Thyroid-stimulating Hormone Binding to Beef Thyroid Membranes

ROLE OF N-ACETYLNURAMINIC ACID*

(Received for publication, January 12, 1976)

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The effect of sugars on \(^{125}\)I-thyroid-stimulating hormone binding to beef thyroid membranes was studied to determine their role in thyroid-stimulating hormone (TSH) binding. At 0.1 M concentration, N-acetylnuraminic acid produced a 3- to 7-fold increase in TSH binding, was the only sugar to enhance TSH binding, and did so whether binding was determined in the cyclase medium or under conditions of optimum binding. The enhanced TSH binding remained after the membranes were removed from the high NeuAc concentration and an effect was observed at concentrations of 10 mM NeuAc. NeuAc did not alter the kinetics of TSH binding but the pH optimum for TSH binding shifted from pH 5.5 to 7.5 in the presence of NeuAc.

Incubation of the membranes with increasing concentrations of NeuAc resulted in increased sialic acid content of the membranes. The NeuAc concentration curve of membrane sialic acid and TSH binding were roughly parallel.

The capacity of the low affinity site increased from 0.74 to 2.5 nmol/mg of protein in the presence of NeuAc. The apparent affinity (0.88 \(\times 10^4\) M\(^{-1}\)) of this site was unaffected by NeuAc. With the high affinity site, NeuAc increased both the apparent affinity and capacity from 9.9 \(\times 10^4\) to 5.5 \(\times 10^4\) M\(^{-1}\) and 1.6 to 3.1 pmol/mg of protein, respectively.

Neuraminidase or neuraminidase plus \(\beta\)-galactosidase incubation of the membranes removed approximately 60% of the sialic acid from the membranes within 15 to 30 min but did not affect TSH binding. Large quantities of sialic acid were detected in the soluble fractions during isolation of the membranes, 4 to 5% of which was ultrafilterable and not associated with high molecular weight proteins.

It is concluded that among the sugars tested, NeuAc exhibits an unique effect on TSH binding that may have physiological significance. The inability to alter TSH binding by enzymatic removal of endogenous sialic acid suggests that either NeuAc resistant to hydrolysis is sufficient to maintain TSH binding or that NeuAc important in TSH binding is removed during membrane preparation but is replaced by incubation with exogenous NeuAc.

Bovine thyroid-stimulating hormone is a glycoprotein with carbohydrate residues of N-acetylglucosamine, mannose, N-acetylglucosamine, fucose, and galactose (1, 2) listed in order of abundance. These sugars probably exist as oligosaccharides in the intact molecule and are distributed between the \(\alpha\) and \(\beta\) subunits. The role of these sugars in TSH\(^1\) binding has not been explored.

Cuatrecasas and Tell (3) explored the role of carbohydrates in insulin binding and adenylyl cyclase activation. They observed that, like insulin, concanavalin A and wheat germ agglutinin enhanced glucose transport and inhibited epinephrine-stimulated adenylate cyclase activity in fat cell and fat cell homogenates, respectively. The effects of the lectins could be reversed by simple sugars which bind specifically to the lectine. From the insulin like effects of these lectine and their binding properties, he concluded that sugars on the membranes are involved in insulin binding and cyclase activation.

Preliminary experiments\(^2\) which dealt with the effect of various lectins on TSH binding indicated that sugars are likely involved in TSH binding and adenylyl cyclase activation of beef thyroid membranes; however, the presence of sugar moieties on both TSH and the membranes led to complicated results that were difficult to interpret.

In this study, the direct effect of various monosaccharides on bovine TSH binding to bovine thyroid plasma membranes is

* This work was supported by United States Public Health Service Grant HD-02528 and the Department of Pediatrics, University of Kansas Medical Center.

1 The abbreviations used are: TSH, thyroid-stimulating hormone; Hepes, N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid.

2 W. Moore and J. Wolff (1974), unpublished observations.
explored to further define the binding process and the role of carbohydrates in TSH binding. We have demonstrated that N-acety neuraminc acid is the only simple sugar (among those studied) which has an effect on TSH binding. The results indicate that NeuAc may be of importance in physiologically meaningful hormone binding.

