Biosynthesis of the Carbohydrate Units of Immunoglobulins. 1. Purification and Properties of Galactosyltransferases from Swine Mesentary Lymph Nodes†

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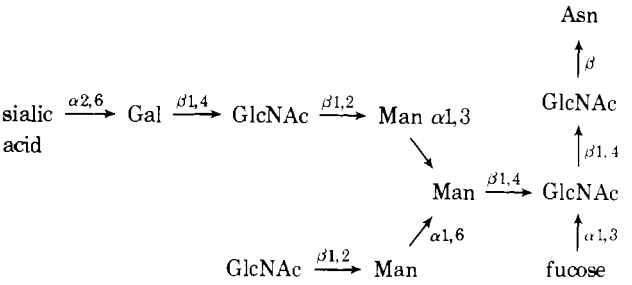
ABSTRACT: Galactosyltransferase (UDPgalactose:glycoprotein galactosyltransferase, EC 2.4.1.22) was isolated from swine mesentary lymph node homogenates using procedures which included affinity chromatography on Sepharose 4B columns containing covalently bound *p*-aminophenyl-β-D-*N*-acetylglucosamine. The homogenous enzyme showed a single band on disc gel electrophoresis and had a specific activity of 35 nmol min^{-1} (mg of protein)$^{-1}$ at 37 °C. A molecular weight of 57 000 was obtained by exclusion chromatography, sucrose density centrifugation, and sodium dodecyl sulfate–gel electrophoresis. The same molecular weight was obtained after reduction and alkylation which indicates that the enzyme is composed of only a single polypeptide chain. The enzyme catalyzed the formation of β1→4 bonds between galactose and free terminal *N*-acetylglucosaminyl residues of soluble preparations of porcine IgG immunoglobulin heavy chain, fetuin, ovalbumin, and ovomucoid. An endogenous glycoprotein, present in particulate subcellular preparations, was also a very good substrate for the enzyme, and it was identified as incomplete IgG immunoglobulin heavy chain. The K_m of the purified enzyme was 2.9×10^{-5} M for fetuin, 5.4×10^{-5} M for ovalbumin, 2.0×10^{-5} M for IgG immunoglobulin heavy chain, and 2.2×10^{-5} M for UDP-galactose. About 20% of the total galactosyltransferase activity in lymph node homogenates was present in the cytosol fraction, and 80% was in the microsomal and Golgi fractions. The kinetic properties of the bound and soluble galactosyltransferases were similar, and both required Mn^{2+} for maximal activity. However, the bound enzyme required the addition of detergents, lysolecithin, GDP-mannose, and UDP-*N*-acetylglucosamine for maximum activity. These compounds did not influence the activity of the soluble transferase. The membrane preparations catalyzed the transfer of galactose from UDP-galactose and *N*-acetylglucosamine from UDP-*N*-acetylglucosamine, to incomplete oligosaccharide chains of endogenous IgG immunoglobulin bound to these particles. The labeled products of these reactions were isolated, and the structures of their oligosaccharide chains were determined and compared with those isolated from the heavy chain of porcine IgG immunoglobulin. The glycopeptide prepared from the endogenous acceptor and the major glycopeptide prepared by proteolytic digestion of the heavy chain of porcine IgG immunoglobulin had identical structures. The following structure for the carbohydrate chains of porcine IgG immunoglobulin was determined by sequential enzymatic hydrolysis and methylation studies.



Glycosyltransferases catalyze the sequential addition of monosaccharide residues to growing oligosaccharide chains in glycoprotein substrates (Spiro, 1970). Many of these enzymes are relatively nonspecific with respect to the nature of the glycoprotein acceptor (Roseman, 1970), requiring only the presence of a suitable monosaccharide residue in the terminal position of the oligosaccharide side chain. Galactosyltransferases can utilize glycoproteins with terminal *N*-acetylglucosaminyl residues, free *N*-acetylglucosamine, and glycosyl derivatives of N-acetylglucosamine, as acceptors (Schachter et al., 1971).

Although the catalytic properties and mechanism of action of soluble galactosyltransferases from colostrum (Brodbeck et al., 1967), mammary gland (Babad and Hassid, 1966), and milk (Hill et al., 1968) have been examined, the intracellular galactosyltransferases in other tissues have not been extensively investigated because these enzymes are tightly bound to subcellular membranes. To the extent that comparisons have been made, it is clear that the galactosyltransferases from different sources are similar. Glucose and *N*-acetylglucosamine are good substrates for lactose synthetase, the galactosyltransferase found in milk. This enzyme is also active with some glycoproteins which terminate in *N*-acetylglucosamine. The rate of reaction with glucose and *N*-acetylglucosamine is dependent on the concentration of α-lactalbumin, which promotes the formation of lactose (Morrison and Ebner, 1971). Particulate galactosyltransferases have been studied in a number of tissues, including porcine submaxillary gland (Schachter et al., 1971), rat kidney cortex (Martensson et al., 1974), and mouse mastocytoma (Helting and Erbing, 1973).

The present communication describes the isolation of a homogeneous preparation of soluble galactosyltransferase from swine mesentary lymph node. The kinetic properties of the enzyme and the structure of the product formed were determined. The properties of a membrane bound enzyme which

† From the Department of Biochemistry, University of Georgia, Athens, Georgia 30602. *Received May 3, 1976.* This work was supported in part by United States Public Health Service Grants AM13150 and CA15215.

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transfers galactose from UDP-galactose to incomplete heavy chains of endogeneous IgG immunoglobulin acceptors in particulate preparations from this same tissue are also described. The primary structure of the oligosaccharide chain of porcine IgG immunoglobulin, the physiological glycoprotein substrate for this enzyme, was determined and compared with the structure of the endogenous substrate. Preliminary studies on galactosyltransferases and glucosaminyltransferases in lymph node tissue have been reported previously (Mendicino et al., 1968; Rao et al., 1976).

