insulin (5700) markers. At this stage of purification, the TGF's had a specific activity approximately 10- to 25-fold higher than that of the acidified ethanol extracts, with a range of recovery of 150,000 to 200,000 colony-forming units per kilogram of tissue. A colony-forming unit is defined as the amount of TGF that will induce the formation of 1000 colonies of normal rat kidney cells $> 3100\ \mu m^2$ under standard assay conditions (*4*), in the presence of EGF (5 ng/ml).

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16. Protein was determined as in (*14*). [^{3}H]Thymidine incorporation and total DNA were determined on portions of tissue that had been dissolved in 1*M* NaOH, precipitated with ice-cold 0.3*M* perchloric acid (PCA), washed with ice-cold 0.2*M* PCA, and finally extracted in 0.5*M* PCA at 70°C. Portions of the final extract were used for determination of radioactive counts in a liquid scintillation counter, and deoxyribose was determined by the method of K. Burton [*Biochem. J.* **62**, 315 (1956)]. Collagen was determined as hydroxyproline after hydrolysis with 6*M* HCl. Typing of the collagen by gel electrophoresis of pepsin digests showed no difference between treated and control chambers; Type I collagen was the predominant form.
17. We thank G. Martin and M. Anzano for helpful suggestions, L. Lamb for assistance with the determinations, C. Brown for advice on the statistical analysis, and R. Morsillo for manuscript preparation.

23 August 1982

Demonstration of a Saturable Binding Site for Thyrotropin in *Yersinia enterocolitica*

Abstract. *Several lines of evidence suggest that there might be immunologic cross-reactivity between the thyroid plasma membrane in humans and antigenic determinants in the enteric pathogen* Yersinia enterocolitica. *Studies were therefore performed to determine whether* Y. enterocolitica, *like the thyroid membrane, contains a thyrotropin binding site. A saturable binding site for bovine thyrotropin was indeed demonstrable, particularly in preparations of the organism that have been treated with ethylenediaminetetraacetate and lysozyme. Hormonal specificity of the binding site, as judged from the inhibition of binding of ^{125}I-labeled bovine thyrotropin, was similar to that of the thyrotropin receptor in human thyroid tissue.*

In this report, we describe the presence of a specific, saturable binding site for the mammalian peptide hormone thyrotropin (TSH) in the pathogenic Gram-negative bacillus *Yersinia enterocolitica*. Several groups of observations prompted us to explore this question. First, patients infected with *Y. enterocolitica* commonly display several disorders thought to be autoimmune in nature, including arthritis, erythrema nodosum, Reiter's syndrome, and iritis (*1*). Further, the serum of such patients contains a variety of antibodies to epithelium (*2*), including some which by immunofluorescent techniques can be shown to bind to the cytoplasm and plasma membrane of human thyroid epithelium (*3*). The second group of observations relates to patients with the autoimmune thyroid diseases, Graves' disease and Hashimoto's disease; serum from these patients contains autoantibodies against a variety of thyroid antigens, including thyroid microsomal antigens and thyroglobulin (*4*). It is currently thought that among these antibodies in Graves' disease is an antibody against the TSH receptor which, like TSH, binds to the thyroid membrane, activates adenylate cyclase therein, and initiates the thyroid hyperfunction characteristic of this disorder (*4*). It seemed of particular interest, therefore, that patients with Graves' disease and Hashimoto's disease display, with inordinate frequency, both circulating agglutinins against *Y. enterocolitica* and evidence of cell-mediated immunity against this organism (*5*). Also of relevance is a report that indicates that rabbits immunized against human or rabbit thyroid tissue develop antibodies that are reactive against *Y. enterocolitica* (*6*).

Together these findings raise the possibility that one or more components of the thyroid membrane and of *Y. enterocolitica* share common or cross-reacting antigenic determinants. To explore this question, we looked for functional homology between *Y. enterocolitica* and the thyroid plasma membrane, reasoning that this might reflect structural similarities that could, in turn, account for immunologic cross-reactivity. The best characterized functional unit of the thyroid membrane is its receptor for TSH, the properties of which have been studied by well-established techniques (*7*). Therefore, we conducted experiments to determine whether *Y. enterocolitica* could be shown to contain any site functionally analogous to the TSH receptor in thyroid plasma membranes, and such indeed proved to be the case.