**EXPERIMENTAL PROCEDURE**

**Materials**

Purified bovine TSH was prepared by Dr. Leonard Kohn, National Institute of Arthritis Metabolism and Digestive Diseases, Bethesda, Md. This TSH was iodinated with ¹²³I by a modification (4) of the lactoperoxidase method of Thorell and Johansson (5). Equimolar quantities (0.2 nmol) of TSH and ¹²³I- (100 μCi/μl) were added to a vial containing 0.4 M acetate buffer (pH 5.6) and 2 μg of lactoperoxidase (Sigma). The reaction was started by adding 20 μg of H₂O₂ and was stopped after 60 s by adding 100 μl of a solution containing 16% sucrose, 1% KI, and 0.02% sodium azide in 0.1% albumin. The iodinated TSH was separated from free ¹²³I- by Sephadex gel filtration. Approximately 40 to 50% iodination was obtained with this method to yield ¹²³I-TSH with a specific activity of approximately 1 μCi/pmol. The initial biological activity of this TSH was 5 to 10 units/mg. The iodinated TSH is equipotent to unlabeled TSH in the iodinated TSH was equilibrated in the Hormone Distribution Program of the National Institute of Arthritis, Metabolism, and Digestive Diseases. This hormone was used to determine nonspecific binding and was equipotent to the purified TSH in this respect.

Sugars were obtained from Sigma Chemical Co., St. Louis, Mo. Stock solutions of the sugars were prepared and buffered to pH 7.4 with N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid. The stock solutions were stored at °80°C until used within 20 to 30 days. Neuraminidase (2.55 units/mg of protein, NANTactose) from Clostridium welchi and β-galactosidase (300 units/mg of protein) from Escherichia coli were obtained from Sigma Chemical Co. and Worthington Biochemical Corp., Freehold, N. J., respectively.

**Preparation of Membranes**

Bovine thyroids were generously provided by Sambol Packing Co., Kansas City, Kansas. Thyroid membranes were prepared in 0.25 mM sucrose as described previously (4, 6) except that 10% glycerol with 10 mM Hepes (pH 7.5), 1 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N′-N-tetraacetic acid, and 1 mM dithiothreitol was used as the final suspending solution. In experiments where N-acety neuraminic acid was assayed, the stock solution of the neuraminidase was 1 mM NaHCO₃ (7) and the final suspending medium was 50 mM Hepes (pH 7.5). These membranes are similar to those prepared by Amir et al. (8) and Tate et al. (9) for study of TSH binding. Comparison of their data with that of Moore and Wolff (4) indicates that the TSH binding properties of the bicarbonate-isolated membranes are equivalent to sucrose-isolated membranes. Additional binding parameters of the two membranes as they pertain to this binding assay are presented in Table I. Membrane protein concentrations are determined by the Lowry method using albumin solutions as standards.

**Analyses of Binding**

Binding was routinely performed under two conditions. **Binding Conditions**—The solution contained ¹²³I-TSH, 1% glycerol, 50 mM Hepes (pH 7.5), 1.25% albumin, and membrane protein in a total volume of 0.1 ml and was incubated at room temperature for 10 min.

**Adenylate Cyclase Conditions**—The solution contained ¹²³I-TSH, 0.33 mM CAMP, 2 mM MgCl₂, 25 mM Tris-HCl (pH 7.5), 0.3 mg/ml of phosphocreatine kinase, 6.7 mM creatine phosphate, 0.1% albumin, 1 mM ATP, 0.05 mM IPT, and membrane protein in a total volume of 0.12 ml and was incubated at 37°C for 10 min. Binding under cyclase conditions is approximately one-third that under binding conditions but is suggested to reflect the binding important in adenylate cyclase activation (4). In each case, binding was initiated by adding the membranes and was terminated by sedimenting the membranes through a layer of 10% sucrose with 1% albumin, 50 mM Hepes (pH 7.5) to a microfuge tube. The amount of ¹²³I-TSH bound to the membranes was determined from the percentage of ¹²³I-TSH in the pellet.