Experimental Procedures

Assay of Galactosyltransferase Activity. The activity of the enzyme was determined by measuring the rate of transfer of galactose from UDP[U-^{14}C]galactose (2.2×10^6 cpm/μmol) to endogenous or exogenous glycosyl acceptors. Reaction mixtures were incubated at 37 °C for 30 min and contained in 0.5 ml: 0.1 M 2-(*N*-morpholino)ethanesulfonic acid, pH 6.5; 10 mM $MnCl_2$; 0.75 mg of fetuin depleted of sialic acid and galactose; 0.4 mM labeled UDP-galactose, and either membrane-bound or soluble enzyme. Triton X-100 (1%) was included in assays of the membrane-bound enzyme. Reactions were terminated by addition of 3.5 ml of 2.8 N perchloric acid containing 2% phosphotungstic acid and the precipitated protein was collected by centrifugation, dissolved in 2 ml of 0.5 N NaOH, and then reprecipitated with 3 ml of the perchloric–phosphotungstic acids mixture. This procedure was repeated twice more and the final pellet was dissolved in 1.0 ml of 0.5 N NaOH. Aliquots of 0.6 ml were transferred to vials containing 10 ml of scintillation solvent and 0.4 ml of 1.0 N HCl. The transfer of galactose to glycopeptides, glucosamine, *p*-nitrophenyl-*N*-acetylglucosamine, and chitobiose was measured by several methods. After incubation under standard conditions, the reaction mixture (0.1 ml) was immediately diluted to 0.5 ml with 0.001 N HCl and applied to a Dowex-Cl column (0.5×4 cm) which had been equilibrated with 0.001 N HCl. The column was washed with 1.5 ml of 0.001 N HCl and the filtrates were collected in a scintillation vial and counted. Very little radioactivity was found in control samples which were incubated in the absence of glycosyl acceptors. The radioactive product, in each case, was further identified by quantitative paper chromatography (Mendicino and Hanna, 1970). The activity measured by these procedures was linear with time of incubation and enzyme concentration. One unit of enzyme activity is defined as the amount of enzyme required to transfer 1 nmol of galactose per min and specific activity is expressed as units per mg of protein.

N-Acetylglucosaminyltransferase activity was determined by measuring the rate of transfer of [1-^{14}C]GlcNAc from UDP[1-^{14}C]GlcNAc (7×10^6 cpm/μmol) to exogenous or endogenous glycosyl acceptors under the same conditions as those described for galactosyltransferase, except that 0.5 mg of ovalbumin was used as the exogenous glycosyl acceptor.

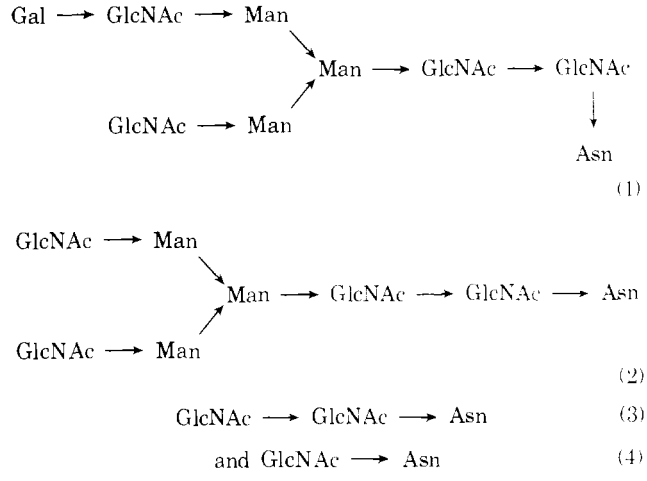
Preparation of Subcellular Fractions. Homogenization and isolation of subcellular particles were carried out according to the method of de Duve et al. (1955). Plasma membranes were purified by the procedure of Fitzpatrick et al. (1969) and Emmelot et al. (1964). Microsomes were fractionated according to the procedure of Dallner et al. (1966). Golgi membranes were prepared by the method of Morré et al. (1970). The particulate galactosyltransferase used in these studies was prepared by sonication of the microsome fraction. The lymph nodes and other tissues were kindly supplied by Gold Kist of Talmo, Georgia.

Preparation of Antisera to Porcine IgG Immunoglobulin. Porcine IgG immunoglobulin (Pentex, Kankakee, Ill.) was extensively purified by chromatography on Sephadex G-200 columns. Partially alkylated heavy and light chains were prepared by the procedure of Small and Lamm (1966). Antisera to IgG and light and heavy chains were then prepared by standard procedures.

Preparation of Galactosyl Acceptors. The alkylated heavy chain of IgG immunoglobulin was insoluble under the conditions of the standard assay system and intact IgG immunoglobulin was not active as a galactosyl acceptor. However, a glycoprotein fragment prepared from intact IgG immunoglobulin by treatment with pepsin was an excellent substrate for the purified enzyme. This soluble fragment which has a molecular weight of 35 000 was prepared by the procedure of Porter (1959) as modified by Utsumi and Karush (1965).

Glycopeptides were prepared by Pronase digestion of heat denatured IgG immunoglobulin. The sample was purified by chromatography on DEAE[1] columns (Kornfeld and Kornfeld, 1970) and by gel filtration on Sephadex G-50 (Spragg and Clamp, 1969).

Various galactosyl acceptors were prepared from glycoproteins and glycopeptides by treating them with highly purified neuraminidase and β-galactosidase. Other samples were oxidized with periodate, then reduced with sodium borohydride, and heated at 80 °C in 0.1 N HCl for 1 h to remove the oxidized fragments. This product was further treated with β-mannosidase and β-*N*-acetylglucosaminidase to remove residual mannose and GlcNAc residues. Glycoproteins and glycopeptides containing the following structures were prepared.



Modified fetuin was prepared by the procedure of Spiro (1964).

Quantitative Analysis of Sugars in Glycoproteins. Samples (0.5 μmol) of glycopeptide or glycoprotein were hydrolyzed in evacuated sealed tubes with 1.5 N H_2SO_4 for 4 to 6 h at 100 °C. These conditions resulted in complete hydrolysis of all the neutral sugars and glucosamine. Exactly 0.30 μmol of D-[^{14}C]galactose, D-[^{14}C]fucose, D-[^{14}C]mannose, and D-[^{14}C]glucosamine with specific activities of about 3×10^6 cpm/μmol were added in each case. After hydrolysis, sulfate ion was removed with Dowex 1-HCO_3^-, and the resin was washed with water. The filtrate and washings were concentrated and applied to Whatman 3 MM paper. The sugars were

[1] Abbreviations used: DEAE, diethylaminoethyl; Tris,tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; GlcNAc, *N*-acetyl-D-glucosamine.

separated by chromatography with ethyl acetate–pyridine–H_2O (8:2:1). The amount of each sugar present in the sample was quantitatively estimated from the extent of dilution of the added radioactive monosaccharide. In this procedure the loss of sugars or hexosamine by destruction during acid hydrolysis or losses incurred upon elution of samples from paper chromatograms does not affect the accuracy of the determination.

