After an additional 10 minutes, paralysis began to return. Neostigmine (0.375 mg) injected intramuscularly produced a similar, but longer lasting effect with a latency of 5 to 10 minutes. Two of the paralyzed animals were used for electromyography. The response from an experimental rabbit fatigues rapidly, while a control, uninjected rabbit shows no fatigue (Fig. 1). Injection of curare (0.8 mg) causes a similar fatiguing effect in a normal rabbit. The response to neostigmine in both experimental and control rabbits is also shown in Fig. 1. The fatigue effect is eliminated in the experimental animal, whereas a characteristic depolarizing block is induced in the control. Both of these results are consistent with a condition of neuromuscular blockade in the injected rabbits.

All the animals tested had precipitating antibodies to eel acetylcholine receptor. (The first animal to become paralyzed died during the night, and no serum was obtained from it.) A typical Ouchterlony pattern for serum collected from each of the other animals is seen in Fig. 2. That the antibodies were directed against eel acetylcholine receptor was demonstrated by the observations that antiserum precipitated the specific toxin binding activity from a purified acetylcholine receptor preparation (*4*), and that no precipitation band was seen with a preparation in which the acetylcholine receptor had been removed by ion-exchange chromatography (see Fig. 2).

The flaccid paralysis and abnormal electromyograms observed in response to injection of purified acetylcholine receptor were alleviated by the anticholinesterases edrophonium and neostigmine. The simplest interpretation of this phenomenon is that injection of eel acetylcholine receptor breaks tolerance of the rabbit to self-acetylcholine receptors, and that the reaction of antibody and receptor results in neuromuscular blockade. Serum from one of the paralyzed rabbits has, in fact, been shown to block the depolarizing response of a rat muscle cell line to iontophoretically applied acetylcholine, although other serums tested lack this activity (*4*). The effect of edrophonium and neostigmine is probably a consequence of inhibiting acetylcholinesterase, thereby raising the acetylcholine concentration enough to overcome neuromuscular blockade.

An autoimmune response to neurotransmitter receptors may be of interest in the study of neuromuscular disease.

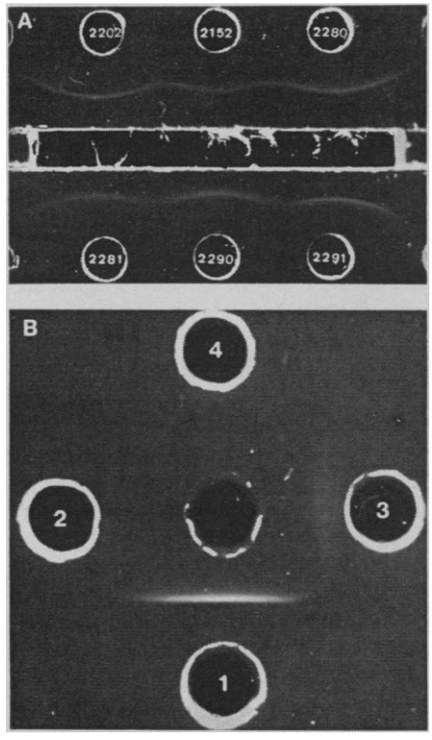

Fig. 2. (A) Ouchterlony patterns of serums obtained from rabbits after the first signs of paralysis. The center trough contains purified acetylcholine receptor at a concentration of 0.8 mg/ml. The wells contain undiluted serum from each rabbit, listed by protocol number; the serum from the rabbit seen in Fig. 1 is in the well numbered 2290. (B) Ouchterlony patterns of serum 2022 reacted with (well 1) purified acetylcholine receptor; fractions from a diethylaminoethyl cellulose chromatogram devoid of (well 2) and containing (well 3) acetylcholine receptor; and 1 percent Triton X100 (well 4), the detergent used to extract acetylcholine receptor from eel membranes.

