



A guanosine diphosphate-L-fucose glycoprotein fucosyltransferase from *Corynebacterium insidiosum*
by Peter Ludwig Sadowski

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE in Microbiology

Montana State University

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Abstract:

Since *Corynebacterium insidiosum* produces a high molecular weight phytotoxic glycoprotein of which 38.7% is fucose, the enzyme catalyzed transfer of fucose to glycoproteins was investigated. Fucosyltransferase activity was demonstrated using a crude cell membrane preparation from *C. insidiosum*. Guanosine diphosphate-L-fucose was used as the sugar nucleotide glycosyl donor. Two glycoproteins were used as fucosyl acceptors: a phytotoxic glycoprotein from *C. insidiosum* subjected to mild acid hydrolysis, and a glycosidase-treated aracid glycoprotein obtained from pooled human plasma. The cell membrane preparation demonstrated a pH optimum of 7.5, and the incorporation appeared to be linear for 1+5 minutes. When the bacterial glycoprotein was used, concentrations in excess of 0.2 μg per μl of reaction mixture appeared to be saturating. The addition of chelating agents and nonionic detergents inhibited transferase activity. The presence of MnCl_2 in the reaction mixture appeared to enhance the incorporation of fucose. The fucosyltransferase could be demonstrated in the soluble fraction.

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A GUANOSINE DIPHOSPHATE-L-FUCOSE GLYCO-
PROTEIN FUCOSYLTRANSFERASE FROM
CORYNEBACTERIUM INSIDIOSUM

by

PETER LUDWIG SADOWSKI

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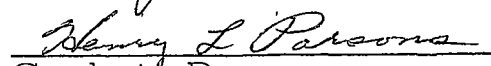
in

Microbiology

Approved:


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Chairman, Examining Committee


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MONTANA STATE UNIVERSITY
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TABLE OF CONTENTS

	<u>Page</u>
VITA	ii
ACKNOWLEDGMENTS.	iii
TABLE OF CONTENTS.	iv
LIST OF TABLES	vi
LIST OF FIGURES.	vii
ABSTRACT	viii
INTRODUCTION	1
EXPERIMENTAL PROCEDURE	9
Culturing	9
Preparation of cell free extract	9
Protein determination	9
Preparation of primer	11
Substrate	11
Fucosyltransferase assay	11
Verification of incorporation of fucose	13
Materials	13
RESULTS.	15
Products of the reaction	15
Location of transferase activity in the subcellular fractions.	15

	<u>Page</u>
Reaction properties.	21
Acceptor specificity studies	31
DISCUSSION	37
SUMMARY.	43
LITERATURE CITED.	44

LIST OF TABLES

	<u>Page</u>
Table 1. The fractionation scheme for <u>C. insidiosum</u> . .	10
Table 2. The location of GDP-fucosyltransferase activity in the various subcellular fractions of <u>C. insidiosum</u>	18
Table 3. The effect of various agents on fucosyl- transferase activity	28
Table 4. The relative incorporation of fucose- ¹⁴ C achieved with various primers	36

LIST OF FIGURES

	<u>Page</u>
Figure 1. Scheme of guanosine diphosphate-L-fucose fucosyltransferase activity from <u>C. insidiosum</u> using partially hydrolyzed phytotoxic glycoprotein as the primer	8
Figure 2. Tracing of radiochromatograms of sugar residues found in unhydrolyzed (X) and hydrolyzed (Y) phytotoxic glycoprotein primers	17
Figure 3. An electron micrograph of the cell membrane fraction.	20
Figure 4. The pH dependence of fucose incorporation	23
Figure 5. The dependence of fucose incorporation on time	25
Figure 6. The dependence of fucose incorporation on the concentration of the primer	27
Figure 7. The column chromatography of reaction mixtures on Sephadex G-25.	30
Figure 8. The affect of acid hydrolysis on the ability of the phytotoxic glycoprotein to serve as a fucosyl acceptor.	33
Figure 9. The use of fucosyltransferase to incorporate fucose into various derivatives of human plasma α_1 -acid glycoprotein	35

ABSTRACT

Since Corynebacterium insidiosum produces a high molecular weight phytotoxic glycoprotein of which 38.7% is fucose, the enzyme catalyzed transfer of fucose to glycoproteins was investigated. Fucosyltransferase activity was demonstrated using a crude cell membrane preparation from C. insidiosum. Guanosine diphosphate-L-fucose was used as the sugar nucleotide glycosyl donor. Two glycoproteins were used as fucosyl acceptors: a phytotoxic glycoprotein from C. insidiosum subjected to mild acid hydrolysis, and a glycosidase-treated α_1 -acid glycoprotein obtained from pooled human plasma. The cell membrane preparation demonstrated a pH optimum of 7.5, and the incorporation appeared to be linear for 45 minutes. When the bacterial glycoprotein was used, concentrations in excess of 0.2 μg per μl of reaction mixture appeared to be saturating. The addition of chelating agents and nonionic detergents inhibited transferase activity. The presence of MnCl_2 in the reaction mixture appeared to enhance the incorporation of fucose. The fucosyltransferase could be demonstrated in the soluble fraction.

INTRODUCTION

In the past decade, membrane associated enzyme systems have been described and implicated in the synthesis of macromolecules in general, and glycoproteins in particular. This work has been reviewed extensively (10,23,28).

The 6-deoxy-L-galactose, L-fucose, is found as a constituent of numerous glycoproteins. It has been found in plasma glycoproteins (7,27), blood group substances (32), glycoproteins of whole brain fractions and nerve endings (18,33), phytohemagglutins from seeds (8), plasma membrane glycoproteins (1), and phytotoxic glycoproteins (25,29).

The transfer of fucose into glycoproteins is catalyzed by a class of enzymes known as fucosyltransferases. Guanosine diphosphate fucose serves as the glycosyl donor in these reactions. The importance of this sugar nucleotide as the glycosyl donor was first demonstrated in vivo by Kornfield et al. (20). They had attempted to detect a nucleotide linked, fucose containing, oligosaccharide intermediate in the synthesis of the heterosaccharide chains of blood group substances. Their attempt to demonstrate the oligosaccharide nucleotide was unsuccessful. They did find large amounts of guanosine diphosphate fucose in the cell fraction, which was shown to contain the newly synthesized blood group substances.

The first workers to demonstrate an enzymatic transfer

of L-fucose from guanosine diphosphate-L-fucose used lactose as the fucosyl acceptor (12). The product of the reaction was the trisaccharide, fucosyllactose, which is the simplest fucose containing oligosaccharide found in milk. The enzyme which catalyzed this reaction was the particulate cell fraction of lactating canine mammary tissue. This particulate preparation would also catalyze the formation of the trisaccharide when incubated with D-glucose, uridine diphosphate-D-galactose, and guanosine diphosphate-L-fucose.

The in vivo incorporation of fucose into the glycoproteins of adult male rats was studied at length by Bekesi and Winzler (3) in 1967. They found that L-fucose-1-¹⁴C was a suitable precursor in their examination of the biosynthesis of the glycoprotein as the fucose was not rapidly converted into other sugars or CO₂. By studying the trichloroacetic acid soluble and insoluble radioactive products from animals sacrificed at various times, the following sequence of reactions was postulated:



More recently, Trujillo and Gan (31) have shown that the sequence of reactions proposed by Bekesi and Winzler can also be predicted from in vitro studies using bovine thyroid gland slices. The in vitro incorporation of fucose-1-¹⁴C was

similarly followed by chromatographic analysis of the trichloroacetic acid soluble and insoluble radioactive products found at various times during incubation.

Grollman and Marcus (13) demonstrated the in vitro incorporation of fucose into soluble blood group H substance. The glycosyl donor was guanosine diphosphate-L-fucose, and the enzyme preparation was obtained from hog gastric mucosa. An immunological technique was available for the identification of the radioactive endproduct as the receptor plus incorporated fucose was identical to the blood group H substance, the H specificity being determined by the terminal α -L-fucosyl-(1 $\rightarrow$ 2)- β -D-galactosyl linkage.

The antigenic determinants of the A, B, O(H) system all have fucose α -(1 $\rightarrow$ 2) linked to a D-galactosyl residue (26). However, the distinguishing feature between the Lewis blood group type a (Le^a) and the Lewis blood group type b (Le^b) is that the Le^b glycoprotein has a fucose (1 $\rightarrow$ 2) linkage whereas the Le^a glycoprotein does not (14).