The sugar content in each case was further confirmed by specific colorimetric or enzymatic assays. Fucose was determined by the cysteine–sulfuric acid method (Dische and Shettles, 1948); *N*-acetylglucosamine and glucosamine were assayed by the procedure of Reissig et al. (1955). Galactose was determined with galactose dehydrogenase and galactose oxidase (Amaral et al., 1963). Mannose was measured with a coupled enzymatic assay (Mendicino and Rao, 1975). All of the values obtained in these assays were corrected for losses occurring during acid hydrolysis by comparison to standards treated in an identical manner. Sialic acid released after treatment with neuraminidase or hydrolysis with 0.1 N H_2SO_4 at 80 °C for 1 h was determined by the thiobarbituric acid method (Warren, 1959).

Quantitative Sequential Hydrolysis of Glycopeptides with Specific Glycosidases. The highly purified glycosidases used in these studies, including neuraminidase (Cassidy et al., 1965), β-galactosidase, 5 units (Li and Li, 1968; Kornfeld and Kornfeld, 1970), β-*N*-acetylglucosaminidase, 10 units (Kornfeld and Kornfeld, 1970; Li and Li, 1970), α-D-mannosidase, 5 units (Li and Li, 1970), β-D-mannosidase (Snaith and Levvy, 1969), and α-L-fucosidase, 2 units (Carlsen and Pierce, 1972), were purchased or prepared by published procedures. The purity of each enzyme was examined with appropriate *p*-nitrophenylglycosyl derivatives. Protease contamination was determined using azocollagen as the protein substrate. Reaction mixtures were incubated at 37 °C for 5 to 8 days under toluene and contained, in 0.2 ml, 0.05 M sodium acetate, pH 5.35, 3 to 0.5 μmol of glycopeptide and purified glycosidase. Afterward the reaction mixture was heated at 100 °C for 1 min to inactivate the glycosidase, which was removed by centrifugation. The supernatant solution was applied to a 1 × 30 cm Bio-Gel P-30 column (200–400 mesh), and elution was carried out with distilled water. The glycopeptide was recovered near the void volume and sugars which eluted in later fractions were assayed by specific spectrophotometric or enzymatic procedures. The isolated glycopeptide was concentrated and treated with another glycosidase.

Analytical Methods and Materials. The concentration of protein was measured by the method of Lowry et al. (1951). Analytical gel electrophoresis was carried out by the procedure of Davis (1964). Sodium dodecyl sulfate gel electrophoresis was performed according to the procedure of Shapiro et al. (1967, 1969). Sucrose density centrifugation was carried out by the procedure of Martin and Ames (1961) with pyruvic kinase and lactic dehydrogenase as standards. Reduction and alkylation of galactosyltransferase was performed by the method of Uyeda (1969). The affinity column containing *p*-aminophenyl-GlcNAc substituents was prepared by reacting activated Sepharose 4B with *p*-aminophenyl-GlcNAc according to the procedure of Cuatrecasas (1970). *p*-Nitrophenyl-GlcNAc was reduced to the corresponding *p*-aminophenyl derivative by catalytic hydrogenation (McBroom et al., 1972). Sepharose 4B was activated with cyanogen bromide at 2 °C for 1.5 h and then mixed with 15 ml of 0.5 M $NaHCO_3$ containing 50 μmol of *p*-aminophenyl-GlcNAc. The reaction mixture was left overnight at 4 °C and afterward the material was washed with 50 volumes of 0.1 M Tris-HCl, pH 8.0, containing 0.1 M ethanolamine and equilibrated with 0.05 M Tris-HCl, pH 7.5. The Sepharose 4B contained 1.3 μmole of *p*-aminophenyl-GlcNAc per ml.

UDP[1-^{14}C]GlcNAc, UDP[U-^{14}C]galactose, and the corresponding unlabeled derivatives were prepared as described previously (Mendicino and Rao, 1975; Mendicino and Hanna, 1970). Paper chromatography was carried out with a number of solvent systems (Mendicino and Hanna, 1970; Hanna and Mendicino, 1970). Glycopeptides were detected with ninhydrin and the periodate–rosaniline sprays (Bonner, 1960). Reducing sugar was analyzed by the procedure of Park and Johnson (1949). The phenol–sulfuric acid method was used to detect carbohydrate in intact glycoproteins and glycopeptides. Periodate oxidations were performed according to the procedure of Smith as described by Spiro (1966), except that the hydrolysis of the oxidized products after reduction with $NaBH_4$ was carried out with 0.1 N H_2SO_4 at 80 °C for 1 h. Radioactivity measurements were carried out as described previously (Mendicino and Hanna, 1970).

The positions of the glycosidic linkages in glycopeptides were determined by permethylation (Hakomori, 1964). The partially methylated samples were subjected to acetolysis with 0.5 N H_2SO_4 in 95% acetic acid at 80 °C. The products were then treated with dilute acid, reduced with sodium borohydride, and acetylated as described by Björndal and Lundblad (1970). Gas–liquid chromatography was carried out with a column (200 × 0.3 cm) containing 3% (w/w) ECNSS-M on Gas Chrom Q (100–200 mesh). The methylated alditol acetates were analyzed at 150 °C as described by Björndal et al. (1967). Methylated glucosaminitol acetates were analyzed in the same columns at a temperature of 190 °C (Stellner et al., 1973). Standard methylated alditol acetates were prepared by chemical synthesis (Siddiqui et al., 1972) and the 3,4-, 4,6-, and 3,4-dimethyl and 3,4,6-trimethyl derivatives of glucosaminitol were prepared by the method of Jeanloz (1958).

Results

Requirements for Maximal Activity with Membrane Bound Transferase Systems. The requirements of bound galactosyl and GlcNAc transferase systems were examined, and the results obtained with microsomes isolated from mesentary lymph node are presented in Table I. The reactions, in each case, required Mn^{2+}, UDP-galactose, UDP-GlcNAc, and GDP-mannose, and omission of any of these components consistently resulted in a loss of transferase activity. The addition of nucleoside di- and triphosphates did not cause a significant increase in transferase activity, nor did they replace the requirement for nucleotide sugars. The stimulation of transferase activity by the addition of other unlabeled nucleotide sugars could be due to either a protective effect on the hydrolysis of UDP-galactose and UDP-GlcNAc by nonspecific hydrolysis or to the synthesis of more endogenous glycoprotein acceptors. In order to obtain evidence for the latter possibility, the particulate preparations were preincubated with unlabeled UDP-GlcNAc and GDP-mannose to obtain enhancement of galactosyltransferase activity by prior addition of other sugars to incomplete endogenous glycosyl acceptors. Afterward the particles were washed and they were incubated in the standard mixture with UDP-D-[^{14}C]galactose in the presence and absence of the other nucleotide sugars. Only a 20% decrease in the stimulation was observed after this treatment, which suggested that the effect was not due solely to the synthesis of new glycosyl acceptors. In other experiments, the rates of transfer

TABLE I: Requirements of the Membrane Bound Galactosyltransferase and GlcNAc Transferase Systems.[a]

Additions or Deletions	Galactosyl-transferase (cpm incorp)	GlcNAc Transferase (cpm incorp)
Complete with UDP [1-^{14}C]galactose	5300	
Complete with UDP[1-^{14}C]GlcNAc		870
Minus $MnCl_2$	70	30
Minus $MnCl_2$, plus EDTA, 20 mM	0	0
Minus $MnCl_2$, plus $MgCl_2$, 10 mM	80	10
Minus UDP-GlcNAc, minus GDP-mannose	1400	
Minus UDP-GlcNAc	3400	
Minus GDP-mannose	3500	630
Minus UDP-galactose		370
Minus UDP-galactose, minus GDP-mannose		350
Minus Triton X-100	2120	350

[a] The standard incubation conditions described in the Experimental Procedures were used.