For example, it has been suggested (*5*) that myasthenia gravis may be a consequence of an autoimmune response to acetylcholine receptors. There appear to be parallels between the paralysis observed in rabbits immunized with eel acetylcholine receptor and victims of myasthenia gravis: alleviation of paralysis by antiesterases, fatiguing seen in electromyographs, and the alleviation of this fatigue by antiesterases. Alternative theories are available for the neuromuscular blockade seen in myasthenia gravis [see (*6*)]; the parallels mentioned above, while consistent with the suggestion of an autoimmune response to acetylcholine receptor, do not serve to distinguish between any of the proposed theories.

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Thyrotropin and Cyclic Nucleotide Effects on Prostaglandin Levels in Isolated Thyroid Cells

Abstract. *Thyrotropin increases prostaglandin levels in isolated thyroid cells. Since comparable results were obtained with butyrated cyclic adenosine monophosphate derivatives as well as with the phosphodiesterase inhibitors quazodine and theophylline, it appears that cyclic adenosine monophosphate mediates this effect of thyrotropin. These observations suggest that intracellular prostaglandins play a role in modulating thyrotropin action on thyroid.*

Previous studies have suggested that prostaglandins (PG's) not only simulate but also modify thyrotropin (thyroid stimulating hormone; TSH) actions on thyroid (*1, 2*). In addition, two unrelated PG antagonists block both TSH and prostaglandin E (PGE_1 and PGE_2) effects on thyroid adenylate cyclase and cyclic 3′,5′-adenosine monophosphate (cyclic AMP) formation (*2, 3*), suggesting that PG's play an important role in regulating thyroid function. In an attempt to validate this thesis, we have shown that TSH increases PG concentrations in isolated bovine thyroid cells and that this effect

is specific for TSH (*4*). We now report studies suggesting that the TSH-induced increase in thyroid cell PG concentrations may be mediated by cyclic AMP.

Antiserums used for PG immunoassay were obtained by immunization of rabbits with PG-protein conjugates by the method of Jaffe *et al.* (*5*). Radioimmunoassay was performed by a double antibody technique (*4*). Since our antiserum to PGE_1 did not effectively distinguish between PGE_1, PGE_2, and PGA_1 while antiserum to $PGF_{2\alpha}$ recognized both $PGF_{1\alpha}$ and $PGF_{2\alpha}$ (*4*), we are reporting PGE_1 and $PGF_{2\alpha}$ concentrations as well as PGA_1 and PGB_1 concentrations (*6*) as PGE_1, $PGF_{2\alpha}$, PGA_1, and PGB_1 "equivalents" (*4*).

Bovine thyroid cells were isolated by an intermittent trypsinization technique (*7*). For PG determinations, 0.2 ml of cell suspension (12×10^6 to 20×10^6 cell/ml) in 5 ml of Krebs-Ringer bicarbonate buffer, *p*H 7.4, was incubated for 15 minutes at 37°C in covered sterile polypropylene tubes with or without added TSH (*8*) or other test agent. For cyclic AMP determinations, 0.5 ml of cell suspension was used, and the buffer also contained glucose (1 mg/ml) and bovine serum albumin (1 mg/ml). After incubation in PG studies, the sedimented cells were frozen in Dry Ice. The PG determinations were then carried out as described elsewhere (*4*). In cyclic AMP studies, after the cells were incubated, the tubes were chilled in an ice bath and centrifuged in the cold, and the medium was decanted. The cell pellet was then extracted with 0.5 ml of trichloroacetic acid and frozen in liquid nitrogen. After homogenization of the pellet, cyclic AMP was measured by the radioimmunoassay procedure of Steiner *et al.* (*9*) with minor modifications as described (*2*).

During 15-minute incubations at 37°C, N^6,$O^{2'}$-dibutyryl cyclic AMP (DBcAMP) effected a concentration-related increase in cell PG concentrations (Table 1). The response to 3 m*M* DBcAMP was comparable to that with 50 milliunits of TSH per milliliter. N^6-Monobutyryl cyclic AMP augmented cell PG concentrations to approximately the same extent as DBcAMP (data not shown). Unsubstituted cyclic AMP and sodium butyrate were ineffective.

