ENZYMATIC INCORPORATION OF FUCOSE INTO BLOOD GROUP H SUBSTANCE*

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The soluble blood group substances found in animal secretions are glycoproteins composed of approximately 85% carbohydrate and 15% protein. Their serological specificity is determined by oligosaccharide side chains covalently attached to a peptide backbone. This communication describes the enzymatic incorporation of fucose from guanosine diphosphate L-fucose (GDP-fucose)into microsomal-bound blood group H substance, catalyzed by a particulate preparation from hog gastric mucosa. As H specificity is determined by an α-L-fucosyl (1——>2)-β-D galactosyl linkage (Rege et al., 1964; Lloyd et al., 1966), this enzymatic reaction represents a terminal step in the biosynthesis of the H determinant.

Materials and Methods: GDP-fucose-C^{14}, fucose 1-phosphate-C^{14} and fucose-C^{14} were prepared as described by Grollman et al., (1965). Hog H substance was a gift from Dr. Elvin Kabat, $Lewis^a$ (Le^a)substance was purified from human ovarian cyst fluid (Marcus and Grollman, 1966), nucleotide triphosphates were obtained from Pabst Biochemicals, GDP-mannose-C^{14} was prepared by the method of Rosen and Zeleznick (1966) and eel serum was kindly provided by Dr. Georg Springer.

Microsomes, prepared from hog gastric mucosa by the procedure described by Kornfeld, et al., (1965), were suspended in a buffer composed of 0.1 M

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TABLE 1

Requirements for the Incorporation of Fucose

Assay Conditions	Incorporation
	μμmoles
Complete System	17.6
-Mg^{++}	1.6
-Mg^{++}; +Mn^{++}	16.7
-GTP	4.3
-GTP; +ATP, UTP and CTP	17.4
-GDP-fucose-C^{14}; +fucose-C^{14}	0.0
-GDP-fucose-C^{14}; +fucose 1-phosphate-C^{14}	0.0
Incubated at 0°	0.1

The complete reaction mixture, in a final volume of 1 ml, contained 100 μmoles Tris HCl, pH 7.2; 8 μmoles $MgCl_2$; 4.2 μmoles GTP; 0.125 mμmole GDP-fucose-C^{14}, specific activity 91 mc/mM and 4 mg microsomal protein. In other reactions $MgCl_2$ was replaced by 8 μmoles $MnCl_2$, GTP by 1.4 μmole each of ATP, CTP and UTP and GDP-fucose-C^{14} by an equivalent amount of fucose 1-phosphate-C^{14} or fucose-C^{14} with specific activities of 91 mc/mM. Reactions were incubated for 90 minutes at 37° and the radioactivity incorporated into the microsomes was determined as described in the text.

Tris-0.008 M $MgCl_2$. Protein concentration was estimated by the biuret reaction (Layne, 1957). Components of the complete reaction mixture are described in the legend to Table 1. Following incubation at 37°, 10 volumes of a buffer containing cold 0.25 M sucrose, 0.025 M KCl and 0.004 M $MgCl_2$ was added and the microsomes were recovered by centrifugation for 60 minutes at 100,000 x g. The pellet was rinsed three times with 0.25 M sucrose, suspended in cold 5% trichloroacetic acid (TCA) and the precipitate collected on a Millipore filter. Radioactivity was determined using a gas-flow detector with an efficiency of 21% for C^{14} or in a scintillation counter with an efficiency of 87% for C^{14}.

Solvent systems employed for paper chromatography and electrophoresis (Grollman, et al., 1965) and the techniques employed for precipitin analysis and hemagglutination inhibition (Marcus and Grollman, 1966) are described elsewhere.

