tomies were performed on day 15, all of the control rats were still pregnant. None of the rats implanted with prolactin–cocoa butter on days 1–6 remained pregnant, but almost all rats remained pregnant when implanted on days 7 or 8 of pregnancy. These results indicate that a prolactin implant in the median eminence, by reducing pituitary prolactin secretion, terminates pregnancy if given during the first 6 days of gestation. When prolactin was implanted in the median eminence of rats on the fourth day of gestation and 2 mg of progesterone were injected daily until day 11, pregnancy was maintained. When progesterone was withdrawn after day 11, and the rats were killed on day 15, it was evident that resorption of the fetuses had occurred. This indicates that the decrease in pituitary prolactin secretion produced by the implant of prolactin into the median eminence, resulted in luteal regression and reduced progesterone secretion.

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Studies of Thyrocalcitonin Inactivation *in Vitro** (33624)

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Recent results of Tashjian and Voelkel (1) indicate the presence of a factor in a serum which inactivates thyrocalcitonin (TC) *in vitro*. Data to be presented below indicate the presence of another factor, or factors, in rat serum which prevents the rapid inactivation of partially purified TC occurring *in vitro* at neutral pH.

Methods. TC was prepared from pork thyroid glands by an acid extraction procedure (Schlueter and Fisher, unpublished). Most experiments were performed with one TC preparation (K322-176) containing 12 Medical Research Council (MRC) U/mg solids, although certain experiments utilized less pure material containing 0.12 U/mg (K322-096), or 0.03 U/mg (K412-045).

TC was incubated *in vitro* in siliconed glass tubes at a concentration of 60 MRC milliunits (mU) per ml in a medium of 0.1 *M* Tris buffer, pH 7.5, 0.1 *M* sodium acetate buffer, pH 5.5, or 0.9% NaCl at various pH levels. Incubation of TC was either for 30 min at 23°, or 4 hr at 37°.

After *in vitro* incubation, TC activity was bioassayed in 100–130-gm female Sprague-Dawley rats, fasted for 16 hr. Three rats were used per group. Subcutaneous injections of 0.5 ml/100 gm body weight were given, with blood sampling 1 hr post-treatment. Serum calcium was determined by EDTA titration (2). The data were analyzed using Student's *t* test.

Results. Inactivation of TC at Neutral pH. In contrast to the data of Tashjian and Voelkel (1) showing stability of TC activity at pH 7.5, preliminary experiments indicated a complete loss of hypocalcemic activity after *in vitro* incubation of TC (12 U/mg) for 4 hr at 37° in 0.1 *M* Tris buffer, pH 7.5. Likewise, hypocalcemic activity was lost when TC was incubated in pH 7.5 Tris buffer for 30 min at 23° (Table I), while TC, incubated in pH 3.2 saline, was not inactivated. Hypocalcemic activity was not recovered by acidifying the inactivated solution to pH 3.0 and incubation at 23° for 1 or 6 hr,

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TABLE I. Effect of TC Incubation in 0.1 *M* Tris Buffer, pH 7.5, for 30 Min at 23°.

	Serum Ca (mg %)[a]	
	Expt 1	Expt 2
TC in pH 3.2 saline	6.88 ± .21[b]	7.22 ± .24[c]
Tris	9.12 ± .24	10.13 ± .58
Tris + TC (12 U/mg)	9.07 ± .22	9.77 ± .17

[a] Values expressed as mean ± SD.
[b] Significantly different ($p < .001$) from Tris.
[c] Significantly different ($p < .01$) from Tris.

with or without the addition of 0.1% bovine serum albumen.

Since no loss of hypocalcemic activity of TC in pH 7.5 Tris buffer was noted by Tashjian and Voelkel (1) using a porcine TC preparation of relatively low specific activity (0.7–0.8 U/mg), experiments were performed comparing TC preparations containing 12 U/mg, 0.12 U/mg, and 0.03 U/mg (Table II). Little or no loss of activity was apparent after incubation of the two lower potency preparations for 30 min at 23° or after incubation of the 0.03 U/mg preparation for 4 hr at 37°. These data indicate that TC preparations of higher specific activity may be more labile at neutral pH than are relatively crude TC preparations.

In order to more clearly determine the effect of pH on TC inactivation *in vitro*, TC (12 U/mg) was incubated for 30 min at 23° in 0.9% saline solutions of varying pH (Fig. 1). At pH 6 or higher, essentially complete loss of hypocalcemic activity occurred, whereas TC solutions at lower pH values were stable.

TABLE II. Effect of Incubation of TC Preparations of Varying Specific Activity in 0.1 *M* Tris Buffer, pH 7.5.