To further explore the role of cyclic AMP in mediating TSH-induced increases in thyroidal PG concentrations, we studied the effects of the phosphodiesterase inhibitors quazodine (*10*) and theophylline alone and in combination with TSH, DBcAMP, and cyclic AMP on PG concentrations and, where feasible (*11*), on endogenous cyclic AMP in isolated thyroid cells. Quazodine caused a parallel concentration-related increase in cell PG and (endogenous) cyclic AMP (Table 1). The response to theophylline was qualitatively similar. When ineffective concentrations of quazodine and TSH were combined, intracellular PG and cyclic AMP were significantly increased. Submaximal concentrations of TSH and quazodine or of TSH and theophylline had additive effects on cell PG and cyclic AMP concentrations. A similar additive effect on cell PG occurred with submaximal concentrations of DBcAMP and quazodine in combination. In the presence of either quazodine or theophylline, exogenous cyclic AMP increased cell PG's to concentrations significantly above those seen with either phosphodiesterase inhibitor alone.

PG's are released into the circulation from various organs as a result of chemical or nerve stimulation, but a high proportion of PGE and PGF compounds are removed after one circulation through the lungs (*12*). This

Table 1. Effects of TSH, cyclic nucleotides, and phosphodiesterase inhibitors on PG and cyclic AMP content of isolated bovine thyroid cells; U, unit.

Test agent	PG content (ng/0.2 ml of cells)*				Cyclic AMP (pmole/0.5 ml of cells)†
	E_1	$F_{2\alpha}$	A_1	B_1	
Basal	10.06 ± 0.53	4.33 ± 0.49	5.12 ± 0.46	1.66 ± 0.19	6.90 ± 0.09
TSH, 5 mU/ml	9.85 ± 0.46	4.38 ± 0.42	5.07 ± 0.31	1.62 ± 0.21	6.86 ± 0.11
TSH, 10 mU/ml	14.76 ± 0.51‡	5.93 ± 0.37‡	8.22 ± 0.36‡	2.47 ± 0.19‡	10.42 ± 0.16‡
TSH, 50 mU/ml	29.55 ± 0.68‡	8.61 ± 0.51‡	13.18 ± 0.57‡	3.84 ± 0.31‡	16.92 ± 0.23‡
DBcAMP, 0.5 m*M*	16.12 ± 0.53‡	5.61 ± 0.45‡	7.81 ± 0.48‡	2.42 ± 0.23‡	
DBcAMP, 1.5 m*M*	21.38 ± 0.64‡	6.83 ± 0.51‡	10.72 ± 0.59‡	2.90 ± 0.25‡	
DBcAMP, 3 m*M*	26.24 ± 0.83‡	7.71 ± 0.59‡	12.96 ± 0.62‡	3.51 ± 0.28‡	
Cyclic AMP, 3 m*M*	9.82 ± 0.58	4.37 ± 0.32	4.83 ± 0.31	1.85 ± 0.23	
Quazodine, 5×10^{-5}*M*	10.13 ± 0.42	4.26 ± 0.38	5.21 ± 0.43	1.81 ± 0.27	6.92 ± 0.08
Quazodine, 10^{-4}*M*	17.50 ± 0.68‡	5.48 ± 0.32‡	7.53 ± 0.39‡	2.52 ± 0.18‡	11.47 ± 0.29‡
Quazodine, 5×10^{-4}*M*	29.83 ± 0.75‡	7.86 ± 0.51‡	11.28 ± 0.42‡	3.31 ± 0.24‡	19.36 ± 0.37‡
Quazodine, 10^{-3}*M*	35.21 ± 0.86‡	9.53 ± 0.58‡	16.14 ± 0.72‡	3.93 ± 0.35‡	23.14 ± 0.39‡
Theophylline, 10^{-3}*M*	22.83 ± 0.71‡	7.84 ± 0.43‡	11.23 ± 0.51‡	3.16 ± 0.30‡	11.99 ± 0.36‡
TSH, 5 mU/ml, plus quazodine, 5×10^{-5}*M*	18.31 ± 0.43§	6.35 ± 0.40§	8.37 ± 0.52§	2.74 ± 0.23§	13.21 ± 0.40§
TSH, 10 mU/ml, plus quazodine, 10^{-4}*M*	21.89 ± 0.57§	7.83 ± 0.39§	10.56 ± 0.43§	3.63 ± 0.21§	16.63 ± 0.48§
TSH, 10 mU/ml, plus quazodine, 10^{-3}*M*	51.12 ± 1.06§	17.23 ± 0.55§	26.19 ± 0.64§	9.02 ± 0.41§	28.09 ± 0.63§
TSH, 10 mU/ml, plus theophylline, 10^{-3}*M*	26.01 ± 0.62§	9.72 ± 0.48§	14.78 ± 0.51§	4.58 ± 0.30§	17.12 ± 0.51§
DBcAMP, 0.5 m*M*, plus quazodine, 10^{-4}*M*	23.98 ± 0.64§	6.76 ± 0.41§	10.49 ± 0.46§	3.35 ± 0.22§	
Cyclic AMP, 3 m*M*, plus quazodine, 10^{-4}*M*	21.72 ± 0.53§	6.12 ± 0.27§	9.86 ± 0.35§	3.08 ± 0.17§	
Cyclic AMP, 3 m*M*, plus theophylline, 10^{-3}*M*	25.16 ± 0.49§	9.35 ± 0.36§	13.62 ± 0.51§	4.23 ± 0.25§	

