4$ + 2 ml n-propanol	Paper	ATP, ADP and P_i	73
H	$CHCl_3$:MeOH:28% NH_4OH, 65:35:5 (v/v)	TLC	Polar compounds (SG, ASG, MGG, etc.) and Phospholipids	54
I	$CHCl_3$:Acetone:MeOH:HOAc:H_2O, 5:2:1:1:0.5	TLC		

* Abbreviations:
SG steryl glycoside
MGG monogalactosyl-glycerol lipid
DGG digalactosyl-glycerol lipid
ASG acylated steryl glycoside

RESULTS AND DISCUSSION

In vivo Studies

Isolation and Identification of Steryl Glycosides (from MVA-2-^{14}C) in Maturing Pea Seeds by Injection or Diffusion at Three Developmental Stages

Two different approaches have been used to label the steryl glycosides of maturing pea seeds. The seeds, removed from the pod, were allowed to absorb MVA-2-^{14}C solution and then incubated in a moist chamber for the designated period. Under these conditions, biosynthesis from MVA-2-^{14}C is favored in the outer part of the pea seeds, i.e. seed coat. The other procedure was to inject MVA-2-^{14}C solution into the center of the seed with a micro-syringe followed by incubating them in the moist chamber. This way, the biosynthetic activity of the inner part of the seeds, i.e. embryo could be examined.

From each of three developmental stages, 11, 15 and 25 days after anthesis, two groups of seeds containing five seeds in a group, were used for the study. One group from each developmental stage was injected with MVA-2-^{14}C [1 μCi, 10.5 mCi/mmole] in 10 μl of water. The other groups of seeds were placed in small beakers containing the same amount of substrate. Seeds were maintained in a moist chamber at room temperature. After allowing the substrate to metabolize for 20 hours, all the seeds were washed free of unmetabolized MVA. The lipids were isolated by four

extractions with hot acetone in a tissue homogenizer. Thin layer chromatography of samples of the acetone extracts were run in solvent system A. Radioactivity at the origin was removed by eluting of the silicic acid scrapings from this zone with $CHCl_3$:MeOH (2:1, v/v). This polar material was subjected to acid hydrolysis.

Acid hydrolysis of polar material: The polar material was redissolved in 2 ml of 0.5 percent H_2SO_4 in 95 percent ethanol and heated under reflux for 20 hours. After neutralization with NaOH solution, the lipids were extracted with ether. The ether extract was subjected to thin-layer chromatography with solvent system A. Distribution of radioactivity was made by area measurements of the peaks traced by a chromatograph scanner. The results are shown in Table 2. Interestingly, although β-amyrin is known to be synthesized from MVA, in seeds that are 25 days after anthesis (5) no ^{14}C is found associated with β-amyrin on these chromatograms.

The aqueous phase from the acid hydrolysis after neutralization with aqueous NaOH was centrifuged to remove the precipitated salt. The supernatant was evaporated to small volume under high vacuum. A sample of this solution was chromatographed on paper in a solvent-saturated tank in solvent system F (Table 1). The products are visualized by spraying with p-anisidine followed by heating at 100°C for five minutes. The major product had R_f 0.17 identical with that of a D-glucose standard.

Table 2. In vivo incorporation of MVA-2-^{14}C into maturing pea seeds.

Sample		Days after anthesis	Method of incorporation of MVA-2-^{14}C*	% ^{14}C of R,S-MVA in acetone ext.	% ^{14}C of R,S-MVA in polar material	% ^{14}C of polar material in sterol after A.H.*+	% ^{14}C of R,S-MVA in sterol After A.H.+
A	1	11	Injection	45.4	7.2	33.3	2.4
	2	15	"	28.0	5.0	40.2	2.0
	3	25	"	5.4	1.4	9.9	0.1
B	1	11	Diffusion	39.5	3.8	30.4	1.2
	2	15	"	36.4	12.5	16.1	2.0
	3	25	"	33.0	8.0	3.4	0.3

* Majority of the rest of the label appeared at the origin of the chromagram sheet. This had been isolated and chromatographed with solvent system D and determined to be unhydrolyzed unknown polar material.

+ A.H., acid hydrolysis.

Isolation and Identification of Steryl Glycoside in Shoot Tissue of Germinating Pea Seedlings

Pea seeds were germinated in the dark for seven days. Five axial shoots were obtained above the cotyledons and incubated in a small vial with MVA-2-^{14}C solution (1 μCi, 10.5 mCi/mmole) in 150 μl of H_2O. After 12 hours of incubation, they were washed free of unmetabolized MVA-2-^{14}C and shredded in hot acetone in a pestle and mortar. The tissue was extracted four times with hot acetone.

Distribution of radioactivity was examined by thin layer chromatography in solvent system C (Table 1) using cholesteryl glucoside as a standard. The radioactive zone corresponding to the standard was scraped from the chromagram sheet and the material eluted from the silica gel with $CHCl_3$:MeOH (2:1, v/v). The ^{14}C content of this material was measured and the results are shown in Table 3.

Table 3. *In vivo* incorporation of MVA-2-^{14}C into the exised shoots of 7 day germinating etiolated pea seedlings.

Sample	% ^{14}C of R,S-MVA absorbed*
Acetone extract	6.5
Steryl glucoside	0.19

* 95.7 percent of the R,S-MVA added was absorbed by the tissue.

Although both maturing pea seeds and germinating pea shoot-root axes can be used to isolate sterol:UDP-glucose glucosyltransferase, the ease and speed with which large

amounts of tissue could be made available for enzyme isolation led to the use of etiolated pea seedlings rather than the immature seeds; the etiolated seedlings were grown for only seven days whereas to obtain the immature seeds as long as seventy days is required from the onset of germination to reach the desired stage of maturity.

In vitro Studies

Introduction

These studies are divided into two parts. A number of factors (pH, reaction temperature, buffer concentration, methanol concentration, seedling tissue and seedling age) were examined regarding their influence on glucosyltransferase activity, also the behavior of the product, steryl glucoside, with respect to its solubility in organic solvents and also its mobility in different solvent systems. For these exploratory studies an 18,000 g particulate fraction of pea seedling tissue homogenate was used and reaction conditions for steryl glucoside synthesis generally in accord with those described by Ongun and Mudd (55) and by Hou et al. (36).

With the findings based on these studies, the second part focuses on the most active glucosyltransferase preparation from the best source material: a 13,000-25,000 g particulate fraction from the shoot-root axes of seven day old etiolated pea seedlings. With this preparation, the

influence of metal ions, ATP and the role of phospholipid component on the enzyme activity was examined. Also presented are attempts to purify the enzyme.

Section I. Exploratory Studies: 1,000-18,000 g Fraction

Preparation of 18,000 g Fraction and Lipid Extraction Procedures

Roots of four to seven day germinated pea seedlings were obtained. After weighing them, they were homogenized in a Waring blender with one volume of 50 mM Tris·HCl (pH 8) containing 0.25 M sucrose for five seconds at low speed and fifteen seconds at high speed. The filtrate after passage of the crude homogenate through one layer of Miracloth was centrifuged at 1,000 g for five minutes. The supernatant was then centrifuged at 18,000 g for 15 minutes. The pellet was resuspended in a volume of buffer (the molarity of which is given for each experiment) equal to one-fifth of that from which it was obtained.

Zero point five ml of 18,000 g fraction (resuspended in 100 mM Tris·HCl, pH 8) was incubated with 0.6 μCi/10 μl high specific activity (275 μCi/μmole) UDP-glucose-^{14}C. The reaction was stopped and extracted with two volumes of $CHCl_3$:MeOH (2:1, v/v). The mixture was further extracted successively with three equal volumes of each of the solvents: (i) $CHCl_3$; (ii) EtOAc; and (iii) EtOAc at pH 2.

Aliquot of extracts from the different extraction procedures were counted. Radiochromatograms of the samples of the extracts after TLC with solvent system C showed that the chloroform extracts contained all the extractable lipid glucoside with R_f value corresponding to cholesteryl glucoside (Figure 1a), i.e., 0.39-0.40. Ethyl acetate extracted at least two more polar materials labelled with glucose-^{14}C (Figure 1b). Under acid conditions, more polar materials were extractable with ethyl acetate (Figure 1c). The total from these three extracts was 25.6 percent and the

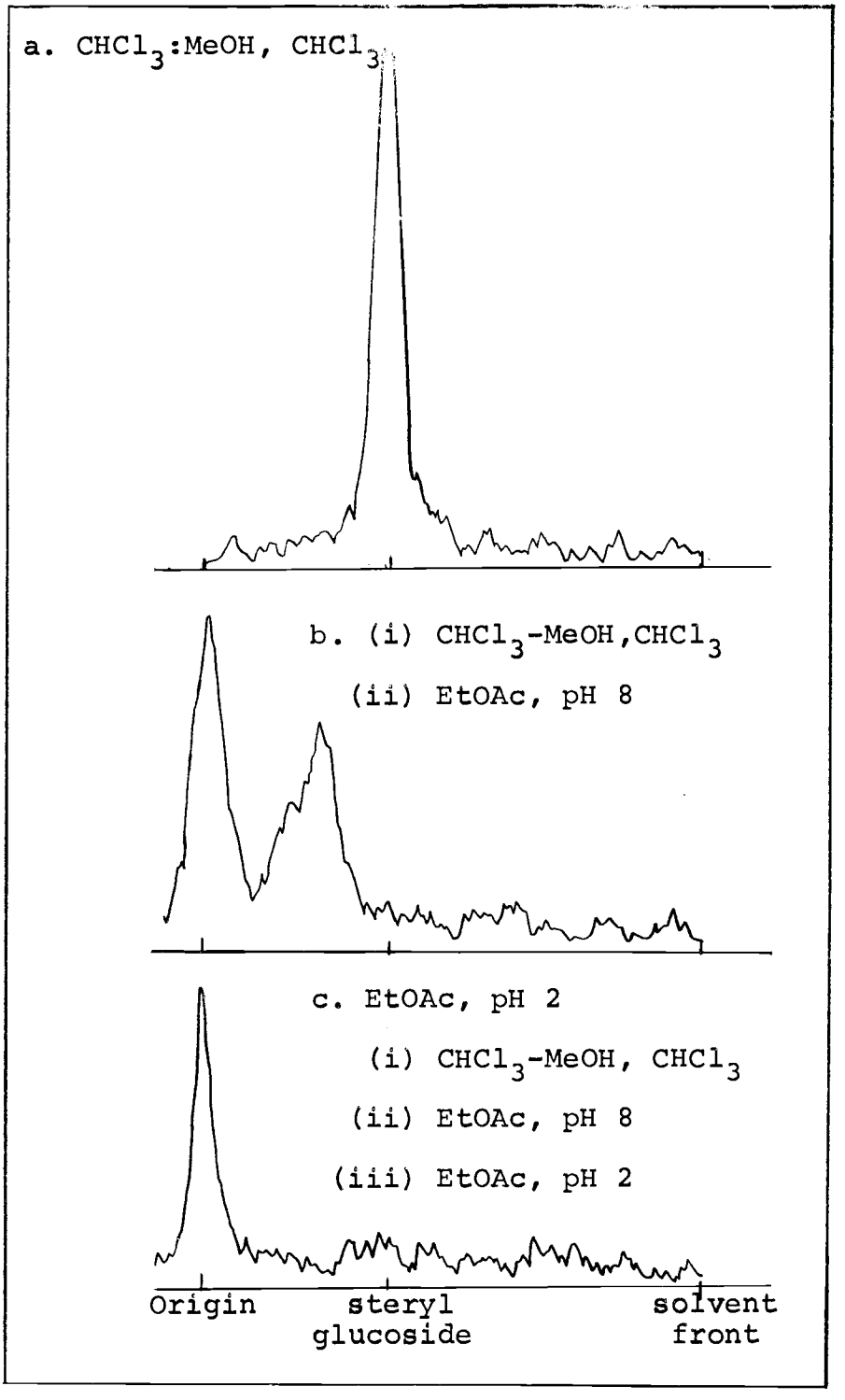


Figure 1. Thin layer chromatograms: different solvent extractions of the enzymatic reaction products.

relative proportion in each extract are shown in Table 4. $CHCl_3$:MeOH extract of the reaction mixture was also thin layer chromatographed with solvent systems D and E and the majority of the label resided at R_f values of 0.72-0.74 and 0.63-0.65, respectively, which corresponded to cholesteryl glucoside.

Table 4. Distribution of radioactivity in different solvent extracts.

Type of extractions	% of total ^{14}C recovered
$CHCl_3$	68.6
EtOAc	12.4
EtOAc at pH 2	19.0

Since the main interest of this study was steryl glucoside biosynthesis, all subsequent reactions were terminated and extracted with equal volumes of $CHCl_3$:MeOH (2:1, v/v) followed by two additional extractions with equal volumes of $CHCl_3$. The other polar lipid glucosides were not investigated further.

Some Factors Influencing the Activity of Sterol:UDP-Glucose Glucosyltransferase

1. Time Course of Glucose-^{14}C Incorporation into Lipid-Extractable Material: The reaction mixture was 1.33 mM each for ATP, Mg^{++} and UDP-glucose-^{14}C (S_A: 0.1 μCi/μmole) with 0.5 ml of the 18,000 g pellet fraction (resuspended in 100 mM Tris·HCl buffer (pH 8.0), giving a

concentration of 3.5 mg protein per ml) from 5-day old root axis in a final volume of 0.75 ml. The reactions were initiated by the addition of enzyme and were run for several time intervals ranging from 0 to 300 minutes at 30°C. The data are shown in Figure 2.

2. Effect of pH and Temperature: The variation of enzyme activity with change of pH and temperature was conducted using four day old root axis as the source of enzyme. The reaction conditions were the same as for 1) above with the exception that the incubations were stopped after 15 minutes. The buffer was 100 mM Tris·HCl in the pH range 7.0 to 9.0 at 30°C. The influence of temperature was conducted with pH 8.0 buffer in the temperature range 20°C to 50°C. Results are shown in Figures 3 and 4.

3. Influence of Methanol Concentration and Buffer Molarity on Sterol:UDP-glucose Glucosyltransferase Activity: The enzyme was from the four day old root axis. Methanol was added to a series of standard assay mixtures in 100 mM Tris·HCl (pH 8.0) buffer to give a series of concentrations ranging from 5 to 20 percent (v/v). The assay conditions were otherwise as for 2) above. Results are shown in Figure 5.

The same assay was also run in Tris·HCl buffer (pH 8.0) ranging in concentrations from 5 mM to 100 mM but without methanol. Results are shown in Figure 6.

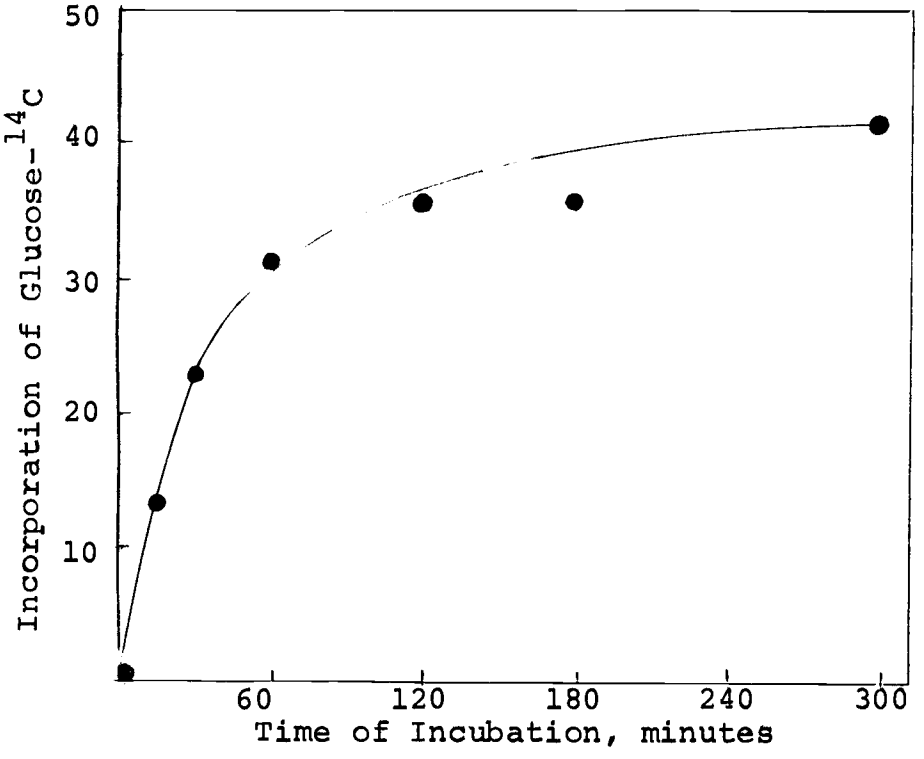


Figure 2. Time course of sterol:UDP-glucose glucosyltransferase (18,000 g fraction from five day-old root axis).

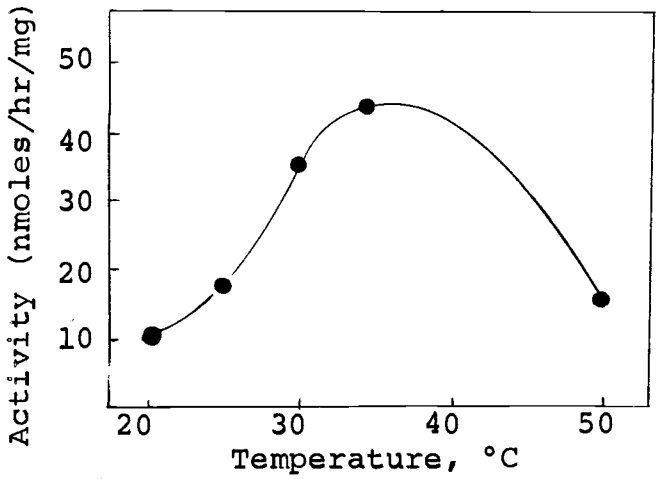


Figure 3. Variation of glucosyltransferase activity with temperature.

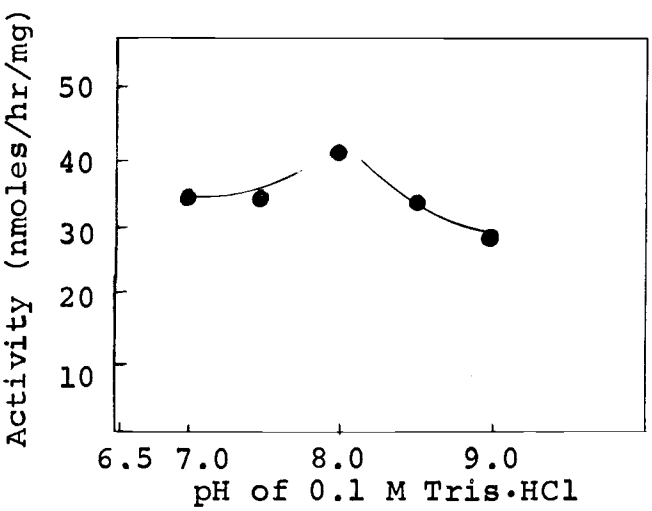


Figure 4. Variation of glucosyltransferase activity with different pH's of 0.1 M Tris·HCl buffer.

