Structural Studies on Carcinoembryonic Antigen Periodate Oxidation†

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ABSTRACT: Periodate oxidation has been applied to examine the carbohydrate structure of carcinoembryonic antigen (CEA) and the possible role of the carbohydrate residues in its antigenic activity. Sialic acid (*N*-acetylneuraminic acid) and fucose were completely destroyed, and galactose and mannose were partially destroyed by a single periodate treatment. Serial periodate treatment (Smith degradation) destroyed additional amounts of galactose and mannose, as well as significant amounts of *N*-acetylglucosamine. Prior removal of sialic acid by neuraminidase treatment led to increased destruction of galactose by periodate. Antigenic activity persisted indicating that the residues destroyed played little, if any, part in the antigenicity of CEA. These results yield an initial view of the structural arrangement of the carbohydrate residues in the CEA molecule.

Carcinoembryonic antigen (CEA)[1] is a tumor associated antigen discovered by Gold and Freedman (1965a,b). It has been detected in a variety of tumors and in fetal gastrointestinal tissue. It may also be present in low concentration in normal adult tissue. The literature on CEA has recently been reviewed by Terry *et al.* (1974).

The finding that CEA could be detected in sera by radioimmune assay (Thomson *et al.*, 1969) raised the hope that CEA might be of value in the detection and diagnosis of cancer. Several investigators (Mach and Pusztaszeri, 1972; Von Kleist *et al.*, 1972; Häkkinen, 1972; Darcy *et al.*, 1973) have isolated from normal adult tissue substances which share antigenic determinants with CEA but differ in chemical composition and molecular size. These and other uncharacterized substances present in patients with various disease conditions render difficult the interpretation of the radioimmune assay results. These difficulties emphasize the need for chemical studies on these materials.

CEA is a glycoprotein (Krupey *et al.*, 1968) with a molecular weight of approximately 180,000 (Slayter *et al.*, submitted for publication). Examination by electron microscopy has shown the molecule to be quite homogeneous in size with a complex secondary structure (Slayter *et al.*, submitted for publication). CEA isolated from individual tumors exhibits a variable chemical composition of 50–65% carbohydrate and the remainder protein (Terry *et al.*, 1974). It exhibits considerable charge heterogeneity derived in part from variation in sialic acid content (Coligan *et al.*, 1973). The N-terminal portion of the protein backbone of CEA extracted from hepatic metastases of colonic adenocarcinoma possesses a constant amino acid sequence (Terry *et al.*, 1972; Chu *et al.*, 1974).

A beginning has been made in elucidating the structure of the carbohydrate portion of CEA. In addition to sialic acid, CEA contains fucose, galactose, and mannose, and the linkage to the protein portion appears to occur through *N*-acetylglucosamine (Terry *et al.*, 1974). Only traces of galactosamine have been detected in highly purified CEA preparations.

The role of the carbohydrate in the antigenicity of CEA is not clear (Morris *et al.*, submitted for publication). Banjo *et al.* (1974) have obtained fractions active in the radioimmune assay by degrading CEA with nagase. These fragments are approximately 90% carbohydrate with 57% of the most active fragment being *N*-acetylglucosamine. The only amino acids present in significant amounts are aspartic and glutamic acids or their amides. These fragments are considerably less active than CEA on a weight basis.

The results of the present studies provide further information on the role of certain monosaccharide residues in the antigenic activity and on the structural arrangements within the carbohydrate groupings of the molecule. A preliminary presentation of this research has been made (Egan *et al.*, 1974), but the present report provides experimental details and provides additional results obtained by serial periodate oxidation (Smith degradation).

Materials and Methods

Preparation of CEA. CEA used in these studies was purified as described by Coligan *et al.* (1972).

Radioimmune Assay for CEA. CEA was measured by double antibody radioimmune assay (Egan *et al.*, 1972; Egan, 1974).

Neuraminidase Digestion. Vibrio cholerae neuraminidase (500 units/ml; 1.6 units/μg; General Biochemicals, Chagrin Falls, Ohio) was added to lyophilized CEA in a ratio of 1 unit of neuraminidase/50 μg of CEA. For each 0.9 ml of neuraminidase, 0.1 ml of sodium citrate buffer (0.2 M sodium citrate–0.01 M $CaCl_2$ (pH 5.5)) was added. After 26 hr at 35°, 95% of the sialic acid was removed as determined by the thiobarbituric acid assay (Warren, 1959). The reaction mixture was exhaustively dialyzed against deionized H_2O at 4°, lyophilized, and dried over P_2O_5. A control sample was handled identically except that neuraminidase was omitted from the reaction.