Highly purified bovine TSH (bTSH), labeled with ^{125}I by a stoichiometric chloramine-T method (*8*), was used as a probe. Cultures of *Y. enterocolitica*, serological type 0:3, preserved in the frozen state, were provided by one of us (S.W.). For each experiment, frozen organisms were thawed out and cultured on blood agar plates, and were then subcultured overnight at room temperature in brain-heart infusion broth.

Initial experiments were directed at determining whether saturable binding of ^{125}I-labeled bTSH to the *Yersinia* organism could be demonstrated. Organisms were harvested by centrifugation and washed once with 10 m*M* tris-HCl buffer, *p*H 7.4. Cells were either studied live, killed by treatment with heat or 0.4 percent Formalin, or treated with lysozyme or lysozyme-EDTA, according to techniques previously described (*9*). Various concentrations of cells were then suspended in 10 m*M* tris-HCl buffer, *p*H 7.4, containing ^{125}I-labeled bTSH (approximately 10^{-11}*M*) (*10*). Some tubes contained, in addition, 1.0 U of unlabeled TSH for measurement of nonsaturable or nonspecific binding. Suspensions were incubated for 60 minutes at room temperature, after which the bacterial residue was sedimented and counted. Under these conditions, nonspecific binding was regularly in the range of 1 to 2 percent of added radioactive bTSH. In all preparations, binding of ^{125}I-labeled bTSH in excess of nonsaturable binding was detected and remained constant for at least 6 hours. This specific binding varied in amount with the content of bacterial protein and the manner in which the bacteria had been treated. The least binding was evident in live and lysozyme-treated bacteria, and greatest binding in bacteria treated with a combination of lysozyme and EDTA, in which preparations more than 20 percent of added ^{125}I-labeled bTSH was bound (Fig. 1). We assume that the differences in tracer binding among bacteria prepared by the various methods were the result of differences in the ease of access of the tracer to the binding site. Because the greatest binding of ^{125}I-labeled bTSH was observed in lysozyme-EDTA preparations, this mode of treating the organisms was used in all subsequent studies.

Characteristics of the saturable binding were studied in comparable experi-

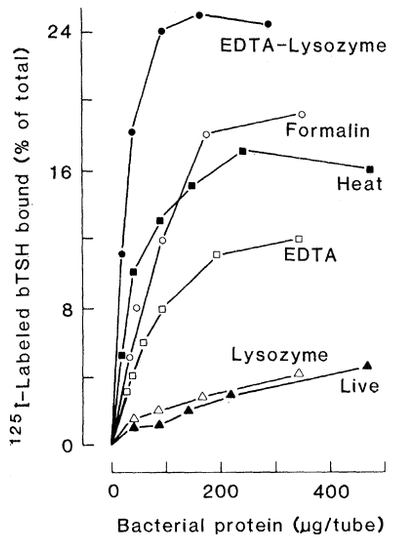

Fig. 1. Binding of ^{125}I-labeled bTSH to *Y. enterocolitica*. Bacteria were examined live, killed with heat (30 minutes at 65°C) or Formalin (0.4 percent for 18 hours), or treated with lysozyme, EDTA, or a combination of the two, according to published methods (*9*). Reaction mixtures (300 μl final volume) were composed of the indicated amounts of bacterial protein and approximately $10^{-11}M$ ^{125}I-labeled bTSH in 10 m*M* tris-HCl, *p*H 7.4. Some tubes contained 1.0 U of unlabeled bTSH for determination of nonsaturable binding. Reactions were carried at room temperature for an hour. Results shown indicate the sedimentable ^{125}I, minus nonspecifically bound ^{125}I, expressed as a percentage of the total ^{125}I in the reaction mixture.