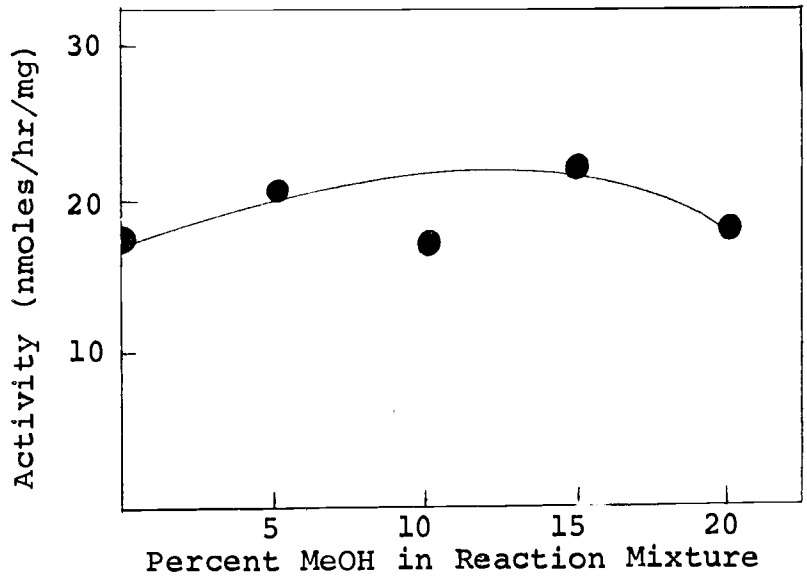


Figure 5. Influence of methanol content in the reaction mixture on glucosyltransferase activity.

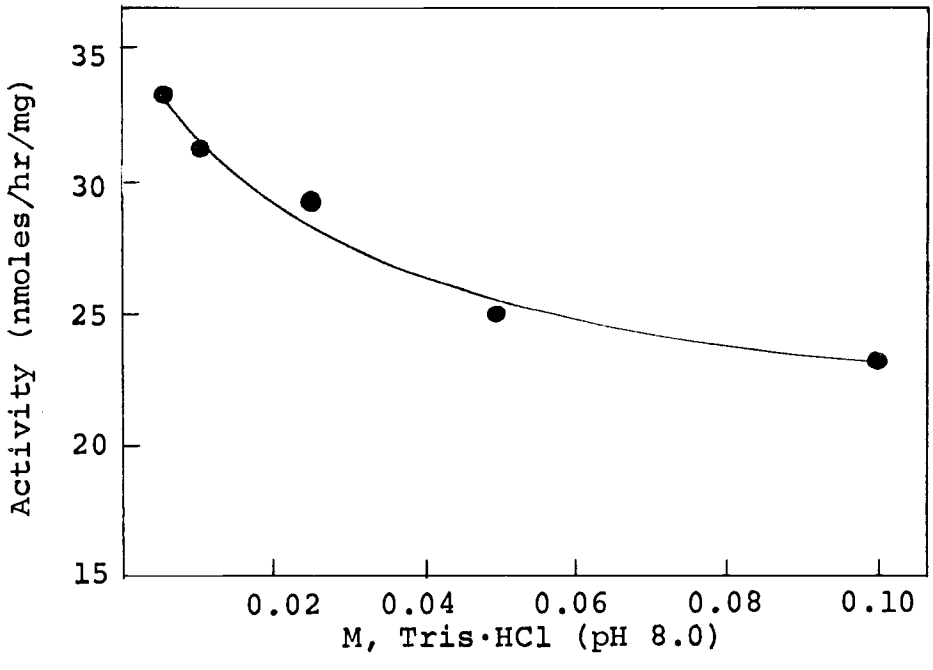


Figure 6. Buffer molarity and glucosyltransferase activity.

Results and Discussion

During the 300 minutes incubation at 30°C, the appearance of ^{14}C in the $CHCl_3$-MeOH extract increased linearly through the first 35 minutes of reaction time (Figure 2).

The enzyme reaction proceeded at an optimal rate between 30°C and 35°C. Sharp inhibition was observed at both extremes. The pH optimum was found to be at 8.0. This was also found by Hou et al. (36) for the microsomal enzyme from soybean and by Ongun and Mudd (55) for the pea-root mitochondrial enzyme when Tris·HCl buffer was used.

In the reactions in which methanol was present, there was a stimulation of activity of sterol:UDP-glucose glucosyltransferase over a broad range though the effect was erratic (Figure 5). Even at 20 percent of this alcohol, little inhibitory effect was observed. A stimulatory effect by ethanol was noticed by Esders and Light (24) in an investigation of the sterol:UDP-glucose glucosyltransferase of the yeast, Candida bogoriensis. Collins et al. (19), in the study of N-acetyl-glucosaminyl transferase, also found that when steroid was added into the reaction mixture in methanol, no inhibition was caused by the presence of this alcohol.

An increase in transferase activity of approximately 40 percent was observed as the buffer molarity decreased through the range of 100 to 5 mM (Figure 6). This observation was consistent with the stimulatory effect of methanol. In the presence of methanol, the effective ionic

strength of the buffer solution decreases. The influence of MeOH is probably more complex than this and probably involves an interaction with membrane lipid. An increase of enzyme activity in the presence of alcohol may be regarded as indicative of the lipoprotein character of the enzyme (31). MeOH is used in Section II of the *in vitro* studies where sterol was introduced into the assay medium.

In the following studies, the standard assay consisted of enzyme resuspended in 25 mM Tris·HCl (pH 8.0) buffer in order to maintain an effective buffering capacity of the reaction mixture without serious loss of activity. The other components of the mixture were as for the time course experiment. In addition, the reaction was stopped after 15 minutes.

Identity of the Endogenous Acceptor

Double labelling of the enzymatic product was accomplished by using an endogenous tritiated acceptor, labelled *in vivo* with MVA-5-^{3}H. Pea seedlings were grown for seven days. Eighteen hours before making the homogenate, ten roots were preincubated with 5 μC_i of MVA-5-^{3}H (S_A 22μCi/μmole). The tritium-labelled pea roots were mixed with unlabelled root to give a total 23 grams from which an 18,000 g pellet was obtained by the procedure described above. One percent (10.5×10^4 dpm) of the R,S-MVA-5-^{3}H was found to be incorporated into the 18,000 g pellet fraction after 18 hours

incubation by counting an aliquot of the pellet suspension in Aquasol. Enzymatic reactions were carried out with 0.5 ml of this ^{3}H-labelled preparation (1.3×10^{4} dpm) and ^{14}C-labelled UDP-glucose (8.4×10^{5} dpm, 1 mole) and with the remaining cofactors as for the previous experiment. The reaction was stopped after 15 minutes and the synthesized steryl glucoside was isolated and purified on TLC with solvent system C. The steryl glucoside was hydrolysed with acid and the ether extract chromatographed by TLC in solvent system A. Tritium was located by scintillation counting of sections of the chromagram sheet.

The $^{3}H/^{14}C$ ratio of the presumed steryl glucoside after purification on TLC was found to be 0.59 (1485 dpm ^{3}H/2522 dpm ^{14}C) with only 0.5 percent deviation in the ratio for two experiments. This value, however, is not meaningful to determine the molar ratio of the two moieties of the synthesized steryl glucoside since the size of the sterol pool is unknown. The endogenuous UDP-glucose available for steryl glucoside synthesis is negligible since samples from incubation of the ^{3}H-18,000 g pellet without UDP-glucose gave a very low ^{3}H count (40 dpm) after purification of the steryl glucoside. After acid hydrolysis of the sample, 47.4 percent of ^{3}H and 2.3 percent of ^{14}C was recovered in the ether extract. Distribution of tritium in this ether extract is shown in Figure 7. Most of the radioactivity was located in an area corresponding to a sitosterol standard. A minor

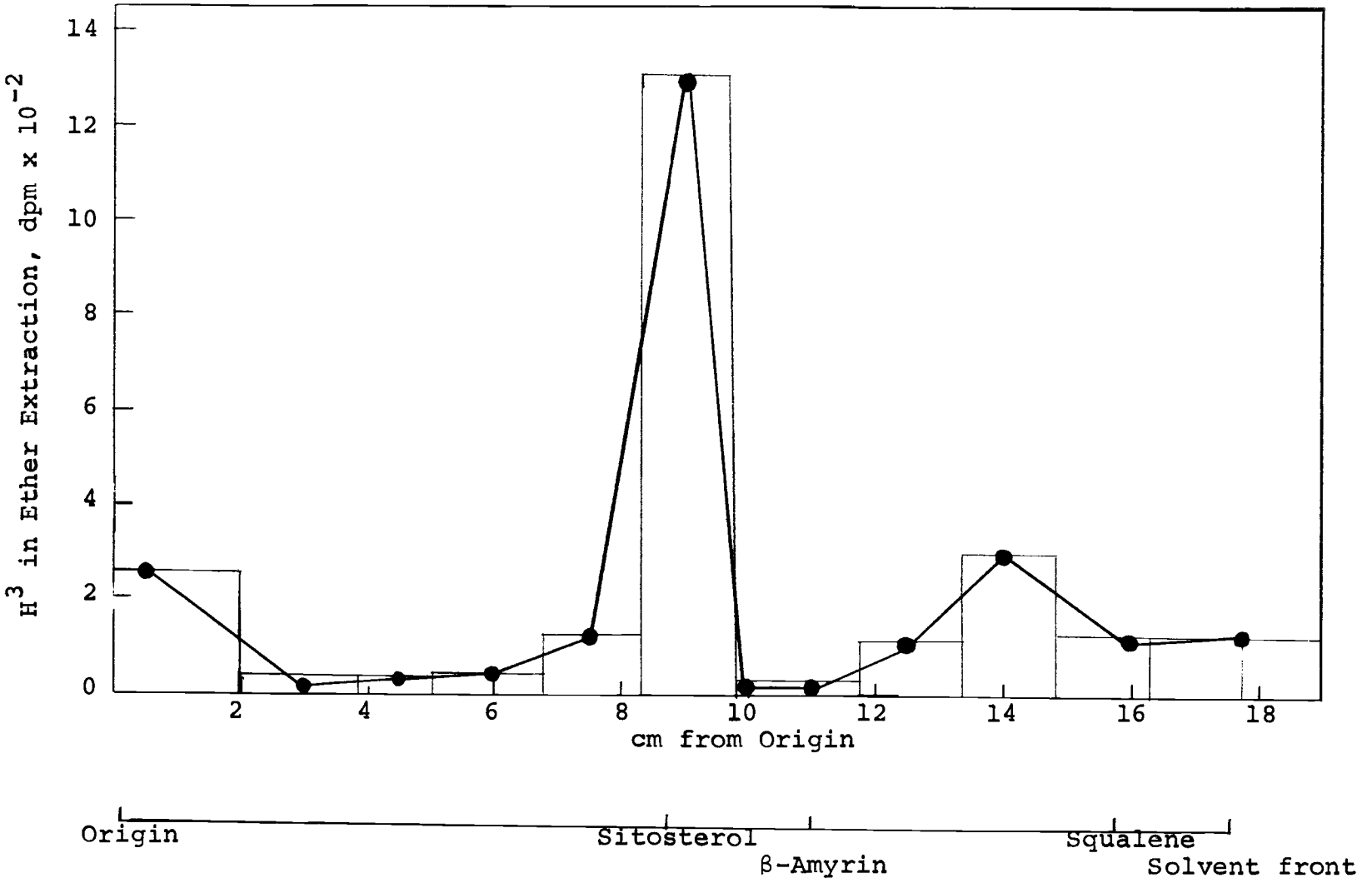


Figure 7. TLC:Ether extract of the acid-hydrolysis product of presumed steryl glycosides.

peak found with R_f slightly less than that for squalene was not identified.

Change of Enzyme Activity with Germination and Its Distribution within the Seedling

In separate experiments, it was found that the enzyme was equally distributed between the upper and lower halves of the shoot axis. Less than one-third of the transferase activity in the shoot axis was found in the cotyledons of four day-germinated pea seeds based on their specific activities (Table 5).

Table 5. Distribution of sterol:UDP-glucose glucosyltransferase activity in four day-old pea seedling tissues.

Tissue	Activity nmoles/hr/mg protein*	Relative Activity
Cotyledon	3.3	0.30
Root axis	7.7	0.69
Shoot axis	11.1	1.00
Upper part of shoot**	10.7	0.96
Lower part of shoot**	11.5	1.04

* Protein concentrations ranged from 3-13 mg/ml for the assay.

** Upper part of shoot includes shoot tip and 2 cm stem below the tip. Lower part of shoot represents stem of the shoot 2 cm from the tip.

Sterol:UDP-glucose glucosyltransferase activity was examined in cell-free extracts of the axis tissues of pea seedlings two to eleven days old. Protein concentrations ranged from 2-8.8 mg/ml for the assays. The higher concentrations were used for the least active preparations. The enzyme was most active in seven day-old seedlings.

Substantial activity was found from the fifth to the ninth day (Figure 8).

Shoot and root tissues were ground up separately in order to compare their glucosyltransferase content. It was found that the variation in the enzyme activity of the shoot paralleled that in the root during the germination period, although the shoot tissue always had higher specific activity than the root (Figure 8). Since the products found by the different axis tissues appeared identical, based upon their chromatographic behavior, the entire axis tissue (root and shoot) was used as enzyme source for all further studies.

Stability of the Enzyme Activity

The enzyme, sterol:UDP-glucose glucosyltransferase, has been found to be reasonably stable upon storage at -20°C. However, the activity is lost after refreezing the thawed enzyme. Interestingly, the activity of a fresh preparation almost doubled after freezing provided the activity comparisons were made in the presence of Mg^{++} (Table 6). Presumably, the freezing process physically disrupted the membrane and made the enzyme more available to substrates.

Table 6. Change of enzyme activity using Mg^{++} (1.33 mM) as cofactor after freezing and thawing.

	Activity nmoles/hr/mg protein	Relative Activity
Freshly prepared	14.6	1.00
2 hours freezing	25.2	1.73
2 days freezing	26.4	1.81
3 days freezing	20.4	1.39

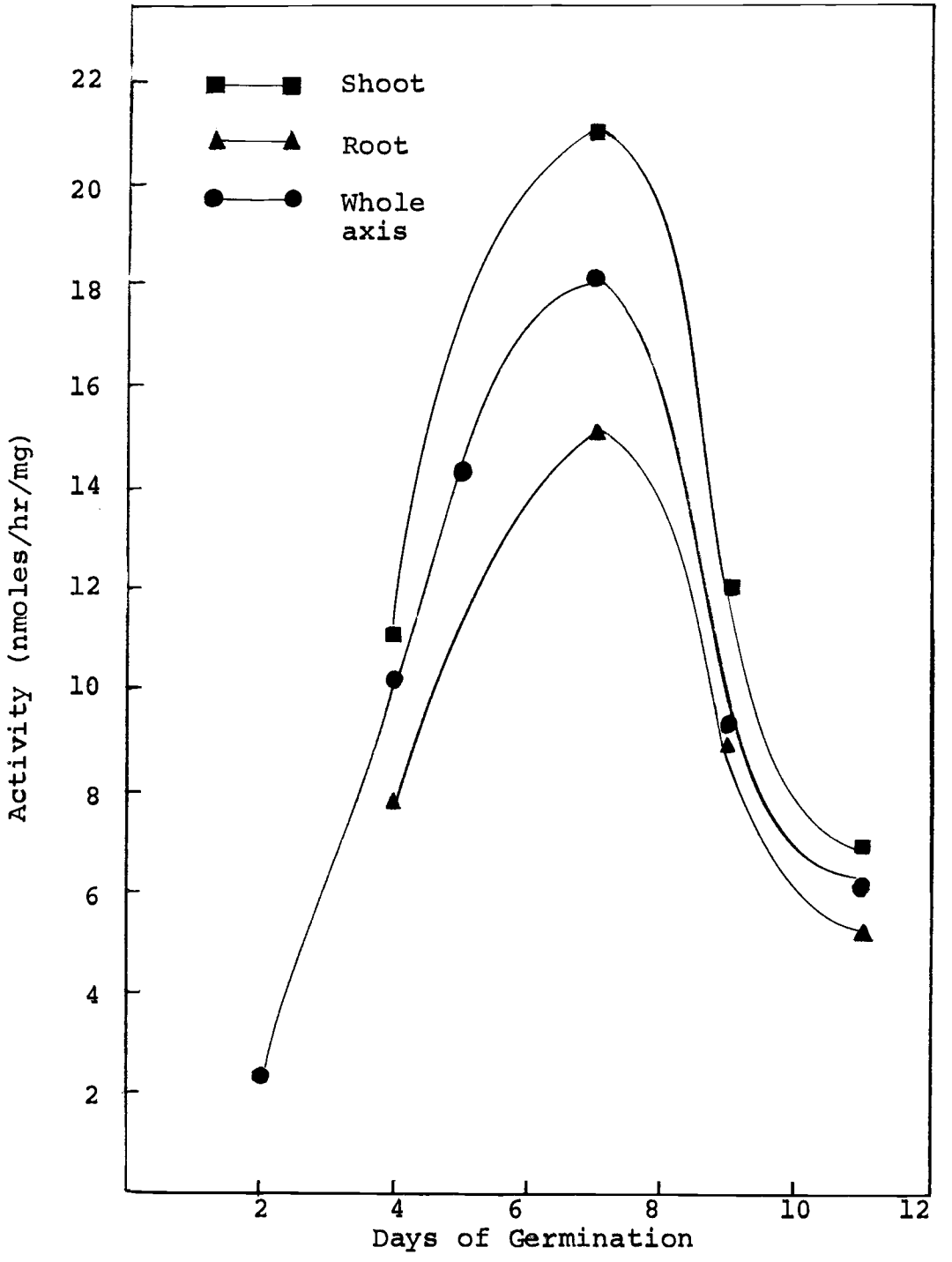


Figure 8. Change of glucosyltransferase activity with germination.

In the studies in section 2 when we routinely used calcium ion in the assay, this stimulation on freezing was no longer observed. No loss of activity was obtained for short period freezing and only 57 percent decrease in activity after three months storage at -20°C.

Localization of Sterol:UDP-glucose Glucosyltransferase in Pea Seedling Homogenate

According to Hou et al. (36), the enzyme for steryl glucoside synthesis from immature soy beans was located in the pellet fraction obtained between 10,000 g to 105,000 g. Ongun and Mudd (55) working with pea roots found the enzyme activity in the 1,000-18,000 g pellet. The distribution of sterol:UDP-glucose glucosyltransferase was made with seven day old pea shoot-root axes. An homogenate was obtained in 25 mM Tris·HCl buffer (pH 8.0) containing 0.4 M sucrose using 1.5 ml buffer for 1 mg of tissue. The use of 0.25 M sucrose as an osmoticum was more suitable for mammalian tissue and a change to 0.4 M concentration was more appropriate.

After filtration through one layer of Miracloth, the crude homogenate was subjected to differential centrifugation at the designated speed and the enzyme activity was assayed under the standard conditions after each centrifugation (Table 7). Experiments also showed that the highest glucosyltransferase activity was located in the lower spin pellet fractions.

Table 7. Sterol:UDP-glucose glucosyltransferase activity in fractions obtained by differential centrifugation of cell-free extracts of seven day-old pea shoot-root axes.

Speed of centrifugation	Time of centrifugation, min.	mg Protein per assay	Activity* nmoles/hr/mg	Relative Activity
0	0	2.0	3.8	1.00
1,000 g	5	5.0	1.4	0.37
6,000 g	15	4.5	10.9	2.87
15,000 g	15	2.0	30.2	7.97
20,000 g	15	1.3	35.6	9.34
50,000 g	30	2.0	6.9	1.81
105,000 g	30	1.3	6.3	1.66
105,000 g supernatant		1.0	0.3	0.08

* Reaction mixture containing 1.33 mM each of UDP-glucose-^{14}C (0.1 µCi), Mg^{++} and ATP and 0.5 ml enzyme to make up a final volume of 0.75 ml with 25 mM Tris·HCl buffer (pH 8.0). The reaction was carried out at 30°C for 15 minutes.