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[1] Abbreviations used are: CEA, carcinoembryonic antigen; the prefixes Mi, Ro, and Si before CEA designate the individuals from whose tumors the CEA was isolated. All three preparations were isolated from liver metastases of primary adenocarcinomas of the colon. These preparations are chemically very similar, but not identical, and antigenically indistinguishable (Terry *et al.*, 1974).

Table I: Periodate Oxidation of MiCEA.

Hr of Oxidation	CEA Activity (mg/ml)	Monosaccharides Recovered (moles/10^5 g of Product[a])				
		N-Acetylneuraminic Acid[b]	Fucose[b]	Mannose[b]	Galactose[b]	*N*-Acetylglucosamine[c]
0	5.13	13.0	35.0	28.4	63.2	85.1
9	4.22					
24	5.49	0	0	23.9	46.9	99.6
48	4.34	0	0	24.1	49.2	98.5

[a] After the described chemical treatment, the CEA was dialyzed against H_2O, lyophilized, and dried over P_2O_5 before analysis. [b] Determined by gas chromatography according to the method of Clamp *et al.* (1972). [c] Determined on the amino acid analyzer by the method of Liu and Chang (1971).

Amino acid analysis was performed by a modification of the method of Liu and Chang (1971). CEA samples, 100–200 μg, were hydrolyzed under vacuum in heavy walled ignition tubes at 110° for 24 and 72 hr in duplicates with 0.5 ml of 3 N *p*-toluenesulfonic acid containing 0.2% 3-(2-aminoethyl)indole. The hydrolyzed samples were analyzed on a Beckman 121 H amino acid analyzer. The basic amino acids and amino sugars were eluted from a 20 × 0.9 cm column of PA35 (Beckman), using sodium citrate buffers (0.4 N sodium), pH 5.27 and pH 5.87. The acidic and neutral amino acids were eluted from a 56 × 0.9 cm column of AA15 (Beckman) with sodium citrate buffers (0.2 N sodium), pH 3.27 and pH 4.55.

Carbohydrate Analysis. Neutral sugars were analyzed by gas chromatography as the trimethylsilyl derivatives of the methyl glycosides according to the procedures of Clamp *et al.* (1972) or of Reinhold (1972).

Amino sugars were determined on the amino acid analyzer during amino acid analyses as described above.

Sialic acid was measured by the method of Warren (1959) or by gas chromatography as described above. The sialic acid liberated from CEA by mild acid hydrolysis (0.1 N H_2SO_4, 80°, 1 hr) was isolated and characterized as described by Spiro (1960). Sialic acid was removed from the hydrolysate by passage through AG1-X8 resin, 50–100 mesh (Bio-Rad Laboratories), which had been converted from the chloride to formate form. The sialic acid was eluted from the column by 0.3 N formic acid and lyophilized. The eluate, along with standards of neutral and amino sugars (Calbiochem), *N*-acetylneuraminic acid (Calbiochem) and *N*-glycolylneuraminic acid (Sigma), were chromatographed on Whatman No. 1 paper in *n*-butyl acetate–acetic acid–water (3:2:1). The sugars were located by the silver nitrate method of Trevelyan *et al.* (1950) using Kodak fixer to remove the silver oxide background (Benson *et al.*, 1952).

Periodate Oxidation. Oxidation was performed with sodium metaperiodate (0.03 M) in 0.05 M sodium acetate buffer (pH 4.5) at 4° in the dark. The CEA concentration was approximately 5 mg/ml. Aliquots were taken at several times for the determination of periodate consumption and for radioimmunoassay of antigenic activity. In the samples taken for radioimmunoassay, the oxidation was terminated by the addition of excess ethylene glycol. Controls were handled in a similar manner except for the addition of periodate. Periodate oxidation of CEA was assumed completed when the periodate consumption had ceased. In most experiments, the oxidation was terminated at approximately 24 hr by the addition of ethylene glycol. The reaction mixtures were then dialyzed exhaustively against deionized H_2O at 4°. Experiments terminated at 48 hr gave results similar to those terminated at 24 hr.

Determination of Periodate Consumption. Periodate consumption was determined spectrophotometrically as described by Hughes and Clamp (1972).