* PG "equivalents," mean ± 1 S.E.M. of four experiments, each experimental determination performed in triplicate. Incubation time is 15 minutes. † Mean ± 1 S.E.M. of four experiments, each experimental determination performed in triplicate. Incubation time is 15 minutes. ‡ Significantly [$P < .02$ to $P < .01$] greater than control. § Significantly [$P < .01$] greater than either compound alone.

may be regarded as evidence against any hypothesis that these compounds could function as hormones. Thus, despite the ability of exogenous PGE and PGF to simulate and modify (exogenous) TSH actions on thyroid, the physiological relevance of these effects is not entirely compelling. Relating these findings, in particular the "competitive interaction" of exogenous PGE and TSH (in combination) on a variety of thyroid functions (*1, 2*), to the demonstration that TSH increases PG in thyroid (*4*) requires reassessment of the properties of adenylate cyclase systems as well as of intracellular regulation of cyclic AMP concentrations and effects.

Rodbell *et al.* (*13*) have proposed a three-component adenylate cyclase system—discriminator, transducer, and amplifier; they have suggested that the amplifier or catalytic component of adenylate cyclase may be located at the inner surface of the membrane. It would seem reasonable to suggest that this tripartite adenylate cyclase complex, particularly the transducer and amplifier components, are susceptible to intracellular as well as extracellular influences.

The data of Shaw and Ramwell (*14*) suggest that, when cyclic AMP is increased within the cell, one of the resultant effects may be an activation of PG synthesis or release (or both). [In this regard, it is relevant to cite the recent work of Jaffe *et al.* (*15*), who found that DBcAMP depressed the release of PG's from cultured cells derived from a carcinoma of the colon; this observation suggested that PG synthesis may be responsive to, if not mediated by, cyclic AMP.] Some of the total PG synthesized will be "active"—that is, active in the sense that it affects the adenylate cyclase system, and would thus represent part of a feedback system. This effect could be exerted intracellularly, perhaps even at the level of the catalytic (amplifier) activity of adenylate cyclase; on the other hand, it is conceivable that the released PG might also interact with a component of the cell membrane facing the exterior.

It has been suggested (*16*) that it is less likely that a feedback mechanism is involved in cells where PG activates adenylate cyclase since this would lead to a positive, and hence potentially unstable, feedback control. If, however, as is the case in thyroid, the PG is less potent a stimulator than the specific tropic hormone (in this instance, TSH), the potential for "competitive interaction"—that is a negative feedback system—still exists.