**RESULTS**

Under binding and cyclase conditions 0.1 mM α-methyl mannoside, mannose, N-acetylmannosamine, galactose, N-acetyl galactosamine (not shown), fucose, glucose, and N-acetyl glucosamine did not alter TSH binding (Figs. 1 and 2). Among the sugars tested, N-acety neuraminic acid was unique in its ability to enhance ¹²³I-TSH binding (Figs. 1 and 2). NeuAc concentrations of 100 mM caused a 3- to 7-fold increase in TSH binding. A significant increase in TSH binding was observed at NeuAc concentrations as low as 10 mM (Fig. 3). TSH binding and displacement in the presence of

| Property | Sucrose-prepared membranes | Bicarbonate-prepared membranes |
|----------|-----------------------------|-------------------------------|
| Apparent affinities | | |
| Low | 0.7-1.1 x 10⁻⁹ M⁻¹ | 0.65 x 10⁻⁹ M⁻¹ |
| High | 2.2-3.1 x 10⁻⁹ M⁻¹ | 3.4 x 10⁻⁹ M⁻¹ |
| Capacities | | |
| Low | 340-740 | 510 |
| High | 0.8-1.6 | 1.1 |
| pH optimum | 5.5 | 5.5 |
| Cyclase activity | 0.8-2 μM | 1.6 μM |

* Ref. 4 and present data.
* Picomoles of TSH bound/mg membrane protein.
* TSH concentration of half-maximal activation.

Non specific (nondisplaceable) binding was determined for each observation by adding 3.5 nmol (1 mg/ml) of unlabeled TSH to the incubation medium. In line with common usage, the nondisplaceable ²²³I-TSH was subtracted from total ²²³I-TSH bound to yield specific binding which is reported unless otherwise specified.

**Assay of Sialic Acid**

Assay of NeuAc by this assay is reported as sialic acid since the assay is not entirely specific for NeuAc, however, NeuAc is suggested to be the major component assayed (10). The sialic acid content of the membranes was determined by the thioarbituric acid assay of Warren (10). Since glycerol and sucrose produced interfering colors with the assay, it was necessary to do one of the following: (a) remove glycerol and sucrose from the membrane suspension as explained under "Preparation of Membranes," (b) perform a spectral analysis of the colored solution and compensate for increased or decreased absorbance at 549 nm resulting from the contaminants, or (c) measure the absorbance at 549 nm before and after treating the colored solution with strong base. The color produced by NeuAc is destroyed by the strong base (10). The residual absorbance at 549 nm due to glycerol or sucrose was 0.05±0.013 which accounted for approximately 30 to 50% of the total absorbance of the solution. The residual absorbance of the NaHCO₃-isolated membranes after strong base was equivalent to that of solvent controls. The approaches gave roughly equivalent results. For convenience, the first approach was used routinely. Prior to the assay, the membranes or solutions were incubated at 80°C with 0.1 M HSO₄ for at least 60 min with intermittent shaking to remove essentially all the sialic acid from the membranes. Then the membranes or solutions were centrifuged at 40,000 × g for 10 min and an aliquot of the supernatant was assayed for sialic acid.

**Table I**

| Property | Sucrose-prepared membranes | Bicarbonate-prepared membranes |
|----------|-----------------------------|-------------------------------|
| Apparent affinities | | |
| Low | 0.7-1.1 x 10⁻⁹ M⁻¹ | 0.65 x 10⁻⁹ M⁻¹ |
| High | 2.2-3.1 x 10⁻⁹ M⁻¹ | 3.4 x 10⁻⁹ M⁻¹ |
| Capacities | | |
| Low | 340-740 | 510 |
| High | 0.8-1.6 | 1.1 |
| pH optimum | 5.5 | 5.5 |
| Cyclase activity | 0.8-2 μM | 1.6 μM |
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Fig. 1. Effect of sugars on TSH binding to bovine thyroid membranes under binding conditions. The medium contained 0.1 M sugar, 50 mM Hepes (pH 7.4), 1.25% albumin, 1% glycerol, and 1.78 pmol/ml of ¹²⁵I-TSH in a final volume of 0.1 ml. Binding was initiated by adding the membranes (22 µg of membrane protein) to the incubation solution. Incubation was at room temperature for 10 min. The results represent the means of three experiments, each in triplicate, and are expressed as picomoles of TSH bound/mg of membrane protein ± S.D. NeuAc occurred within 2 to 3 min and was stable for at least 90 to 120 min.