Sodium Borohydride Reduction of Oxidized CEA. An equal volume of 0.2 M, pH 8.0, sodium borate buffer containing 0.2 M sodium borohydride was added to the oxidized and dialyzed CEA to give a final concentration of 0.1 M for both the buffer and borohydride. The reaction was performed at 0° for 12 hr and terminated by lowering the pH to 5 by the addition of acetic acid. Aliquots were removed and neutralized at timed intervals for radioimmunoassay. Subsequently, the reaction mixture was dialyzed at 4° against deionized H_2O. In control studies, CEA not subjected to periodate oxidation was treated with sodium borohydride in a similar manner.

Acid Hydrolysis of Oxidized–Reduced CEA. After reduction and dialysis, samples were subjected to mild acid hydrolysis by one of the following methods: (1) 0.05 N H_2SO_4 for 1 hr at 80° or (2) 0.5 N HCl for 24 hr at 25°. Samples were removed and neutralized at timed intervals for radioimmunoassay. After hydrolysis, the reaction was terminated by neutralization with NaOH followed by dialysis at 4° against deionized H_2O.

Serial Periodate Oxidation. The consecutive application of periodate oxidation, sodium borohydride reduction, and mild acid hydrolysis as described above in repeated cycles is referred to as serial periodate oxidation or Smith degradation (Smith and Unrau, 1959).

Results and Discussion

Periodate Oxidation of MiCEA. Treatment of MiCEA with periodate resulted in the destruction of a significant portion of the carbohydrate of CEA (Table I). As expected from their probable terminal position, all of the sialic acid and fucose residues were susceptible to periodate oxidation. In addition, approximately 16% of the mannose and 24% of the galactose were susceptible. No *N*-acetylglucosamine[2] was destroyed. The small amount of *N*-acetylgalactosamine present in this sample prevented accurate quantitation of this sugar during the experiment. The periodate oxidation

[2] It has been assumed in these studies that all amino sugars are in their acetyl form, as is usually the case in glycoproteins.

Table II: Smith Degradation of RoCEA.

	Sugars Present (moles/10^5 g of Product[a])					
	Untreated	Expt I after 1st Cycle[b-d]	Expt I after 2nd Cycle[b,c]	Experiment II after 1st Cycle[b,c,e]	$NaBH_4$ Only[c]	Hydrolyzed Only[c,e]
N-Acetylneuraminic acid[f]	12.3	0	0	Trace	11.2	12.7
Fucose[f]	41.9	0	0	0	36.3	38.6
Mannose[f]	47.9	36.4	24.1	39.3	44.6	50.4
Galactose[f]	64.9	23.3	19.2	24.0	61.5	67.4
N-Acetylglucosamine[g]	107.8	123.0	64.7	132.5	110.0	102.0
N-Acetylgalactosamine[g]	22.7	22.3	29.8	11.6	21.8	18.7
% dry weight recovered including amino acids	91	68	54	84	78	79

[a] After the described chemical treatment, each reaction was dialyzed against H_2O, lyophilized, and dried over P_2O_5 prior to analysis. [b] The first cycle consists of periodate oxidation, $NaBH_4$ reduction, and acid hydrolysis. In the second cycle the acid hydrolysis was not performed. [c] Values are normalized to 91% dry weight recovery. The actual dry weight recoveries are shown in the bottom line. [d] 0.05 *N* H_2SO_4 at 80° for 1 hr was used during the hydrolysis step. [e] 0.5 *N* HCl at 25° for 24 hr was used during the hydrolysis step. [f] Determined by gas chromatography by the method of Clamp *et al.* (1972). [g] Determined on the amino acid analyzer by the method of Liu and Chang (1971).

had no significant affect on the antigenic activity[3] of CEA.