Glucan synthetase was also assayed in the different centrifugation fractions according to the method of Chambers and Elbein with some modifications (17). This and IPDase have been used as a marker enzyme for the Golgi apparatus (17, 65). The pellets from each centrifugation up to and including 20,000 g were suspended in 1 ml buffer containing 100 mM Tris·HCl, 4 mM EDTA and 1 mM DTT, and the remaining pellets were suspended in 0.5 ml of the same buffer. The reaction mixture contained 5 mM UDP-glucose-^{14}C (S_A:0.1 μC_i/ μmole), 20 mM $MgCl_2$, 10 mM cellobiose and 100 μl of the enzyme preparation. The final volume was made up to 200 μl with buffer and pH was adjusted to 8.0. The reaction was carried out in a hot water bath at 26-27°C for 15 minutes and was stopped by heating the reaction mixture in a boiling water bath for 5 minutes.

After the addition of 2 mg cellulose powder into the reaction mixture, it was extracted three times with 1 ml of hot water (approximately 95-100°C). Insoluble polysaccharides were removed and discarded by means of centrifugation at 25,000 g for 12 minutes at room temperature. Ten mg of Ficoll was added into the supernatant. The mixture was then combined with 8.4 ml of 95 percent EtOH to make the final concentration of EtOH approximately 75 percent in order to precipitate hot water-soluble polysaccharides. The pellet resulting from centrifugation at 25,000 g for 30 minutes was redissolved in seven drops of hot water. To this solution

Aquasol was added to obtain the ^{14}C content. Quenching was corrected by spiking the sample with ^{14}C-toluene standard.

The results (Table 8) indicated that a peak of glucan synthetase activity was found in the 15,000 g and 20,000 g pellet fractions, which corresponds to the peak of glucosyltransferase activity. This indicated that the glucosyltransferase may be associated with the Golgi apparatus. The high

Table 8. Glucan synthetase activity in fractions obtained by differential centrifugation of cell-free extracts of seven day-old seedlings.

Speed of centrifugation	Time of cent., min.	mg Protein per assay	Activity nmoles/hr/mg
1,000 g	5	0.7	121
6,000 g	15	0.5	108
15,000 g*	15	0.3	146
20,000 g	15	0.2	310
50,000 g	30	0.3	169
105,000 g	30	0.2	221
105,000 g	supernatant	0.2	338

* This is a minimum figure as a part of the preparation was accidentally lost.

glucan synthetase activity observed in the 105,000 g supernatant was unexpected. There is, however, a soluble plant glucan synthetase activity which uses ADP-glucose (61). Conceivably, glucose-^{14}C-1-phosphate derivable from UDP-glucose-^{14}C might undergo a transferase reaction with added ATP to give the ADP glucose-^{14}C donor in the 105,000 g supernatant.

Location of Endogenous Steryl Glucoside in Various Fractions of the Cell Free Extract

One ml sample of each fraction shown in Table 7 was repeatedly extracted with equal volumes of $CHCl_3$-MeOH (2:1, v/v). The evaporated extracts were analyzed qualitatively by thin layer chromatography with solvent system C using cholesteryl glucoside as the standard. Endogenous steryl glucoside was found to be associated only with the 15,000 g, 20,000 g and 50,000 g fractions.

Section II. Further Studies of the Sterol:UDP-glucose Glucosyltransferase: 13,000-25,000 g Fraction

Distribution of Sterol:UDP-glucose Glucosyltransferase

Based upon the observations made in Section I, it is clear that the physiologically active stage for steryl glucoside synthesis is at the seventh day of germination and the most active cell fraction is between 13,000-25,000 g centrifugation. Seven day old pea seedlings are homogenized in 25 mM Tris·HCl buffer (pH 8) which was 0.4 M in sucrose. The volume used was 1.5 times the seedling weight. The homogenate was obtained by blending the tissue for five seconds at low speed and 15 seconds at high speed in a Waring blender. The blended tissue was filtered through one layer of Miracloth to obtain the crude homogenate, which is then subjected to centrifugation at 1,000 g for five minutes, at 13,000 g for 15 minutes in a Sorvall centrifuge with an

HSG rotor when large amounts of enzyme were obtained, or in a Spinco Model L centrifuge with a 30 rotor when smaller amounts of enzyme were prepared. The supernatant was further centriguated at 25,000 g for 15 minutes. The 13,000 and 25,000 g were resuspended in 25 mM Tris·HCl. The data is shown in Table 9.

The most active fraction, that sedimenting between 13,000 and 25,000 g, also contains slightly more than one-half the total activity. The activity resident in the supernatant fraction may represent a combination of microsomal (82) and soluble glucosyltransferase activities (57). The 13,000-25,000 g fraction was used in all subsequent investigations of the enzyme.

The pellet obtained was routinely washed once with the buffer used for the homogenization and centrifuged again at 25,000 g for 15 minutes. All the centrifugations were carried out under refrigeration (0-4°C). The washed pellet was resuspended in 25 mM Tris·HCl, pH 8. The protein content was adjusted to 2.4 mg per ml and determined by the Biuret method (26). Typically, 100 gm of fresh plant tissue would give about 20 ml of enzyme preparation.

The resuspended 25,000 g pellet was stored in 3-5 ml volumes at -20°C. Under such conditions the preparation only lost ten percent of the sterol:UDP-glucose glucosyltransferase activity after a month. They were used only once after thawing.

Table 9. Isolation of sterol:UDP-glucose glucosyltransferase activity in seven day-old pea seedlings.

Centrifugation fraction (g)	Volume (ml)	Protein (mg/ml)	Specific activity (nmoles/hr/mg)	Relative activity	Total Activity units (nmoles/hr)
1,000 supernatant	395	6.20	4.5	1.00	11,030
13,000	20	5.94	15.8	3.51	1,880
25,000	20	5.75	49.4	10.98	5,680
Supernatant	385	5.48	2.5	0.54	5,170

* Reaction mixtures contained 1 µmole each of Mg^{2+} and ATP; 0.5 µmoles of UDP-glucose-[U-^{14}C](0.05 µCi); 90 nmoles of sitosterol in 37 µl of MeOH, 0.5 ml enzyme extract (containing 1 mg protein) and 16.5 µmoles Tris·HCl buffer in a total volume of 0.75 ml. The pH was 8.0; the reaction time was 15 minutes and the temperature was 30°C.

Enzyme Properties

Metal Ion Requirement of Glucosyltransferase

Several metal ions were tested for their effect on the enzyme activity. The enzyme activity in the 25,000 g and the 105,000 g pellets fractions was examined. The results are shown in Table 10.

In Table 10 is shown the influence of several ions at two different concentrations on the sterol:UDP-glucose glucosyltransferase activity. It is evident that the transferase does not require the addition of metal ion in order to function. However, Ca^{2+} is slightly stimulatory at the low concentration and more so at the higher concentration. The stimulation by Ca^{2+} is supported by the inhibition of the enzyme activity in the presence of EGTA. The binding of Ca^{2+} by EGTA is six orders of magnitude greater than that for Mg^{2+} (52). Interestingly, in column I of Table 10 the inhibition by EDTA exceeds that by EGTA and suggests the possibility that Mg^{2+} may also act in a stimulatory fashion. This is found to be the case at the higher concentration of Mg^{2+}. The activity that persists in the presence of the high concentration of either EGTA or EDTA might suggest the presence of two different glucosyltransferases: one dependent and the other independent of metal ion stimulation. Conceivably,

Table 10. Effect of metal ions on sterol:UDP-glucose glucosyltransferase.

Fraction	Addition	Relative Activity* I 1.3 mM adduct	Relative Activity* II 13 mM adduct
25,000 g	None	1.00	1.00
	Boiled enzyme	0.02	
	$MgCl_2$	1.02	1.57
	$MnCl_2$	0.97	0.57
	$CaCl_2$	1.11	1.67
	$BaCl_2$	1.02	1.33
	$ZnCl_2$	0.11	0.02
	EDTA	0.36	0.33
	EGTA	0.58	0.39
105,000 g	None	1.00	
	Mg^{++}	1.13	
	Ca^{++}	1.16	

* Reaction mixtures contained 1 µmole of ATP, either 1 µmole or 10 µmole of the metal ion, 0.5 µmoles UDP-glucose-[U-^{14}C](0.05 µCi), 90 nmoles of sitosterol in 37 µl of MeOH, 0.5 ml enzyme extract and 16.5 µmoles Tris·HCl buffer in a total volume of 0.75 ml. The pH was 8.0; the reaction time 15 minutes and the temperature 30°C. The activities of the samples with no addition were 90 and 33 nmoles/hr/mg protein for I and II, respectively. These were two different preparations of the enzyme.

this difference in properties might also be displayed by one transferase present in two different environments in the membrane. Ba^{2+} is a member of the same group (IIa) of elements as Ca^{2+} in the Periodic Table and its stimulatory effect may be a consequence of this fact. The inhibition by Mn^{2+} and Zn^{2+} has been reported for the soluble glucosyltransferase of germinating Phaseolus aureus seeds (82). The extent of inhibition by these two ions on the particulate transferase of the pea seedling system however is far greater.

As previously shown, the 25,000 g fraction is much more active than the microsomal fraction and the stimulation was much more significant for the 25,000 g preparation than for the microsomal enzyme.

Usually, activity one finds is that shown in II. In column I an extremely active glucosyltransferase was found thus the significance of activation by metal ions may be attributable to the preparation at the state of which normally obtained only attributed by the addition of Mg^{++} and Ca^{++}.

Optimal Magnesium and Calcium Concentration for Glucosyltransferase Activity

The concentration of calcium ion in cytosol or free cytoplasm of animal cells is in the range of 10^{-5} to 10^{-8} M (64). In plants, its concentration is around 5,000 ppm of dry weight,which would approximate to 12 mM on a fresh

weight basis. The physiological concentration is usually taken as appropriate for *in vitro* studies, but large variations may always occur.

The influence of a range of calcium concentrations from 0.66 to 133 mM on the transferase activity was examined in the presence of 1.33 mM ATP on the 25,000 g enzyme preparation. A similar concentration range was used for the study of magnesium. The results are shown in Figure 9.

Calcium ion stimulates the enzyme activity over a very broad range with little inhibition evident at a concentration as high as 133 mM. The optimal activity was observed at 13.3 mM and this concentration is used in all subsequent assays.

The influence of magnesium ion on the enzyme activity was similar up to about 40 mM but consistent with the data in Table 10, its effect is as great as that for Ca^{++}.

Optimal ATP Concentration

In preliminary studies with a crude cell-free preparation of root axis and with magnesium ion providing the metal ion requirement, ATP was found almost to double the incorporation of glucose from UDP-glucose into lipid (Table 11). A stimulation of ATP was also found by Hou *et al.* (36) using an immature soy bean seed preparation and also by Bush and Grunwald (14) using tobacco seedlings.

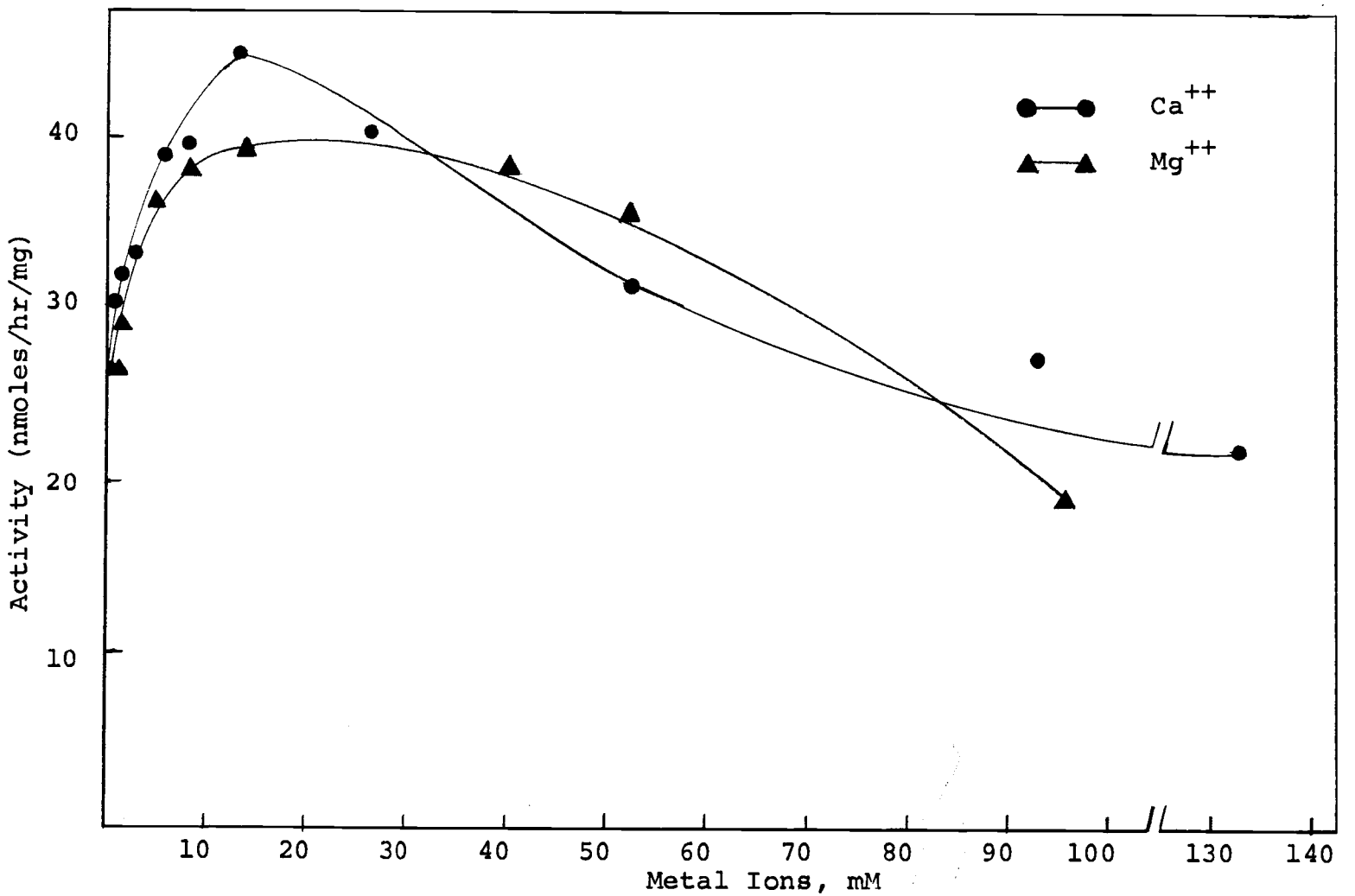


Figure 9. Influence of Ca^{++} and Mg^{++} concentrations on glucosyltransferase activity.

Table 11. ATP stimulation of glucosyltransferase activity on the 1,000-18,000 g pellet.

Sample	Activity nmoles/hr/mg	Ratio
Enzyme + 1.33 mM Mg^{++} + 1.33 mM ATP	14.9	1.00
Enzyme + 1.33 mM Mg^{++}	7.9	0.53

The effect of ATP was also investigated over a range of concentrations using the 25,000 g enzyme fraction and again allowing Mg^{++} to fill the metal ion requirement. Figure 10 shows ATP provides a slight stimulation of the transferase activity over the range 0.65 mM to 4 mM, but at 13.3 mM the activity is essentially lost. Also shown in Figure 11 is the influence of ATP using 13.3 mM Mg, and again substantial stimulation is shown at low ATP concentrations. Slight inhibition of the transferase activity was observed at 4 and 5.3 mM ATP. Complete loss of transferase activity was obtained at ATP concentrations of 7.3 mM and above.

The influence of ATP on transferase activity in the presence of Ca^{++} is shown in Figure 12, although some stimulation of transferase activity was observed in the presence of less than 0.67 mM ATP. Severe inhibition of the enzyme activity occurred with ATP concentrations higher than 1 mM. It has been found, however, that the stimulation has not been consistently reproducible. The degree of stimulation varied from preparation to preparation. This may be a consequence of the fact that the enzyme is membrane bound

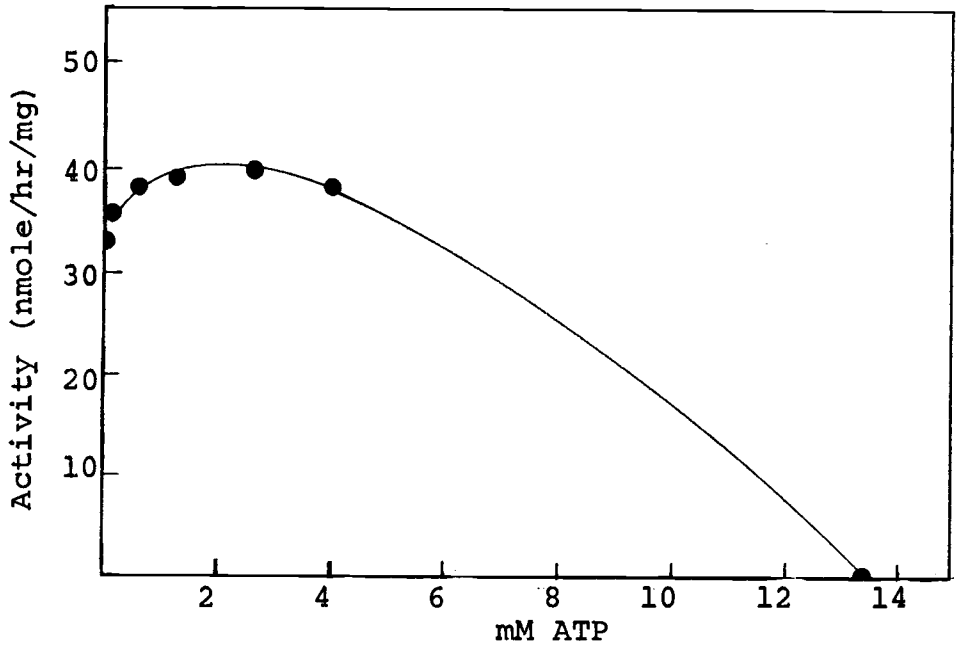


Figure 10. Effect of ATP on glucosyltransferase activity of 25,000 g fraction with 1.33 mM Mg^{++} as cofactor.

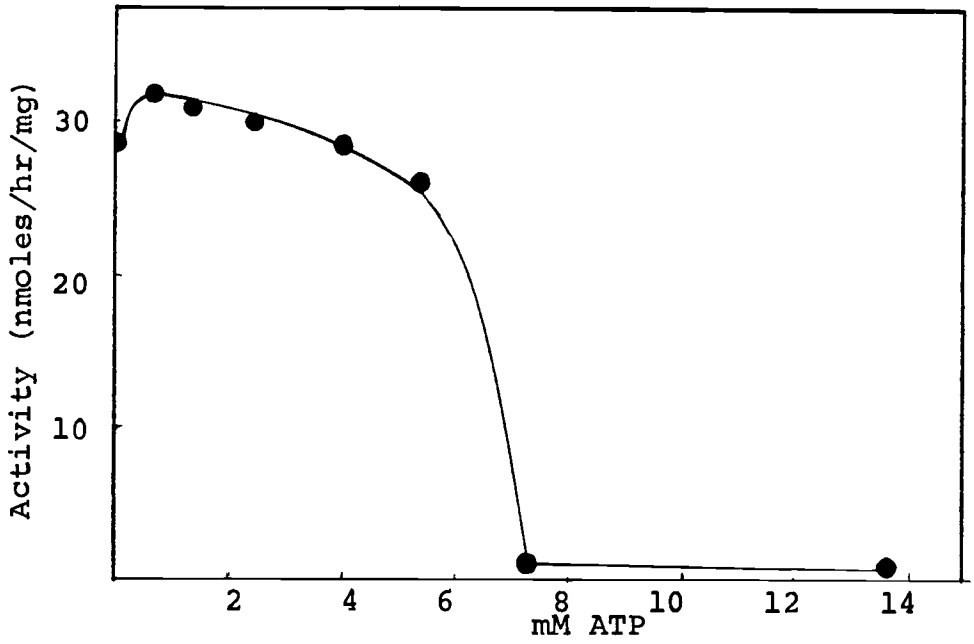


Figure 11. Effect of ATP on glucosyltransferase activity of 25,000 g fraction with 13.3 mM Mg^{++} as cofactor.