	Serum Ca (mg %)[a]	
	30 min 23°	4 hr 37°
Tris	9.12 ± .24	9.24 ± .24
Tris + TC (12 U/mg)	9.07 ± .22	—
Tris + TC (.03 U/mg)	7.42 ± .34[b]	7.58 ± .43[b]
TC in pH 3.2 saline	7.36 ± .10[c]	
Tris	10.41 ± .31	
Tris + TC (12 U/mg)	10.16 ± .31	
Tris + TC (.12 U/mg)	8.16 ± .36[b]	

[a] Values expressed as mean ± SD.
[b] Significantly different ($p < .01$) from Tris.
[c] Significantly different ($p < .001$) from Tris.

Effect of rat serum on TC inactivation. Although the above experiments indicated that TC activity was stable in saline solutions below pH 6 for short intervals (30 min at 23°) it was found that incubation of TC (12 U/mg) for 4 hr at 37° in 0.1 *M* sodium acetate, pH 5.5, resulted in complete inactivation (Table III). However, the addition of normal rat or dog serum to TC *in vitro* resulted in retention of hypocalcemic activity in inverse proportion to the concentration of serum added. Also, when serum previously heated for 20 min at 60° was utilized (heat-treated serum), hypocalcemic activity was retained. Tashjian and Voelkel (1) have reported that heating of rat serum for 20 min at 60° destroys the TC inactivation factor which is present in normal serum. We interpret our results to indicate the presence of a factor, or factors, in normal and heat-treated serum which prevents *in vitro* TC inactivation resulting from incubation for 4 hr at 37° at a pH of 5.5.

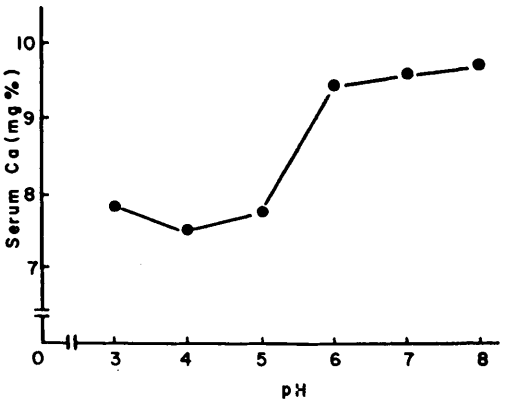

FIG. 1. Incubation of TC for 30 min at 23° in 0.9% saline solutions of varying pH.

To study further the effect of serum on TC inactivation, TC was incubated at pH 5.5 in acetate medium for 4 hr at 37° with a wide range of dilutions of both normal serum and heat-treated serum (Fig. 2). Complete loss of hypocalcemic activity occurred without the addition of serum. When normal nonheated

TABLE III. Effect of the Addition of Serum to TC Incubated in 0.1 *M* Sodium Acetate Buffer, pH 5.5, for 4 Hr at 37°.

	Serum dilution	Serum Ca (mg %)[a]		
		Rat serum	No serum	Dog serum
TC in pH 3.2 saline	—		7.67 ± .07[d]	
Na acetate	—		9.33 ± .28	
Na Ac + TC	—		9.51 ± .50	
Na Ac + TC + normal serum	1:10	8.96 ± .28		7.95 ± .15[c]
Na Ac + TC + normal serum	1:20	7.92 ± .17[c]		7.81 ± .20[c]
Na Ac + TC + normal serum	1:50	7.47 ± .43[c]		7.36 ± .28[d]
Na Ac + TC + heat-treated[b] serum	1:10			7.55 ± .07[d]

[a] Values expressed as mean ± SD.
[b] Heated 20 min at 60°.
[c] Significantly different ($p < .01$) from Na acetate.
[d] Significantly different ($p < .001$) from Na acetate.

rat serum was added, hypocaclemic activity was retained to a degree inversely proportional to the dilution of serum, up to dilutions of about 1:100 or 1:1000. At these dilutions essentially maximal activity was present compared to similar incubation of TC in pH 3.2 saline. The addition of serum at dilutions higher than 1:1000 was ineffective in preventing loss of hypocalcemic activity. When heat-treated serum was added using similar incubation conditions, little or no TC inactivation was apparent at high concentrations of serum, and essentially complete hypocalcemic activity was retained with serum dilutions of 1:100 to 1:1000. Thus, it appears that the ability of serum to prevent TC inactivation at pH 5.5, after incubation at 37° for 4 hr, is relatively great and that the apparent lack of protective activity with higher concentrations of normal serum is likely due to the TC inactivator described by Tashjian.