Smith Degradation of RoCEA. The sugars present after two successive Smith degradation cycles of RoCEA are shown in Table II, experiment I. The first cycle was repeated in experiment II. Each cycle refers to successive periodate oxidation, $NaBH_4$ reduction, and acid hydrolysis. Values for untreated CEA and control samples which have undergone $NaBH_4$ reduction or acid hydrolysis only are also included. These results are similar to those obtained for MiCEA (Table I) except that larger portions of the mannose (24%) and galactose (64%) were found to be susceptible to the initial periodate oxidation in RoCEA (experiment I) when compared to the untreated control. As with MiCEA, no *N*-acetylglucosamine was susceptible to the initial periodate oxidation. The second cycle in experiment I resulted in the additional destruction of 33% of the remaining mannose and 16% of the remaining galactose. Also, in the second cycle 47% of the *N*-acetylglucosamine was now susceptible to periodate. The control samples which had undergone only $NaBH_4$ reduction or hydrolysis showed little if any nonspecific carbohydrate losses. The values in Table II are normalized to the 91% dry weight recovery obtained with the untreated control. The low recoveries obtained for the other samples, especially in experiment I, are attributed to the difficulties in weighing accurately small amounts of material (<1.5 mg). Justification for this normalization of the values is obtained by comparing cycle 1 of experiments I and II. These should give identical values since they differ only in the hydrolysis step. The values are essentially identical after normalization to equal dry weight recovery.

The control hydrolysis in Table II was performed with 0.5 N HCl for 24 hr at 25°. Similar results were obtained using 0.05 N H_2SO_4 for 1 hr at 80° except that the sialic acid was lost during the hydrolysis and subsequent dialysis.

[3] In regard to antigenic activity, one must be cautious in extrapolating the results obtained with our goat anti-CEA to include all antisera used in other laboratories. However, clinical data obtained with our antiserum are in general agreement with those obtained in other laboratories (Kupchik *et al.*, 1973).

The increase in moles/10^5 g (17%) of the *N*-acetylglucosamine after the first cycle in experiment I (Table II) compared to the untreated CEA agrees with the same approximate increase in the per cent by weight of amino acids (19%). This increase is expected, since the Smith degraded material which has been dialyzed and lyophilized prior to analysis should be enriched in sugar residues and amino acids which are not susceptible to periodate oxidation. The decrease in moles/10^5 g of the *N*-acetylgalactosamine in experiment II, cycle 1, is probably spurious since this loss was not detected in experiment I, cycle 1.

In agreement with results obtained with MiCEA (Table I), no loss in antigenic activity occurred during the initial periodate treatment of RoCEA (Table III). In addition, no loss in antigenic activity occurred during the subsequent $NaBH_4$ reduction step. However, after the hydrolysis step, the activity dropped to 0.96 mg/ml which represents a 68% loss relative to the original activity. The loss in antigenic activity in experiment II, cycle 1, using different hydrolysis conditions (0.5 N HCl, 25°, 24 hr) was essentially identical (73%).

As expected, the $NaBH_4$ reduction control CEA in Table III, which was initially treated with pH 4.5 buffer not containing periodate, lost no antigenic activity. However, a control CEA preparation which had undergone hydrolysis only (Table III) lost approximately the same amount of antigenic activity (61%) as samples during the hydrolysis step of the Smith degradation. Both methods of acid hydrolysis resulted in the loss of the same amount of antigenic activity in CEA preparations subjected to hydrolysis only.

The loss in antigenicity during hydrolysis probably was not due to carbohydrate destruction since none was evident, but more likely to breakdown in the polypeptide backbone. This would give rise to CEA fragments which may not be as effective inhibitors as intact CEA in the radioimmunoassay. Sephadex G-200 chromatography of CEA that had been treated with dilute acid under conditions identical with those used in these experiments indeed indicates that extensive fragmentation is occurring. Also, thin layer chromatog-

Table III: Effect of Smith Degradation and $NaBH_4$ Reduction or Acid Hydrolysis Only on RoCEA Antigenic Activity.

	Treatment Prior to Assay	CEA Activity[a] (mg/ml)
Experiment I	None	2.99
	0.03 M $NaIO_4$, pH 4.5, 24 hr	2.34
	Dialysis	2.93
	0.1 M $NaBH_4$, 11 hr	3.47
	Dialysis	3.06
	0.05 N H_2SO_4, 80°, 1 hr	0.96
	Dialysis	0.72
	0.03 M $NaIO_4$, pH 4.5, 24 hr	0.83
$NaBH_4$ reduction control	None	3.82
	pH 4.5, 24 hr	3.42
	Dialysis	2.68
	0.1 M $NaBH_4$, 11 hr	3.48
Hydrolysis control	None	3.39
	0.5 N HCl, 25°, 24 hr	1.33

[a] Values are corrected for any changes in the volume of the reaction mixtures.

Table IV: Periodate Oxidation of SiCEA.