TABLE II: Subcellular Distribution of Galactosyltransferase in Swine Mesentary Lymph Node.[a]

Fraction	Total Act. (nmol min^{-1} (g tissu)$^{-1}$)	Spec Act. (nmol min^{-1} (mg protein)$^{-1}$)	Spec Act. Rel to Soluble Fraction
1. Plasma membranes	0.217	0.017	1.2
2. Golgi membranes	0.200	0.087	6.2
3. Microsomes	0.260	0.090	6.5
4. Supernatant fraction	0.183	0.014	1.0

[a] The subcellular fractions were isolated from a 20% homogenate prepared from 5 g of lymph node as described in the text. Each of the particulate fractions was washed twice with 10 volumes of 0.02 M Tris (pH 7.0)–0.25 M sucrose–2 mM $MnCl_2$. The final pellet was suspended in this buffer and assayed for galactosyltransferase activity by the standard procedure.

of sugar from UDP-GlcNAc and GDP-mannose into the endogenous particulate acceptors were measured. The rates of transfer under the standard assay conditions were found to be 870 and 640 cpm, respectively. The results of these experiments clearly indicated that the amount of new endogenous acceptor which was formed on incubation with various nucleotide sugars could not account for all of the increase in galactosyltransferase activity observed in the presence of other nucleotide sugars, in spite of the fact that the amount of glycosyl acceptor was probably rate limiting under these conditions. However, when sonicated particulate preparations were first treated with β-galactosidase and β-*N*-acetylglucosaminidase, then prior incubation with UDP-GlcNAc markedly increased the subsequent transfer of galactose from UDP-galactose to endogenous acceptor. These results indicate that more acceptor sites can be made available for the transfer of galactose in the membrane-bound system.

The addition of detergents, such as 1% Triton X-100 or 0.5% deoxycholate, stimulated the transferase reactions in particulate membrane systems, as seen in Table I. The apparent K_m's for UDP-galactose or UDP-GlcNAc were not influenced by the addition of the detergents. These compounds had no effect on the activity of soluble purified galactosyltransferase. It is probable that detergents increase the accessibility of substrate to the active sites of the bound enzymes by disrupting the structure of the lipid–protein matrix of these membranes.

Subcellular Distribution of Galactosyltransferase in Mesentary Lymph Node. The distribution of galactosyltransferase activity in various subcellular fractions isolated from lymph node homogenates is shown in Table II. The transferase activity was examined with an excess of exogenous acceptor, in each case, to ensure that only the concentration of enzyme was rate limiting. The supernatant fraction contains very little endogenous acceptor, and low activities are observed in this fraction in the absence of added acceptor. The activities in all of the subcellular fractions were increased by the addition of exogenous acceptor. The Golgi and microsome fractions contain most of the galactosyltransferase activity. Following subfractionation of the microsomes by the method of Dallner et al. (1966), most of the transferase activity appeared in the smooth membrane fraction. In contrast to the results obtained with many other tissues, a significant amount of galactosyltransferase was consistently found in the supernatant fraction. The possibility that this activity represented enzyme which was removed from the membranes of particulate fractions during homogenization was examined. Washed particulate fractions were preincubated under various conditions and then the high-speed supernatant was examined for solubilized transferase activity. Little or no galactosyltransferase activity was detected in these supernatants which suggested that the transferase activity found in the soluble fraction was not derived from the particulate membrane fraction. It was also observed that different homogenation conditions did not affect the yield of soluble galactosyltransferase. This evidence further indicated that galactosyltransferase is present in both the soluble and particulate fractions in lymph node tissue.

Purification of Galactosyltransferase. The enzyme present in the soluble fraction was purified to homogeneity. All operations were carried out at 3 °C and centrifugation steps were performed at 27 000*g*. Swine mesentary lymph node (1000 g) was homogenized with 2 l. of a standard buffer (0.05 M sodium acetate, pH 5.0, and 2 mM 2-mercaptoethanol) in a Waring Blendor at maximum speed for 2 min. The suspension was centrifuged and the pellet was extracted once more with 2 l. of buffer. The extracts were combined, filtered through cheesecloth, and dialyzed overnight against 20 l. of buffer. The turbid solution was centrifuged and the supernatant was filtered through glass wool to remove floating fatty material (fraction 1, Table III).

Solid ammonium sulfate (326 g per l.) was added and the resulting precipitate was collected by centrifugation and dissolved in 250 ml of 0.01 M Tris-HCl (pH 7.5)–2 mM 2-mercaptoethanol and dialyzed exhaustively against the same buffer (fraction 2, Table III).

The enzyme was adsorbed to a DEAE-Bio-Gel A column (8 × 14 cm) which was previously equilibrated against 0.01 M Tris-HCl, pH 7.5. The column was washed with 0.01 M Tris-HCl (pH 7.5)–2 mM 2-mercaptoethanol until the OD at 280 nm decreased to 0.05. The enzyme was eluted with 0.05 M Tris-HCl, pH 7.5, and the activity was found in a protein peak which emerged from the column between 400 and 1400 ml (fraction 3).