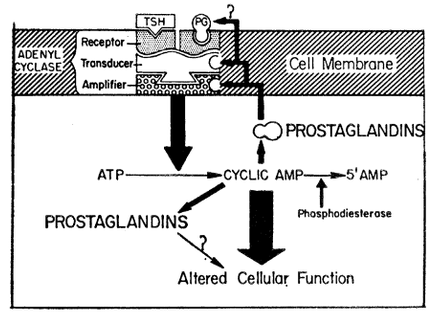

Fig. 1. Hypothetical role of intrathyroidal PG's in modulating TSH effects on thyroid.

Although the studies of Kuehl *et al.* (*17*) as well as our own (*2*) with the PG antagonist 7-oxa-13-prostynoic acid led to the initial hypothesis that a PG may be an obligatory intermediate in the actions of hormones on adenylate cyclase, findings by Levey (*18*) suggest that an alternate explanation is equally appropriate. He has shown that, in contrast to a number of hormones, PG's stimulate solubilized adenylate cyclase in the absence of added phospholipids, suggesting that PG's behave like acidic phospholipids in that they alter the conformational state of the transducer or coupler subunit. Hence, 7-oxa-13-prostynoic acid, a compound structurally related to the PG's (*19*) could also interfere with hormonal effects on cyclase in this manner, a mechanism more consistent with our current working postulate (as discussed below) than that advanced by Kuehl and co-workers (*17*).

Our studies indicate that TSH-induced increase in intracellular PG in thyroid is mediated by way of cyclic AMP. The hypothesis we would advance to account for these findings and those reported earlier (*1, 2, 4*) is represented in Fig. 1. TSH activates membrane adenylate cyclase resulting in an increase in intracellular cyclic AMP. Although rapid inactivation of cyclic AMP by phosphodiesterase should ensure that the response to TSH is evanescent, the precise role of this enzyme in modulating TSH effects on thyroid remains unsettled (*20*). The increase in intracellular cyclic AMP leads to an increase in intracellular PG, presumably as a consequence of increased PG synthesis (*21*). The intracellular PG's then interact with either the transducer, or amplifier, or both. Although the interaction is diagrammatically shown to mimic the interaction occurring at the external cell surface, that is, via specific receptor sites, this need not be the case (*22*). In any event, the intracellular PG's are envisioned as inducing a configurational or functional change (or both) in the transducer or amplifier, resulting in a diminished response to membrane-bound TSH—that is, a form of "negative feedback," mimicked by the competitive TSH-PG interaction described in earlier experiments and resulting in decreased cyclic AMP production. Also shown diagrammatically is the possibility that released PG's also interact with a component of the cell membrane facing the exterior.

We propose, therefore, as a working postulate that, in thyroid, there exists an intracellular cyclic AMP–PG-mediated negative feedback system modulating TSH effects on thyroid function and that this represents a "redundant" (*23*) alternative to cyclic AMP inactivation by phosphodiesterase.

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Osteopetrosis Cured by Temporary Parabiosis

Abstract. *The excessive accumulations of spongiosa in the long bones of congenitally osteopetrotic mice permanently disappeared after a brief parabiotic union to normal littermates. Most of the bone removal was accomplished long after interruption of parabiosis. It is proposed that, during parabiosis, progenitors of competent osteolytic cells were recruited from the blood of the normal mouse.*

Signs of osteopetrosis in microphthalmic mice disappear within 6 weeks after parabiotic union is made between mutant and normal littermate (*1*). However, the parabiotic union need exist for only 2 weeks to obtain a permanent cure of the congenital bone disease.

Parabiosis was performed at 10 (six pairs) or 45 (two pairs) days of age (*2*). In each instance, the subcutaneous union was made between a mutant and normal sibling of the same sex. Two weeks after parabiosis, the animals were disjoined, and the right hind limb was amputated below the knee in order to provide a tibia for histologic examination. Two to 8 months later, when mice were 84 (six pairs) or 270 (two pairs) days of age, respectively, the animals were killed and the remaining long bones were prepared for histologic study (*3*).