Using this information and the fact that "secretors" secrete A, B, O(H) soluble blood group substances and "nonsecretors" secrete Le^a glycoprotein, Shen et al. (26) demonstrated that blood group substance specificity can be accounted for by a specific fucosyltransferase. From the information about the

fucosyl linkages in antigenic determinants of the various blood groups, it could be predicted that one should find a specific α -(1 $\rightarrow$ 2)-fucosyltransferase in "secretors" and an absence of this transferase in "nonsecretors". This was established to be the case.

Further work on α -L-fucosyltransferases and their relationships to blood group characters was done by Chester and Watkins (6) and by Grollman et al. (14) in 1969. In the former paper, 2-, 3-, and 4- α -L-fucosyltransferases were demonstrated in persons who are "secretors" of soluble blood group substances; "nonsecretors" were found to have 3- and 4- α -L-fucosyltransferases. The latter paper distinguishes enzymatically between women who are $Le^a(+)$ or $Le^b(+)$ and women who are $Le^a(-)$ or $Le^b(-)$. The structural determinants of Le^a and Le^b contain fucose linked α -(1 $\rightarrow$ 4) to N-acetylglucosamine. An α -(1 $\rightarrow$ 4)-fucosyltransferase was demonstrated in the milk of women who had Lewis type antigens. More recently, Jarkovsky (17) also did some work on fucosyltransferases in human milk which were specified by the Lewis blood type.

During this time, not all work on fucosyltransferases was done using the blood group substances. In 1968, Bosmann et al. (5) found two fucosyltransferases in HeLa cells. The

objections to some of the previous work on transferases using particulate enzyme preparations was avoided in this work by solubilizing the transferases with the nonionic detergent, Triton X-100. The evidence for two fucosyltransferases is based on specificity. Two primers showed successful incorporation of fucose: fetuin, from which sialic acid and galactose were removed exposing a terminal N-acetylglucosamine, and porcine submaxillary glycoprotein from which sialic acid and fucose were removed exposing a terminal galactose. Glycoprotein fucosyltransferase activity was sought in HeLa cells since 94% of the fucose in the cell is not extractable by organic solvents and therefore presumably bound in glycoproteins.

In the early 1970's, fucosyltransferases responsible for the incorporation of fucose into glycoproteins have been found in many systems. Grimes and Burge (11) have identified a fucosyltransferase in chick embryo fibroblast cultures responsible for the incorporation of fucose into the membrane glycoprotein of Sindbis virus. This activity can be demonstrated in infected and uninfected cells implying some sort of host modification. A fucosyltransferase has been isolated from rat small intestine mucosa responsible for α -(1 $\rightarrow$ 2) linkages in blood groups (4). Jabbal and Schachter (16) have found two fucosyltransferases in pork liver. They are differentiated not only by

primer specificity, but also by kinetic data. Parkhouse and Melchers (24) and Melchers (22) have found a fucosyltransferase responsible for the synthesis of the carbohydrate portion of immunoglobulin M (IgM) obtained from mouse plasma-cell tumor MOPC 21. The incorporation of fucose was necessary for the secretion of IgM. Zatz and Barondes (33,34) have identified fucosyltransferases in rat brain which will incorporate fucose into porcine plasma glycoproteins after they have been subjected to mild acid hydrolysis as well as into endogenous receptors.

No bacterial fucosyltransferases have been reported even though Ginsburg has identified an enzyme from Aerobacter aerogenes responsible for the formation of guanosine diphosphate fucose (9).

Since Corynebacterium insidiosum produces a high molecular weight phytotoxic glycoprotein of which 38.7% is fucose (25), it would seem reasonable that this bacterium possesses guanosine diphosphate-L-fucose fucosyltransferase activity. Therefore, the purpose of this report is twofold: 1) to demonstrate fucosyltransferase activity (Fig. 1), and 2) to establish some of the parameters of this activity.

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WASHINGTON, D. C.

Figure 1. Scheme of guanosine diphosphate-L-fucose fucosyltransferase activity from C. insidiosum using partially hydrolyzed phytotoxic glycoprotein as the primer.

GDP-L-FUCOSE+PRIMER+
CEII MEMBRANE ENZYME →

PRIMER-L-FUCOSE + GDP +
CEII MEMBRANE ENZYME

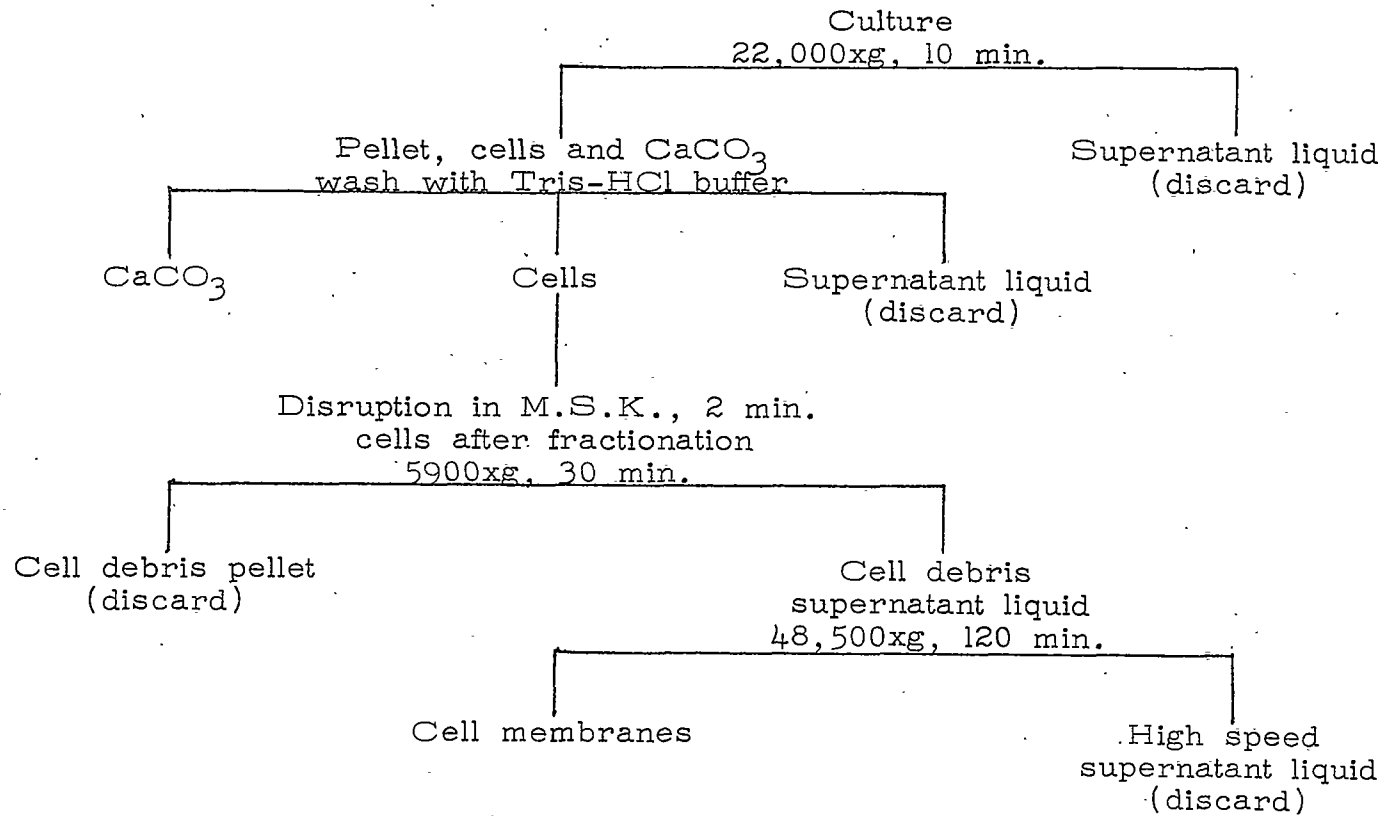
EXPERIMENTAL PROCEDURE

Culturing: The culture of Corynebacterium insidiosum (McCull.) Jensen (courtesy of F. I. Frosheiser, University of Minnesota) was maintained and cultured as described by Ries and Strobel (25) except that sucrose was substituted for glucose as the carbon source.