Results: Requirements for maximum incorporation of fucose are shown in Table 1. Mg^{++} is required as a cofactor but can be replaced by Mn^{++}. The presence of GTP or other nucleotide triphosphates increased the incorporation of fucose, presumably by decreasing the breakdown of GDP-fucose (Grollman et al., 1965). GDP-fucose cannot be replaced as the fucosyl donor by fucose 1-phosphate or by fucose. Addition of 1 mg of hog H substance, hog A substance or human Le^a substance or of the 100,000 x g supernatant fraction did not stimulate the reaction. A sharp pH optimum was observed at pH 7.2.

The rate of incorporation of fucose and mannose into microsomal-bound TCA-insoluble material is illustrated in Figure 1. Since mannose is not a constituent of porcine blood group substances, (Kabat, 1956), the mannose-labelled product serves as a control representing microsomal-bound carbohydrate devoid of blood group specificity. Twenty-five percent of the added GDP-fucose was incorporated into microsomal-bound form and the remaining radioactivity was recovered and identified by previously described methods

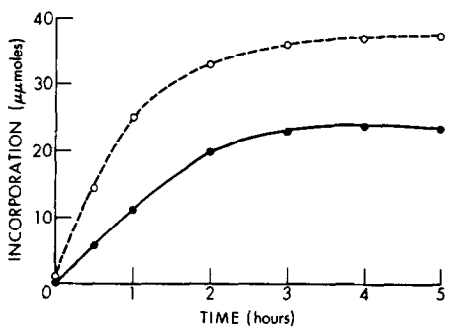

Fig. 1. Incorporation of fucose and mannose into microsomal-bound form. Reaction mixtures contained, in a final volume of 10 ml, 1.0 mmole Tris HCl, pH 7.2; 80 μmoles GTP; and 1.25 mμmole GDP-fucose-C^{14}, specific activity, 91 mc/mM, (●——●) or GDP-mannose-C^{14}, specific activity 91 mc/mM (○——○) and 40 mg microsomal protein. Incubations were carried out at 37° and, at the indicated intervals, 1.0 ml aliquots were added to cold buffer, centrifuged and precipitated with TCA as described in the text.

Hydrolysis of the intact microsomes or of the DOC-soluble, H_2O-soluble fraction (cf Table 3) in 1 N H_2SO_4 at 100° for one hour released a single radioactive compound from each of the reaction products which had the chromatographic mobility of fucose and mannose, respectively.

TABLE 2

Incorporation of Fucose into Microsomes Prepared from Hogs of Different Blood Group Specificity[1]

Hog	A titer[2]	H titer[2]	Incorporation
			μμmoles
1	0	2048	20.1
2	0	2048	12.4
3	0	1024	9.4
4	0	1024	8.7
5	2048	64	0.3
6	16	2	0.1
7	32	8	0.5
8	16	0	0.4

[1] Composition of the reaction mixture and the conditions for incubation are described in the legend to Table 1.

[2] Tested on 100,000 x g supernatant solution of homogenates of hog gastric mucosa. Titer expressed as reciprocal of highest dilution producing complete inhibition of hemagglutination.

(Grollman et al., 1965) as fucose 1-phosphate and fucose. The extent of fucose incorporation depended on the blood group of the hog from which the particles were prepared. Microsomes prepared from hogs of type H incorporated 20 times as much fucose as those prepared from hogs whose mucin exhibited both A and H activity (Table 2). The microsomal-bound radioactivity in the fucose-labelled and mannose-labelled products was released by suspending the particles in DOC. This procedure is known to release newly-synthesized blood group material from microsomes prepared from hog gastric mucosa (Kornfeld, et al., 1965). Following removal of DOC by dialysis, some of the radioactivity was sedimented by centrifugation at 100,000 x g. Subsequent characterization was performed only on the soluble material. If endogenous blood group material was not present in sufficient quantity to obtain an immune precipitate purified hog H substance was added as carrier.