ments in which lysozyme-EDTA treated organisms were incubated with ^{125}I-labeled bTSH and various concentrations of unlabeled bTSH. Progressive, and ultimately almost complete, inhibition of binding of the labeled bTSH by unlabeled bTSH was evident. Saturation of binding sites was achieved at bTSH concentrations above 0.5 U/ml. Scatchard plots of the data indicated that there was a single binding site with a binding maximum (B_{max}) of 2.9×10^{-8} mole per milligram of bacterial protein and a dissociation constant (K_d) of $4.2 \times 10^{-8}M$ (Fig. 2). This value of the K_d indicates that the affinity of the bTSH binding site in *Y. enterocolitica* is less than that of the high-affinity site (*11*), but greater than that of the lower affinity site, in human thyroid membranes.

Additional experiments were performed to define the hormonal specificity of the bTSH binding site. In these experiments, the binding of ^{125}I-labeled bTSH by lysozyme-EDTA treated preparations of *Y. enterocolitica* was assessed in the presence and absence of widely varying concentrations of unlabeled bTSH, bovine luteinizing hormone (LH), follicle-stimulating hormone (FSH), human chorionic gonadotropin (hCG), bovine serum albumin, and a single high concentration of porcine insulin, porcine glucagon, and synthetic adrenocorticotropin-(1–24) (ACTH). Bovine LH, bovine FSH, and hCG proved capable of inhibiting the binding of ^{125}I-labeled bTSH, but were far less potent than bTSH itself in doing so (Table 1). Bovine serum albumin, ACTH, insulin, and glucagon were without effect at the high concentration tested ($10^{-5}M$). The relative potency of these hormones (and of bovine serum albumin), or lack thereof, in inhibiting the binding of the radioactive bTSH to *Y. enterocolitica* was quite similar to their reported potency in inhibiting the binding of bTSH to its receptor in thyroid membranes (*12*). As in human thyroid membranes (*11*), the percentage binding of ^{125}I-labeled bTSH was greatly decreased when studies were conducted in medium containing 50 m*M* NaCl, rather than 10 m*M* tris. For this reason, hormonal specificity of the bTSH binding site was not tested under the former conditions.

The finding that the Gram-negative enteric pathogen *Y. enterocolitica* contains a specific, saturable binding site for the mammalian peptide hormone TSH is consonant with the earlier cited evidence that antibodies against this organism interact with the human thyroid epithelial cell, and has potential ramifications in two areas of current interest. The first is the role of infection with organisms that bear heterophilic antigens in the genesis of autoimmune disease (*13*). Defined examples of this relationship include the role of infection with β-hemolytic streptococcus in the etiology of rheumatic fever (*14*) and of *Mycoplasma* infection in cold agglutinin-mediated hemolytic disease (*15*). In the case of autoimmune thyroid disease, it has been assumed that lymphocytes autoreactive against thyroid antigens develop as a result of random mutations, and that these are allowed to persist and to initiate the autoimmune disease because of an underlying, genetically determined, defect in immune surveillance (*4*). The present findings raise the possibility, however, that the initiating event in susceptible individuals is infection with an organism such as *Y. enterocolitica*, that bears antigens cross-reactive with those in the thyroid gland.

A second area of apparent relevance of the present observations is the growing body of evidence that suggests that

Table 1. Effect of hormones and bovine serum albumin on the binding of ^{125}I-labeled bTSH to membranes of *Y. enterocolitica*. Various concentrations of bTSH (Armour), bovine LH (NIH), bovine FSH (NIH), hCG (Ayerst), and single concentrations of porcine insulin (Eli Lilly), porcine glucagon (Eli Lilly), ACTH-(1–24) (Organon), and bovine serum albumin (Calbiochem) were incubated with 80 μg of bacterial protein [EDTA-lysozyme residue (*9*)] and with approximately $10^{-11}M$ ^{125}I-labeled bTSH in 300 μl of 10 m*M* tris-HCl, *p*H 7.4. After 60 minutes of incubation at room temperature, bound radioactivity was determined after centrifugation and subtraction of nonsaturable binding (1 U of bTSH per tube). Except in the case of those hormones ineffective at the highest concentration tested, the results are expressed as the concentration needed to produce half-maximum inhibition of binding of ^{125}I-labeled bTSH in tubes containing only the radioactively labeled hormone (percent of total).