Under binding conditions, the pH optimum for TSH binding to thyroid membranes was approximately pH 5.5 (Fig. 4). In the presence of 30 mM NeuAc, the pH optimum for TSH binding was approximately pH 7.5 (Fig. 4). At pH 7.5, the amount of TSH bound to the NeuAc-treated membrane was approximately 3-fold higher than to control membranes at the same pH. The peak of TSH binding with NeuAc at pH 7.5 was approximately 30% higher than the peak of TSH binding at pH 5.5 without NeuAc. The two pH curves on TSH binding were qualitatively similar with a fairly narrow pH optimum for binding.

The aggregation of the membranes as measured by light scattering (absorbance at 700 nm) was determined as a function of pH. Aggregation of the membranes at pH less than 6.5 in the presence of 30 mM NeuAc was half-maximal at pH 5.7 to 5.9. This was not significantly different from aggregation in the absence of NeuAc.

The membranes were exposed briefly to high NeuAc concentrations to determine if high concentrations of NeuAc result in increased sialic acid content of the membranes. Membranes were incubated with increasing concentrations of NeuAc at 22° for 30 min and sedimented by centrifuging at 40,000 × g for 10 min. The surface of the pellet was washed and the membranes were resuspended in 50 mM Hepes. An increase in membrane sialic acid content was detected at NeuAc concentrations above 10 mM in the incubation medium (Fig. 5). This NeuAc concentration curve of membrane sialic acid content is roughly parallel to the NeuAc concentration curve of TSH binding.

Analysis of the ¹²⁵I-TSH binding by Scatchard plots revealed two orders of binding sites with affinities of 0.88 × 10⁸ M⁻¹ and 2.2 × 10⁹ M⁻¹. In the presence of 30 mM NeuAc, the apparent affinity of the low affinity site was unchanged at 0.88 × 10⁸ M⁻¹ while the capacity of this site increased from 0.74 nmol/mg of protein to 2.5 nmol/mg of protein (Fig. 6). NeuAc concentration was established empirically from the NeuAc concentration curve of membrane sialic acid content as one which produced significant enhancement of TSH binding but did not require expenditures of large quantities of NeuAc.

Both the apparent affinity and capacity of the high affinity site increased in the presence of NeuAc with 9 mM NeuAc in the incubation medium. The apparent affinity of the high affinity site increased from 2.2 × 10⁹ M⁻¹ to 5.5 × 10⁹ M⁻¹ and the capacity increased from 1.6 to 3.1 pmol/mg of protein (Fig. 7). A lower NeuAc concentration was required to study the high affinity site since 30 mM NeuAc concentrations produced maximum TSH binding at low TSH concentrations. The 9 mM

Fig. 2. Effect of sugars on TSH binding to bovine thyroid membranes under cyclase conditions. The incubation solution contained 0.22 mg/ml of membrane protein, 0.33 mM cAMP, 2 mM MgCl₂, 1 mM ATP, 0.1% albumin, 0.05 mM ITP, 6.7 mM creatine phosphate, 0.03 mg/ml of creatine phosphokinase, 25 mM Tris-HCl (pH 7.5), 1% glycerol, and 750 pmol/ml of ¹²⁵I-TSH. Binding was initiated by adding the membranes and was performed at 37° for 10 min. The results are the means of three experiments, each in triplicate, and are expressed as picomoles of TSH bound/mg of membrane protein ± S.D.
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The incubation solution for binding conditions (D) contained 0.155 mg/ml of membrane protein, 0.33 mM cAMP, 2 mM ATP, 0.1% albumin, 0.05 mM ITP, 6.7 mM creatine phosphate, 0.3 mg/ml of creatine phosphokinase, 25 mM Tris-HCl (pH 7.5), 1.75 pmol/ml of 125I-TSH, and the desired NeuAc concentration. The binding was started by adding the membranes and performed for 10 min at room temperature. The incubation solution for cyclase conditions (C) contained 0.133 mg/ml of membrane protein, 0.33 mM cAMP, 2 mM ATP, 0.1% albumin, 0.05 mM ITP, 6.7 mM creatine phosphate, 0.3 mg/ml of creatine phosphokinase, 25 mM Tris-HCl (pH 7.5), 1.75 pmol/ml of 125I-TSH, and the desired NeuAc concentration. The binding was started by adding the membranes and was performed for 10 min at 37°. The results are expressed as picomoles of TSH bound/mg of membrane protein ± S.D. Results are the mean ± S.D. of three experiments, each in triplicate. The standard deviations do not reflect the true sensitivity of the assay but the variability between experiments. When the binding at 4 mM NeuAc is compared to control binding by determining the significance of the difference between the means of paired observations, the difference between control binding and binding at 4 mM NeuAc is statistically significant with p < 0.05.

concentration was chosen as that concentration which was roughly twice the NeuAc concentration required to produce a significant increase in TSH binding.