Immunochemical identification of part of the soluble fucose-labelled material as blood group H substance is shown in Table 3. In three represen-

TABLE 3

Distribution and Identification of Fucose-labelled Reaction Product

Hog	Nucleotide Added	Microsomal-bound Radioactivity			Fraction DOC-soluble, H_2O soluble Fucose-C^{14} Precipitated by	
		Total	DOC-soluble, H_2O-insoluble	DOC-soluble, H_2O-soluble	Eel Serum	Eel Serum & Fucose
		$\mu\mu$moles			%	
1	GDP-fucose-C^{14}	165	72	63	43	1
2	GDP-fucose-C^{14}	218	76	69	10	1
	GDP-mannose-C^{14}	349	97	99	0	-
3	GDP-fucose-C^{14}	95	8	28	36	2
	GDP-mannose-C^{14}	62	4	46	0	-

Reaction mixtures contained, in a final volume of 10 ml, 1 millimole Tris HCl, pH 7.2; 80 μmoles $MgCl_2$; 42 μmole GTP; 100 mg microsomes and either 2.25 mμmoles GDP-fucose-C^{14}, specific activity 91 mc/mM or 2.25 mμmoles GDP-mannose-C^{14}, specific activity 91 mc/mM. Incubations were carried out at 37° for 6 hours. 40-80% of the total microsomal-bound radioactivity was released from the microsomes by treatment with 0.3% sodium desoxycholate (DOC). Exhaustive dialysis to remove DOC was followed by centrifugation to remove membranes and H_2O-insoluble material. 50 micrograms of carrier H substance was added in Experiments 2 and 3.

Precipitin reactions contained 0.9% NaCl; 0.02 M Na_2PO_4, pH 7.2; radioactive blood group substance and sufficient eel serum to provide an excess of antibody as tested against Type O human erythrocytes. L-fucose was present in a final concentration of 100 mg/ml.

tative experiments, 10-50% of the radioactivity was precipitated by an eel serum previously characterized as an anti-H reagent (Springer and Williamson, 1963). This precipitation was inhibited by fucose. A caprine antiserum prepared against purified hog H substance[1] also precipitated the radioactive product. The mannose-labelled reaction products were not precipitated by either antiserum.

Discussion: Inasmuch as the gastric mucosa secretes a variety of mucins and glycoproteins, many of which contain fucose, mannose and hexosamine, the biosynthetic products of glycosyl-transfer reactions in this tissue require precise characterization. The microsomes used for these studies incorporated not only fucose and mannose from their respective nucleotide-sugars but also could be shown to incorporate galactose from UDP-galactose-C^{14} and hexosamine from UDP-hexosamine-C^{14}. Tuppy and Staudenbauer (1966) reported that a microsomal preparation from hog gastric mucosa incorporated radioactivity from a mixture of UDP-N-acetylglucosamine and UDP-N-acetylgalactosamine. These authors showed that all of this radioactivity could be released by treatment with N-acetylgalactosaminidase but did not characterize the reaction product by specific immunochemical methods. In the presently reported studies, identification of part of the soluble fucose-labelled product as H substance was established by precipitation with well-characterized anti-H reagents. The remaining soluble and insoluble fucose-labelled material may represent other fucose-containing mucins or glycoproteins. Trapping of radioactive material or nonspecific sedimentation with the immune precipitate was excluded by the absence of radioactivity in the precipitin reaction containing the mannose-labelled product and carrier H-substance.

The preferential incorporation of fucose by type H microsomes is of interest since both hog A substance and hog H substance contain fucose in a similar α-fucosyl linkage. As these experiments utilize endogenous acceptors,

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[1] Marcus, D. M. - manuscript in preparation.

the results suggest that there are more available sites for fucose in microsomes from type H hogs. Human Le^a substance, which contains an available site and could potentially serve as an acceptor for the fucosyl transferase in question (Grollman, 1966), failed to stimulate the reaction when added exogenously. Because of the tightly bound character of the fucosyl transferase, Le^a substance may not gain access to the vicinity of the enzyme. Efforts to solubilize the enzyme which would allow testing of Le^a substance and other potential acceptor molecules are in progress.

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