Preparation of cell free extract: The cells were harvested at specified times. The cell membranes and other subcellular fractions were obtained as described in Table 1. The cells were collected by subjecting the suspension to centrifugation at 22,000xg and 4°C. Subsequent steps were conducted at 4°C unless otherwise specified. The cells were washed 3-5 times with a buffer which consisted of Tris-HCl, 0.05M, pH 7.5 and 0.4M sucrose. The cells were disrupted in an Apparatebau Melsungen (referred to as M.S.K.) using glass beads (Glasperlen, 0.11-0.12 mm) previously washed in buffer. The cells were disrupted in 20 second bursts to a total of 2 minutes to avoid overheating. The cell membrane pellet, obtained by subjecting the cell debris supernatant liquid to centrifugation at 48,500xg for 120 minutes, was resuspended in a solution consisting of 0.05M Tris-HCl, and 0.4M sucrose, pH 7.5, using a 15 ml Pyrex glass homogenizer.

Protein determination: Protein was determined by the method of

Table 1. The fractionation scheme for C. insidiosum



Lowry et al. (21). Bovine serum albumin was used as a standard.

Preparation of primer: The phytotoxic glycoprotein from C. insidiosum was obtained as described by Ries and Strobel (25). The toxin was stored at -20°C until used. The primer was routinely prepared by dissolving 100 mg lots of glycoprotein in 2N HCl, 3-5 ml, in a sealed test tube and heating the solution in an oven at 100°C for 30 minutes. The product was dialyzed against frequent changes of distilled deionized water at 4°C for a minimum of 72 hours. The glycoprotein which came out of solution was removed by centrifugation and the resulting supernatant dried using hot air. When primer was needed, the dried supernatant was resuspended in distilled deionized water. The glycosidase-treated α_1 -acid glycoprotein was a gift from Dr. H. Schachter, Department of Biochemistry, University of Toronto.

Substrate: Guanosine diphosphate-L-fucose- ^{14}C (U), (GDP-fucose- ^{14}C), ammonium salt, 195 mCi/mmol, was obtained from Amersham/Searle and stored at -20°C until used. The solution used for enzyme assay was prepared by dissolving the GDP-fucose- ^{14}C in distilled water (55,500 cpm/ μl ; 128 p moles).

Fucosyltransferase assay: The enzyme preparation, primer,

and substrate were combined on a parafilm strip and then incubated in a small diameter glass tube at 20°C. The concentration of the various reactants was as specified. The reactions were terminated by spotting the reaction mixtures on Whatman no. 1 paper. The unreacted substrate was removed by high voltage paper electrophoresis at 24 v/cm in 0.05M sodium tetraborate, pH 9.1. Under these conditions, the substrate (and breakdown products of the substrate) will migrate but polymeric products will remain at the origin. The amount of fucose incorporated into glycoprotein was determined by cutting out the origin and measuring the radioactivity in a Packard Liquid Scintillation Spectrometer (Model 3320). The origin strip was immersed in a liquid scintillation cocktail consisting of 1.5 ml of absolute methanol and 13.5 ml of scintillation solution containing 5.0 g of 2,5-diphenyloxazole and 100 mg of p-bis-2(5-phenyloxazolyl)-benzene per liter of toluene. The channels ratio method was used to correct for quenching.

One experiment was conducted using a Sephadex G-25 column to separate the unreacted substrate from the reaction mixture. The reaction was incubated in a small test tube at 20°C and 35 revolutions per minute. The reaction was terminated by placing the test tube in a boiling water bath and drying the reaction mixture with a stream of nitrogen gas. The

product mixture was resuspended in 400 μ l of distilled water and fractionated by chromatography on a Sephadex G-25 column, 31 cm x 1.9 cm. The 0.5 ml fractions were collected, and then placed in counting vials containing 15 ml of Aquasol universal liquid scintillation cocktail (New England Nuclear).

Verification of incorporation of fucose: The reaction products were eluted from the origin with 10 ml of water per origin after electrophoresis of 13 standard reaction mixtures. The eluant was collected and dried using hot air, and then further dried under vacuum over P_2O_5 at room temperature. The radio-labeled glycoprotein was dissolved in distilled water to give a final concentration of 3640 dpm in a 100 μ l. A 30 μ l aliquot of glycoprotein was dried in a test tube and resuspended in 2.0 ml of 1N TFA (trifluoroacetic acid) and subjected to hydrolysis in a sealed test tube for 2 hours at 120°C.

Sugars were identified by descending paper chromatography in ethyl acetate-acetate-formic acid- H_2O (18:3:1:4) v/v/v/v. The sugar standards were located using the methods of Trevelyan (30). The radiolabeled primer and monosaccharides were located using a Packard Radiochromatogram Scanner (Model 385).

Materials: Triton X-100 and tris(hydroxymethyl)aminomethane

(Trizma) were obtained from Sigma Chemical Company. The sodium cacodylate and the EDTA were obtained from K&K Laboratories, Inc. All other chemicals used were reagent grade.

RESULTS

Products of the reaction: Electrophoresis of any reaction mixture containing primer and active enzyme yielded two radioactive peaks. One was at the origin and the other was a broad peak extending 10 cm to 16 cm off the origin relative to the movement of the picric acid marker at 20 cm. The components which remained at the origin were subjected to descending paper chromatography following elution. The results are shown in Figure 2. Chromatography of the unhydrolyzed product showed all the radioactivity at the origin. The hydrolyzed reaction product showed a trace of radioactivity at the origin and a majority of the radioactivity approximately four inches from the origin. Only trace amounts of the radioactivity incorporated appeared to be soluble in the liquid scintillation cocktail.

Location of transferase activity in the subcellular fractions:

Subcellular fractions were assayed for fucosyltransferase activity. The results from a typical cell preparation procedure are shown in Table 2. The high speed supernatant liquid demonstrated a higher specific activity than the cell membrane precipitate, however, inasmuch as the activity associated with the membrane fraction could be easily concentrated by pelleting at 48,500xg, it was used as a subject of this study. Dr. William Hess kindly supplied the electron micrograph (Fig. 3) of the cell

I6

Figure 2. Tracing of radiochromatograms of sugar residues found in unhydrolyzed (X) and hydrolyzed (Y) phytotoxic glycoprotein primers. Each complete reaction mixture contained: phytotoxic glycoprotein subjected to mild acid hydrolysis, 3.7 μg ; GDP-fucose- ^{14}C , 26.2 p moles; Tris, pH 7.5, 1.25 micromoles; sucrose, 10 micromoles; and 20 μg of enzyme protein in a total volume of .035 ml. The cells were cultured for 50 hours prior to being harvested.