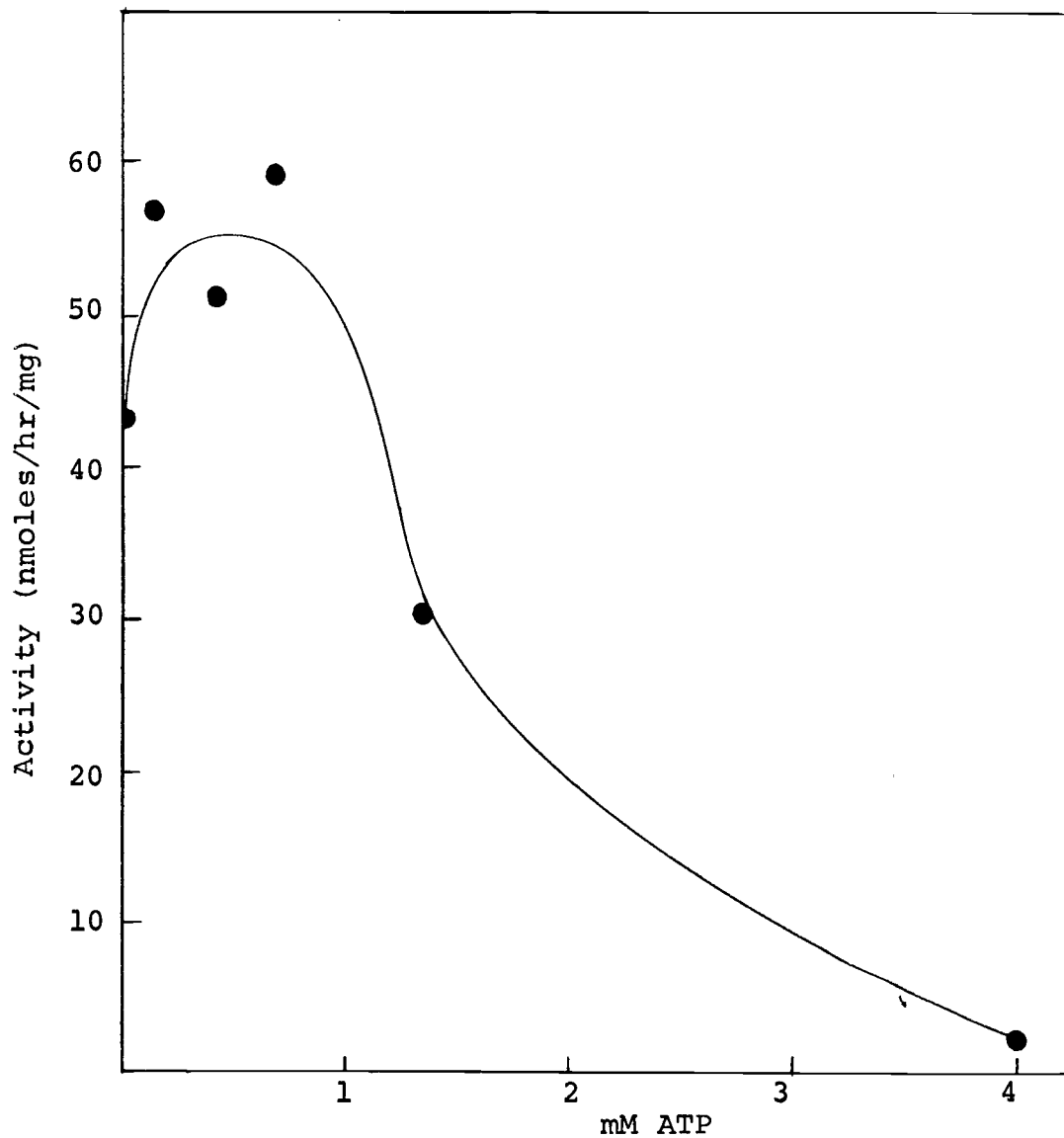


Figure 12. Effect of ATP on glucosyltransferase activity of 25,000 g fraction with 13.3 mM Ca^{++} and sitosterol in MeOH. (The final MeOH concentration in the reaction mixture is five percent.)

and that the degree of fragmentation of the membrane might vary from preparation to preparation.

From the mechanism of the enzymatic reaction studied Equation (1), no energy requirement appears to be necessary.

$$\text{UDP - glucose} + \text{sterol} \rightleftarrows \text{UDP} + \text{steryl glucoside} \quad (1)$$

Therefore, the stimulation of glucosyl transferase at low ATP concentration was left as an open question at this juncture.

Exogenous Sterol Acceptor

Sterol is a co-substrate of the enzyme reaction. Therefore, to examine the influence of sterol to the reaction was necessary. The result is shown in Table 12. It is clear that no severe inhibition was observed when 100 nmoles

Table 12. Effect of addition of sterol on sterol:UDP-glucose glucosyltransferase activity.

Addition	Relative Activity**
0	1.00
37 µl MeOH	1.75
50 nmoles sitosterol*	1.73
100 " "	1.69
150 " "	1.60
200 " "	1.65
250 " "	1.48

* Sitesterol was added into the reaction mixture in 37 µl MeOH. Other cofactors used were 0.67 mM ATP, 13.3 mM Ca^{++} and 0.67 mM UDPG. Reaction was carried out at 30°C for 15 minutes.

** The activity of the sample with no addition was 55.5 nmoles/hr/mg protein.

of sitosterol was added. From the activity of some of the preparations, it is known that as much as 30 nmoles (12 µg) of sterol is glucosylated in the course of reaction. One routinely added 90 nmoles to the incubation to avoid sterol limiting the reaction. In a similar experiment, stimulation has been noticed. Some sort of variation from different preparations was observed with ATP effect.

Time-Course and Substrate Saturation Level of the 25,000 g Fraction

Based upon previous findings, the standard assay mixture is 0.5 ml of enzyme preparation containing 1.2 mg protein, 0.67 mM ATP, 13.3 mM Ca^{++} ($CaCl_2$), and the substrates were 0.67 mM UDP-glucose-^{14}C (specific activity: 0.1 mCi/mmole) and 90 nmole sitosterol dissolved in 37 µl of MeOH. The final volume of the reaction mixture was adjusted to 750 µl with the 25 mM Tris.HCl buffer, pH 8.

A time-course experiment was carried out in one large test tube containing a reaction mixture scaled up six-fold. 0.75 ml of the reaction mixture was taken out at 1, 5, 10, 20, 40, and 80 minutes of incubation at 30°C. The results are shown in Figure 13. Linear incorporation was maintained in excess of twenty minutes, which was used for subsequent assays.

The influence of protein concentration in the incorporation of glucose is shown in Figure 13a. In the range of 0.5 to 1.7 mg of protein per assay volume, the

incorporation is linear. The deviation from linearity at low protein concentration may be a consequence of dissociation at high dilution. The deviation at concentrations in excess of 1.7 mg of protein per assay may be a result of a favorable aggregation of the membrane. Alternatively, an enhanced turnover of steryl glucoside may occur through acylation which is made favorable by the presence of a critical concentration of acyl CoA at high concentration of the 25,000 g fraction.

For the substrate saturation experiment, the reaction mixture was made as previously with varying UDP-glucose concentrations from 0.67 to 10.0 mM. The result (Figure 14)

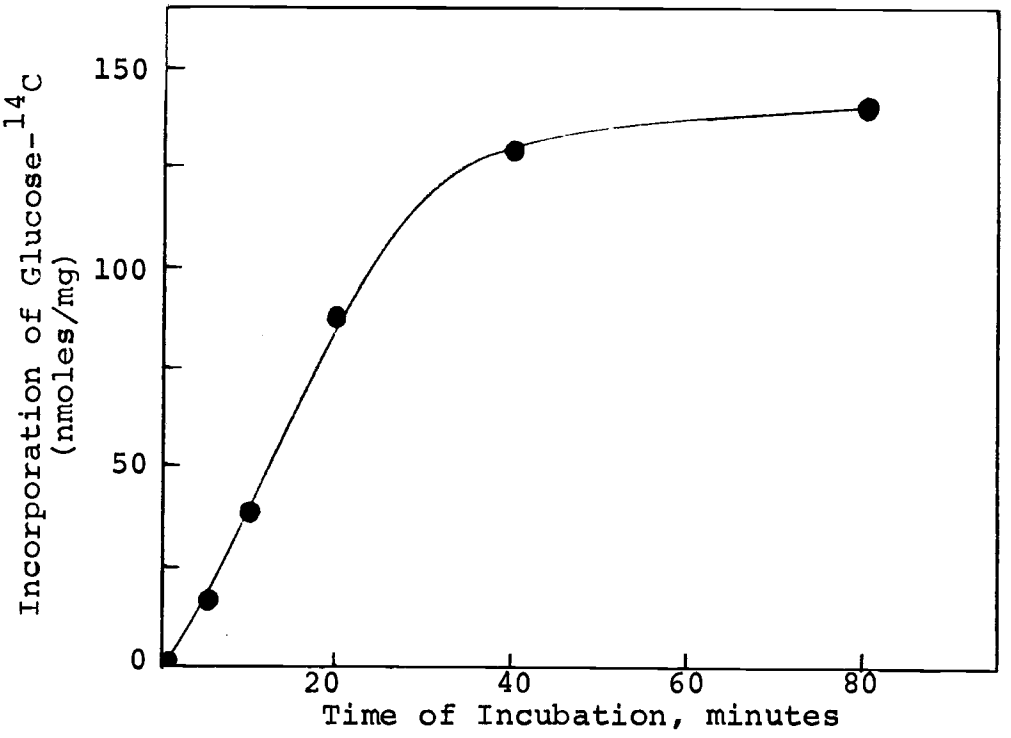


Figure 13. Time-course of glucosyltransferase activity of the 25,000 g fraction.

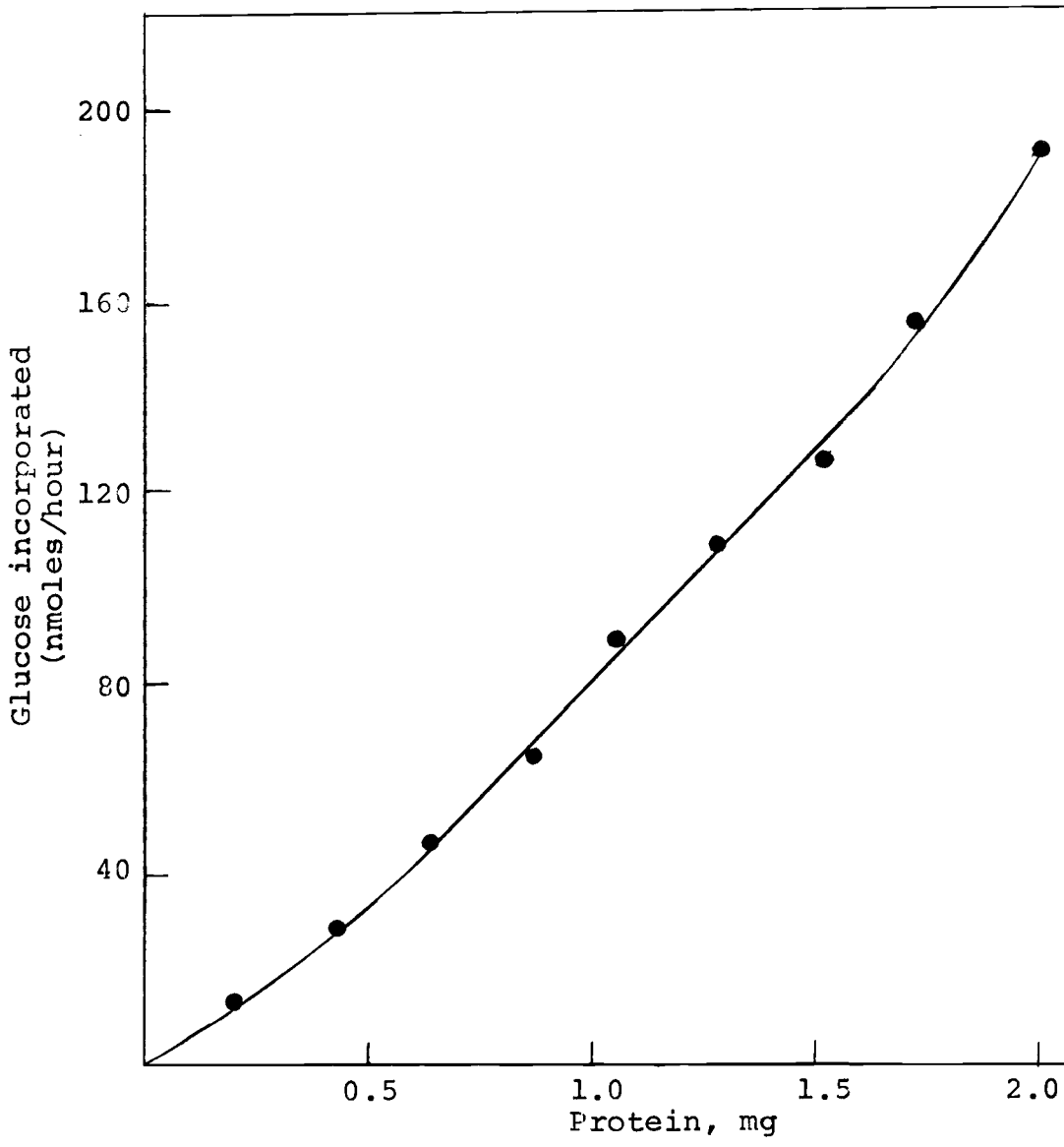


Figure 13a. Influence of the 25,000 g protein on the incorporation of glucose-[^{14}C] into lipid.

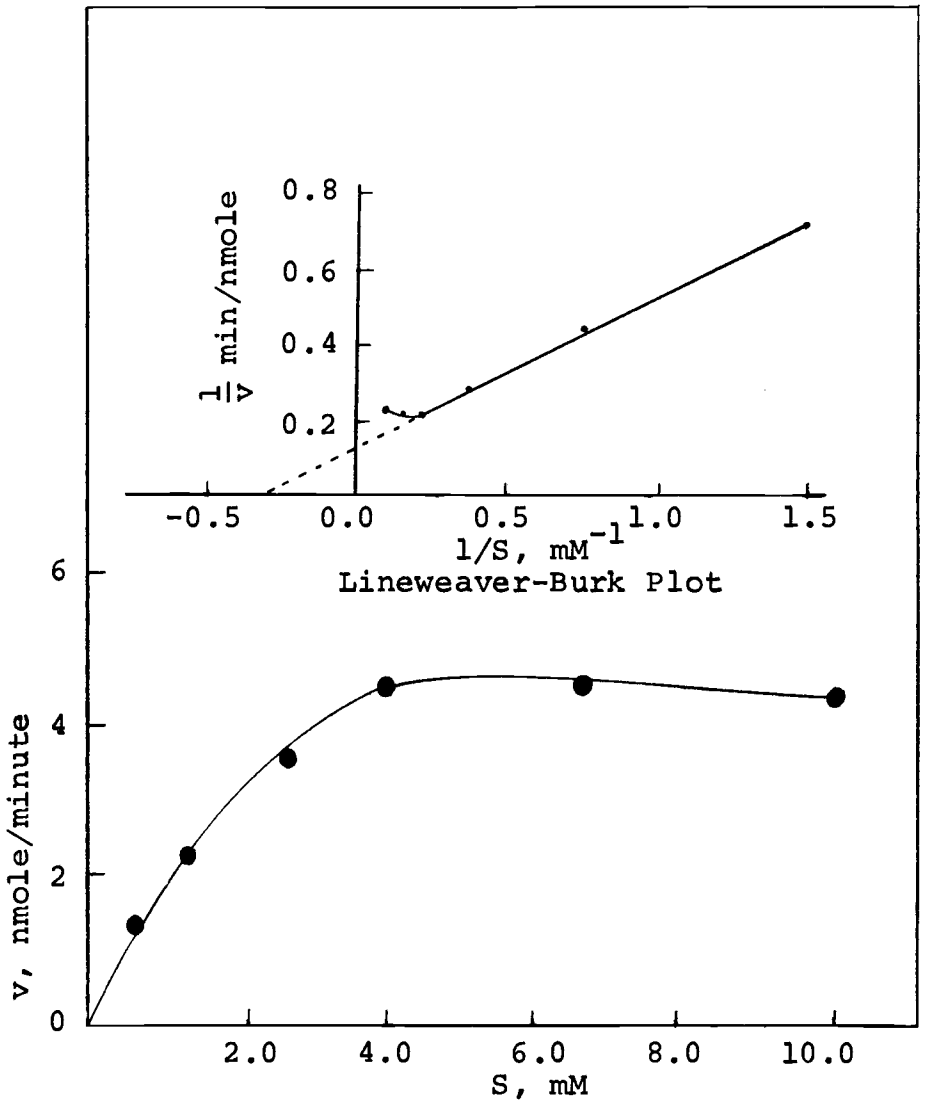


Figure 14. Substrate saturation curve of glucosyltransferase activity of the 25,000 g fraction.

suggests that the enzyme activity is inhibited at substrate concentration higher than 4 mM. Extrapolation from a Lineweaver-Burk plot gave a value for Km of 3.33 mM and V_{max} is 8 nmole per minute.

Effect of Cofactors on the Glucosyltransferase Activity

After the optimal conditions for the enzyme reaction were established, the influence of each individual cofactor on the enzyme reaction was examined by subtracting one cofactor at a time from a standard assay mixture, and also by addition to another enzyme preparation of one cofactor at a time to give its final concentration of that cofactor in the standard assay. The results are shown in Table 13.

Table 13. Effect of cofactors on sterol:UDP-glucose glucosyltransferase activity.

Reaction Mixture+	Activity nmoles/hr/mg	Relative Activity
Complete	68.5	1.00
with boiled enzyme	1.0	0.01
-Ca^{++}	29.2	0.43
-ATP	68.8	1.01
-MeOH*	60.3	0.88
-sitosterol	69.4	1.01
Enzyme preparation	21.0	1.00
+Ca^{++}	50.6	2.41
+ATP	33.8	1.61
+sitosterol/MeOH	37.2	1.77

* Sitosterol was dispersed into the reaction mixture by sonication.
+ The assay mixtures were made to the standard volume by the addition of buffer.

It is clear that calcium ion is the most important co-factor for the glucosyltransferase activity and that MeOH is also stimulatory. As previously mentioned, the influence of ATP is frequently variable.

Glucosyl Donor Specificity of the Glucosyltransferase

In the case of starch synthesis in several plants, E. Recondo and L. F. Leloir (67) have reported that ADP-glucose is a glucosyl donor far superior to UDP-glucose. In more recent studies (84), TDP-glucose and GDP-glucose have been shown to act as glucosyl donors in certain α-1.4-glucan-synthesizing systems. However, ADP-glucose was the superior glucosyl donor in all starch grain systems.