Several experiments were performed in an attempt to determine the nature of the factor, or factors, in serum which protects TC from inactivation at a pH above 5.0. In these experiments TC was incubated in 0.1 *M* Tris buffer, pH 7.5, for 30 min at 23°. These conditions resulted in essentially complete loss of hypocalcemic activity, compared to incubation in pH 3.2 saline, and the addition of heat-treated serum partially prevented the loss of activity.

Since it has been shown by Parsons (4) that the presence of albumin in TC solutions enhances biological activity, the possibility of a protective effect of serum proteins, during *in vitro* incubation, was studied. The addition of 1 or 4% bovine serum albumin to the medium did not prevent TC inactivation. Similarly, it was found that addition to the medium of human albumin or alpha, beta, or gamma globulin, in concentrations similar to those in blood, also failed to protect TC from inactivation. In addition, coating the incubation tubes with 1% porcine albumin (Pentex PK 0262) or gamma globulin (Pentex Lot 15), prior to incubation of TC in pH 7.5 Tris for 30 min at 23°, did not prevent TC inactivation. These data, as well as the very small concentrations of added serum required for protection of TC, indicate that glass adsorption of TC is unlikely to explain the spontaneous inactivation which occurs at neutral pH.

The effect of preincubation of TC for 15 mins with 1% aqueous solutions of porcine (Pentex PK 0262), bovine (Armour K370-295A), human (Armour K322-253), or egg (Pentex Lot 12) albumin, prior to the addition of 0.1 *M* pH 7.5 Tris buffer, and incubation for 30 min at room temperature was studied (Table IV). The final concentration of albumin in the medium was 0.1%. It was found that preincubation of TC with porcine and egg albumin, but not bovine or human albumin, prevented TC inactivation

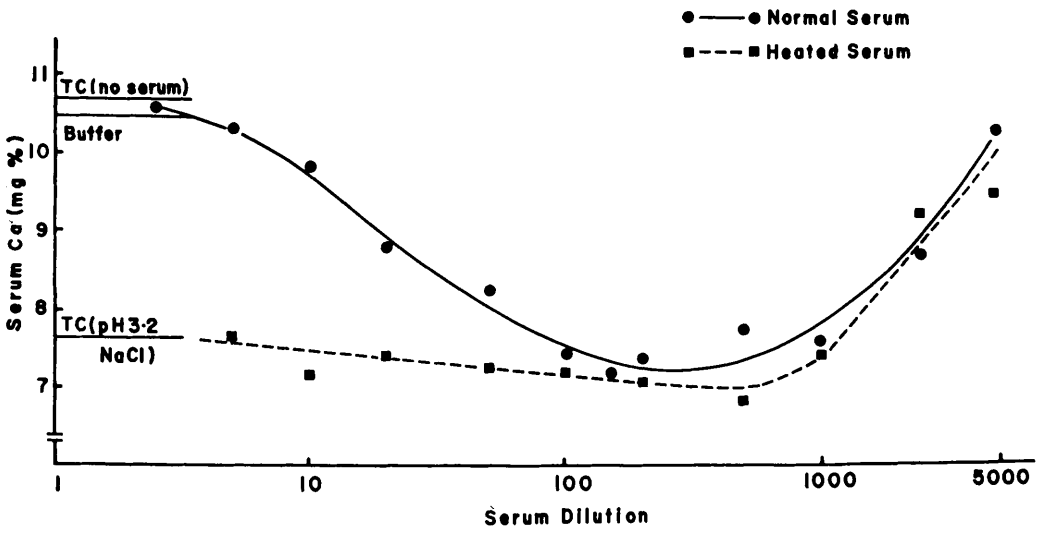

FIG. 2. Effect of the addition of normal or heat-treated serum to TC incubated in pH 5.5 sodium acetate buffer for 4 hr at 37°.

comparable to that found with heat-treated rat serum. Addition of porcine or egg albumin directly to Tris medium, however, did not prevent TC inactivation. It has not yet been determined if rat albumin will protect TC from inactivation at neutral pH.

Other materials which have been utilized unsuccessfully, in an attempt to prevent *in vitro* TC inactivation at neutral pH, include reducing agents such as cysteine, mercaptoethanol, and dithiothereitol, an enzyme inhibitor (epsilon amino caproic acid), several carbohydrates, 0.1% gelatin, and magnesium and manganese salts.

Discussion. Tashjian and Warnock (3) have reported the loss of hypocalcemic activity of TC preparations *in vitro* after heating at 100° for 1 hr in dilute HCl or pH 7.5 Tris buffer, or when incubated with hydrogen peroxide. No significant loss of activity occurred when TC was heated in dilute HCl at 100° for 15 min, or in 0.1 *M* Tris, pH 7.5, when heated at 37° for 4 hr. They found that inactivation was more rapid at neutral than at acidic pH. They also reported no significant differences in the stability of hypocalcemic activity of TC preparations of widely differing specific activity (0.4, 1.0, and 12 MRC U/mg).