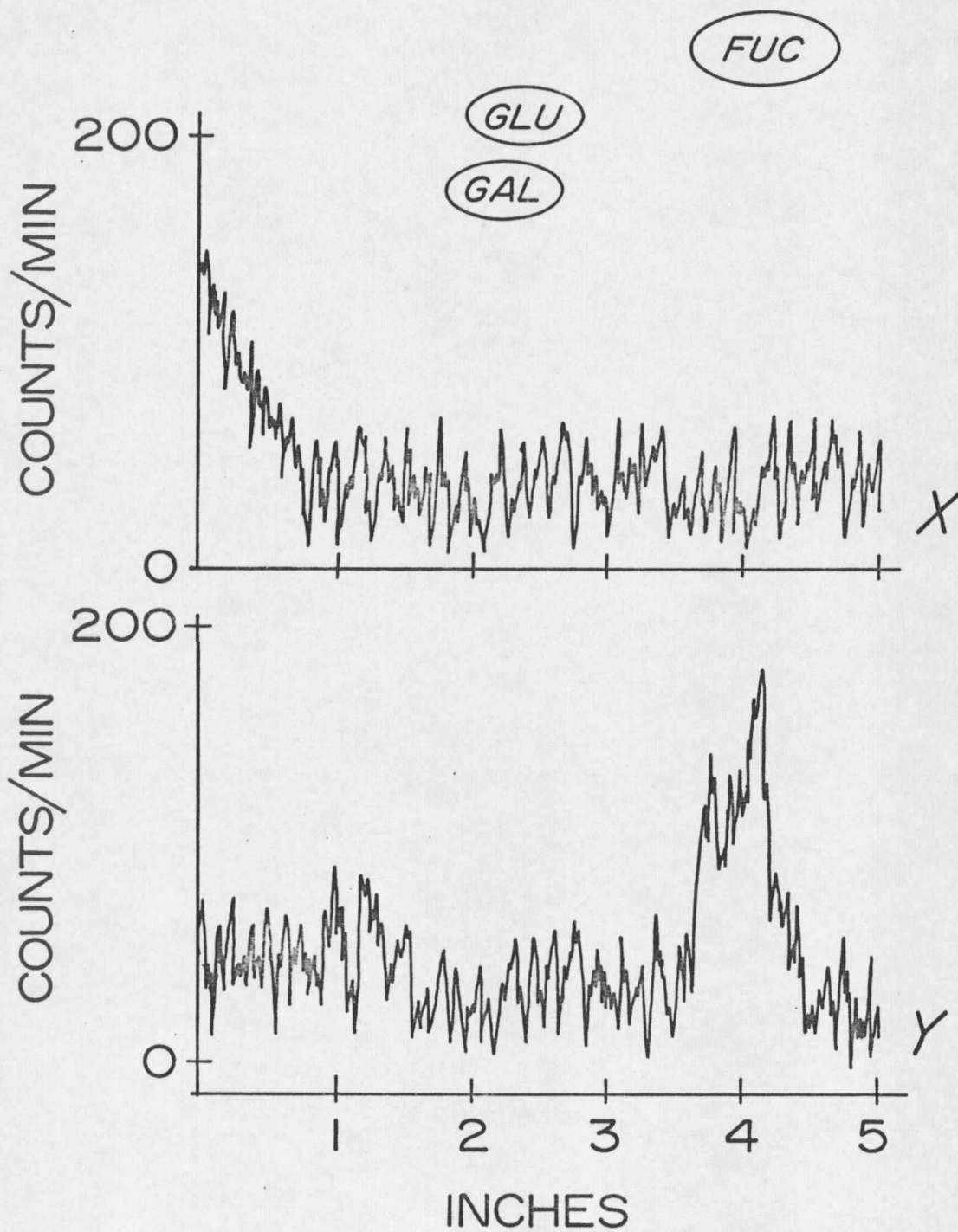


Table 2. The location of GDP-fucosyltransferase activity in the various subcellular fractions of C. insidiosum

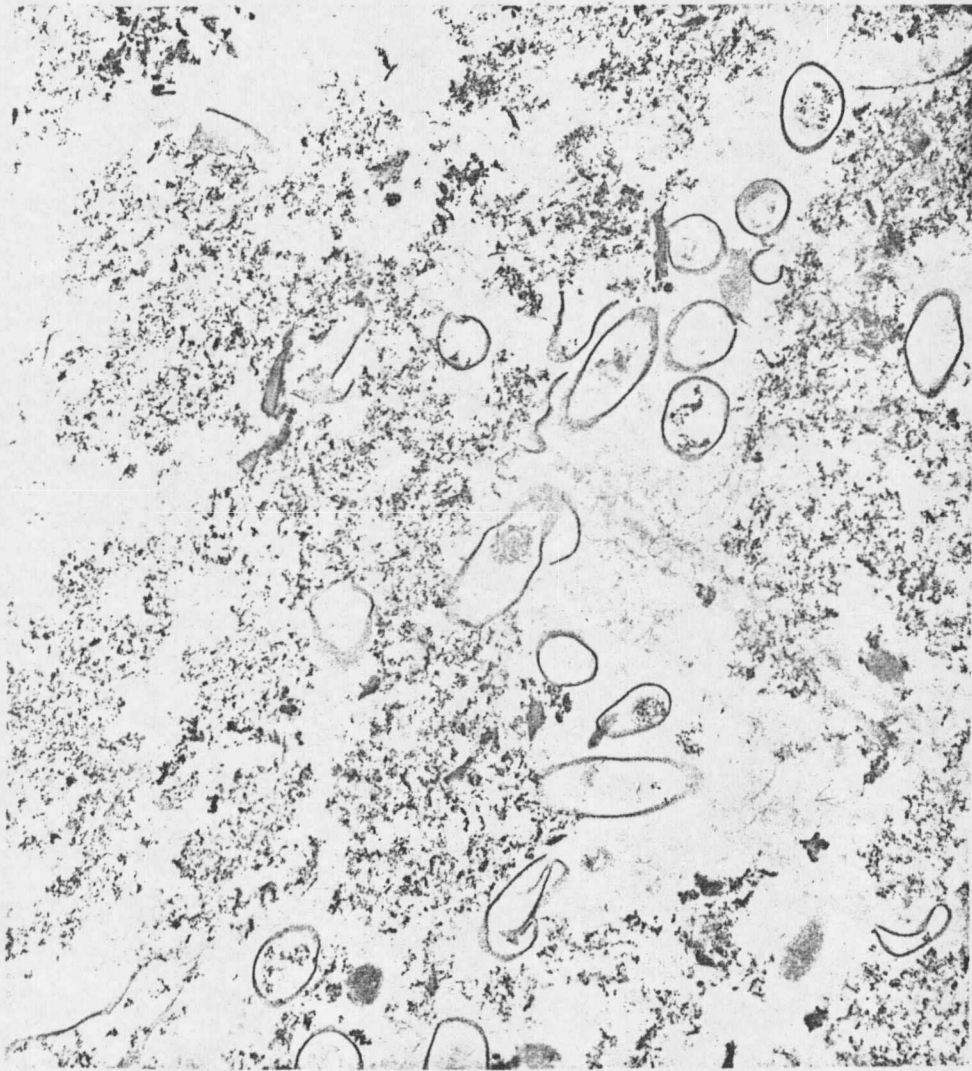
Subcellular fraction	Volume ml	Total ^a activity	Specific ^b activity
Cells after fractionation (crude preparation)	245	4900	8.0
Cell debris (supernatant liquid) after 5900xg	150	8550	47.5
Cell debris (precipitate)	45	4320	16.0
High speed supernatant liquid after 48,500xg	115	2783	22.0
Cell membranes	8	270	13.0

^a The number of p moles of fucose incorporated per ml of subcellular fraction.

^b The number of p moles of fucose incorporated per mg of protein.

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Figure 3. An electron micrograph of the cell membrane fraction. The field is magnified 10,200 X.

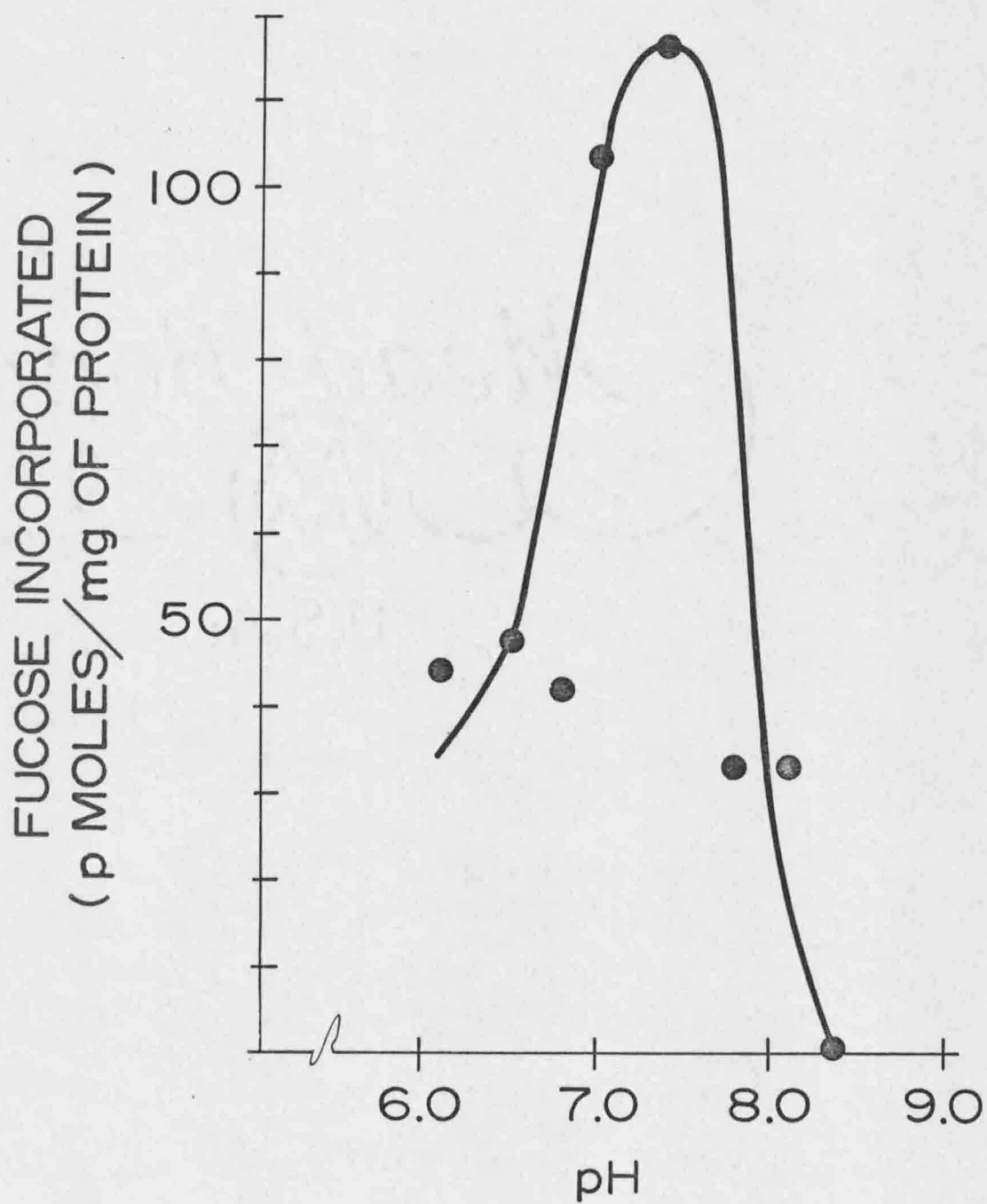


membrane fraction.