In order to determine the preference among the glucosyl donors, labelled UDP-glucose was used in the standard assay reaction mixture in the presence of increasing amounts (0.1 to 3.0 μmoles) of each of the unlabelled nucleoside diphosphoglucoses. If the added unlabelled nucleoside diphosoglucose acts as an effective donor of glucose, it will compete with the labelled UDP-glucose and therefore, a decrease in the incorporation of ^{14}C into the chloroform extract will be observed as the concentration of competing donor increases. In a control series, unlabelled UDP-glucose was added over the same concentration range. In the absence of any competing effect the same amount of ^{14}C should be found in the lipid product in the experimental series as in the controls. The results are shown in Figure 15.

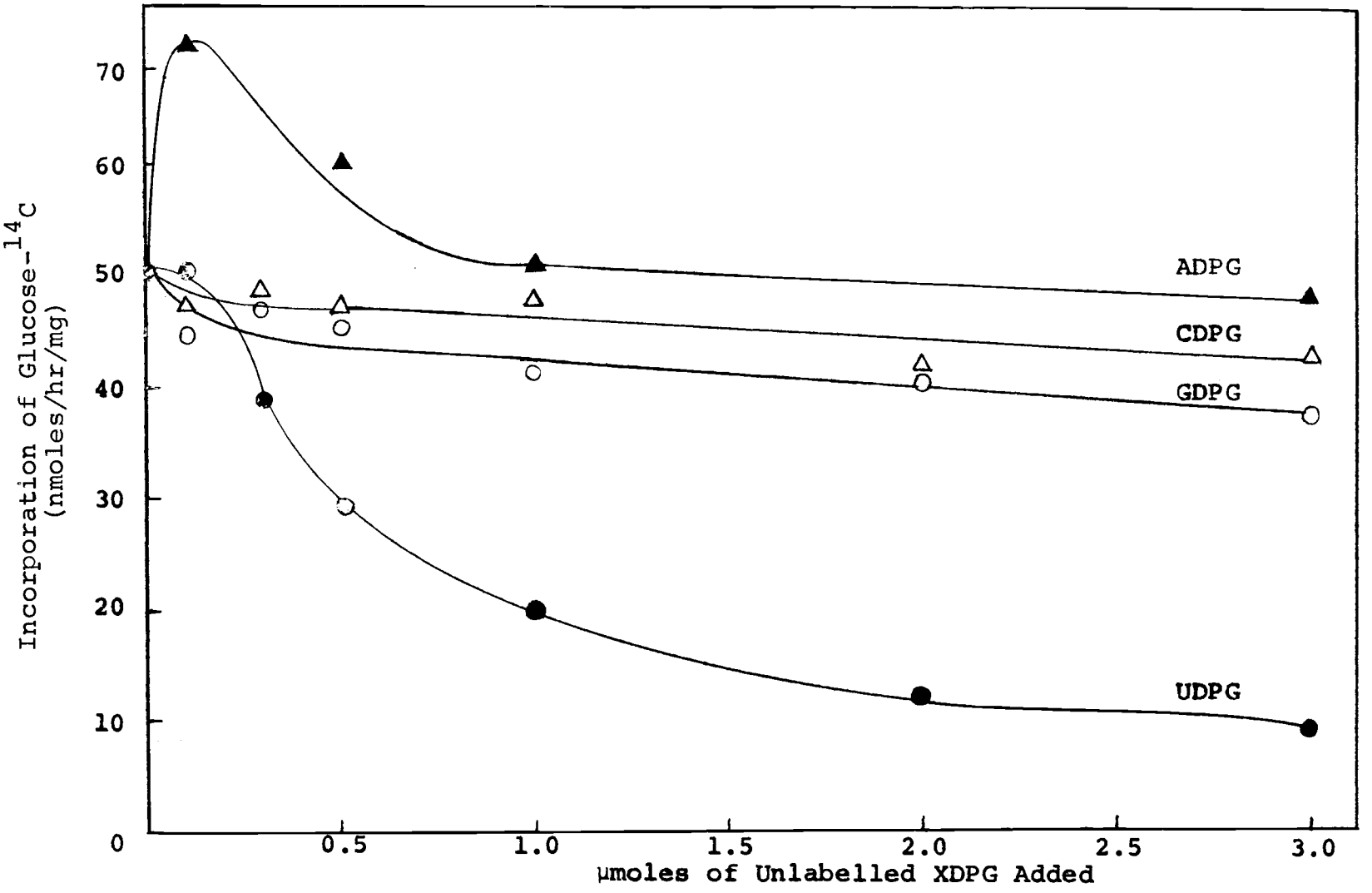


Figure 15. A standard reaction mixture containing labelled UDPG (0.5 µmole) and unlabelled glucosyl donors.

Of the three nucleoside diphosphoglucoses used, CDP-glucose and GDP-glucose have very little effect. No more than ten percent inhibition of the enzyme activity is observed when they are present in concentration six times that of the labelled UDP-glucose. Clearly, they are ineffective donors of glucose as compared with UDP-glucose. ADP-glucose, on the other hand, behaves quite differently. Wide fluctuations occur at concentrations up to 0.67 mM (ADP-glucose/UDP-glucose $\leq$ 1) with up to a 50 percent enhancement of glucose-^{14}C into the $CHCl_3$-MeOH extract. It appears that UDP glucose is made more available to the transferase, possibly because an enzyme competing with the transferase for UDP-glucose will preferentially accept ADP-glucose (e.g., glucan synthetase which is known to be present and will accept UDPG as a substrate). Consequently, under the first order condition used for measurement of glucosyltransferase activity, the concentration of UDP-glucose available for the glucosyltransferase is higher in the presence of ADP-glucose than in its absence. At higher concentrations of ADP-glucose (ADP-glucose/UDP-glucose >> 1) it acts as an inhibitor of the sterol:UDP-glucose glucosyltransferase.

Decline of Enzyme Activity during the Time-Course Study

Inspection of the time-course curve (Figure 13) reveals that the rate of incorporation of glucose into lipid

declines to approximately one-half the initial rate after 30 minutes, and approximately one-twentieth after 60 minutes. At this stage only ten percent of the available substrate (UDP-glucose) has been utilized. Similar observations have been made by Hou et al. (36), Ongun and Mudd (55) and Bush and Grunwald (14). Reasons for this decline might be that products of the reaction are inhibitors; that UDP-glucose is being used in competing reactions (e.g., polysaccharide, other glycolipids and glycoprotein synthesis), or that denaturation of the enzyme occurs during the assay period.

The immediate products of the enzymatic reaction are steryl glucoside and UDP. The latter has been found to be an inhibitor of glucosyltransferase activity: Hou et al. (36) found that at one-twentieth concentration of UDP-glucose there was a 50 percent inhibition by UDP.

1. Effect of UDP on the Enzyme Reaction

UDP was prepared in 25 mM Tris·HCl (pH 8.0) solution at a concentration of 0.02 moles per 10 µl. The transferase was assayed using 0.67 mM of UDP-glucose and UDP ranging from zero to 0.67 mM. The results are shown in Figure 16. It was found that only when equal amounts of UDP and UDP-glucose were present that there was a 50 percent inhibition of the enzyme activity. The difference between these results and those of Hou et al. (36) may be attributed

to the fact that a different plant tissue and also a different cell fraction were used. Thus, the 25,000 g fraction contains IDPase which may act on UDP. This activity is not known to be present in Hou's soybean preparation.

The 40 percent loss of activity at 0.067 mM UDP clearly must be a contributing factor to the decline of activity in the time course experiment. However, the 95 percent loss of activity found at the end of that experiment (Figure 13), even if all of the UDP glucose had been transformed to give UDP, cannot be accounted for by such an inhibition.

2. Effect of Steryl Glucoside on the Glucosyltransferase Activity

Another product of the enzymatic reaction is steryl glucoside. After a 30-minute incubation of the standard assay mixture, it can be calculated that 45 nmoles of steryl glucosides are formed. A suspension of cholesteryl-β-glucoside containing 1 μmole / ml was made by sonication in 25 mM Tris·HCl buffer (pH 8.0). Ten, twenty, thirty and fifty μl of this suspension was added into the standard reaction mixture. The influence on the incorporation of ^{14}C into lipid is shown in Figure 17.

Cholesteryl glucoside inhibits the transferase activity 17.6 percent when 50 nmoles are present at the beginning of the reaction. Such a degree of inhibition by exogenous cholesteryl glucoside would not account for the decrease of enzyme activity after 20 minutes of incubation. However,

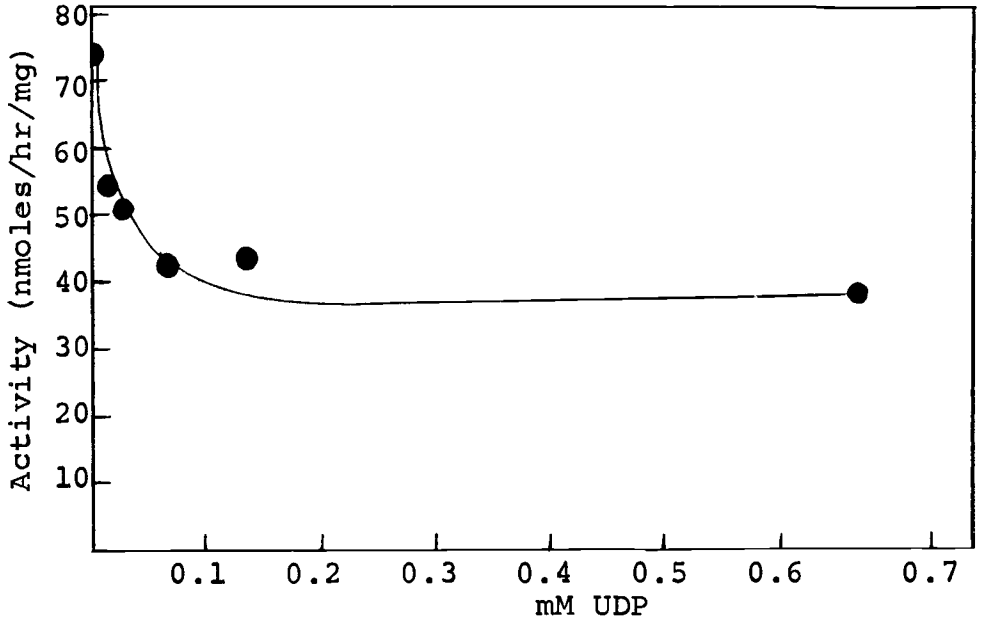


Figure 16. Inhibition of glucosyltransferase activity in the presence of UDP.

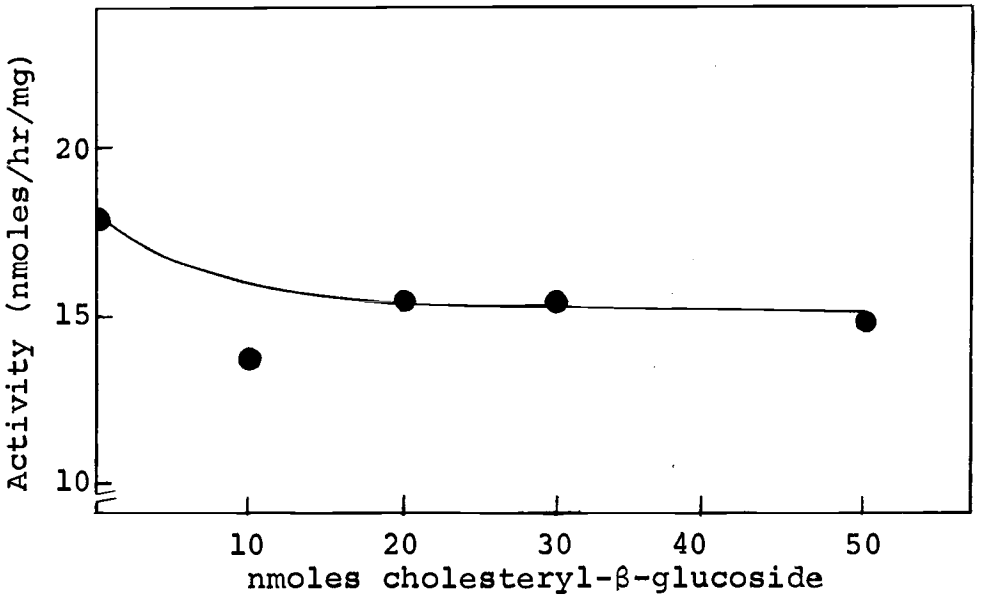


Figure 17. Effect of cholesteryl-β-glucoside on glucosyltransferase activity.

the possibility exists that the steryl glucoside that is synthesized by the enzyme is not readily released from the enzyme active site. Consequently, the above experiment does not conclusively rule out product inhibition and the effect may be limited because of the means by which the sterol glucoside is added.

3. Denaturation of the Enzyme during Incubation

A standard assay mixture without UDP-glucose-^{14}C was preincubated for 20, 40 and 60 minutes. A 20 minute assay was initiated by addition of the UDP-glucose-^{14}C at the end of the preincubation period. The results are shown in Figure 18.

Almost 50 percent loss of activity was observed after 20 minutes of preincubation. This activity measurement actually represents the incorporation of ^{14}C between the 20th and 40th minute of incubation in the time course study (Figure 13). Clearly, the transferase activity is not only inhibited by UDP, steryl glucoside and by competing reaction for UDPG, but is also sensitive to the combination of other cofactors.

4. Distribution of Label in the Aqueous Phase Enzyme Reaction

To the residual reaction mixture after $CHCl_3$:MeOH extraction, TCA was added to give a final concentration of 10 percent. The precipitated protein was spun down in a

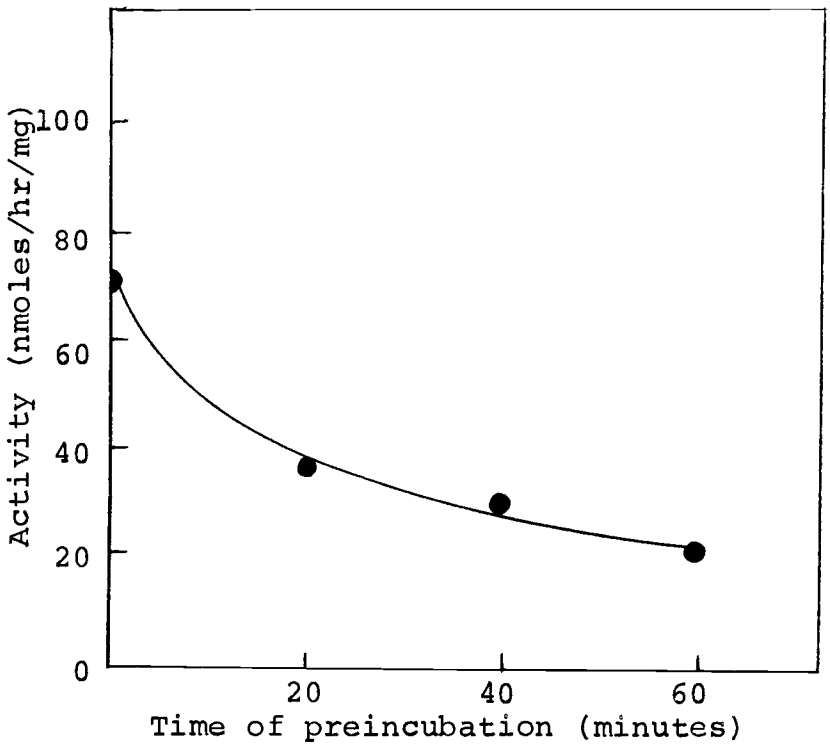


Figure 18. Glucosyltransferase activity after preincubation of the enzyme with cofactors: Ca^{++}, ATP, sitosterol/MeOH.

clinical centrifuge at high speed. It was found that 96 percent of the UDP-glucose-^{14}C added for the glucosyltransferase reaction remained in the supernatnat.

An aliquot (10,000 dpm) of the supernatant was subjected to paper chromatography in solvent system F. Sixty-five percent of the radioactivity was associated with a UDP-glucose standard with R_f 0.05. Fifteen percent of the radioactivity remained at the origin and the rest of the label was distributed in a broad band between R_f 0.1 and 0.25.

Phospholipid Composition and Phospholipase Treatment of the 25,000 g Fraction

1. Phospholipid Composition

If the transferase has a requirement for a membrane environment in order to function then destruction of this by degradation of the phospholipid component of the membrane should result in a loss of activity of the transferase. If the transferase does not suffer irreversible alterations in the process, recovery of the activity might be accomplished by adding back specific phospholipids or a lipid extract of the membrane itself. Similar experiments have been carried out particularly with membrane-bound glucose-6-phosphatase (22) and ATPase (21).

The phospholipid composition of the 25,000 g fraction was determined on different enzyme preparations by the

method of Raheja et al. (62). A chromogenic solution was made by first dissolving 16 g of ammonium molybdate in 120 ml of water to give solution I. Then, 40 ml of concentrated HCl and 10 ml of mercury are shaken with 80 ml of solution I for 30 minutes to give, after filtration, solution II. Two hundred ml of concentrated H_2SO_4 is added carefully to the remainder of solution I. To the resultant solution is added solution II to give solution III. Forty-five ml of methanol, 5 ml of chloroform, and 20 ml of water are added to 25 ml of solution III to give the chromogenic solution, which is stable for at least three months when stored at 5°C.

Three ml of the 25,000 g fraction was extracted repeatedly with $CHCl_3$:MeOH (2:1, v/v). An aliquot of the extract containing approximately 10 µg P_i was evaporated to dryness. To this 0.4 ml of $CHCl_3$ and 0.1 ml of the chromogenic solution was added. The sample was placed on a boiling water bath for 1-1.5 min after allowing the mixture to cool for 10 minutes 5 ml of $CHCl_3$ was added to the mixture and shaken gently. The mixture was centrifuged in a clinical centrifuge and the lower $CHCl_3$ phase pipetted into a cuvette and the absorbance read at 710 nm against a blank. Values of 65, 72, 86 and 89 µg phospholipid per mg protein were formed for four different preparations.

Phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl serine were identified in the $CHCl_3$-MeOH extract of the 25,000 g preparation by thin-layer

chromatography in a tank saturated with the solvent B:$CHCl_3$:MeOH:HOAc (65:25:8, v/v). Phospholipids were visualized as blue spots by spraying the chromatogram with Cu/MoO_4 solution (27). R_f values for standards of phosphatidyl choline, phosphatidyl serine and phosphatidyl ethanolamine in this system are 0.15, 0.22 and 0.49 respectively. Zones corresponding to these three standards were scraped from the chromatogram of the $CHCl_3$:MeOH extract of the sample containing 72 µg phospholipid per mg protein. The phospholipids were eluted with 2 ml of $CHCl_3$:MeOH followed by one volume of H_2O. The extract was shown to contain 15 µg P-serine, 16 µg P-ethanolamine and 25 µg P-choline per mg protein. The nature of the remaining phospholipids is unknown.

2. Phospholipase Treatment

The 25,000 g fraction was subjected to separate enzymatic digestions with phospholipases A, C and D. The bonds cleaved by these enzymes are shown in Figure 19. The amount used differed for each phospholipase because of differences in their specific activity. For treatment of 2 ml of the enzyme preparation (4.8 mg protein), either 4.65 units of phospholipase A* or 0.2-0.4 units of phospholipase C* or 4-6 units of phospholipase D* were used in the presence of

* To cleave lechithin, one unit of
1. phospholipase A releases 1µ mole of fatty acid per minute at 37°C, pH 7.3-7.4.
2. phospholipase C releases 1µ mole of fatty acid per minute at 37°C, pH 7.3.
3. phospholipase D releases 1µ mole of fatty acid per hour at 26°C, pH 5.6.