The effect of neuraminidase and β-galactosidase incubation of the membranes was studied to determine if endogenous membrane NeuAc is involved in TSH binding. Membrane sialic acid was quickly removed by neuraminidase digestion (Table III). Approximately 60% of the membrane sialic acid was removed after 15 to 30 min of incubation. Neuraminidase was not due to selective loss of membrane protein during sedimentation and resuspension, but approximately 10% of the membrane protein was lost during the procedure. The surface of the pellet was washed twice with 50 mM Hepes and then resuspended in 0.1 M H2SO4. The membranes were incubated at 80° for 60 min in H2SO4 prior to assay of the sialic acid content by the thiobarbituric acid assay. The results indicate the nanomoles of sialic acid/mg of membrane protein after sedimentation and resuspension. Results are the means of two experiments each in triplicate.

Fig. 3. Effect of NeuAc concentration on TSH binding to bovine thyroid membranes. The incubation solution for binding conditions (D) contained 0.165 mg/ml of membrane protein, 50 mM Hepes (pH 7.5), 1.25% albumin, 1% glycerol, 1.75 pmol/ml of 125I-TSH, and the desired NeuAc concentration. The binding was started by adding the membranes and performed for 10 min at room temperature. The incubation solution for cyclase conditions (C) contained 0.155 mg/ml of membrane protein, 0.33 mM cAMP, 2 mM ATP, 0.1% albumin, 0.05 mM ITP, 6.7 mM creatine phosphate, 0.3 mg/ml of creatine phosphokinase, 25 mM Tris-HCl (pH 7.5), 1.75 pmol/ml of 125I-TSH, and the desired NeuAc concentration. The binding was started by adding the membranes and performed for 10 min at 37°. The results are expressed as picomoles of TSH bound/mg of membrane protein ± S.D. Results are the mean ± S.D. of three experiments, each in triplicate. The standard deviations do not reflect the true sensitivity of the assay but the variability between experiments. When the binding at 4 mM NeuAc is compared to control binding by determining the significance of the difference between the means of paired observations, the difference between control binding and binding at 4 mM NeuAc is statistically significant with p < 0.05.

Fig. 4. Effect of pH on the binding of TSH to bovine thyroid membranes in the presence and absence of NeuAc. The incubation solution contained 50 mM acetate buffer for pH 3.5 to 5.5, 50 mM Hepes buffer for pH 5.5 to 8.5, or 50 mM Tris-HCl buffer for pH 8.5 to 10.5, 0.22 mg/ml of membrane protein, 1% glycerol, 1.25% albumin, and 1.78 pmol/ml of 125I-TSH in a final volume of 0.1 ml. No buffer effect was observed at pH 5.5 or 8.5. Incubation was at room temperature for 10 min and was initiated by adding the membranes. Results are expressed as picomoles of TSH bound/mg of membrane protein ± S.D. in the absence (O) and presence (D) of 30 mM NeuAc. Results are the means of three experiments each in triplicate.