Reaction properties: The fucosyltransferase activities have a pH optimum at pH 7.5 (Fig. 4). The time course of fucose incorporation appeared to be linear after the first two minutes (Fig. 5) and continued to be linear up to 45 minutes. Two points were obtained at 30 and 45 minutes using a cell membrane preparation obtained by sonication and are therefore not shown. Concentration of the primer in the reaction mixtures did affect the incorporation of fucose (Fig. 6) with the saturating amount of primer needed for maximum activity at 7-10 μg (0.2 μg per μl of reaction mixture). The addition of nonionic detergents and chelating agents did not enhance transferase activity (Table 3). The presence of MnCl_2 at high concentrations appeared to increase the amount of fucose incorporation but the addition of MgCl_2 completely inhibited the reaction, even at low concentrations (Table 3). Guanosine triphosphate did not enhance incorporation of fucose (Table 3).

Several changes were made in the reaction mixture, incubation procedure, and method of separating the reaction product from unreacted substrate yielded a considerably higher amount of incorporation (Fig. 7). The difference between the control and the active preparation of 50,000 counts is equivalent to 116

Figure 4. The pH dependence of fucose incorporation. The pH's denoted are of the reaction mixture. The three reactions having acid pH contain sodium cacodylate buffer; the remaining five contain Tris buffer. Each complete reaction mixture contained: phytotoxic glycoprotein subjected to mild acid hydrolysis, 3.7 μ g; GDP-fucose- ^{14}C , 26.2 pmoles; buffer, 1.25 micromoles; sucrose, 10 micromoles; and enzyme protein in a total volume of .035 ml. The cells were cultured for 50 hours prior to being harvested.



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Figure 5. The dependence of fucose incorporation on time. Each complete reaction mixture contained: phyto-toxic glycoprotein subjected to mild acid hydrolysis, 3.7 μ g; GDP-fucose- ^{14}C , 26.2 p moles; Tris, pH 7.5, 1.25 micromoles; sucrose, 10 micro-moles; and 20 ug of enzyme protein in a total volume of .035 ml. The cells were cultured for 50 hours prior to being harvested.

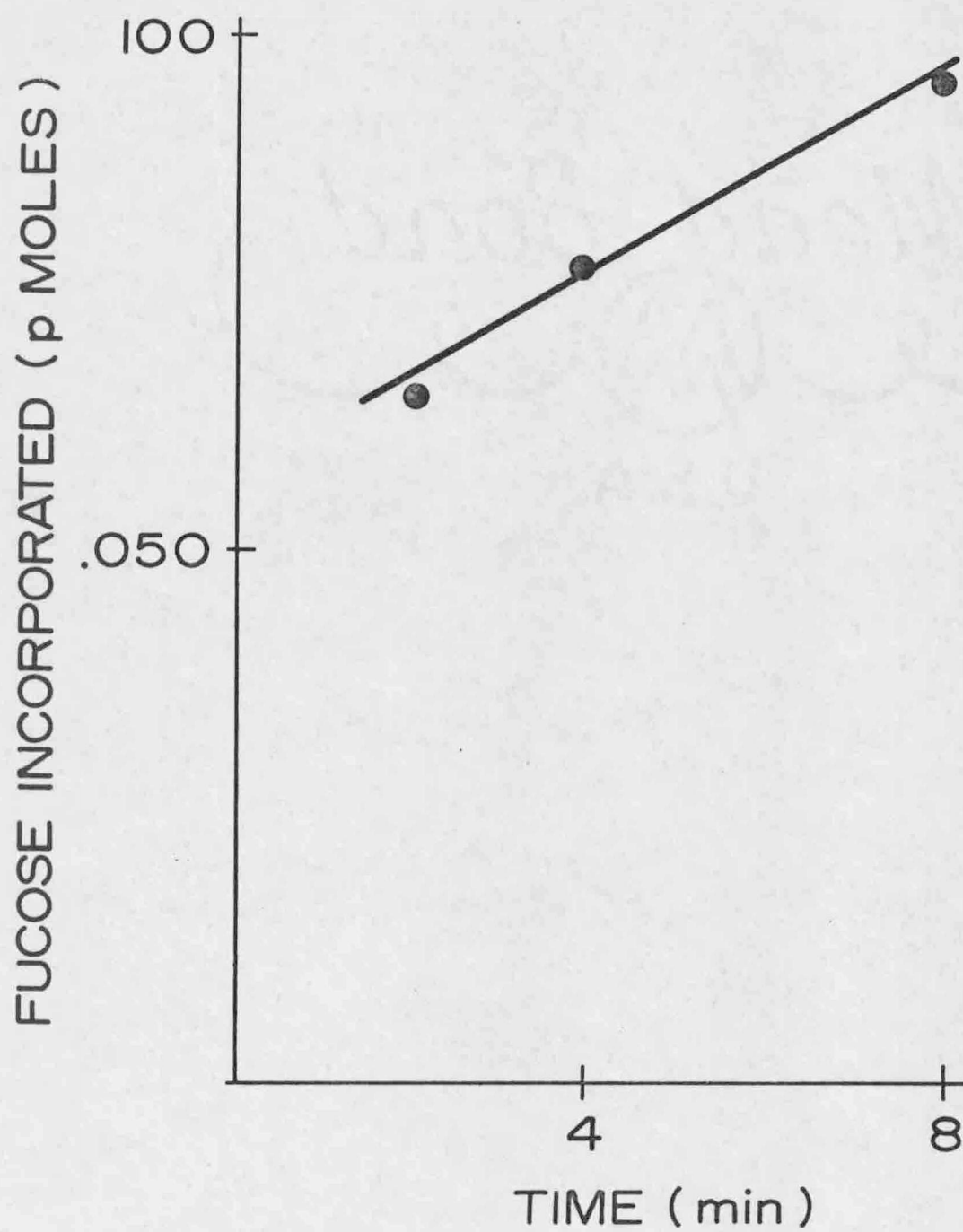


Figure 6. The dependence of fucose incorporation on the concentration of the primer. Each complete reaction mixture contained: GDP-fucose-¹⁴C, 26.2 p moles; Tris, pH 7.5, 1.25 micromoles; sucrose, 10 micromoles; enzyme protein, 20 µg; and the phytotoxic glycoprotein subjected to mild acid hydrolysis in a total volume of .050 ml. The cells were cultured for 50 hours prior to being harvested.