	Sugars Present (moles/10^5 g of Product[a])			
	Untreated		Neuraminidase Treated	
	Before $NaIO_4$	After $NaIO_4$	Before $NaIO_4$	After $NaIO_4$
N-Acetylneuraminic acid[b]	21.3	0	1.1	0
Fucose[c]	36.8	0	39.4	0
Mannose[c]	27.4	17.7	25.5	15.4
Galactose[c]	63.6	37.7	63.3	19.6
N-Acetylglucosamine[d]	105.4	83.0	95.6	88.3
N-Acetylglucosamine[d]	9.9	8.9	9.2	7.4

[a] After the described chemical treatment, each reaction was dialyzed against H_2O, lyophilized, and dried over P_2O_5 prior to analysis. [b] Determined by the thiobarbituric acid assay (Warren, 1959). [c] Determined by the gas chromatographic procedure of Reinhold (1972). [d] Determined on the amino acid analyzer by the method of Liu and Chang (1971).

raphy of the dialysate from experiment II, cycle 1 of Table II, indicated the presence of several peptide fragments (Coligan and Pritchard, unpublished). Mild acid conditions are known to hydrolyze the Asp–Pro peptide bond (Fraser *et al.*, 1972). Considering that a high percentage of the amino acids in CEA are Pro and Asx, it is possible that at least some of the fragmentation is due to the occurrence of this sequence in CEA. Despite the loss in antigenic activity on a weight basis in Smith degraded samples and in the acid hydrolysis only controls, all the samples were capable of inhibiting in the radioimmunoassay to the 94% level. This implies that no antigenic determinants have been destroyed completely by mild acid treatment.

It is interesting that in the second cycle (Table III), when 50% of the *N*-acetylglucosamine was destroyed along with significant additional amounts of mannose and galactose, no further loss in antigenic activity occurred. Although the acid hydrolysis step was not performed in the second cycle, other experiments have shown the antigenic activity to be stable to hydrolysis at this step. Thus, after destroying 100% of the fucose and sialic acid and approximately 75% of the galactose, 50% of the mannose, and 50% of the *N*-acetylglucosamine, essentially no loss in antigenic activity can be detected.

Identification of the Sialic Acid. Paper chromatography of the sialic acid fraction obtained by mild acid hydrolysis showed only a single spot corresponding to *N*-acetylneuraminic acid. Therefore, for quantitative determinations, all sialic acid detected by our analytical procedures was assumed to be *N*-acetylneuraminic acid.

Position of Attachment of the Sialic Acid in CEA. In order to examine the mode of attachment of sialic acid in CEA, a preparation of SiCEA was divided, and one-half was treated with neuraminidase while the other half served as a buffer control. Table IV shows that 95% of the sialic acid was removed from the neuraminidase treated sample. Both samples were then treated with periodate. Removal of the sialic acid resulted in a much greater percentage of the galactose being susceptible to periodate; 69% in the neuraminidase treated *vs.* only 41% in the untreated CEA preparation. None of the other carbohydrates show significant differences. Since the amount of sialic acid (20.2 mol/10^5 g) removed by neuraminidase treatment is essentially equal to the additional amount of galactose (18.1 mol/10^5 g) destroyed by periodate in the neuraminidase-treated sample, a single sialic acid substitution must be protecting each of these galactose residues from periodate oxidation. Therefore, this substitution must be on the 3 position of galactose. This assignment is supported by the identification of the 2,4,6-trimethyl derivative of galactose in our mass spectrometric studies (Coligan and Schnute, unpublished results).

Examination of SiCEA (Table IV) indicated that approximately 35% of the mannose and 41% of the galactose were susceptible to a single periodate oxidation. Also, a significant amount of the *N*-acetylglucosamine of SiCEA was apparently oxidized by periodate; 20% in the untreated and 8% neuraminidase treated. The discrepancy in these values can be attributed to the difference in moles per 10^5 g of *N*-acetylglucosamine detected in untreated (105.4) *vs.* neuraminidase-treated samples (95.6) prior to periodate treatment. As shown with the other sugars in these reactions, these values should have been approximately equal. We have found quantitation of the amino sugars to be more difficult than the neutral hexoses and are inclined to attribute these differences to experimental variation.