Fig. 5. Effect of NeuAc on the sialic acid content of bovine thyroid membranes. The membranes (1.36 mg of membrane protein) were incubated in the indicated NeuAc concentration with 50 mM Hepes (pH 7.4) for 10 min at room temperature. The incubation solution was then diluted 15-fold with 50 mM Hepes and followed by sedimentation of the membranes by centrifuging at 40,000 × g for 10 min. Complete recovery of membrane protein is assumed so that the increase in NeuAc is not due to selective loss of membrane protein during sedimentation and resuspension, but approximately 10% of the membrane protein was lost during the procedure. The surface of the pellet was washed twice with 50 mM Hepes and then resuspended in 0.1 M H2SO4. The membranes were incubated at 80° for 60 min in II2SO4 prior to assay of the sialic acid content by the thiobarbituric acid assay. The results indicate the nanomoles of sialic acid/mg of membrane protein after sedimentation and resuspension. Results are the means of two experiments each in triplicate.
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**Fig. 6.** Effect of NeuAc on TSH binding to the low affinity site of bovine thyroid membranes. Top, the incubation solution contained 0.099 mg/ml of membrane protein, 50 mM Hepes (pH 7.5), 1% glycerol, 1.25% albumin, and the appropriate concentration of 125I-TSH. The binding was started by adding the membranes and was performed at room temperature for 10 min. The amount of TSH bound (nmoles/mg of membrane protein) is plotted versus the ratio of amount bound to concentration of free hormone (BOUND/FREE). Bottom, the conditions of incubation are the same as above except the incubation solution contained 30 mM NeuAc. The affinity of this site was unchanged by NeuAc at 0.88 ± 0.1 x 10^-9 M^-1. The capacity of the site was 0.74 and 2.5 nmol/mg of protein in the absence and presence of NeuAc, respectively. The very low affinity site depicted on each figure was not studied further.

Membrane degradation since a similar release was observed from control membranes. Incubation with 50 μg/ml of β-galactosidase did not cause release of membrane sialic acid until 3 to 4 hours of incubation. Incubation of the membranes with β-galactosidase did cause the release of a substance with peak absorbance at 532 nm in the thiobarbituric acid assay. This compound was not identified further.

In contrast to their effect on membrane sialic acid content, incubation of the membranes with 50 μg/ml of neuraminidase or neuraminidase plus β-galactosidase at 22° for 30 to 60 min did not alter TSH binding. Decrease in binding was observed at 3 to 4 hours of incubation but was attributed to membrane degradation (Table IV).

The sialic acid content of the fractions obtained during membrane isolation was assayed to determined if significant NeuAc is removed from the membranes during isolation. Analysis of the sialic acid content of the soluble and membrane fractions during preparation of the membranes revealed that a large quantity of sialic acid was present in the soluble fractions (Table V). Approximately 4 to 5% of the soluble sialic acid was ultrafilterable through an Amicon membrane with exclusion molecular weight of greater than 1000. Consequently, this portion of the soluble sialic acid was not associated with high molecular weight proteins such as thyroglobulin which is a rich source of NeuAc in the thyroid. This free sialic acid may represent endogenous NeuAc that is removed from the membrane during preparative procedures. If the sialic acid content of the filtrate is expressed in terms of nanomoles of NeuAc/mg of protein, there was a 60-fold increase between the filtrate of the initial homogenate and the filtrate of the final membrane suspension while the nanomoles of NeuAc/mg of protein in the soluble fraction remains fairly constant throughout the isolation procedure.

**DISCUSSION**

Previous studies by Moore and Wolff (4) have described the binding of TSH to bovine thyroid membranes and the relation of binding to adenylate cyclase activation. TSH binding to cells (11-13), slices (14), and membranes (8, 9, 12, 13) have been described by other investigators. The studies of TSH binding have been predominantly descriptive and the mechanism of TSH binding remains unknown. Several points concerning the binding process are known. Through a study of the effect of pH, cations, and anions, Moore and Wolff (4) suggested that electrostatic interactions may be important in the binding process. The effect of phospholipase A and filipin on TSH binding indicated that membrane lipids are involved.
in the binding process (4). Wolff et al. (15) demonstrated that the binding capacity of TSH resides mainly in the $\beta$ subunit.

The effect of selected lectins on TSH binding indicated that the carbohydrate moieties of the hormone and/or membrane may be involved in the binding process. In this report, we demonstrate that NeuAc is unique among the sugars in its ability to enhance bovine TSH binding to bovine thyroid membranes. In fact, filipin and phospholipase A are the only other compounds which have demonstrated ability to enhance TSH binding (4). The increased TSH binding in the presence of NeuAc results from an increase in the capacity of the low affinity receptors rather than from an increase in their affinity and from an increase in both the

### Table III

*Effect of neuraminidase and $\beta$-galactosidase incubation on membrane sialic acid content*

| Incubation time | Neuraminidase | $\%$ of control | $\beta$-galactosidase | Buffer |
|-----------------|---------------|-----------------|-----------------------|--------|
| **min**         | Neuraminidase | $\%$ of control | $\beta$-galactosidase | Buffer |
| 0               | 100           | 100             | 100                   | 100    |
| 15              | 39            | 40              | 89                    | 98     |
| 30              | 38            | 42              | 94                    | 101    |
| 60              | 48            | 48              | 88                    | 95     |
| 180             | 41            | 41              | 66                    | 94     |
| 240             | 27            | 36              | 65                    | 73     |