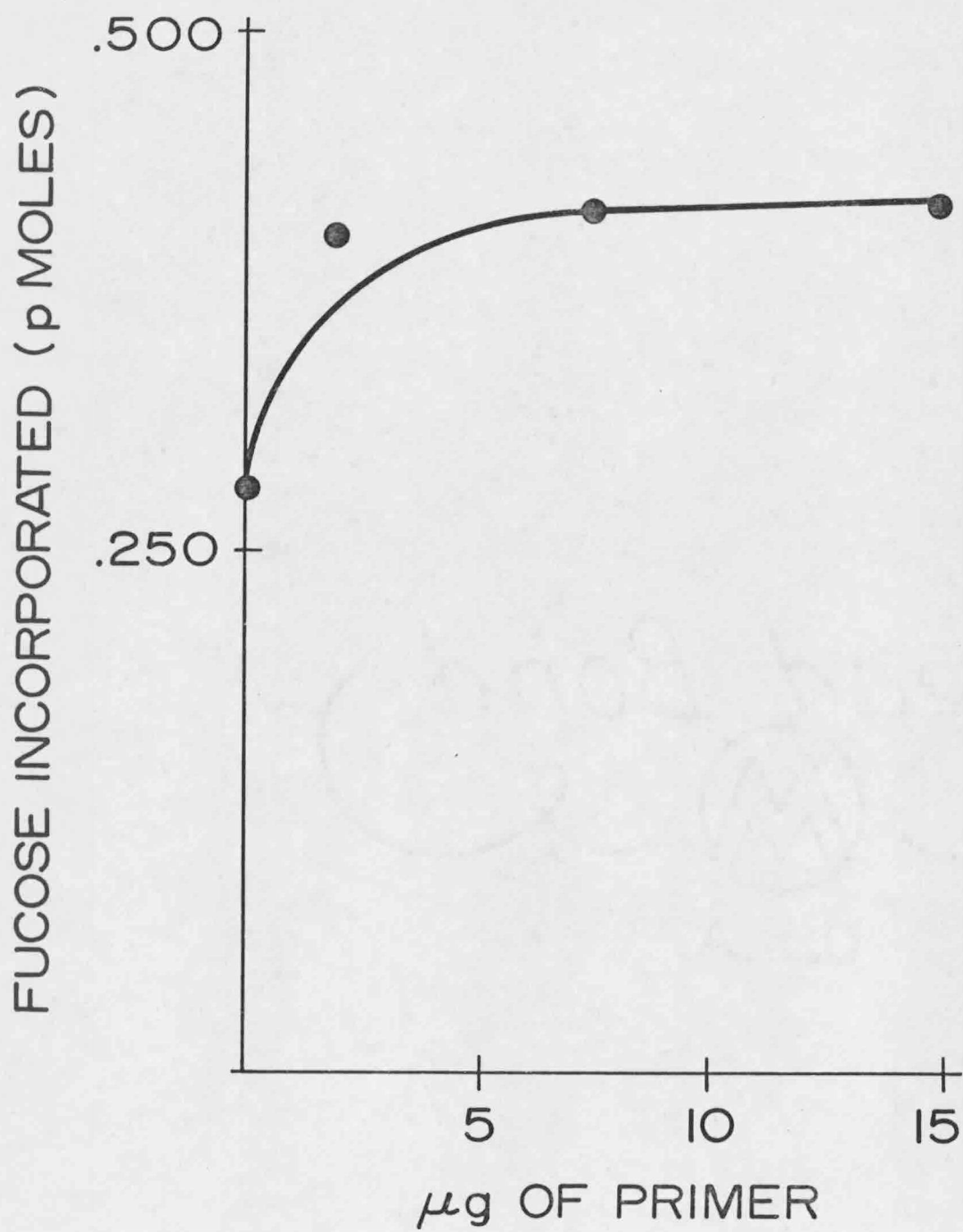
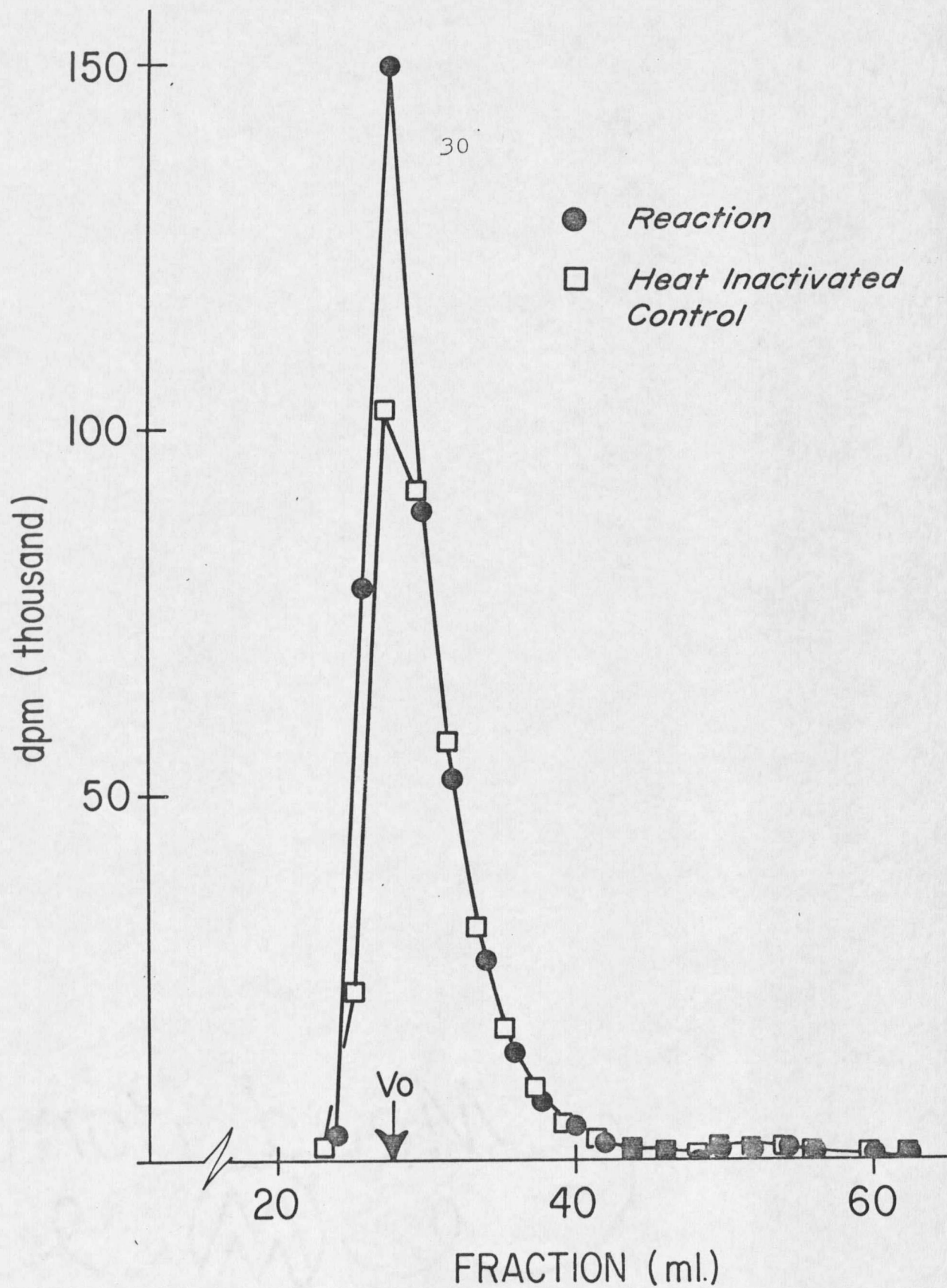


Table 3. The effect of various agents on fucosyltransferase activity

Changes in the normal incubation mixture	Relative fucosyltransferase activity
	%
None	100
1 μ l of Triton X-100, 1.0%	6
0.1%	67
EDTA, 1.0 mM	22
0.1 mM	67
MgCl ₂ , 10 μ moles	0
5 μ moles	0
2 μ moles	0
MnCl ₂ , 10 μ moles	306
5 μ moles	0
2 μ moles	0
GTP, 0.025 μ moles	33
None, enzyme preparation from cells fractionated in presence of 1 mM EDTA	50
None, enzyme preparation from cells fractionated in presence of 0.1% Triton X-100	0

Figure 7. The column chromatography of reaction mixtures on Sephadex G-25. Each complete reaction mixture contained: phytotoxic glycoprotein subjected to mild acid hydrolysis, 20 μ g; GDP-fucose-¹⁴C, 1920 p moles; Tris, pH 7.5, 2.75 micromoles; sucrose, 22 micromoles; and 247.5 μ g of enzyme protein in a total volume of .100 ml. The cells were cultured for 65 hours prior to being harvested.