13.3 mM calcium ion at room temperature for 15 minutes. These conditions of protein and phospholipase concentration and duration of hydrolysis are similar for these reported (30, 77, 88, and 91). A control sample containing Ca^{++} alone was made and left at room temperature for the same time. The enzyme mixture was then spun at 25,000 g for 15 minutes and the pellet resuspended in the original volume of buffer. The sample was divided into four equal volumes for transferase assay in the presence of phospholipids. It was not possible to use EDTA to stop the phospholipase action without severely inhibiting the glucosyltransferase.

Phospholipid (10 µg) was added back into the reaction mixture by first evaporating under N_2 a $CHCl_3$ solution of the lipid in the assay tube and dispersing it by sonication in an appropriate volume of 25 mM Tris·HCl buffer (pH 8). The phospholipase-treated enzyme was added followed by the remaining components for the standard assay. The results of these experiments are shown in Table 14.

Additions of combinations of phospholipids (1:1:1, w/w), combined weight (10 µg) and also a $CHCl_3$-MeOH extract from 0.5 ml of the pellet fraction was added to the reaction mixture in the same way as for the individual phospholipids. The results are shown in Table 15.

Experiments in which the individual phospholipids are added to the 25,000 g pellet fraction before phospholipase treatment produce a stimulation of glucosyltransferase

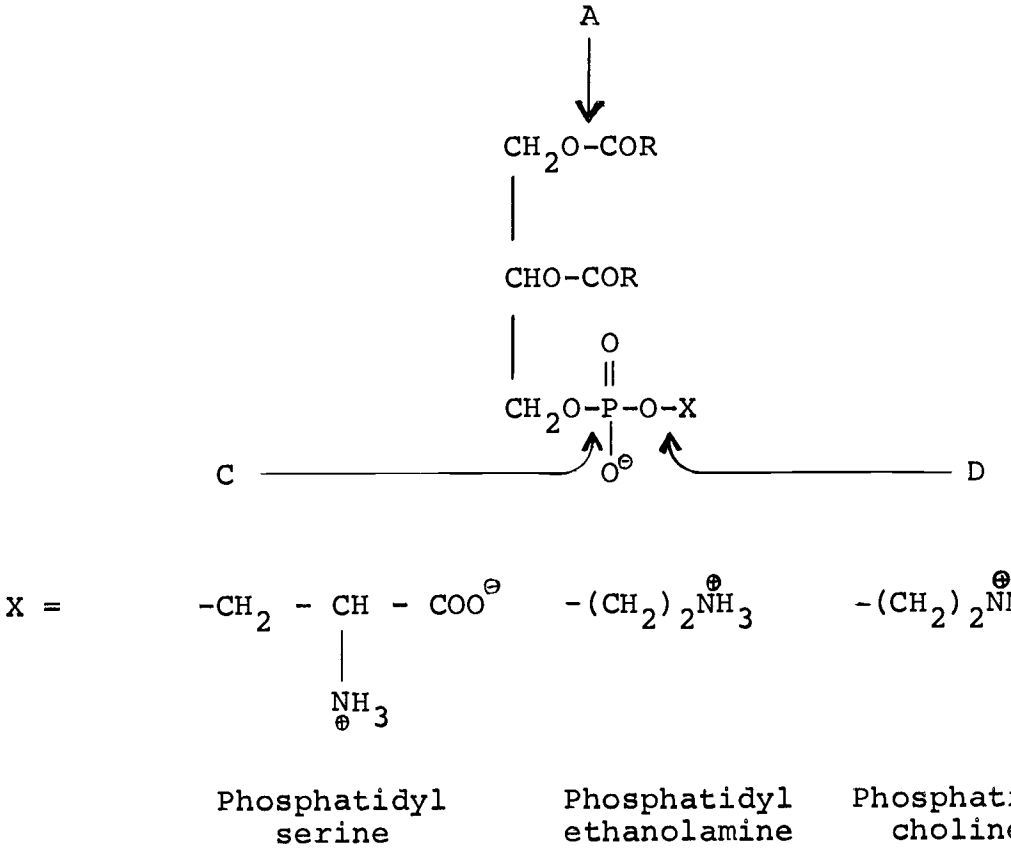


Figure 19. Bonds cleaved by phospholipases A, C and D.

Table 14. Recovery of glucosyltransferase activity by the addition of phospholipids after phospholipase treatment.

Phospholipase	Phospholipid	Activity (nmoles/hr/mg)	Relative activity
None	-	7.5	1.00
	P. choline	10.0	1.33
	P. ethanolamine	16.8	2.25
	P. serine	9.9	1.33
A	-	5.3	0.71
	P. choline	5.4	0.73
	P. ethanolamine	17.2	2.30
	P. serine	5.8	0.77
C	-	5.8	0.78
	P. choline	7.3	0.97
	P. ethanolamine	9.8	1.31
	P. serine	7.1	0.95
D	-	5.9	0.79
	P. choline	7.3	0.98
	P. ethanolamine	11.9	1.59
	P. serine	7.2	0.96

Table 15. Recovery of glucosyltransferase activity by a mixture of phospholipids or 25,000 g fraction $CHCl_3$-MeOH extract after phospholipase treatment.

Phospholipase	Phospholipid	Activity (nmoles/hr/mg)	Relative activity
None	-	27.7	1.00
	PL mixture	27.4	0.99
	Plant ext.	28.3	1.02
A	-	9.9	0.36
	PL mixture	11.3	0.41
	Plant ext.	13.7	0.49
C	-	13.3	0.48
	PL mixture	14.3	0.52
	Plant ext.	18.1	0.65
D	-	14.6	0.53
	PL mixture	15.6	0.56
	Plant ext.	24.5	0.88

activity in each case (Table 14). Phosphatidyl ethanolamine is much more stimulatory than either of the other two. This either reflects a relatively non-specific detergent effect or is a consequence of the loss of a phospholipid-dependent transferase activity during preparation of the enzyme which is then recovered by the addition of phospholipid. The fact that phospholipase treatment of the transferase produces a loss of 21-29 percent of this activity indicates a requirement for phospholipid, and by implication, a membrane environment for the transferase.

Of the phospholipase-treated samples, addition of phosphatidyl choline or phosphatidyl serine brings about the full recovery of glucosyltransferase activity after digestion with either phospholipase C or D, but not after digestion with phospholipase A. Presumably, the formation of lysophospholipids in the membrane by the action of phospholipase A seriously disrupts the membrane structure and results in losses of glucosyltransferase activity. Such losses are irretrievable by the phosphatidyl choline or phosphatidyl serine. Phosphatidyl ethanolamine addition is the most interesting in that in each case a substantial stimulation of activity is observed beyond that present before digestion with the phospholipases. Its effect on the phospholipase A-treated sample is more significant than on the phospholipase C- or D-treated samples and is comparable to the control sample.

A comparison of the influence of the three phospholipids on the recovery of glucosyltransferase activity based upon the net charge carried by the molecule at pH 8 suggests that a phospholipid bearing a net negative charge, phosphatidyl serine, is poorly effective. Phosphatidyl ethanolamine and phosphatidyl choline both bear a net zero charge, but whereas phosphatidyl choline is no more effective than phosphatidyl serine, phosphatidyl ethanolamine is capable of stimulating transferase activity beyond that of the enzyme before phospholipase treatment. It is clear that not only the net charge on the phospholipid is important but also the size of the basic moiety (c.f. $-\overset{+}{N}H_3$/$-\overset{+}{N}Me_3$).

The effectiveness of phosphatidyl ethanolamine over phosphatidylcholine might also suggest that the glucosyltransferase is located on the inside rather than the outside of the membrane. The asymmetry of membranes with respect to their phospholipid components has recently been discussed (10).

Data from experiments in which a mixture of the three phospholipids was used are shown in Table 15. The experiments were conducted on a different 25,000 g preparation from that used in the previous experiment. The absence of any stimulation by phospholipid on the transferase before phospholipase treatment may be a consequence of the membrane preparation being less fragmented and approximately more than the original state of the membrane in the plant tissue

than the previous preparation. The loss of transferase activity by the phospholipases is more extensive than before and, again, may also be a consequence of the different states of the two membrane preparations. After each phospholipase treatment there is only poor recovery of transferase activity by the addition of the phospholipid mixture. This may be a consequence of the fact that a critical level of phosphatidyl ethanolamine is necessary to produce reactivation. The recovery is better with the natural lipid, the $CHCl_3$-MeOH extract of the 25,000 g fraction, no doubt because the amount of phospholipid in the plant extract is much greater than the 10 μg of phospholipids added. There may be a specificity for the acyl side-chain of the phospholipid component for optimizing the transferase activity. Alternatively, there may be some additional natural lipid component required other than the three phospholipids investigated.

The Effect of Addition of ATP on Glucosyltransferase Activity during the Assay

It has been shown that ATP is not an absolute requirement for the transferase activity. However, to investigate further the role of ATP on this activity the influence of repeated additions of ATP during the course of the reaction was examined. A time course of enzyme activity had shown a linear incorporation of glucose into lipid for approximately 25 minutes after which the reaction markedly slowed.

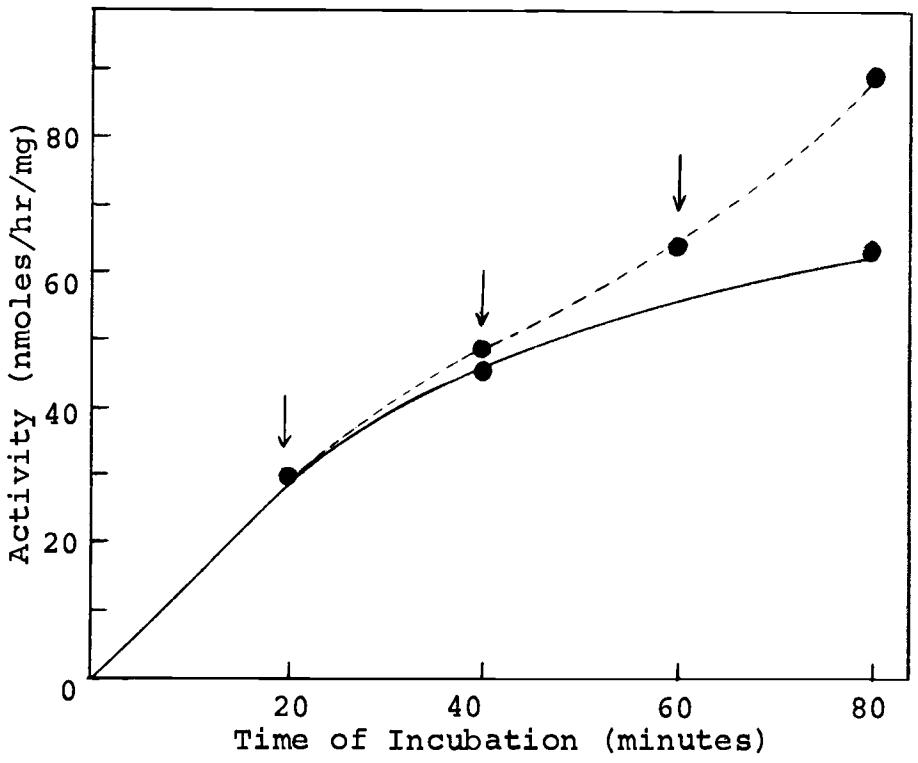
Addition of 0.5 µmoles of ATP into the reaction mixture at 20 minute intervals during the assay gives the incorporation data shown in Figure 20. The repeated addition of ATP permits a recovery of activity to occur after 40 minutes. Furthermore, no inhibition was observed by the total addition of two µmoles of ATP in this way,which is observed if present at the beginning of the reaction. Most importantly, this phenomenon was only found in a freshly prepared enzyme preparation but not the thawed preparations. This lack of response by the thawed preparations to repeated ATP addition during the incubation suggests the presence of a cold-labile component in the membrane fraction which is closely associated with the maintenance of transferase activity.

Other Properties of the 25,000 g Fraction

Determination of Pi Released during the Reaction

The maintenance of activity by ATP suggested an enzyme activation is linked to the turnover of ATP. To examine this further, the fate of ATP during the assay was investigated.

Release of inorganic phosphate was measured during the assay in the absence and in the presence of ATP. The reaction mixture contained all the components for the standard assay except that unlabelled UDP-glucose was used. At the end of the incubation, 0.75 ml of 20 percent TCA was added and the precipitated protein spun down. P_i was determined



●——● Incubation with 0.5 μmoles ATP at the beginning of the reaction.

●·····● Incubation with 0.5 μmoles ATP at the beginning of the reaction and added at arrows.

Figure 20. Glucosyltransferase activity with repeated addition of ATP during incubation.

spectrophotometrically according to the modified Fisk-SubbaRow method (48). The method is sufficiently sensitive to detect P_i at the nmole level.

The results are shown in Figure 21. A maximum of 0.09 μmoles of endogenous P_i was released after 10 minutes. When the reaction mixture contained 0.5 μmole ATP, P_i was released at a faster rate. All the added ATP was converted into P_i after 10 minutes and, as a check on the analytical procedure, it can be seen that the total P_i measured represents the sum of the exogenous and endogenous levels.

The release of P_i in the absence of ATP arises either from endogenous ATP or from phosphorylated protein by the action of a phosphatase. The capacity of the enzyme preparation to phosphorylate protein was examined using ATP-γ-^{32}P.

The experiments were carried out with the cofactor concentration used for the measurement of glucosyltransferase activity but replacing UDP-glucose-^{14}C and unlabelled ATP with UDP-glucose and ATP-γ-^{32}P (spec. act. 55,000 dpm/0.5 μmoles) respectively. At the end of the incubation, the reaction mixture was spun in a clinical centrifuge for one minute and the pellet, after washing twice with water, was dissolved in Aquasol and counted. The supernatant was also counted and subjected to paper chromatography in a solvent system (G) composed of 100 ml of 0.1 M phosphate buffer (pH 6.8) containing 60 mg $(NH_4)_2SO_4$ and 2 ml of n-propanol (73).

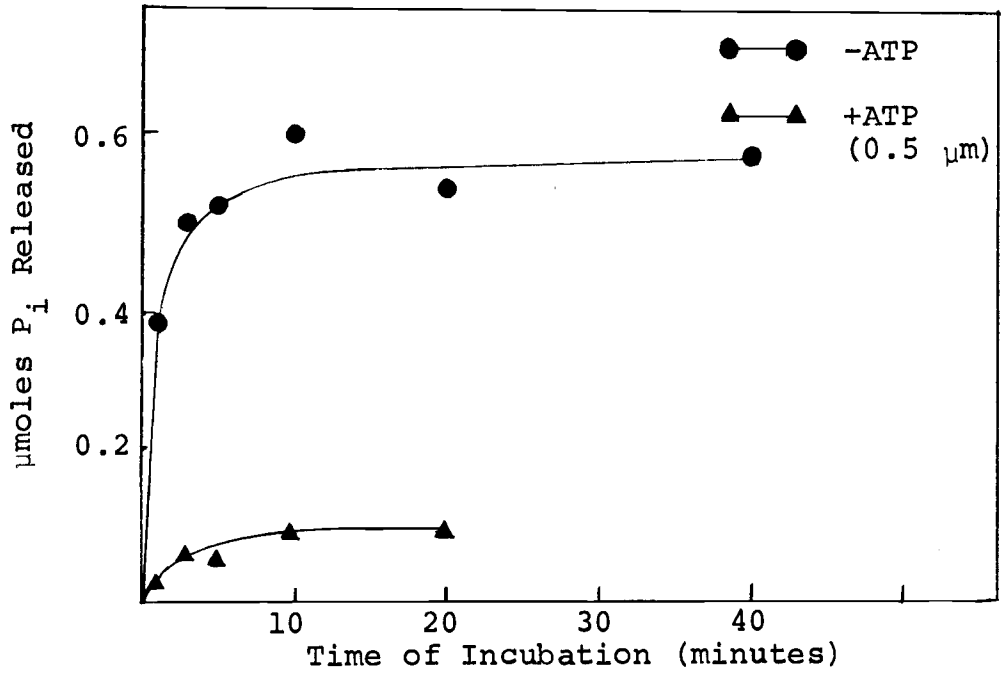


Figure 21. Release of P_i during the progress of glucosyltransferase assay.

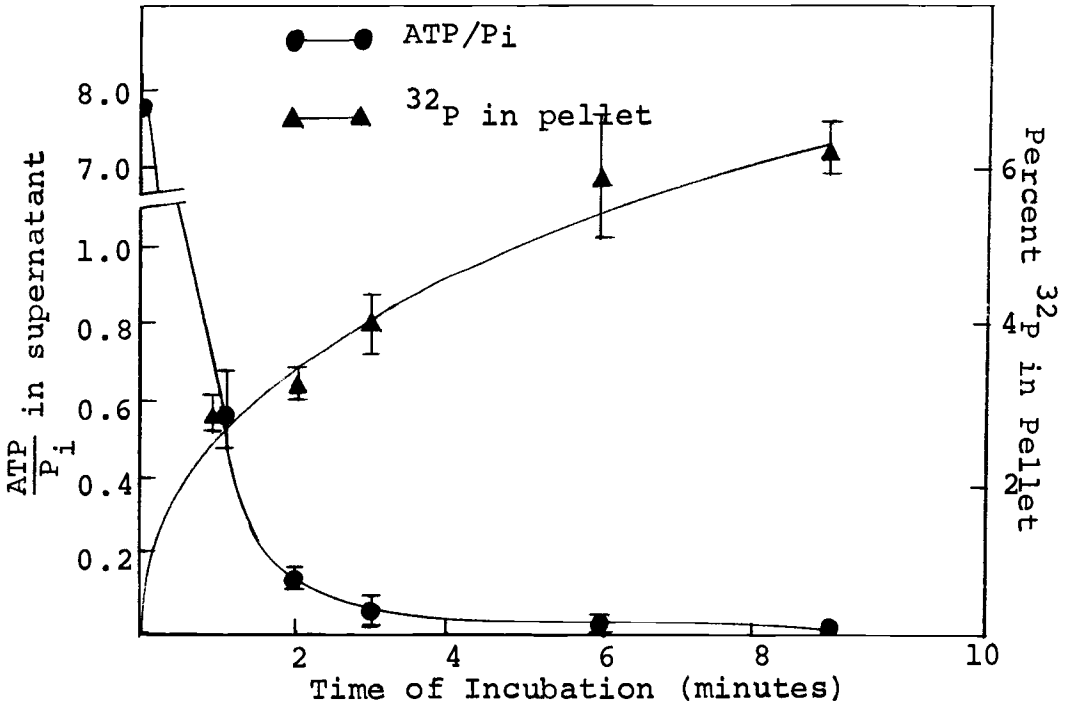


Figure 22. Release of P_i and phosphorylation of the 25,000 g protein during incubation.

The ratio of ATP-γ-^{32}P to $^{32}P_i$ was obtained by measuring the areas under the corresponding peaks on radiochromatographic scans of the paper strips. It was found that the release of P_i occurred immediately after the start of the reaction (Figure 22). After 6 minutes, 99 percent of the ATP-γ-^{32}P was in the form of $^{32}P_i$. This activity was also retained by preparations which had been frozen.

Incorporation of ^{32}P into the pellet fraction increased as the incubation progressed (Figure 22). The nature of the binding of ^{32}P in the pellet was further investigated by subjecting it to boiling in neutral buffer for five minutes. After centrifugation 26 percent of the ^{32}P was retained. Boiling a sample in 1 N NaOH solution for five minutes only retained one percent of the original ^{32}P in the pellet after centrifugation. According to Tao et al. (85), this indicated that 26 percent of the ^{32}P found in the washed pellet represented phosphoprotein. This suggested the existence of a protein kinase in the enzyme preparation.