### Table IV

*Effect of neuraminidase and $\beta$-galactosidase incubation on TSH binding to bovine thyroid membranes*

| Incubation time | Neuraminidase | $\%$ of control | $\beta$-galactosidase | Buffer |
|-----------------|---------------|-----------------|-----------------------|--------|
| **min**         | Neuraminidase | $\%$ of control | $\beta$-galactosidase | Buffer |
| 0               | 100           | 100             | 100                   | 100    |
| 15              | 81            | 92              | 94                    | 100    |
| 30              | 101           | 84              | 94                    | 100    |
| 60              | 111           | 88              | 86                    |        |

### Table V

*Sialic acid content of fractions obtained during bovine thyroid membrane preparation (7)*

The membrane isolation was performed with 0.1 M NaHCO$_3$ as the suspending solution. Thyroid slices were disrupted by a polytron (Polytron homogenate) and then homogenized with 5 to 8 strokes in a loose-fitting Dounce homogenizer. The homogenate was filtered through four layers of cloth mesh to yield the Dounce homogenate. This was centrifuged at 3,000 rpm for 10 min in a SS-34 rotor to yield the 3K pellet and supernatant. The 3K supernatant was centrifuged at 18,000 rpm for 10 min to yield the 18K pellet and supernatant. The 18K pellet was resuspended and centrifuged at 11,000 rpm for 10 min to yield the 11K pellet. The top of this pellet (11K (top)) was removed by gentle resuspension of the loose upper portion of the pellet. The 11K top is usually applied to a discontinuous sucrose gradient to yield the membrane fraction for the binding studies. Considerable material was lost between the Polytron and Dounce homogenates by filtering through the gauze. The particulate suspensions (Polytron homogenate, Dounce homogenate, 3K, 18K, and 11K supernatants and 11K pellet (top)) were centrifuged at 40,000 × g for 20 min to sediment the particulate material. The resulting supernatants (soluble) were filtered through an Anmicon filter with exclusion molecular weight of greater than 1,000 to form the filtrate. The 3K pellet is a loose pellet and probably contains soluble material which resulted in more sialic acid in this pellet than in the particulate fractions of the homogenates. Approximately 97% of the sialic acid of the Dounce homogenate was recovered in the 3K fractions. Each fraction was incubated with 0.1 M H$_2$SO$_4$ for 60 min at 80°C prior to the thiobarbituric acid assay. The results are the means of six experiments using three different membrane preparations each in triplicate. The results are expressed as percentage of TSH binding to control membranes at zero time. The average control binding was 0.68 pmol/mg of membrane protein.

| Fraction                  | Particulate | Soluble | Filtrate |
|---------------------------|-------------|---------|----------|
| Polytron homogenate        | 40.5 (7.4 ± 0.6)* | 353 (13.7 ± 0.5) | 4.0 (62 ± 6) |
| Dounce homogenate          | 21.9 (6.2 ± 1) | 274 (13.3 ± 0.3) | 5.7 (103 ± 24) |
| 3K pellet                  | 44.3 (14.3 ± 0.6) | | |
| 3K supernatant             | 7.3 (4.8 ± 0.9) | 235 (15.3 ± 0.04) | 7.1 (153 ± 6) |
| 18K supernatant            | 4.7 (13.2 ± 0.1) | 219 (16.4 ± 0.3) | 6.1 (141 ± 12) |
| 18K pellet                 | 13.7 (11.9 ± 3) | | |
| 11K supernatant            | 2.0 (13.3 ± 0.2) | 8.1 (16.0 ± 0.1) | 0.7 (220 ± 6) |
| 11K pellet (bottom)        | 0.1 (47.9 ± 5) | 0.8 (10.1 ± 0.2) | 0.1 (400 ± 5) |
| 11K pellet (top)           | 1.7 (5.2 ± 0.6) | | |

* Sialic acid concentration in nanomoles/mg of protein.
affinity and capacity of the high affinity sites. Amir et al. (8) failed to observe an increase in TSH binding with 1 and 30 mM NeuAc concentrations. The difference between their observations and the present data is difficult to explain. The effect of NeuAc on binding was consistently observed throughout these experiments from 10 to 100 mM NeuAc concentration.