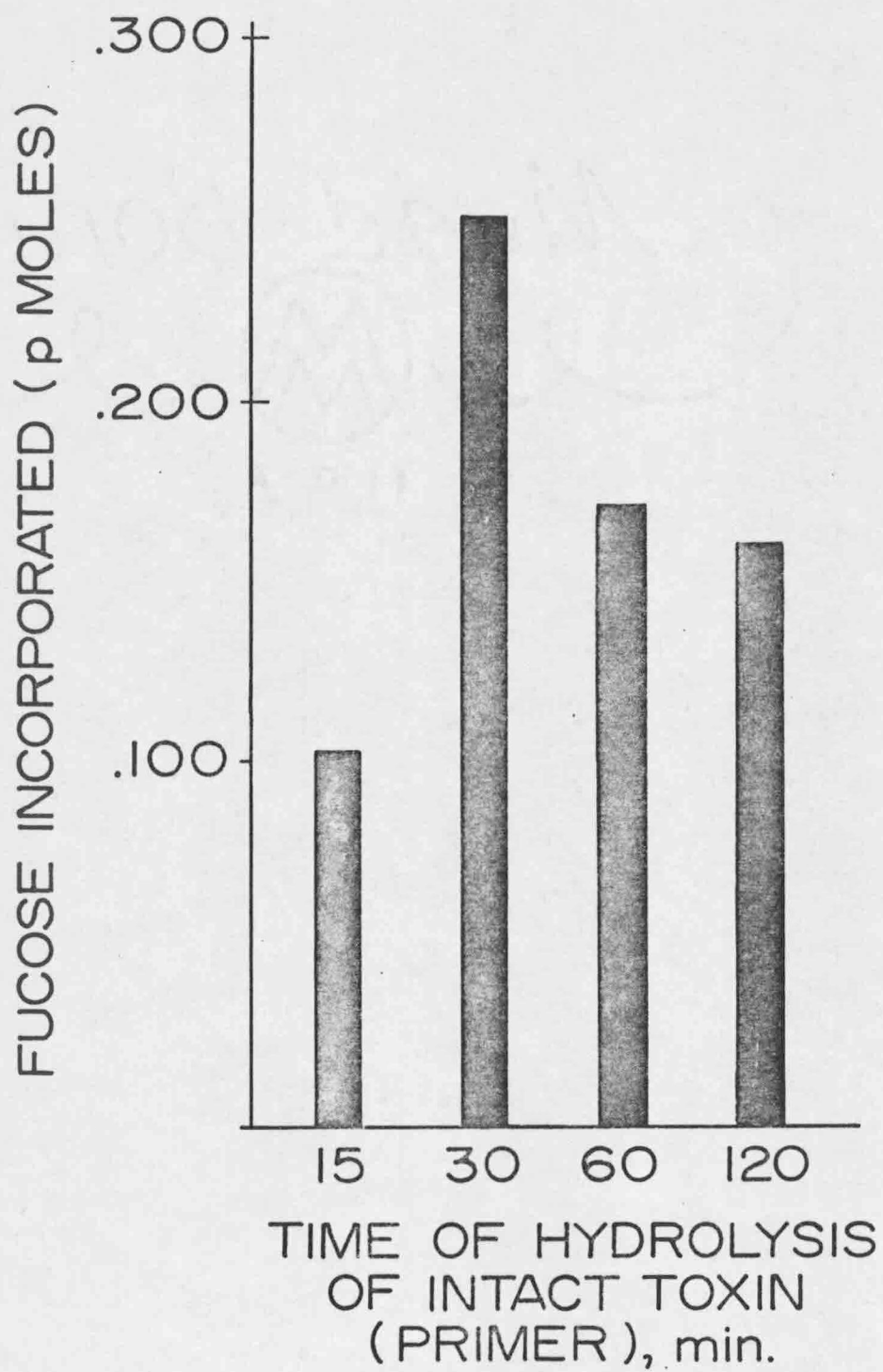


p moles of fucose incorporated.

Acceptor specificity studies: The phytotoxic glycoprotein from C. insidiosum was hydrolyzed for varying amounts of time according to the procedure described previously. The toxin hydrolyzed for 30 minutes served as the most suitable acceptor for fucose (Fig. 8). Transferase activity could be successfully demonstrated using enzymatically treated α_1 -acid glycoprotein as a primer (Fig. 9). The amount of fucose incorporated was inversely proportional to the amount of primer present up to a concentration of 0.8 μ g of primer per μ l of reaction mixture. The α_1 -acid glycoprotein treated with sialidase appeared to be the best primer for the enzymatic incorporation of fucose (Table 4).

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Figure 8. The affect of acid hydrolysis on the ability of the phytotoxic glycoprotein to serve as a fucosyl acceptor. Each complete reaction mixture contained: phytotoxic glycoprotein subjected to mild acid hydrolysis, 1 μ g; GDP-fucose-¹⁴C, 128 pmoles; Tris, pH 7.5, .25 micromoles; sucrose, 2.0 micromoles; and 13 μ g of enzyme protein in a total volume of .010 ml. The cells were cultured for 64 hours prior to being harvested.



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Figure 9. The use of fucosyltransferase to incorporate fucose into various derivatives of human plasma α_1 -acid glycoprotein. Each complete reaction mixture contained: phytotoxic glycoprotein subjected to mild acid hydrolysis, 1 μ g; GDP-fucose- ^{14}C , 128 p moles; Tris, pH 7.5, .25 micromoles; sucrose, 2.0 micromoles; and 13 μ g of enzyme protein in a total volume of .010 ml. The cells were cultured for 64 hours prior to being harvested.

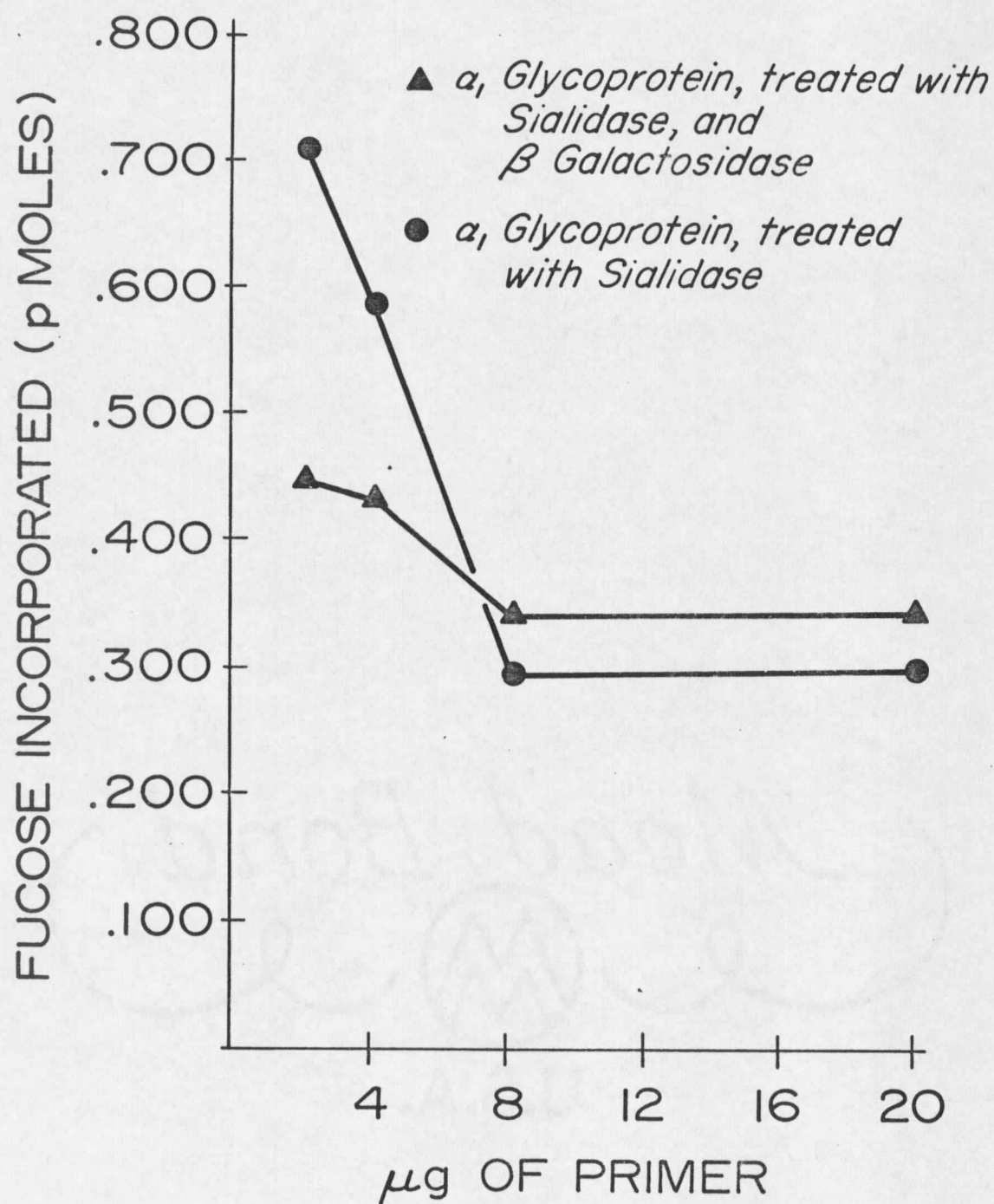


Table 4. The relative incorporation of fucose- ^{14}C achieved with various primers