In contrast, in the present studies, complete loss of hypocalcemic activity occurred when partially purified TC (12 U/mg) was incubated for as little as 30 min at 23° in pH 7.5 Tris buffer. This loss of activity was found to be pH dependent, with little inactivation occurring below pH 5. In addition,

TABLE IV. Effect of Preincubation of TC with Albumin prior to Incubation in 0.1 *M* Tris Buffer, pH 7.5 for 30 Min at 23°.

	Serum Ca (mg %)[a]	
	Expt 1	Expt 2
TC in pH 3.2 saline	7.71 ± .37[d]	7.39 ± .37[c]
Tris	10.05 ± .39	9.52 ± .11
Tris + TC only	9.92 ± .39	9.73 ± .17
Tris + TC + heated serum (1:50)	8.64 ± .47[c]	7.73 ± .95[c]
Tris + TC + porcine albumin[b]	8.39 ± .48[d]	8.20 ± .25[c]
Tris + TC + bovine albumin[b]	10.23 ± .17	9.47 ± .12
Tris + TC + human albumin[b]	9.70 ± .23	9.73 ± .15
Tris + TC + egg albumin[b]	8.45 ± .34[c]	7.66 ± .49[d]

[a] Values expressed as mean ± SD.

[b] Preincubated 15 min in 1 ml 1% albumin, diluted to 0.1% with pH 7.5 Tris.

[c] Significantly different ($p < .05$) from Tris.

[d] Significantly different ($p < .01$) from Tris.

[e] Significantly different ($p < .001$) from Tris.

crude TC preparations (0.03 or 0.12 U/mg) were found to be much more stable in neutral pH, with little inactivation occurring after incubation for 4 hr at 37°. The utilization of a crude TC preparation (0.7–0.8 U/mg) by Tashjian and Voelkel (1) in their studies of TC inactivation by serum may be the reason they did not find significant inactivation of TC in control solutions of pH 7.5 buffer not containing serum. The greater stability of crude TC preparations at neutral pH might be related to the presence of material other than thyrocalcitonin in the preparation.

Although the mechanism of inactivation of TC at neutral pH cannot be determined from the present experiments, the inability of such reducing agents as cysteine, mercaptoethanol, or dithiothreitol to prevent inactivation suggests that a factor, or factors, other than oxidation may be responsible.

The inactivation of crude TC by normal serum has been reported by Tashjian and Voelkel (1). In their experiments, serum dilutions of 1:100 or greater did not result in TC inactivation even after incubation of TC for 4 hr at 37°. In the present experiments, relatively high concentrations of normal serum (1:2 to 1:50) resulted in loss of hypocalcemic activity, probably due to the serum inactivator described by Tashjian and Voelkel, while greater dilutions of normal serum (1:100 to 1:1000) reduced the inactivation of TC which occurred after incubation for 30 min at 23° in pH 7.5 Tris buffer alone, and prevented inactivation resulting from incubation for 4 hr at 37° in pH 5.5 acetate buffer. Serum was only partially effective, however, in preventing inactivation occurring after incubation of TC for 4 hr at pH 7.5. Utilization of serum heated at 60° for 20 min in place of normal serum gave similar results, except that high concentrations of heat-treated serum did not inactivate TC, as did similar concentrations of normal serum. Heating serum for 20 min at 60° has been reported by Tashjian and Voelkel (1) to destroy the TC inactivating factor.

The protective ability of porcine and egg albumin, when preincubated with TC prior to exposure to neutral pH, while bovine or human albumin, or human serum protein fractions are ineffective, is thus far unexplained. Although it has not been determined if rat albumin will prevent TC inactivation, the necessity of preincubation of TC with porcine and egg albumin, but not with rat serum, may indicate that albumin is not the only protective factor in serum.

Summary. A preparation of porcine TC containing about 12 MRC U/mg solids was found to lose all hypocalcemic activity when incubated in 0.1 *M* Tris buffer, pH 7.5, for 30 min at 23°. Crude TC preparations (0.03 U/mg or 0.12 U/mg) did not lose hypocalcemic activity under these conditions. Inactivation of TC was found to be pH dependent with little or no loss of activity occurring at pH 5 or below. Both normal serum and serum heated at 60° for 20 min decreased TC inactivation which occurred above pH 5.0, when added to the medium at a concentration of 0.1–1.0%. High concentrations of normal serum, however, inactivated TC. Of several agents utilized, in attempts to prevent the loss of hypocalcemic activity which occurred *in vitro* at neutral pH, porcine albumin and egg albumin (but not bovine or human albumin) were found effective to an extent similar to that found with serum.

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