The enzyme from the previous step was concentrated by precipitation with ammonium sulfate (326 g per l.) dialyzed against 0.05 M Tris-HCl (pH 7.5)–2 mM mercaptoethanol and then adjusted to 10 mM $MgCl_2$ and 1 mM UMP. It was adsorbed to an affinity column (2.2 × 5 cm) which contained

TABLE III: Purification of Galactosyltransferase from Mesentary Lymph Node.[a]

Fraction	Volume (ml)	Protein (mg)	Total Units (nmol/min)	Spec Act. (nmol min^{-1} mg^{-1})	Yield (%)	Purification
1. Crude extract	4100	11 480	160	0.0159	100	1
2. Ammonium sulfate fractionation	250	4 760	128	0.0301	80	2
3. Chromatography on DEAE Bio-Gel A	1000	970	96	0.11	60	3.5
4. Affinity chromatography	50	1.62	40	35	30	2200

[a] The enzyme was assayed by the standard procedure, with oxidized fetuin as the substrate.

p-aminophenyl-GlcNAc covalently attached to Sepharose 4B. The column was previously equilibrated against 0.05 M Tris-HCl (pH 7.5)–10 mM $MnCl_2$–1 mM UMP. Afterward the column was washed with 75 ml of 0.05 M Tris-HCl (pH 7.5)–10 mM $MgCl_2$–1 mM UMP. The enzyme was then eluted with 50 ml of 0.05 M Tris-HCl, pH 7.5, containing 0.05 M EDTA, pH 7.5. The final purified preparation was dialyzed against 0.02 M Tris-HCl, pH 7.5 (fraction 4). The procedure outlined in Table III for a typical preparation of galactosyltransferase permits the isolation of a homogenous enzyme with a yield of 30%. A final specific activity of 35 nmol min^{-1} mg^{-1} was obtained with modified IgG heavy chain as the glycosyl acceptor.

Properties of the Purified Enzyme. The enzyme was stable for at least several months when frozen at −20 °C. The enzyme showed a relatively broad pH optimum between pH 5.5 and 7.5, with a maximum at pH 6.8.

The analysis of the purified enzyme by polyacrylamide gel electrophoresis at both pH 8.9 and 4.3 showed only a single protein band (Figure 1). The enzyme had a relatively low mobility at both pH's and the band was more diffuse at pH 8.9. When the enzyme was dissociated with 2-mercaptoethanol and sodium dodecyl sulfate (gel C) or reduced and alkylated (gel D, Figure 1), only one band was observed on polyacrylamide gel electrophoresis.

The molecular weight of the purified enzyme was determined by centrifugation in a 5 to 20% linear sucrose gradient. A molecular weight of 60 000 was estimated using the procedure of Martin and Ames (1961). Similar values were obtained by chromatography on Sephadex G-200 and gel electrophoresis. When the elution volume of the enzyme on a Sephadex G-200 column (2.2 × 35 cm) was compared with those of reference proteins as described previously (Abou-Issa and Mendicino, 1973), a molecular weight of 57 000 ± 2 500 was obtained. The subunit structure of lymph node galactosyltransferase was also examined by polyacrylamide gel electrophoresis after treatment with 2-mercaptoethanol and sodium dodecyl sulfate. A single component with a molecular weight of 57 500 was found, which indicated that the enzyme contained only a single polypeptide chain. When the enzyme was dissociated by more rigorous procedures, the same results were obtained. Only one band with a molecular weight of 57 000 was found when the native enzyme was reduced and alkylated as described in a previous report (Mendicino and Kratowich, 1972).

Influence of UDP-Galactose and Glycosyl Acceptors on Galactosyltransferase Activity. The effect of the concentration of UDP-galactose on the initial rates of bound and soluble galactosyltransferase in the presence of saturating amounts of glycosyl acceptor was examined. The apparent K_m of the soluble enzyme for UDP-galactose was 2.2×10^{-5} M, and the K_m of the particulate enzyme was 1.7×10^{-5} M with modified fetuin as the glycosyl acceptor.

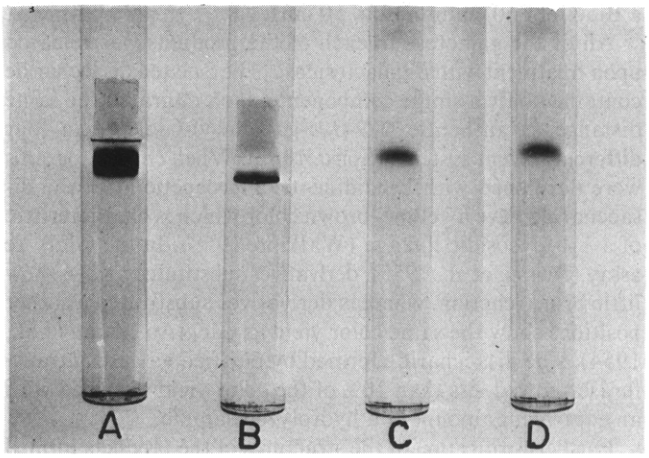


FIGURE 1: Polyacrylamide gel electrophoresis of purified galactosyltransferase. Gel A was run at pH 8.9 in Tris-glycine buffer in 7.5% acrylamide gel for 3 h, and gel B was run under the same conditions in sodium acetate buffer at pH 4.3 with electrodes reversed. The enzyme was treated with 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol for 2 h at 37 °C and the pattern in gel C was obtained upon electrophoresis for 4 h in the presence of 0.1% sodium dodecyl sulfate. Gel D was obtained after reduction and alkylation of the enzyme. In each case, 75 μg of enzyme with a specific activity of 35 nmol min^{-1} (mg of protein)$^{-1}$ was applied to the gel.

The effects of increasing concentrations of glycosyl acceptors were examined by using modified fetuin, ovalbumin and soluble preparations of porcine IgG immunoglobulin heavy chains which were deficient in galactose. In the presence of saturating amounts of UDP-galactose, a K_m of 2.9×10^{-5} M was obtained with fetuin, 5.4×10^{-5} M was obtained with ovalbumin, and 2.0×10^{-5} M was found with modified porcine IgG heavy chain. The best IgG heavy chain acceptor had oligosaccharide chains with structure 2 described in the Experimental Procedures. Acceptors having structures 1, 3, and 4 showed at least threefold lower activities and had higher K_m's. In other experiments the effect of increasing concentrations of added glycosyl acceptor on the velocity of the reaction as a function of the concentration of UDP-galactose was examined. The K_m of the soluble enzyme for UDP-galactose decreased with increasing concentrations of glycosyl acceptor.

The K_m's for low-molecular-weight acceptors were significantly higher. The K_m for free GlcNAc, *p*-nitrophenyl-GlcNAc, and a glycopeptide prepared from porcine IgG immunoglobulin, calculated from double-reciprocal plots, were 5.8×10^{-3}, 6.6×10^{-4}, and 2.5×10^{-4} M, respectively. *p*-

Nitrophenyl-GlcNAc, GlcNAc, UMP, and UDP competitively inhibited the transfer of galactose to glycoprotein substrates. The addition of 1 mM GlcNAc or 1 mM UMP resulted in about 25% inhibition, whereas 1 mM UDP caused a 75% inhibition of the reaction.