Amino Acids and Periodate Oxidation. Analysis of amino acids before and after periodate oxidation of CEA is shown in Table V. These data are for the untreated SiCEA in Table IV. Analyses of other preparations gave similar results. Cysteine and tryptophan are completely destroyed in

Table V: Amino Acids before and after Periodate Oxidation of SiCEA.[a]

	Mole Per Cent	
	Before $NaIO_4$	After $NaIO_4$
Lys	2.4	2.1
His	1.7	1.9
Arg	3.2	4.0
Asp	15.8	16.3
Thr	9.5	9.6
Ser	12.4	13.8
Glu	9.9	10.7
Pro	8.7	5.3
Gly	6.3	7.7
Ala	7.0	7.6
1/2-Cys	1.9	0.0
Val	3.8	4.6
Met	0.1	0.3
Ile	2.8	2.5
Leu	8.2	7.6
Tyr	4.1	3.7
Phe	2.3	2.2
Trp	0.8	0.0

[a] Samples were hydrolyzed in 0.5 ml of 3 N *p*-toluenesulfonic acid for 24 and 72 hr in duplicates at 110° *in vacuo*. The hydrolysates were analyzed on a Beckman 121 H amino acid analyzer.

the periodate-treated samples. In this sample 40% of the proline was destroyed, although in most other samples much less was destroyed (<15%). It is possible that some of the methionine in CEA is oxidized by periodate to the corresponding sulfoxide and sulfone (Lee and Montgomery, 1961); however, during amino acid analysis a significant and variable amount of methionine is converted to the sulfoxide and detected as such. The other amino acid residues do not appear to be significantly changed by periodate oxidation.

The complete destruction of cysteine and tryptophan and the partial destruction of proline by periodate oxidation had no affect on the antigenic activity of CEA.

Heterogeneity and Nature of the Carbohydrate Portion of CEA. CEA preparations isolated from different tumors were used in the various experiments reported here. The results obtained in these experiments with three different preparations of CEA were very similar but not identical. In all three preparations, a single periodate treatment removed all of the fucose and sialic acid. Variable amounts of mannose (16, 24, and 35%) and galactose (24, 64, and 41%) were destroyed after a single periodate oxidation, whereas only SiCEA showed a detectable destruction of *N*-acetylglucosamine. In experiments where *N*-acetylgalactosamine could be quantitated, it was resistant to oxidation. Thus, it appears that different tumors yield CEA of varying carbohydrate complexity. This individuality is not unexpected considering the current understanding of the enzymatic mechanisms involved in the biosynthesis of the carbohydrate units of glycoproteins (Spiro, 1970).

These data are in agreement with observations on other mammalian glycoproteins, reviewed by Heath (1971), which indicate that fucose and sialic acid are always terminal sugars. In confirmation, mass spectrometric examination of the sugar derivatives (Björndal *et al.*, 1970) obtained by methylation, hydrolysis, reduction, acetylation, and gas chromatography (Hakomori, 1964; Stellner *et al.*, 1973) has identified the 2,3,4-trimethylated derivative of fucose in CEA (Coligan and Schnute, unpublished results). We have also identified the 2,3,4,6-tetramethyl derivatives of both galactose and mannose indicating the existence of terminal galactose and mannose residues in CEA. These residues as well as the 3,4,6-trimethyl derivative of mannose (an intrachain residue we have identified) would be susceptible to periodate oxidation. These data also show that much of the mannose and galactose in CEA must be nonterminal and substituted to exclude vicinal hydroxyl groups. Indeed, mass spectrometric studies have identified the 2,4,6-trimethyl derivative of galactose (Coligan and Schnute, unpublished results). Since in only one preparation was any significant amount of *N*-acetylglucosamine destroyed by the initial periodate oxidation, it is unlikely that a significant amount of the monosaccharide occupies a terminal nonreducing position in the polysaccharide chains.

We can begin to visualize the carbohydrate structure in CEA as beginning with *N*-acetylglucosamine attached to asparagine in the peptide chain (Terry *et al.*, 1974). Fucose and *N*-acetylneuraminic acid are present at nonreducing ends. *N*-Acetylneuraminic acid is attached to the 3 position in galactose. Galactose and mannose constitute the intermediate portion of the carbohydrate groupings, but some are also terminal. Obviously, much additional work remains to be done before this picture can be completed. Significantly, the sites of the antigenic activity have remained elusive.