Protein added	Half-maximum inhibitory concentration (*M*)
Bovine TSH	4.2×10^{-8}
Bovine LH	1.9×10^{-6}
Bovine FSH	1.5×10^{-5}
Human CG	8×10^{-6}
Porcine insulin	Inactive*
Porcine glucagon	Inactive*
Adrenocorticotropin-(1–24)	Inactive*
Bovine serum albumin	Inactive*

*Inactive at $10^{-5}M$.

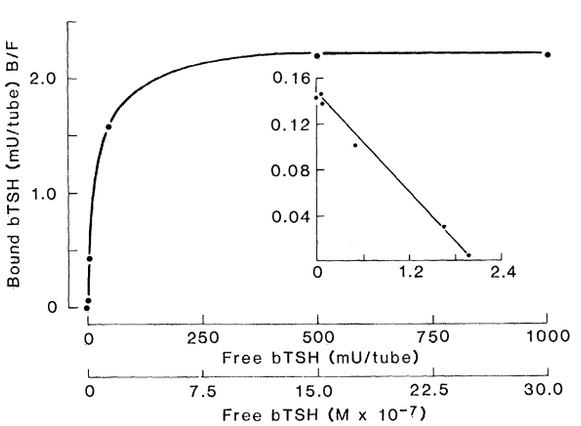

Fig. 2. Saturable binding of bTSH to membranes of *Y. enterocolitica*. Eighty micrograms of bacterial protein [EDTA-lysozyme treated bacteria (*9*)] was incubated with approximately $10^{-11}M$ ^{125}I-labeled bTSH and various amounts of unlabeled bTSH (Thytropar, Armour) in 300 μl of 10 m*M* tris-HCl, *p*H 7.4. Results are expressed as the amount of unlabeled TSH bound in each tube in relation to the amount that remained free after 60 minutes of incubation at room temperature. Bound and unbound ^{125}I-labeled bTSH were separated by centrifugation. Scatchard plot analysis of the data, shown in the inset, indicated a single binding site with a B_{max} of 2.9×10^{-8} mole per milligram of bacterial protein and a K_d of $4.2 \times 10^{-8}M$.

certain mammalian peptide hormones reflect the evolutionary conservation of compounds that are present in lower organisms and that may subserve some function therein. A variety of peptide hormones has been identified in certain protozoa or fungi, and insulin and secretin have been identified in the prokaryote *Escherichia coli* (*16*). Seemingly analogous to our findings are those of Richert and Ryan (*17*), who identified a saturable binding site for the glycopeptide hormone hCG in *Pseudomonas maltophilia* and our own finding of a binding site for bTSH in certain strains of *E. coli*. The functional significance, if any, of the bTSH binding site in *Y. enterocolitica* is uncertain, as is that of the hCG binding site in *Pseudomonas*, but remains to be evaluated.

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16 September 1982; revised 6 December 1982

Fourier Transform Infrared Difference Spectra of Intermediates in Rhodopsin Bleaching

Abstract. *The membrane protein rhodopsin is the primary light receptor in vision. Fourier transform infrared difference spectroscopy is sensitive to conformational changes in both the protein and the retinylidene chromophore of rhodopsin. By blocking rhodopsin bleaching at specific intermediates, it is possible to elucidate some of the primary molecular events of vision.*

The central problem in vision research is understanding how light absorption by a single rhodopsin molecule in the photoreceptor membrane leads to neural excitation (*1, 2*). Despite extensive research on the primary photochemistry and molecular mechanisms in rhodopsin, only limited information is available. This problem is typical of the difficulty of obtaining information about the structure and conformational changes of membrane proteins involved in important cellular processes such as ionic transport.