Evidence for Formation of a 4-O-β-D-Galactosyl-N-acetylglucosaminyl Linkage. In order to examine the specificity of the purified galactosyltransferase, it was necessary to prepare large amounts of products. Standard incubation mixtures were prepared with the quantities increased 60-fold. Both GlcNAc and modified porcine IgG heavy chain devoid of galactose were used as substrates. The disaccharide formed with GlcNAc as the substrate was isolated by paper chromatography in butanol–ethanol–water (10:1:2). The product formed with the modified porcine IgG heavy chain was separated on a Bio-Gel P60 column (2 × 50 cm).

All of the galactose in each of the products was released upon treatment with β-galactosidase. The isolated disaccharide contained only a single component which migrated the same distance as authentic 4-*O*-β-D-galactosyl-GlcNAc in four different solvent systems (Spiro, 1962). When chromatograms were developed with benzidine–trichloroacetic acid, the disaccharide gave a yellow-brown color which is characteristic of a 1,4-glycosidic linkage (Watkins, 1958). In the GlcNAc assay (Reissig et al., 1955), derivatives substituted at C-4 show little or no reaction, whereas derivatives substituted at other positions show the same color yield as GlcNAc (Kuhn et al., 1954). The disaccharide formed by purified galactosyltransferase showed less than 10% of the color yield obtained with an equivalent amount of a hydrolyzed sample.

Further verification of the structures of the products formed in these reactions was obtained in methylation studies. The reduced disaccharide and glycopeptides prepared from the product formed with modified porcine IgG heavy chain were subjected to permethylation and examined by gas chromatography. As seen in Figure 3, curve 5, a peak corresponding to 3,6-dimethyl-1,4,5-triacetyl-*N*-methylacetylglucosaminitol was formed from the disaccharide. When the IgG glycopeptide was examined by this method, an increase in the amount of the 3,6-dimethyl-1,4,5-triacetyl-*N*-methylacetylglucosaminitol peak corresponding to the amount of radioactive galactose incorporated into the glycopeptide was found. The galactosyltransferase isolated from mesentary lymph node catalyzes the formation of a β1→4 bond between galactose and the terminal GlcNAc residue in IgG immunoglobulins. No evidence for the formation of β1→3 or β1→6 linkages could be demonstrated even when large samples were analyzed.

Composition and Structure of the Oligosaccharide Chains in Porcine IgG Immunoglobulins. The membrane fractions isolated from swine mesentary lymph node catalyzed the transfer of galactose and GlcNAc to IgG immunoglobulin acceptors which were tightly bound to these structures. The arrangement of sugars in the oligosaccharide chains of swine IgG was determined in order to prepare specific glycosyl acceptors and aid in the identification of the bound endogenous glycosyl acceptors in this tissue. The carbohydrate composition of the glycopeptide prepared from porcine IgG immunoglobulin was determined by acid and enzymatic hydrolysis. The glycopeptide contained mannose, GlcNAc, galactose, fucose, and sialic acid in the molar ratio of 3.0:3.85:0.85:0.75:0.60 based on the amount of mannose present. The values obtained by exhaustive enzymatic hydrolysis with highly purified glycosidases were in good agreement with those obtained by hydrolysis with acid. Only 2.9 residues of GlcNAc were released by enzymatic hydrolysis compared with 3.85 by treatment with acid. However, this difference would be expected since one GlcNAc residue which is attached to asparagine through an amide linkage would not be released by treatment with glycosidases.

TABLE IV: Sequential Enzymatic Hydrolysis of Glycopeptide Prepared from Porcine IgG Immunoglobulin.[a]

Treatment	Amount of Each Sugar Released			
	Galactose	GlcNAc	Mannose	Fucose
Acid hydrolysis	0.85	3.85	3.00	0.75
A. 1. β-Galactosidase	0.75			
2. α-Fucosidase				0.65
B. β-*N*-Acetylglucosaminidase		1.90		
C. α-Mannosidase			2.10	
D. Acid hydrolysis of glycopeptide C	0	2.25	0.85	0.70
E. α-Fucosidase				0.65
F. Periodate oxidation of glycopeptide A	0	2.1	1.1	0

[a] The analysis was performed on asialoglycopeptides which were prepared as described in the text. Assays for sugars and the conditions for incubation with glycosidases are described in the Experimental Procedures. Experiment A, 1.3 μmol of asialoglycopeptide was treated with β-galactosidase and 0.2 μmol was treated with α-fucosidase. Experiment B, 1.1 μmol of the asialoglycopeptide reisolated after treatment with β-galactosidase was used. Less than 0.05 μmol of sugar was released from this glycopeptide upon incubation with either α-mannosidase or β-galactosidase. Experiment C, 0.8 μmol of the asialoglycopeptide recovered in experiment B was used. Less than 0.05 μmol of sugar was released when this glycopeptide was treated with β-*N*-actylglucosaminidase or β-galactosidase. Experiment E, 0.2 μmol of the glycopeptide reisolated in experiment C was treated with α-fucosidase.

The glycopeptide was treated with neuraminidase to remove sialic acid and it was then treated sequentially with highly purified β-galactosidase, β-*N*-acetylglucosaminidase, α-mannosidase, and α-fucosidase. The glycosidic linkages between the sugars in each of the resulting glycopeptides were then determined by permethylation and analysis by gas chromatography. The results of sequential hydrolysis with glycosidases are presented in Table IV. The spectra obtained by gas chromatography are shown in Figures 2 and 3.

The spectra of the intact glycopeptide, curve 1, Figures 2 and 3, showed the presence of eight components. When the glycopeptide was treated with neuraminidase, peak E, 2,3,4-trimethylgalactose, was converted to peak C, 2,3,4,6-tetramethylgalactose. Thus, all of the sialic acid is attached to galactose by a 2→6 linkage. Treatment of the asialoglycopeptide with β-galactosidase, curve 2, resulted in the release of 0.75 μmol of galactose (Table IV) and the loss of peak C, 2,3,4,6-tetramethylgalactose. The relative size of peak G, 3,4,6-trimethylglucosaminitol increased compared with peak H, 3,6-dimethylglucosaminitol, after this treatment. These results show that galactose is attached β1→4 to GlcNAc.