ADP and Glucosyltransferase Activity

It will be recalled that both ^{32}P labelled ATP and the release of P_i determined in the early experiment indicated that almost all the available ATP (0.6 μmoles) had been hydrolyzed after ten minutes of incubation (Figure 22). The glucosyltransferase activity proceeded linearly for approximately another twenty minutes. For this reason, if the

reaction was under the influence of ATP, it may not be a direct effect. A product of ATP hydrolysis (ADP) or a phosphosylated component with moderate lifetime may be the component responsible for the maintenance of glucosyltransferase activity.

The standard glucosyltransferase assay was carried out with the exception that a range of ADP concentrations (0-4 mM) was used instead of ATP. The incorporation of glucose into the lipid fraction for a twenty minute incubation period was measured and is shown in Figure 23. It was found that glucosyltransferase activity is essentially unaffected by ADP concentrations up to 1.33 mM of ADP. At 4 mM ADP there was a 50 percent inhibition. When ATP was used, inhibition became significant at 1.33 mM and the activity was almost completely blocked at 4 mM (Figure 12).

From these observations it is clear that ADP does not affect glucosyltransferase as much as ATP either in its capacity to stimulate or to inhibit the reaction. It is important to note that what appears to be an active ATPase has been found in this system, sufficiently active to hydrolyse 99 percent of available ATP within ten minutes. Therefore, why does one find that at 4 mM ATP the transferase is virtually completely inhibited, whereas in the presence of 4 mM ADP only 50 percent inhibition occurs?

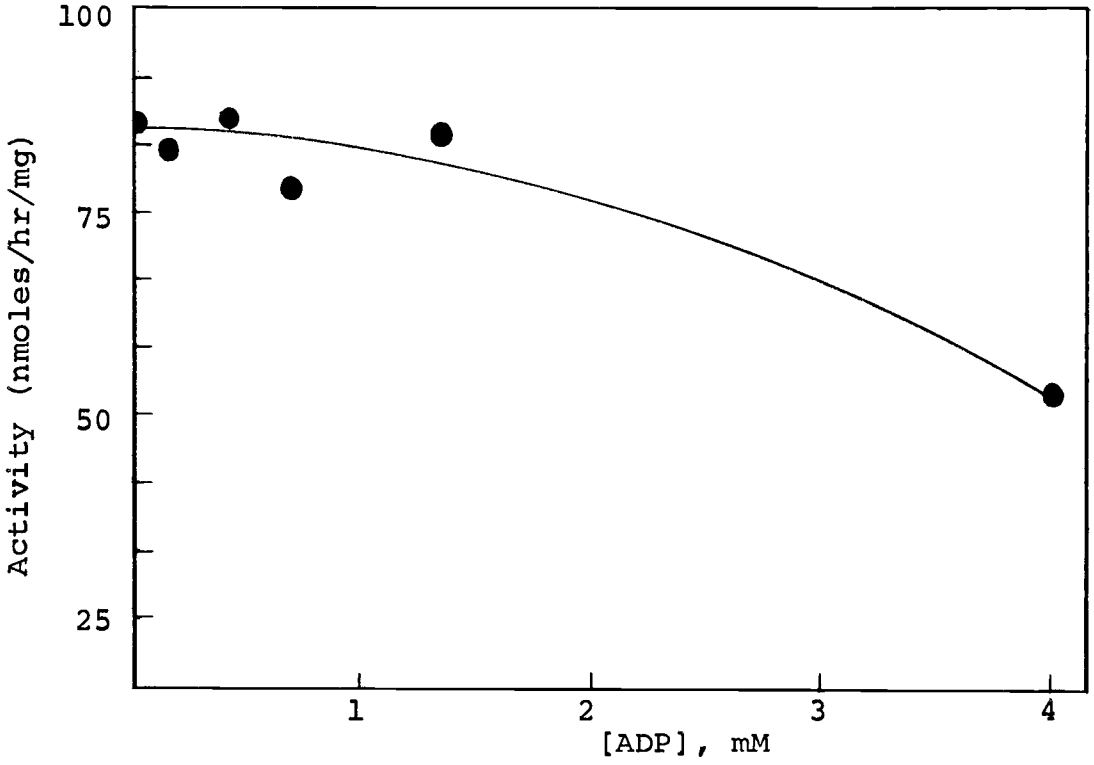


Figure 23. ADP and glucosyltransferase activity.

The Influence of Oligomycin, Ouabain, 2-4-Dinitrophenol and Amytal on Glucosyltransferase Activity

The release of P_i from ATP by the 25,000 g fraction (Figure 22) was previously suggested as most likely arising through the action of an ATPase. The maintenance of glucosyltransferase activity by repeated additions of ATP (Figure 20) further suggested that ATPase may have a role in governing the transferase activity. Because the composition of the 25,000 g fraction was not known but may include mitochondria and/or mitochondial fragments, the influence on glucosyltransferase activity of a number of agents known to affect ATP levels, was examined. Amytal is a specific inhibitor of oxidative phosphorylation and 2,4-dinitrophenol (DNP) is an effective uncoupler in mammalian systems (80). Oligomycin inhibits mitochondrial ATPase at a low concentration (1 µg/mg protein) and microsomal ATPase at a high concentration (100 µg/mg protein) (87), also in mammalian systems. Ouabain inhibits magnesium, sodium, potassium dependent ATPase allosterically (2).

Each agent was added into the standard glucosyltransferase reaction mixture in a low and a high concentration, shown in Table 16. At the end of a twenty minute incubation period incorporation of glucose into the lipid fraction was measured.

The two uncouplers of oxidative phosphorylation, DNP and amytal, produce small inhibitions. Oligomycin produces

Table 16. Effect of ouabain, oligomycin, DNP and amytal on glucosyltransferase activity.

Samples	ATPase Inhibitors		Activity nmoles/mg/hr	Percent Inhibition
Control	-		38.4	0
1a	Ouabain	36.5 µg	53.0	-37.9
1b	Ouabain	365 µg	37.2	3.0
2a	Oligomycin	1 µg	47.3	-23.2
2b	Oligomycin	100 µg	13.2	65.6
3a	DNP	9 µg	29.1	24.3
3b	DNP	90 µg	30.6	20.3
4a	Amytal	5 µg	32.3	15.9
4b	Amytal	50 µg	31.3	18.6

the severest inhibition at a high concentration, suggesting a microsomal location of the transferase if ATPase and the transferase activity are linked.

The stimulation of transferase activity of oligomycin and ouabain at low concentration is not understood. The inhibition at high concentration by oligomycin led to the investigation of the influence of oligomycin on transferase activity over a range of concentrations using another transferase preparation. The data are shown in Figure 24. Again, a stimulation is seen at a low concentration of the inhibitor, though it is less than was observed for the previous 25,000 g preparation. The transferase activity declines, though not linearly, with an increase in oligomycin concentration. The role that ATPase may play in glucosyltransferase activity is unclear. Interestingly, in sucrose

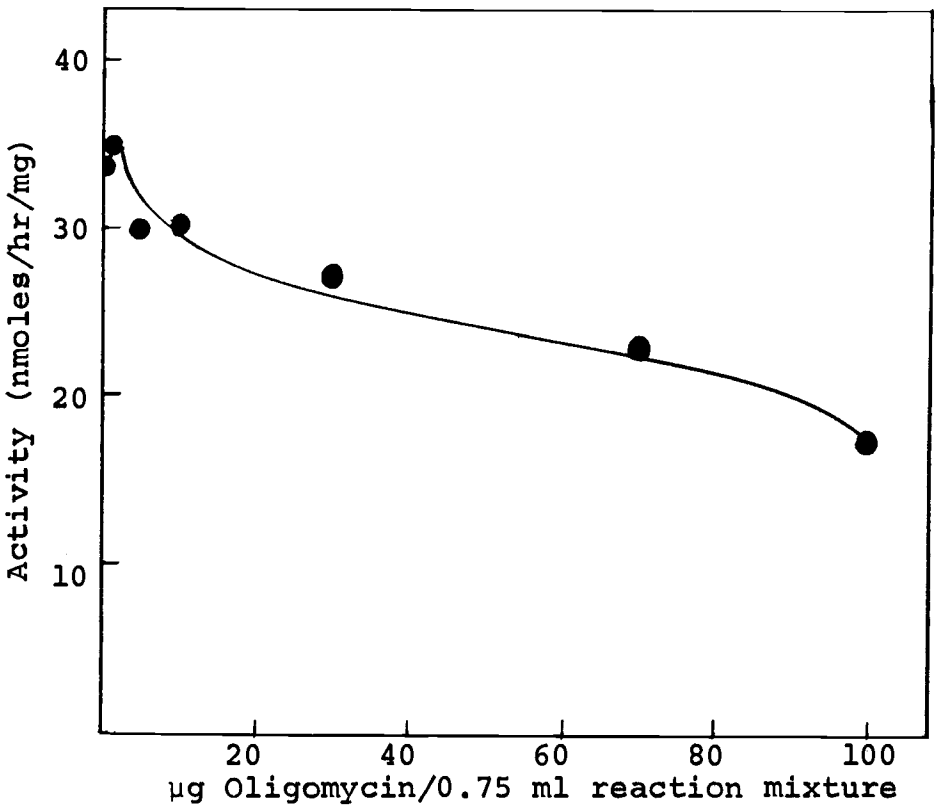


Figure 24. Oligomycin inhibition curve on glucosyltransferase activity.

density gradient studies of the 25,000 g preparation, it is shown that ATPase is separable from the glucosyltransferase.

Protein Kinase Activity

1. c-AMP and Protein Kinase Activity

The suggestion of a protein kinase, and the stimulation of the transferase by Mg^{++}, Ca^{++} and ATP, suggested the possible participation of c-AMP in governing glucosyltransferase activity. It has been suggested that the relative concentrations of Ca^{++} and c-AMP in a biological system provide a regulatory mechanism for many biological transmissions at the cellular level (63). Most importantly, c-AMP has been claimed to be found in plants (11, 66), though these claims have been challenged recently (3, 41).

As many protein kinases are activated by c-AMP (76, 4) protein kinase activity of the 25,000 g fraction in the presence and in the absence of c-AMP was assayed.

Protein kinase activity was measured according to the method of Albin (1) with some modifications. The activity of the 25,000 g particulate enzyme preparation was determined as the transfer of ^{32}P from ATP-γ-^{32}P to the added protein acceptors, histone II-A and protamine. Endogenous protein kinase activity was determined as ^{32}P transfer in the absence of exogenous protein acceptor. The reaction mixture contained 50 mM Tris·HCl (pH 7.0), 5 mM $MgSO_4$, 5 mM DTT, 30 mM KF, 250 µg exogenous protein acceptor, and 2 mM

ATP-γ-^{32}P in a total volume of 0.2 ml. When c-AMP was used, it was present in 10 μM concentration. The specific activity of labelled ATP was 1.61 μCi/mmole. The amount of enzyme added is 0.12 mg. For the determination of the background ^{32}P, the enzyme protein was added into a reaction mixture containing 10 percent TCA. The reactions were conducted for five minutes as determined from the time course (Figure 25). At the end of the reaction, the test tubes were transferred into an ice-bath and two ml of 20 percent TCA in 1 M H_3PO_4 was added. Unlabelled ATP (0.1 M, 50 μl) was then added and the mixture was allowed to stand in ice for 10-60 minutes. After standing, the reaction mixture was filtered through a Millipore cellulose ester filter (0.45 μ) followed by rapid washing with 20 ml ice-cold ten percent TCA solution. After briefly drying the filter under vacuum, it was dissolved and counted in ten ml Aquasol. The results are shown in Table 17.

The column "+c-AMP/-c-AMP" demonstrates the presence of c-AMP-independent protein kinase(s) in the 25,000 g fraction. This kinase activity also shows a preference for the endogenous rather than the exogenous proteins and of histone II-A rather than of protamine of the latter proteins. This order of specificity was found in many separate experiments. In the presence of c-AMP there is a marked inhibition of this activity on endogenous protein acceptors, but the degree of inhibition varied with the enzyme preparation.

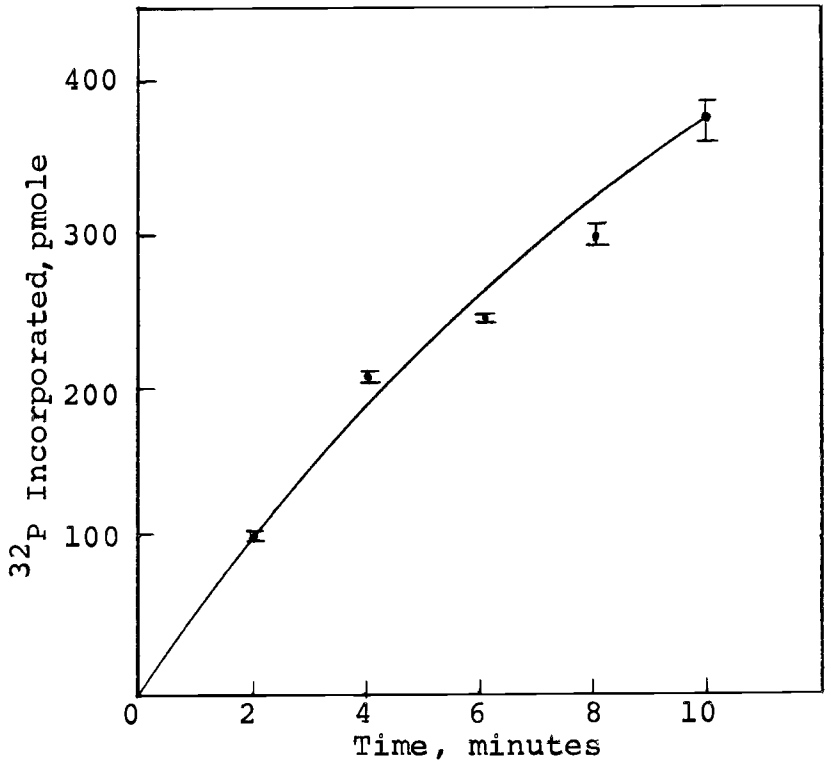


Figure 25. Time-course of protein kinase assay.

Table 17. Protein kinase activity in the presence and absence of c-AMP.

Protein Acceptor	S_A (unit/mg protein)[1,2] +c-AMP	-c-AMP	Relative Activity +c-AMP/-c-AMP
Endogenous	1052 ± 142	1766 ± 142	0.60 ± 0.14
Protamine	659 ± 64	678 ± 146	0.97 ± 0.39
Histone II-A	1298 ± 95	1110 ± 64	1.17 ± 0.16

1 One unit of protein kinase activity is the transfer of one picomole of P_i from ATP-γ-^{32}P onto the protein acceptor at 30°C in five minutes. S_A of exogenous proteins is calculated by subtraction of the endogenouse kinase activity. The background samples contribute 31 units which is subtracted from each sample.

2 The results are the average of duplicate samples with experimental error shown.

The kinase loses twenty percent of its activity after freezing and thawing.

The kinase activity determined by ^{32}P in the TCA precipitate may really represent the net result of both kinase and phosphoprotein phosphatase in the 25,000 g fraction. The inhibition of kinase activity by c-AMP, i.e., the ratio of <1 for +c-AMP/-c-AMP (Table 17) suggests the presence of a c-AMP - dependent phosphoprotein phosphate acting in opposition to a c-AMP - independent protein kinase. A c-AMP dependent phosphoprotein phosphatase has recently been isolated from synaptic membrane fractions of rat cerebrum (50). However, no such protein has yet been isolated from a plant system. Cyclic AMP-independent protein kinase activity has been found in six day old etiolated pea shoots (40) and associated with ribosomes prepared from five day old etiolated pea shoots and cultures of Lemna minor (42, 86).

The phosphorylation of protein may result as the presence of ATPase in the preparation. It is known that K^+ and Na^+ dependent ATPase undergoes Na^+ dependent phosphorylation and K^+ dependent dephosphorylation (21).

2. c-AMP and ATP on Glucosyltransferase Activity

The influence of ATP in the presence of c-AMP on the transferase activity was investigated. Of many experiments conducted with different c-AMP concentrations, the data shown in Table 18 is representative. It is interesting to observe that over the concentration range of ATP, there is no effect of c-AMP on glucosyltransferase activity, whereas from Figure 12 it can be seen that a moderate stimulation of the transferase activity is observed in the absence of c-AMP.

Table 18. Effect of the presence of c-AMP and various concentrations of ATP on glucosyltransferase activity.

Samples containing 0.26 mM c-AMP	mM ATP	Glucosyltransferase activity nm/mg/hr	Relative activity
1	0	39.6	1.00
2	0.03	39.5	0.99
3	0.08	37.8	0.95
4	0.13	39.3	0.99
5	0.67	39.5	0.99

Attempted Enzyme Purifications

Several attempts have been made to purify the 25,000 g fraction, but with little success.

Preparation of Acetone Powder

According to Neufeld and Hall (53) and Ongun and Mudd (55) acetone powders of cell-free extracts of spinach leaf chloroplasts and pea root mitochondria, respectively, were able to catalyze the reaction of glucosyl transfer from UDP-glucose to a sterol, lipid acceptor. Several attempts modeled after the procedures described by these authors to prepare an active acetone powder of the pea seedling extract were unsuccessful. Only trace amounts, one to two percent, of the original enzyme preparation could be recovered.

Detergent Treatment

The purification of membrane-bound proteins often requires "solubilization" of the protein or the release of the protein from the membrane lipid by freezing. Among the procedures used to solubilize such proteins, detergents are the most commonly used. Collins et al. (20) has purified steroid glycosyltransferases of rabbit liver microsomes three-five fold by solubilization with Triton X-100 followed by precipitation with ammonium sulfate. Similarly, Peaud-Lenoel and Axelos (57) have solubilized a sterol UDP-glucose glucosyltransferase from wheat seedling extract with Triton X-100.

Two percent Triton X-100 or five percent sodium deoxycholate was added into 3.8 ml samples of the 25,000 g fraction to make a final concentration of 0.1 percent of the detergents. The mixtures were left in ice water for 30 minutes and then centrifuged at 25,000 g for 15 minutes. The resulting pellets were resuspended in 25 mM Tris·HCl buffer (pH 8.0). Supernatants after centrifugation were passed through a column of five ml of pre-washed Amberlite XAD-4 to remove the detergents (78). Sterol:UDP-glucose glucosyltransferase activity was measured in the pellets and supernatants. The results (Table 19) showed that neither detergent was very effect in releasing the glucosyltransferase activity from the membrane. The neutral detergent,

Table 19. Sterol:UDP-glucose glucosyltransferase activity after solubilization with detergents.

Sample	Activity (nmoles/hr/mg)	Protein mg	Total Act. nmoles/hr	Relative Sp. Act.
Before treatment	45.5	9.1	415.0	1
Triton X-100 pellet	58.8	3.6	211.7	1.29
Triton X-100 supernatant	29.2	1.6	46.7	0.64
DOC pellet	17.5	3.3	38.6	0.39
DOC supernatant	14.6	1.4	20.4	0.32

Triton X-100, did produce about 30 percent increase in specific activity in the pellet fraction. This is not a very

encouraging approach because of the very considerable loss of total activity (50 percent) by the process. The loss may be due to binding of protein to the XAD. The anionic detergent, DOC, is clearly inhibitory to the transferase.