At the end of the 2-week period of parabiosis, little, if any, of the excess bone had been removed from the medullary cavities of the long bones (Fig. 1, A and B). However, during the subsequent observation period when the animals were no longer united, signs of osteopetrosis vanished from the mutants (Fig. 1C). In the unoperated mutants that survived for 10 months or longer (five microphthalmic mice), spontaneous remission was never seen (Fig. 1D).

During parabiosis bone remodeling in the mutant was initiated by cells or hormones (or other factors) derived from the normal mouse. Perhaps among the mononucleated elements normally in circulation are progenitors of osteolytic cells. It seems unlikely that anything but cells could survive long after interruption of parabiosis to have accomplished the result reported here. Calcitonin and parathormone activities are elevated in the gray-lethal and microphthalmic mice (*4*). However, these endocrine effects are probably secondary to an unresponsive osseous target tissue. Primary hyperparathyroidism and hypercalcitonism in the etiology of congenital osteopetrosis in mice is not borne out by the therapeutic failure of thyroidectomy and parathyroidectomy (with L-thyroxine replacement therapy) in long-term follow-up studies (*5*).

A practical implication of the present report is that congenital osteopetrosis may be curable by an appropriate series of blood transfusions.

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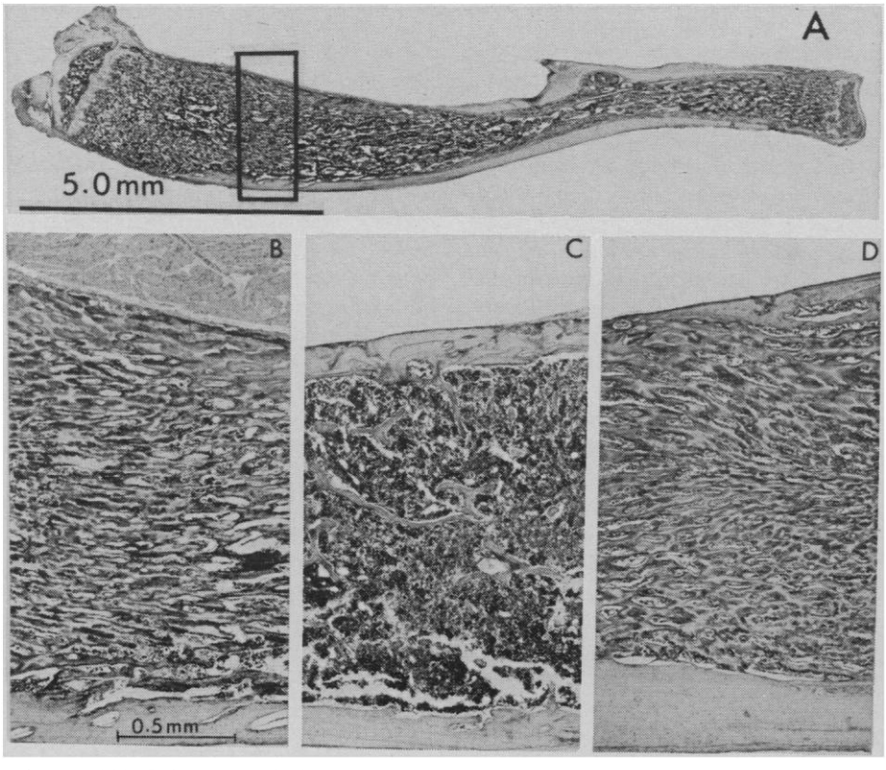

Fig. 1. (A) When parabiosis was terminated 2 weeks after onset, the amputated right tibia showed no evidence of bone remodeling. (B) Area enclosed in A is enlarged to reveal the presence of spongy bone at the interior of the shaft. (C) Eight months later the left tibia of the same mutant is of normal histologic appearance. (D) Tibia of an unoperated osteopetrotic mouse 10 months of age.

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