Further treatment with β-*N*-acetylglucosaminidase, curve 3, caused the release of 1.9 μmol of GlcNAc, Table IV, and the loss of peak G, 3,4,6-trimethylglucosaminitol. Peak D, 3,4,6-trimethylmannitol, was converted to peak B, 2,3,4,6-tetramethylmannitol. These results show that 2 mol of GlcNAc are attached β1→2 to mannose. When the resulting glycopeptide was treated with α-mannosidase, 2.1 μmol of mannose was released. Peak B, 2,3,4,6-tetramethylmannitol (curve 4, Figure 2), decreased and peak F, 2,4-dimethylmannitol, was

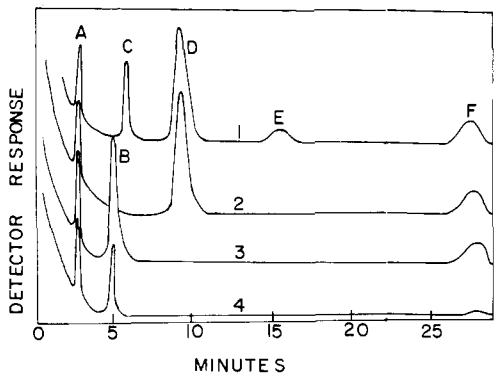


FIGURE 2: Identification of the methylated alditol acetates formed after treatment of glycopeptides with purified glycosidases. Separation by gas chromatography was carried out as described in the text. Peaks correspond to the following standards: peak A, 2,3,4-trimethyl-1,5-diacetyl-L-fucitol; peak B, 2,3,4,6-tetramethyl-1,5-diacetyl-D-mannitol; peak C, 2,3,4,6-tetramethyl-1,5-diacetyl-D-galactitol; peak D, 3,4,6-trimethyl-1,2,5-triacetyl-D-mannitol; peak E, 2,3,4-trimethyl-1,5,6-triacetyl-D-galactitol; peak F, 2,4-dimethyl-1,3,5,6-tetraacetyl-D-mannitol. Curve 1 was obtained with the intact isolated glycopeptide; curve 2, after treatment with neuraminidase and β-galactosidase; curve 3, after further treatment with β-*N*-acetylglucosiminidase; curve 4, after treatment with α-mannosidase.

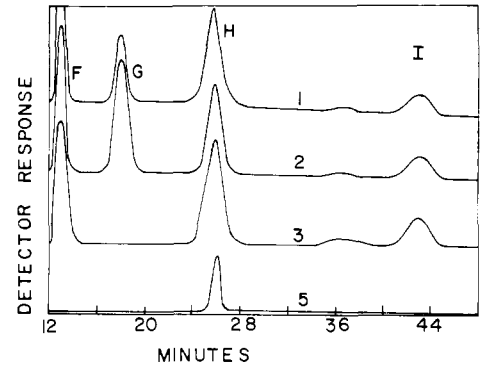


FIGURE 3: Identification of methylated acetylated *N*-acetylglucosaminitols after treatment of glucopeptides with purified glycosidases. Separation by gas chromatography was performed as described in the text. Peaks correspond to the following standards. Peak F, 2,4-dimethyl-1,3,5,6-tetraacetylmannitol; peak G, 3,4,6-trimethyl-1,5-diacetyl-*N*-methylacetylglucosaminitol; peak H, 3,6-dimethyl-1,4,5-triacetyl-*N*-methylacetylglucosaminitol; peak I, 6-methyl-1,3,4,5-tetraacetyl-*N*-methylacetylglucosaminitol. Curve 1 was obtained with the intact glycopeptide; curve 2, after treatment with neuraminidase and β-galactosidase; curve 3, after treatment with β-*N*-acetylglucosiminidase; curve 5 was obtained by methylation and acetylation of the product formed after incubation of purified galactosyltransferase with UDP-galactose and GlcNAc.

lost. These results show that 2 mannose residues are attached through α1→3 and α1→6 linkages to a third mannose to form a branch point in the oligosaccharide chain.

The glycopeptide still contained 2.25, 0.85, and 0.70 residues of GlcNAc, mannose, and fucose, respectively (treatment D, Table IV). Hydrolysis with α-fucosidase caused the release of 0.65 μmol of fucose and the loss of peak A, 2,3,4-trimethylfucitol. Peak I, 6-methylglucosaminitol was converted to peak H, 3,6-dimethylglucosaminitol, by this treatment. These results show that fucose is attached by an α1→3 linkage to one of the remaining GlcNAc residues.

Exhaustive treatment of the resulting glycopeptide with β-mannosidase and β-*N*-acetylglucosiminidase resulted in the release of most of the mannose and 1 equiv of GlcNAc. These results suggest that the glycopeptide contains a terminal mannose residue which is linked by a β1→4 bond to GlcNAc, which in turn is attached to a second GlcNAc residue linked to asparagine in the protein chain. All of the residual GlcNAc was released by treating the glycopeptide with proteases and then with highly purified 4-L-aspartylglucosylamine amidohydrolase which was isolated from swine kidney (Kohno and Yamashina, 1972). The data obtained in these studies are consistent with the structure of the carbohydrate chains of porcine IgG shown in the abstract.

Characterization of Endogenous Glycosyl Acceptors Present in Membrane Fractions. To isolate the bound glycosyl acceptor lymph node membrane fractions were incubated with UDP[1-^{14}C]galactose or UDP[6-^{3}H]GlcNAc in the standard reaction mixture with amounts increased 30-fold. Afterward the membranes were removed by centrifugation at 100 000*g* for 30 min and they were washed once with 0.05 M Tris-HCl, pH 7.0. The final pellet was suspended in 15 ml of 0.05 M Tris-HCl, pH 7.0, containing 0.5% deoxycholate, and the suspension was disrupted by sonication for 20 s at maximum intensity with a Biosonik II instrument. Insoluble material was removed by centrifugation at 100 000*g* for 1 h and the supernatant solution was dialyzed, concentrated, and passed through a Sephadex G-50 column (2.5 × 30 cm) to remove deoxycholate.

The eluted sample was concentrated, reduced with dithiothreitol, alkylated with iodoacetamide and fractionated by gel filtration on Sephadex G-100 (2.5 × 100 cm) in 1 M propionic acid as described previously (Garver et al., 1975). A single radioactive peak was eluted from the column in a position which corresponded to the heavy chain of porcine IgG immunoglobulin. The same radioactive peak was obtained when UDP[1-^{14}C]galactose or UDP[6-^{3}H]GlcNAc was used as the substrate. Analysis by gel diffusion with rabbit antibody, specific for intact porcine IgG and light and heavy chains, revealed the presence of a radioactive band which corresponded to the heavy chain of porcine IgG immunoglobulin. The results summarized in Table V show that galactose was transferred to the terminal GlcNAc residues of the oligosaccharide chains and the GlcNAc was transferred to the mannose residues in the outer chains. These results suggest that an appreciable

TABLE V: Localization of Radioactivity in Sugar Residues of Endogenous IgG Glycosyl Acceptors following Incubation with UDP[1-^{14}C]galactose or UDP[6-^{3}H]GlcNAc.[a]

	Amount and Spec Act. of Sugar Removed			
	UDP[1-^{14}C]galactose		UDP[6-^{3}H]GlcNAc	
Treatment	(μmol/μmol)	(cpm/μmol)	(μmol/μmol)	(cpm/μmol)
Isolated glycopeptide	1	810	1	720
A. β-Galactosidase	0.9	730	0.8	0
B. β-Acetylglucosaminidase	2.0	10	1.9	680
C. α-Mannosidase	2.0	0	1.8	0
D. GlcNAc isolated after acid hydrolysis of glycopeptide C	1.7	0	1.9	20

[a] The labeled glycosyl acceptor was solubilized and isolated as described in the text. Labeled glycopeptides prepared by digestion with Pronase were isolated and sequential enzymatic hydrolysis was carried out by the procedure described in Table IV.

amount of the endogenous IgG heavy chain bound to these membrane preparations still lacks galactose and GlcNAc.