The pH optimum for TSH binding shifts from approximately pH 6.5 to pH 7.6 in the presence of NeuAc. The decrease in binding at pH below 5.5 in the absence of NeuAc had previously been attributed to degradation of either TSH or the receptor (4). However, TSH binding to the NeuAc-treated membranes decreases rapidly at pH less than 7.5 so that the contours of the pH curve of the control and NeuAc-treated membranes are qualitatively similar. The significance of this similarity is not known at the present time. Moore and Wolff (4) demonstrated that thyroid membranes aggregate at pH less than approximately 6.5, being half-maximal at pH 5.6. Aggregation when measured by light scattering (absorbance at 700 nm) in the presence of 30 mM NeuAc was half-maximal at pH 5.8 to 5.9 and did not differ significantly from aggregation of control membranes.

Concentrations of NeuAc in the incubation solution which promote TSH binding also result in increased sialic acid content of the membranes which is retained after several sedimentations and resuspensions in solutions free of NeuAc. These membranes continue to exhibit increased TSH binding even after the membranes are removed from the high NeuAc concentration which indirectly suggests that NeuAc binding to the membranes is important in the enhancement of TSH binding.

The biological significance of these observations is open to conjecture. Neuraminidase and neuraminidase plus β-galactosidase treatment of the membranes effectively removed approximately one-half of the NeuAc from the membranes without affecting the TSH binding. This suggests the following possibilities: (a) endogenous membrane NeuAc is not involved in TSH binding, (b) the NeuAc resistant to hydrolysis is sufficient to maintain TSH binding, or (c) membrane NeuAc that is physiologically important in TSH binding is removed during membrane isolation but is replaced by incubation with high concentrations of NeuAc.

This research and other recent reports indicate that carbohydrates and more specifically NeuAc are involved in the binding and activation process so that it is impossible to eliminate a role of NeuAc in TSH binding. Winand and Kohn (16) have recently reported that the soluble TSH receptor is rich in carbohydrate. Their 15,000 to 30,000 molecular weight receptor fragment contains approximately 30% carbohydrate and 10% sialic acid. Using this soluble receptor system, Tate et al. (17) observed that TSH binding to the soluble receptor is inhibited by preincubation with Sepharose-linked concanavalin A or neuraminidase. When compared to the present data, this indicates the presence of NeuAc residues on the intact membrane which are resistant to neuraminidase hydrolysis and thereby maintain TSH binding following neuraminidase incubation. These same NeuAc residues might become available to neuraminidase through conformational changes in the receptor occurring during solubilization and account for the observations in the soluble receptor system. One might expect NeuAc residues resistant to neuraminidase in the intact membrane to be unavailable to TSH binding, but this assumes that NeuAc is directly rather than indirectly involved in the binding process such as by electrostatic attraction.

The appropriateness of the third possibility is difficult to determine from the present data. Even though considerable amounts of sialic acid become soluble during isolation of the membranes, its exact source is not known. In most receptor systems, the measured affinities of the hormones for their membrane receptor are rather low compared to the affinity that might be expected from concentrations of the hormone that produce a biological response. This discrepancy varies from 1 to several orders of magnitude (14, 19, 19) and may result from alterations of the receptor during preparative procedures or storage (20). Exposure of the thyroid membranes to NeuAc results in a significant increase in both the affinity and capacity of the high affinity site and may represent partial "repair" of a damaged receptor or replacement of a component lost during membrane preparation. Whether this repair results in an increased sensitivity of TSH stimulation of adenylate cyclase is presently under investigation.

In conclusion, treatment of bovine thyroid membranes with NeuAc results in an increase in TSH binding which appears to result from an increase in the capacity of the low affinity receptors and also from an increase in the affinity and capacity of the high affinity receptors. The enhancement of TSH binding by NeuAc seems related to NeuAc binding to the membranes but removal of endogenous NeuAc from the membranes by neuraminidase digestion does not affect TSH binding.

The biological significance of these observations is obscure, but the unique qualities of NeuAc and its role in membrane receptor systems suggests that its role in TSH binding may be physiologically meaningful.

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