We report here on the application of Fourier transform infrared (FTIR) difference spectroscopy to study the conformational changes that occur in the photoreceptor membrane. As demonstrated with purple membrane from *Halobacterium halobium* (*3–5*), alterations of individual groups in the bacteriorhodopsin molecule can be detected with this new method. Previous FTIR studies of the photoreceptor membrane have focused on the secondary structure of rhodopsin and the orientation of the rhodopsin α-helices (*6*). Kinetic infrared spectroscopy has revealed time-dependent changes in the infrared bands of photoreceptor membrane on light exposure (*7*). This approach can be used to obtain the infrared difference spectrum of the metarhodopsin (meta) I and II transitions, although a new sample is required for each data point (*7*). We have found that changes in both the protein and the chromophore of rhodopsin at different stages of bleaching can be easily detected by FTIR difference spectroscopy. Specific findings include confirmation that an isomerization of the chromophore oc-

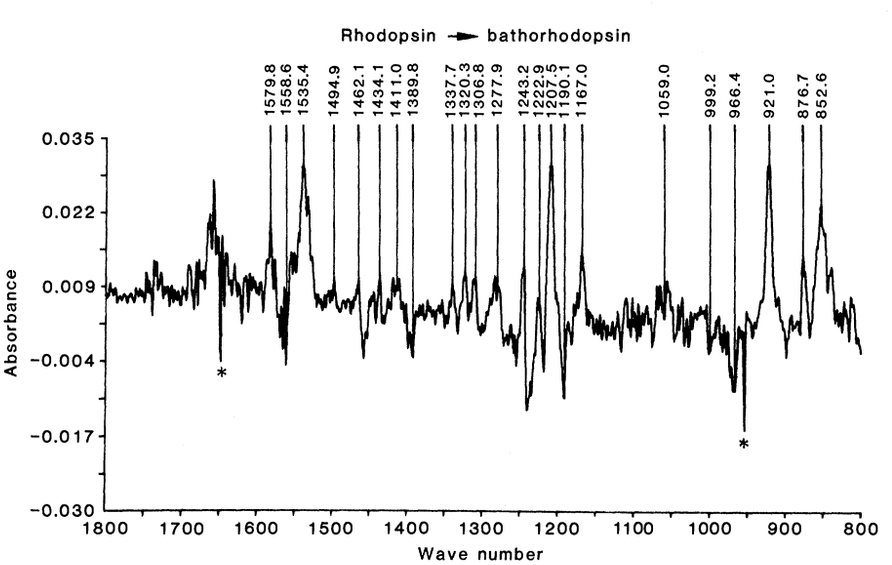

Fig. 1. Fourier transform difference spectra of photoreceptor membrane film deposited on AgCl, measured at 77 K while fully humidified. Photoreceptor membrane was isolated from bovine retinas (*24*), and films were formed by the isopotential spin-dry method (*25, 26*). Films had an absorbance of 0.4 to 0.5 at 500 nm and appeared optically transparent. Films were humidified by exposing a blank AgCl window to saturated water vapor while cooling the back of the window with a flow of cold N_2 gas for 15 to 30 seconds, then immediately placing the window in a cell containing an identical window with the deposited photoreceptor membrane film and sealing the cell. The amount of water vapor present was monitored from the H_2O bands at 3400 cm^{-1}. The cell was mounted on the copper tail of a low-temperature dewar (Janis Research Company, Stoneham, Massachusetts) equipped with a KBR and Ge window. The FTIR measurements were made with a Nicolet MX-1 spectrometer at a resolution of 2 cm^{-1}. Difference spectra were obtained by subtracting a reference spectrum recorded in the dark from a sample spectrum after 5 minutes of illumination with 500-nm light from a 600-W incandescent source filtered with Kodak Wratten filter 47. The reference and sample spectra were recorded with 300 scans (a total of 5 minutes). Four spectra from different samples were co-added to yield the spectrum shown. The asterisks mark peaks due to instrument-related artifacts.