Discussion

The galactosyltransferases examined in these studies are probably involved in the biosynthesis of the oligosaccharide chain of IgG immunoglobulin. The enzymes catalyzed the transfer of galactosyl groups to glycoproteins containing terminal GlcNAc residues and a solubilized preparation of porcine IgG devoid of galactose was an excellent substrate for both the soluble and particulate enzymes. The best macromolecular substrates for the enzyme contained branched oligosaccharide chains which terminated in GlcNAc. The structure of the protein moiety did not significantly influence the activity of the transferase. However, a macromolecular structure is required, since the glycopeptides prepared from these glycoproteins and free GlcNAc had much higher K_m's.

Most of the tightly bound endogeneous glycoprotein acceptors present in particulate mesentary lymph node preparations are precursors of IgG immunoglobulin in which the core portion of the oligosaccharide moiety may be complete. The outer galactose and penultimate *N*-acetylglucosamine residues of the two branches of the oligosaccharide chain are incomplete and glycosyltransferases incorporate galactose and *N*-acetylglucosamine mainly into these terminal positions. The main structural features of the carbohydrate chains of porcine IgG heavy chain were similar to those of other immunoglobulins and plasma glycoproteins attached through an asparagine–amide linkage. The structure of human IgG immunoglobulin has been determined (Kornfeld et al., 1971).

The observation that about 20% of the galactosyltransferase is present in a soluble form in this tissue is interesting, in view of the fact that in most tissues this enzyme is tightly bound to membranes present in the microsomes and Golgi apparatus. The principal step in the purification of galactosyltransferase was chromatography on Sepharose 4B columns containing bound GlcNAc. UMP and Mn^{2+} were essential for binding to the affinity column and the enzyme could be eluted with solutions containing *p*-nitrophenyl-GlcNAc; however, better yields were obtained with EDTA. The final purified enzyme had a specific activity of 35 nmol min^{-1} mg^{-1} with glycoprotein substrates. Much higher specific activities were obtained with low-molecular-weight substrates; however, the concentrations of these substrates required for activity were an order of magnitude greater than the corresponding physiological concentrations. Based on these properties, the enzyme would function principally in the synthesis of glycoproteins in lymph node tissue.

A single homogeneous species of intracellular galactosyltransferase with a molecular weight of 57 000 was isolated from lymph node extracts. Three species with molecular weights ranging from 43 000 to 54 000 were isolated from milk by Barker et al. (1972). The low-molecular-weight forms of the milk enzyme may be formed by partial proteolysis.

The results obtained in kinetic studies suggest that the activity of bound galactosyltransferase may be limited by the concentration of bound galactosyl acceptor, as well as the concentration of UDP-galactose. The cooperative interaction between these substrates would lead to a situation in which at high concentrations of UDP-galactose only low concentrations of glycoprotein acceptor would be required for maximum activity. However, when the concentration of UDP-galactose was low, the activity of galactosyltransferase would decrease and more bound glycosyl acceptor lacking galactose might accumulate in the membrane fractions. This mechanism could provide a very effective control point for coordinating the regulation of the synthesis of UDP-galactose in the soluble fraction with the synthesis and secretion of the bound glycoprotein in the membranes of the endoplasmic reticulum.

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Potentiometric Determination of Ionizations at the Active Site of Papain†

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ABSTRACT: The ionization behavior of groups at the active site of papain was determined from the pH dependence of the difference in proton content of papain and the methylthio derivative of the thiol group at the active site of papain (papain-S-SCH_3). This difference in proton content was determined directly by two independent methods. One method involved potentiometric measurements of the protons released on demethylthiolation of papain-S-SCH_3 with dithiothreitol, as a function of pH. The other method involved analogous measurements of the protons released on methylthiolation of papain with methyl methanethiolsulfonate. The methylthio pH-difference titrations generated by these measurements indicate that ionization of the thiol group at the active site of papain is linked to the ionization of His-159. The pK of the thiol group changes from 3.3 to 7.6 on deprotonation of His-159 at 29 °C, $\Gamma/2$ 0.05. Similarly, the pK of His-159 shifts from 4.3 to 8.5 when the active site thiol group is deprotonated. The microscopic ionization constants determined in this work for Cys-25 and His-159 indicate the equilibrium constant for transfer of a proton from Cys-25 to His-159 is 8–12, and that in the physiological pH range the active site thiol group exists mainly as a thiol anion.

Recently, much discussion has centered around the relationship between the pH dependence of the catalytic efficiency of papain and the state of ionization of the thiol and imidazoyl groups at the active site of this enzyme (Chaiken and Smith, 1969; Polgar, 1973, 1974; Shipton et al., 1975; Drenth et al., 1975; Lowe, 1976). At the heart of this controversy is a failure to resolve an ambiguity which always arises when one attempts to determine a microscopic ionization constant of a group on a polybasic molecule from the pH dependence of some spectral or kinetic property. This ambiguity occurs because neighboring ionizations could perturb the pK of the group in question as well as the response factor being used to measure the degree of ionization of the group under study. A relationship which illustrates such an ambiguity is given in the Appendix. One way around the ambiguity is to determine directly the pH dependence of the proton content of the group in question from a potentiometric pH-difference titration between the polybasic substance and a derivative of the substance, wherein the ionization of the group under study is blocked by a small group which does not perturb other ionizations. For example, pH-difference titrations, wherein one potentiometrically deter-

† From the Department of Biological Chemistry, The University of Michigan Medical School, Ann Arbor, Michigan 48109. *Received June 2, 1976.* This study was made possible by Research Grant AM-09276 from the National Institutes of Health and Training Grant GM00187. Part of this work is described in a Ph.D. dissertation to be submitted by F. A. Johnson to the Graduate School of The University of Michigan.