Fucosyl acceptor	Relative fucosyltransferase activity
	%
Phytotoxic glycoprotein	
15 min. hydrolysis	15
30 min. hydrolysis	36
60 min. hydrolysis	24
120 min. hydrolysis	23
Endogenous primer ^a	23
α_1 -acid glycoprotein, treated with sialidase ^b	100
α_1 -acid glycoprotein, treated with sialidase: β -galactosidase ^c	61

^a Native glycoprotein in the cell membrane preparation

^b Optimum concentration of 4 $\mu\text{g}/\mu\text{l}$ of reaction

^c Optimum concentration of 2 $\mu\text{g}/\mu\text{l}$ of reaction

DISCUSSION

In the standard assay for enzyme activity, the amount of fucose incorporated into the glycoprotein primer was considered to be the amount of radioactivity at the origin after electrophoresis and subtraction of the heat inactivated control. Ries and Strobel (25) found that the phytotoxic glycoprotein from C. insidiosum would not migrate in an electrophoretic field. The radioactivity found in the peak which had migrated off the origin was presumed to contain unreacted substrate and substrate breakdown products. This heterogeneity and the reverse osmotic flow as a result of subjecting the paper to electrophoresis for four hours would account for the broadness of the peak.

The data obtained by eluting the reaction product which remained at the origin and then subjecting it to descending paper chromatography indicated that the primer which bound the fucose was homogenous in respect to its tendency to remain at the origin. The chromatography of the reaction product which was hydrolyzed indicated that the radioactivity was incorporated in an acid labile bond, and that this radioactivity demonstrated a similar R_{glucose} as fucose. It was established that the radioactivity which remained at the origin after electrophoresis was incorporated and that this radioactivity corresponded to fucose.

Any fucose remaining at the origin in the standard reaction mixtures apparently is not the result of a fucolipid inter-

mediate since only traces of the radioactivity became soluble in the toluene liquid scintillation cocktail.

The transferase activity was found to be concentrated in the membranes and high speed supernatant liquid cell fractions. The transferase was not consistently found in the soluble fraction. Bauer et al. (2) found that mannosyltransferase could only be demonstrated in Dictyostelium discoideum soluble fractions at certain times in its growth cycle. The cell membranes were used throughout the work as the preliminary parameters for transferase activity were established with that fraction. The activity was more concentrated in the cell membrane preparation, and this was an important consideration in regard to reaction volumes. No ready technique was available for reduction of the high speed supernatant liquid volume.

The fucosyltransferase activity appeared to have a pH optimum of 7.5 which is consistent with the optimum observed by other investigators (6,13,16). The reaction appeared to demonstrate a linear time course up to 45 minutes.

The amount of primer that will saturate the reaction is quite low compared to what other investigators have observed. For example, Jabbal and Schachter (16) routinely employed 100 μg of α_1 -acid glycoprotein in a 10 μl reaction mixture. Their reactions also contained 5.2 μg of protein and 2400

p moles of substrate per μ l of reaction mixture. The disparity in protein concentration (approximately 2.0 μ g/ μ l in my reactions) is not as great as the variance in substrate levels (12.6 p moles per μ l in my reactions). The comparatively small amount of primer necessary to achieve saturation might be related to the low substrate levels employed.

Jabbal and Schachter (16) showed that the presence of the chelating agent EDTA caused a 65% decrease in activity. However, Hudgin and Schachter (15) found only a 10% decrease in the activity of sialyltransferase with the addition of EDTA. The addition of EDTA to the reaction mixture, or the disruption of the cells in the presence of the chelating agent was found to be deleterious to the activity of the transferase (Table 3).

The presence of Triton X-100 in some particulate preparations has been found to enhance enzyme activity (4,11,16). This was not true in the case of this enzyme since increasing the amount of detergent in the reaction mixture caused a decrease in the activity (Table 3). This is in contrast to Jabbal and Schachter (16) who found that Triton X-100 was desirable in the reaction mixture and that addition of the detergent beyond optimum concentration did not adversely affect the activity; the optimum concentration in their work was 0.1% and they increased the concentration up to 2.0%. The detergent apparently acts on

the lipid constituents of the membrane causing it to unfold with the result that more enzyme is exposed. Other workers have employed Triton X-100 to solublize membrane bound fucosyltransferases. Bosmann et al. (5) described the characterization of two such transferases in HeLa cells. The disruption of C. insidiosum cells in the presence of the detergent failed to yield a detectable soluble fucosyltransferase.

The addition of MnCl_2 to the reaction mixture produced a rather dramatic increase in the activity. But, MgCl_2 appeared to totally inhibit the reaction. These data, although reproducible, are suspect due to the very high background levels of incorporation encountered in the heat inactivated controls.

A significant increase in the amount of detectable fucose incorporation was obtained by changing the reaction mixture, incubation procedure, and the method of separating unreacted substrate from reacted substrate (Fig. 7). No large changes in the relative concentration of the ingredients in a typical reaction were made. There was a twofold increase in the amount of primer, and a 1.5 fold increase in the substrate concentration. The total volume was increased to 100 μl and this made it possible to agitate the reaction mixture during incubation. The significant difference necessary to account for an approximately 250 fold increase in demonstrable fucosyltransferase

activity is difficult to explain. The constant mixing of the reaction ingredients seems to be worthy of notice. The method of separating the reacted substrate from the incorporated fucose should also be considered. The constant stirring may have significantly increased the chance collisions between substrate and enzyme. Possibly, a glycoprotein fragment capable of binding fucose which eluted at the void volume of the Sephadex G-25 column, will migrate from the origin on the electrophoresis. Maybe long exposure to the sodium borate buffer caused incorporated fucose to eventually migrate from the origin. The high amount of incorporation obtained in the heat inactivated control is hard to explain but not unusual in this type of system. Other methods are generally found before the information is published (19).

The phytotoxic glycoprotein characterized by Ries and Strobel (25) proved to be a suitable acceptor for fucose after mild acid hydrolysis. The glycoprotein contains five monosaccharides: L-fucose, mannose, glucose, galactose, and rhamnose in a ratio of 10:1:5:5; rhamnose was only present in trace amounts. The removal of fucose as well as the other sugars has been verified by paper chromatography and by gas-liquid chromatography. The toxin hydrolyzed for 30 minutes showed the optimum ability to incorporate fucose. Hydrolysis for that

length of time removed the optimum amount of fucose without destroying specific binding sites for the sugar and the enzyme. Although the α_1 -acid glycoprotein kindly supplied by Doctor Schachter showed a greater amount of incorporation, a comparison of binding efficiencies is not appropriate as the plasma glycoprotein's binding sites were prepared enzymatically which is a more specific and gentler technique. The higher amount of incorporation into the sialidase treated α_1 -acid glycoprotein might imply the predominance of a fucosyltransferase specific for fucose α -(1 $\rightarrow$ 3)-galactose linkages. However, incorporation was also seen in the sialidase- β -galactosidase treated glycoprotein implicating a transferase specific for fucose:N-acetylglucosamine linkages; there is no N-acetylglucosamine present in the phytotoxic glycoprotein. Also, the decrease in the amount of fucose incorporated with an increase of the α_1 -acid glycoprotein concentration needs some clarification. Possibly, there are binding sites available for the enzyme where no binding sites for the sugar exist. Here again, low substrate levels would aggravate the condition.

SUMMARY

Guanosine diphosphate-L-fucose fucosyltransferase activity has been found in a crude cell membrane preparation from Corynebacterium insidiosum. The importance of this observation lies in that no previous report of a fucosyltransferase in bacteria has been made. The fucosyl acceptor utilized to establish some of the parameters of the reaction was the phyto-toxic glycoprotein produced by the organism. The glycoprotein was first subjected to mild acid hydrolysis and then used as an acceptor. Incorporation of fucose into human plasma α_1 -acid glycoproteins by this fucosyltransferase implies a specificity determined by terminal and penultimate sugars.

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