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Partial Characterization of the Cytosol 3α-Hydroxysteroid: NAD(P)$^+$ Oxidoreductase of Rat Ventral Prostate†

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ABSTRACT: The cytosol 3α-hydroxysteroid dehydrogenase of rat ventral prostate has been partially purified. The rates of both the oxidation and reduction by crude and partially purified enzymes have been measured with a variety of radioactive substrates, and the effects of several inhibitor steroids have been assessed. Four conclusions have been drawn from the study. First, no detectable 3β-androstanediol was formed from dihydrotestosterone, and the oxidation of 3β-androstanediol was undetectable. Second, the cytosol enzyme exhibits a distinct and unique substrate specificity in that steroids with keto or hydroxyl substitution on the 11th carbon of the steroid cannot serve as substrates or as inhibitors of the enzyme. Third, either 5α or 5β reduction of Δ^4,3-keto steroids must take place before the steroids can serve as substrates of the enzyme. Fourth, many Δ^4,3-keto steroids that cannot act as substrates for the enzyme inhibit the enzyme competitively and may well serve as physiological regulators of the reaction in the intact cell.

Enzymes that interconvert the 3-keto group of steroids containing saturated A,B rings with both 3α- and 3β-hydroxysteroids have been characterized in a variety of bacteria (Talalay and Marcus, 1954, 1956; Marcus and Talalay, 1956; Ringold *et al.,* 1967) and in several mammalian tissues (Tomkins, 1956; Koide, 1963, 1965a,b, 1969; Gustafsson *et al.,* 1968; Unhjem, 1970; Rommerts and van der Molen, 1971; Shimazaki *et al.,* 1972; Björkhem *et al.,* 1973; Martin and Nicholas, 1973; Levy *et al.,* 1974; Mowszowicz and Bardin, 1974). Either di- or triphosphopyridine nucleotides can serve as coenzymes, and in most instances the enzymes are located predominantly in the cytosol (soluble) fraction of the cell. In addition to their role in the metabolism of steroids the enzymes may participate in transhydrogenase reactions in some tissues (Hurlock and Talalay, 1958; Baron *et al.,* 1963; Koide *et al.,* 1962; Koide, 1964).

In androgen target tissues, such as the ventral prostate of the rat, the 3α-hydroxysteroid oxidoreductase[1] may play a

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[1] Trivial names used are: testosterone, 17β-hydroxy-4-androsten-3-one; dihydrotestosterone, 17β-hydroxy-5α-androstan-3-one; androsterone, 3α-hydroxy-5α-androstan-17-one; 3α-androstanediol, 5α-androstane-3α,17β-diol; 3β-androstanediol, 5α-androstane-3β,17β-diol; androstenedione, 4-androstene-3,17-dione; androstanedione, 5α-androstane-3,17-dione; epitestosterone, 17α-hydroxy-4-androsten-3-one; progesterone, 4-pregnene-3,20-dione; deoxycorticosterone, 21-hydroxy-4-pregnene-3,20-dione; cortexolone, 17α,21-dihydroxy-4-pregnene-3,20-dione; 19-nortestosterone, 17β-hydroxyestren-3-one; corticosterone, 11β,21-dihydroxy-4-pregnene-3,20-dione; cortisol, 11β,17α,21-trihydroxy-4-pregnene-3,20-dione; cortisone, 17α,21-dihydroxy-4-pregnene-3,11,20-trione; methyldihydrotestosterone, 17α-methyl-17β-hydroxy-5α-androstan-3-one; allopregnanedione, 5α-pregnane-3,20-dione; 5α-dihydrodeoxycorticosterone, 21-hydroxy-5α-pregnane-3,20-dione; 5α-dihydrocortexolone, 17α,21-dihydroxy-5α-pregnane-3,20-dione; 5α-dihydrocorticosterone, 11β,21-dihydroxy-5α-pregnane-3,20-dione; 5α-dihydrocortisol, 11β,17α,21-trihydroxy-5α-pregnane-3,20-dione; 5α-dihydrocortisone, 17α,21-dihydroxy-5α-pregnane-3,11,20-trione; etiocholanolone, 3α-hydroxy-5β-androstan-17-one; 5β-dihydrotestosterone, 17β-hydroxy-5β-androstan-3-one; 5β-androstanedione, 5β-androstane-3,17-dione; epidihydrotestosterone, 17α-hydroxy-5α-androstan-3-one; 17-hydroxyprogesterone, 17α-hydroxy-4-pregnene-3,20-dione; 17-hydroxyallopregnanedione, 17α-hydroxy-5α-pregnane-3,20-dione; 3α-hydroxysteroid oxidoreductase, 3α-hydroxysteroid:NAD(P)$^+$ oxidoreductase (EC 1.1.1.50).