Enzyme Aggregates in the Reaction Mixture

It had been noted that during the assays that protein precipitated in the reaction mixture. Recent findings of Kamath and Ananth (38) and Kupfer and Levin (45) concerning the rapid sedimentation of rat liver microsomes at 1,500 g in the presence of Ca^{++}, and the retention of monooxygenase drug-metabolizing activity in such Ca^{++}-aggregated microsomes led us to examine this aggregation phenomenon with a possible view of taking advantage of it in the purification of the transferase.

Different combinations of cofactors of the same concentration as that used for the standard assay were preincubated with the enzyme preparation for 15 minutes at 30°C. The results are shown in Table 20. Only sample seven showed aggregate formation during the 15 minute preincubation. It was later determined that the presence of sitosterol was not required for this aggregation. The transferase loses 47 percent of its activity if preincubated alone for the same time and at the sampe temperature (compare Expt. 1 and 2). However, this loss of enzyme activity can be partially or completely prevented if the enzyme is preincubated with any

Table 20. Preincubation of the enzyme preparation with different cofactors.

Experiment	15 minute preincubation with	Cofactors added for incubation in addition to UDP-glucose-^{14}C	Activity (nmoles/hr/mg)	Ratio
1	Control: no preincubation	Ca^{++}, ATP, sito/MeOH	31.1	1.9
2	Control: no cofactors	Ca^{++}, ATP, sito/MeOH	16.8	1.0
3	Ca^{++}	ATP, sito/MeOH	11.0	0.7
4	ATP	Ca^{++}, sito/MeOH	23.4	1.4
5	sito/MeOH	Ca^{++}, ATP	17.9	1.1
6	Ca^{++}, sito/MeOH	ATP	32.5	1.9
7	Ca^{++}, sito/MeOH, ATP	--	24.8	1.5

of the combinations of cofactors shown with the exception of a preincubation with Ca^{++}.

Transferase activity could be retained completely if the enzyme preparation is preincubated with Ca^{++} and sitosterol/MeOH (experiment 6). However, as the aggregates are only found under the condition of experiment 7, in which there is partial protection of enzyme activity, the pellet and supernatant from this experiment were investigated further.

Transferase activity was measured in the supernatant and in a suspension of the pellet obtained either by addition of the supernatant or a volume of buffer to give the original volume. The influence of freezing of the two fractions and of various combinations of the fresh and frozen components was also examined. The data are shown in Table 21.

Essentially, no transferase activity is found in the supernatant. Not surprisingly, transferase in the pellet is enhanced by the addition of cofactors if it is resuspended in buffer, but is inhibited if resuspended in supernatant (Experiments 5-8). This can be seen to be the case throughout the table if one compares resuspension of an appropriate pellet in the buffer with resuspension in supernatant. The supernatant retains cofactors from the initial preincubation, consequently, addition of cofactors to a combination of pellet and supernatant effectively doubles

Table 21. Glucosyltransferase activity of the pelleted aggregates obtained by preincubating the enzyme preparation with cofactors Ca^{++}, ATP, and sitosterol/MeOH.

Experiment	Enzyme (0.5 ml)*	Addition of cofactors for glucosyltransferase assay	Activity (nmoles/mg/hr)
1	Control: no preincubation	+	23.0
2	Control: preincubated	+	15.4
3	S	+	0.4
4	S	-	0.4
5	P + Buffer	+	17.5
6	P + Buffer	-	12.0
7	P + S	+	8.8
8	P + S	-	12.5
9	P + FS	+	6.9
10	P + FS	-	15.3
11	FP + Buffer	+	9.5
12	FP + S	+	0.5
13	FP + S	-	2.3
14	FP + FS	+	10.4
15	FP + FS	-	17.0

* Abbreviations: P: pelleted aggregates
S: supernatant fraction after the aggregation
FP: frozen P (6 hours)
FS: frozen S (6 hours)

the cofactor concentration and produces the inhibition. Several general observations can be made: comparison of experiment 5 with 11 shows freezing of the pellet is deleterious. The activity shown by resuspension of the fresh pellet in buffer cannot be obtained by using either fresh or frozen supernatant (c.f. experiment 5 with 8 and 10), although the frozen supernatant is better in this respect. Seemingly a cold-labile inhibitor of transferase is present in the supernatant. A similar conclusion can be drawn comparing experiments 11, 13 and 15. The most effective recovery of activity was obtained by combining a frozen pellet with a frozen supernatant (comparing experiment 15 with 11). Clearly, to pursue a purification of the enzyme further, the fresh pellet should be resuspended in buffer. However, it should not be forgotten that a sacrifice of activity has already been made to obtain the pelleted aggregate in the first place. A reduction in the time of aggregation may be a solution to this problem.

Attempts to solubilize the aggregates found over a range of preincubation periods using 0.1 percent Triton X-100 in the Tris buffer were made in the hope of furthering the purification of the transferase by subjecting a solubilized aggregate to gel filtration. The 25,000 g fraction was preincubated as before and it was observed that after seven minutes aggregation appeared to be complete as evidenced by a clear solution above the aggregate. It was

separated by centrifugation in the clinical centrifuge. Attempts to solubilize the aggregate with the detergent in concentrations from 0.1 percent to 2 percent in the Tris buffer were completely unsuccessful.

Sucrose Density Gradient Centrifugation

Sucrose density gradient centrifugation has frequently been used to fractionate membrane proteins. Ray et al. (65) have been able to isolate β-glucan synthetase particles from pea seedlings and concluded that they are Golgi membranes. Peaud-Lenoel and Axelos (57) used a linear sucrose gradient to separate particulate preparations from wheat and found an active fraction for sterylglucoside synthesis.

A freshly prepared 25,000 g pellet fraction was further purified by sucrose density gradient centrifugation in a linear gradient from 20-50 percent (gm/100 ml). Sucrose solutions were prepared in 25 mM Tris.HCl buffer (pH 8.0). Twenty-five ml of gradient was layered on top of 1 ml of 50 percent sucrose solution in a cellulose nitrate ultracentrifuge tube (Beckman No. 302237). Two ml of freshly prepared 25,000 g pellet fraction containing 10-11 mg of protein was used for each tube. The centrifugation was carried out at 0-4°C in a 27.1 swinging bucket rotor in a Beckman model L2-65B ultracentrifuge for two hours.

The contents of each tube were fractionated into ten fractions by piercing the bottom of the centrifuge tube and

counting drops. One-hundred drop fractions were collected above the pellet for eight fractions. The ninth fraction contained 200 drops and the rest were pooled in the tenth fraction which ranged from 250-350 drops. The pellet at the bottom of the tube was resuspended in 1.0 ml of 25 mM Tris·HCl buffer solution. The collected fractions were pelleted by centrifugation in a No. 40 rotor at 30,000 g for 30 minutes and resuspended in 1 ml 25 mM Tris·HCl buffer. The standard glucosyltransferase assay was carried out on each fraction. A 10-fold dilution of a sample of each fraction was made in order to determine ATPase activity and latent IDPase activity. According to Ray et al. (57), IDPase activity reaches its maximum after four days storage at 0°C.

Assay procedures for ATPase and IDPase are described as follows:

1. ATPase

ATPase activity was determined according to the method of Ray et al. (65) with some modifications. The obtained enzyme preparation (25,000 g) used for the sterol:UDP-glucose glucosyltransferase assay was diluted ten-times with 25 mM Tris·HCl buffer, pH 8. The reaction mixture contained 0.3 ml of this diluted preparation, 2 mM ATP and 2 mM of either Ca^{++} or Mg^{++} in a final volume of 0.5 ml. The reaction was carried out at 37°C for five minutes and was stopped by adding 0.5 ml of 20 percent TCA. The

precipitated protein was spun down at 2500 rpm for 10 minutes. The released inorganic phosphate was determined spectrophotometrically by a modified Fiske-SubbaRow Method (48).

2. IDPase

Inosine diphosphatase (IDPase) activity was measured on the ten-times diluted enzyme preparation according to the method of Ray et al. (65). The reaction mixture contained 0.3 ml diluted enzyme preparation, 2.4 mM IDP and 2 mM Mg^{++} in a final volume of 0.5 ml. The reaction was carried out at 37°C for five minutes and was stopped by adding 0.5 ml of 20 percent TCA. The precipitated protein was removed by centrifugation and the released inorganic phosphate was determined spectrophotometrically,as described before.

Results of the fractionation are shown in Figure 26. Protein was found predominantly in a diffuse opaque band in fractions 3, 4, and 5 (Figure 26a) and also in the pellet. Glucosyltransferase activity was located in two places. One coincided with the major protein band and the other in fraction 8 which gave a very high specific activity (Figure 26b).

Separation of IDPase (Figure 26c) was not as clear as glucosyltransferase. Interestingly, the highest specific activity of IDPase was also found in fraction 8. Mg^{++}-stimulated and Ca^{++}-stimulated ATPase were also identified (Figures 26d and e, respectively). Ca^{++}-stimulated ATPase is more readily sedimentated than the opaque band and Mg^{++}-

Figure 26. Distribution of enzyme activities after sucrose density gradient centrifugation.

a. Protein content

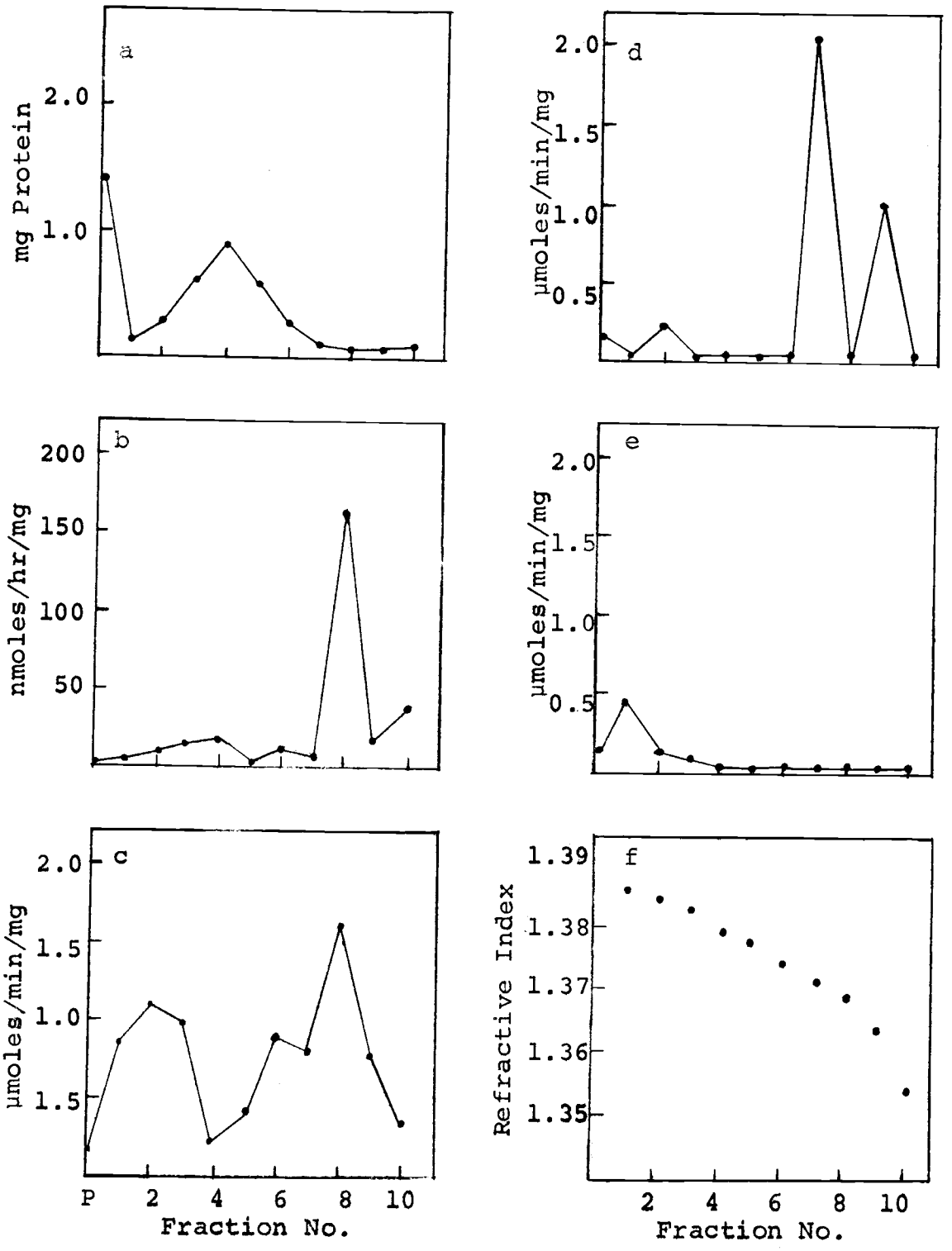
b. Glucosyltransferase activity

c. IDPase activity

d. Mg^{++} dependent ATPase activity

e. Ca^{++} dependent ATPase activity

f. Refractive index at 26°C


a
mg Protein
2.0
1.0
d
µmoles/min/mg
2.0
1.5
1.0
0.5
b
nmoles/hr/mg
200
150
100
50
e
µmoles/min/mg
2.0
1.5
1.0
0.5
c
µmoles/min/mg
2.0
1.5
1.0
1.5
P
2
4
6
8
10
Fraction No.
f
Refractive Index
1.39
1.38
1.37
1.36
1.35
2
4
6
8
10
Fraction No.

stimulated ATPase can be found in low activity close to the Ca^{++} stimulated ATPase and in high specific activity in two fractions, peaks at fractions 7 and 9, neither of which was associated with the high specific activity glucosyltransferase band.

Although fractionation of the 25,000 g fraction was not carried further, the results obtained suggest that glucosyltransferase is associated with a high specific activity IDPase and can be separated from both ATPases. This suggests that the glucosyltransferase involved in steryl glucoside synthesis is associated with Golgi membranes. Ray et al. (65) indicated that the Golgi membranes isolated from germinating pea seedlings exhibit latent IDPase activity and weak ATPase activity in addition to the polysaccharide synthetase they studied. The two peak activity bands as well as the broad distribution of IDPase activity in the gradient suggests fragmentation of the Golgi bodies has occurred during the preparation. It has been noted that intact Golgi vesicles can only be obtained with careful chopping of the tissue (51, 65), suggesting that the blending technique would give highly fragmented Golgi membranes.

GENERAL DISCUSSION

Sterol:UDP-glucose glucosyltransferase of plants has been studied by Hou et al. (36), Ongun and Mudd (55), Bush and Grunwald (14), and some others (39, 47, 57) using various biological materials. Although enzyme activity has been demonstrated with preparations of broad centrifugation cuts, the rate of reaction is low (14, 55) and the reaction conditions vary with the different preparations.

In the present study, this enzyme has been characterized in more detail. A more active enzyme preparation was obtained by using a 13,000-25,000 g fraction of seven-day pea shoot-root axes.

The enzyme has apparent kinetic constants of Km 3.33 mM and V_{max} 480 nmoles/hr for UDP-glucose. Values of 2.21 x 10^{-1} mM for Km and 2.44 nmoles/hr for V_{max} were found for the soybean enzyme by Hou et al. (36) and 2.8 x 10^{-2} mM for Km for the wheat enzyme by Peaud-Lenoel and Axelos (57). Both Km and V_{max} are one-two orders of magnitude greater for the pea seedling enzyme. This may be due to the fact that the 25,000 g fraction used is a relatively pure enzyme preparation free of endogenous inhibitors. The marked increase of enzyme activity in the presence of MeOH and high concentrations of Ca^{++} has not been observed before.

Several lines of evidence lead to the conclusion that the glucosyltransferase is a membrane-associated enzyme. It

is sedimented in a relatively low speed fraction. It is partially inactivated by short-term treatment with phospholipases A, C and D and is stimulated by PS, PC and PE. Recovery of activity of the phospholipase-treated enzyme may be accomplished by adding back each of the three phospholipids but especially by the addition of PE. Also consistent with the transferase being associated with a lipid environment is its increased activity after freezing (31). The stimulation of activity by the addition of MeOH may also be regarded as a reflection of the hydrophobic nature of the enzyme.

Sucrose density gradient centrifugation produces a light and a heavy band of glucosyltransferase activity. The light band contains a glucosyltransferase which is four times as active as that of the heavy band. Each band also coincides with peak activities of IDPase, an enzyme claimed to be a marker for plant Golgi membranes (65). The method of preparation of the 13,000-25,000 g fraction most likely leads to a heterogenous population of membrane fragments both in size and origin. The broad distribution of IDPase activity then suggests that this enzyme is not specific for one membrane. The narrower distribution of the glucosyltransferase implies a more localized occurrence of this activity.

It is interesting to speculate that the two bands of glucosyltransferase activity from the density gradient

represent either two different transferases or the same transferase in two different environments. From the stability study of the enzyme (Figure 18), it is apparent that it undergoes a relatively rapid inactivation, approximately 50 percent loss of activity after a 20 minute preincubation with Ca^{2+}, ATP and sitosterol-methanol. Over the next 40 minutes there is a decline of an additional 20 percent of the original activity. Thus, after one hour, approximately 30 percent of the activity is retained. It is interesting to compare this with the influence of the chelators EDTA and EGTA on the enzyme (Table 10). In the presence of 13 mM of the chelators approximately 33-39 percent of the enzyme activity is retained. If endogenous metal ion is readily released and is not buried in the membrane, then it appears that there is a metal ion-dependent and a metal ion-independent glucosyltransferase present.

The stimulation of the transferase by Ca^{2+} was unexpected. Mg^{2+} and Mn^{2+} have frequently been used for this enzyme reaction (14, 36, 39), but Ca^{2+} stimulation of the plant enzyme has not been observed before. The inhibition by Zn^{2+} may be a result of a competitive inhibition with either Mg^{2+} or Ca^{2+}.

The most intriguing feature of this enzyme is the role that may be played by ATP in its functions. As previously mentioned, there should be no energy requirement for this enzyme activity. It was observed that P_i was released from

the enzyme preparation under the glucosyltransferase assay conditions but omitting ATP (Figure 21). The release of P_i could be augmented if ATP were present (Figure 21). This prompted an examination of the fate of ATP with ATP-γ-^{32}P under the glucosyltransferase assay conditions. Figure 22 shows the rapid disappearance with time of ATP-γ-^{32}P and the much slower appearance of ^{32}P in the membrane. The phosphorylation of the membrane led to an investigation of protein kinase activity of the membrane fraction. The membrane exhibits activity for phosphaylation of both endogenous protein and the exogenous protein histone and protamine. A comparison of the incorporation of ^{32}P under the glucosyltransferase assay conditions (Figure 22) with that under the protein kinase assay conditions (Figure 25) shows the former to be more effective. In a previous experiment (Figure 20), it had been shown that the decline in activity exhibited by the transferase during the assay could be offset by repeated additions of ATP. This could be demonstrated in fresh preparations only. These experiments lead one to suggest that phosphorylation of the transferase or some membrane component may be involved in the maintenance of cold-labile glucosyltransferase activity. Further experiments to examine the nature of the phosphorylated component are necessary to clarify these suggestions.

The function of UDPG:sterol glucosyltransferase is unknown. The fact that the acceptor molecule of glucose may

be a membrane component provides the transferase with a potentially important role in modifying the membrane in which it is located. The pea seedling enzyme does not appear to have a requirement for sterol which implies that the sterol acceptor is endogenous.

A knowledge of this endogenous sterol is important if a role for the enzyme is to be suggested. If the sterol is an integral component of the membrane, then its glucosylation and possible release from the membrane will affect the membrane fluidity and the activity of the enzymes located in the membrane.

It has also been shown that steryl glucoside is associated with particulate fractions of the cell. It is conceivable that the glucosylation of membrane-associated sterol is an important step in decreasing the hydrophobicity of membrane vesicles to enhance their transport properties.

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