Table 4. Filter test of RNA fractions eluted from column III of Table 2; the experimental details are as described in Tables 1 and 3. The RNA was not treated with deoxyribonuclease.

RNA eluted from column	Filters (count/min)		$\frac{r^+-r1231}{r^+}$
	T4r+	T4r1231	
III—T4r1231	152	148	0.03
III—T4r+	138	18	0.87

RNA fraction of experiment 1 (first line of Table 1), which was adsorbed to the portion of the column containing the r+ DNA, represents some 40 percent of RNA homologous to the DNA region deleted by mutant r1272.

In experiment 1, the purified rII RNA fraction still contains a considerable number of sequences nonspecific for the rII region. In order to trap the nonspecific RNA more effectively in the upper layer of the column, and to minimize its adsorption to the bottom layer, we prepared a column with an upper layer 20 cm long containing 12 mg of T4r1272 DNA and a lower layer, 5 cm in length, containing only 3 mg of T4r+ DNA. Passage of a preparation of T4r+ RNA through this column produced an RNA fraction which appears to be specific by more than 90 percent for the DNA region absent in mutant r1272, but present in T4r+ DNA (Tables 2 and 3). This RNA was fractionated further by hybridization with DNA of mutant r638, which lacks cistron B, but possesses cistron A. RNA molecules corresponding to the two cistrons could thus be separated (Table 3 and Fig. 1).

Since the right ends (that is, those distal to the A cistron) of both mutants, r1272 and r638, are genetically not defined [r1272 extends some ten recombination units beyond the B cistron (*7*)], we attempted to purify an RNA fraction not accepted by the DNA of a deletion mutant terminating on both sides within the rII region. For this purpose RNA eluted from the upper layer, containing T4r638 DNA, of the column in run II (638/W) in Fig. 1 was passed through a column containing T4r1231 DNA and T4r+ DNA. The RNA excluded by the r1231 DNA but bound by the r+ DNA was expected to be equivalent to the right half of the rII A cistron. Hybridization tests with filters containing T4r+ and T4r1231 DNA's confirmed this expectation (Table 4).

When nitrocellulose filters not containing DNA were incubated with RNA eluted from either column a considerable amount of labeled material resistant to ribonuclease became attached to the filters. Treatment of the RNA with deoxyribonuclease (electrophoretically purified, Worthington) before incubation diminished most of the activity in the control filters. This observation suggests that, upon elution from the DNA-nitrocellulose column, some DNA was released and became adsorbed to the test filters and was then able to bind some of the RNA to the filters.

The high purity of the RNA specific for the rII region (Table 3) suggests that we would have been able to identify RNA specified by a similar-sized region in a genome at least ten times as large as the genome of phage T4. Therefore it seems likely that this deletion method might be successfully applied to the isolation of operon- or even gene-specific bacterial messengers with but little improvement of our technique.

The smallest segment of the T4 genome whose complementary RNA we have succeeded in purifying is that of approximately half a cistron with an estimated length of some 1000 nucleotides. Preliminary evidence indicates that RNA molecules of even smaller sizes can be fractionated almost equally well. If so, the deletion method would provide a unique opportunity to place unequivocally small fragments of messenger molecules on the genetic map according to their sites of transcription. With the availability of such a genetically well-defined system as the rII region in bacteriophage T4, the deletion method meets the first of two principal requirements for the elucidation of the primary structure of an mRNA, namely, the orientation in a linear fashion of RNA fragments of the size of transfer RNA. Whether the deciphering of these fragments, obtainable in extremely small quantities only, will ever become possible, remains to be shown (*8*).

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5. Low-salt buffer consists of a 1 : 200 dilution of high-salt buffer; high-salt buffer consists of 0.3*M* NaCl and 0.03*M* sodium citrate.
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Thyrocalcitonin Inhibition of Bone Resorption Induced by Parathyroid Hormone in Tissue Culture

Abstract. *Added thyrocalcitonin greatly diminished parathyroid-hormone–induced resorption of bone in tissue culture. The results indicate that bone is a primary site of action of thyrocalcitonin.*

Thyrocalcitonin, the hypocalcemic, hypophosphatemic principle of mammalian thyroid gland discovered by Hirsch *et al.* (*1*) is now generally recognized as a hormone of major importance in the regulation of the amount of calcium in plasma. Bone was suspected as its principal site of action because of the hypocalcemic effect of thyrocalcitonin in rats even after parathyroidectomy (*1*), nephrectomy (*2*), or removal of the gastrointestinal tract (*3*) and because of the parallellism of the observed hypocalcemia and hypophosphatemia (*2*). We now report that thyrocalcitonin added in vitro to bone undergoing resorption in tissue culture under the influence of parathyroid hormone acts directly on isolated bone and we suggest that bone is a principal site of action of the newly recognized thyroid hormone.

The essentials of the procedure for the bone cultures (*4*) are as follows. Calvariae of Swiss albino mice of the Webster strain, aged 4 to 5 days, were removed aseptically and attached to coverslips with a mixture of chicken plasma and chicken embryo extract

(2:1). Each calvaria was inserted into the well of a Leighton tube and was covered with 2 ml of the appropriate medium. The tubes were gassed with a mixture of oxygen and nitrogen (1:1), stoppered, and placed horizontally into an incubator at 37°C, where they were continuously rotated. The media were changed daily (Sunday excluded), and at the time of each change the extent of resorption in the living cultures was scored (microscopic examination). After a 7-day incubation period, the calvariae were fixed with 10 percent neutral formalin and stained by the Von Kossa reaction, which stains the remaining bone mineral black. The stained calvariae were mounted on microscopic slides and photographed under transmitted light.

Results of one of two essentially identical experiments are shown in Fig. 1. Eighteen calvariae were divided by random into six groups of three each and cultured individually. The calvariae shown in Fig. 1*A* were cultured in 2 ml of medium composed of heated horse serum (80 percent) and Gey's balanced salt solution (20 percent), containing 200 units of penicillin and 200 μg of streptomycin. The remaining groups were cultured in the same medium with the following additions per milliliter: (*B*) 0.5 unit of Lilly parathyroid extract; (*C*) 0.5 unit of parathyroid extract plus 13 units of partially purified hog thyrocalcitonin [product of first chromatography on Sephadex G-50; specific activity was 460 ± 53 units/mg of nitrogen (*2*)]; (*D*) 13 units of thyrocalcitonin; (*E*) vehicle for thyrocalcitonin—0.05 ml of 0.05*M* sodium acetate buffer, *p*H 3.8; (*F*) 0.5 unit of Lilly parathyroid extract plus vehicle for thyrocalcitonin.

The stability of thyrocalcitonin during incubation in the culture medium at 37°C was examined by biological assay in rats (*2*). There was no apparent loss of activity at 1, 3, or 6 hours, relatively little loss at 24 hours, but by 48 hours little activity remained. Accordingly, as stated above, the media were changed daily.

Figure 1*B* illustrates the inducing effect of parathyroid hormone on bone resorption as compared with Fig. 1*A*, in which no parathyroid hormone was added. Figure 1*C* shows the greatly diminished loss of bone mineral when both thyrocalcitonin and parathyroid hormone were added. The effect, if any, of thyrocalcitonin alone (Fig. 1*D*) was minimum in this system. The addition of sodium acetate buffer, the vehicle for thyrocalcitonin, did not alter the effect of the control medium (Fig. 1*E*) nor did it have any apparent effect on the resorptive action of parathyroid hormone (Fig. 1*F*).

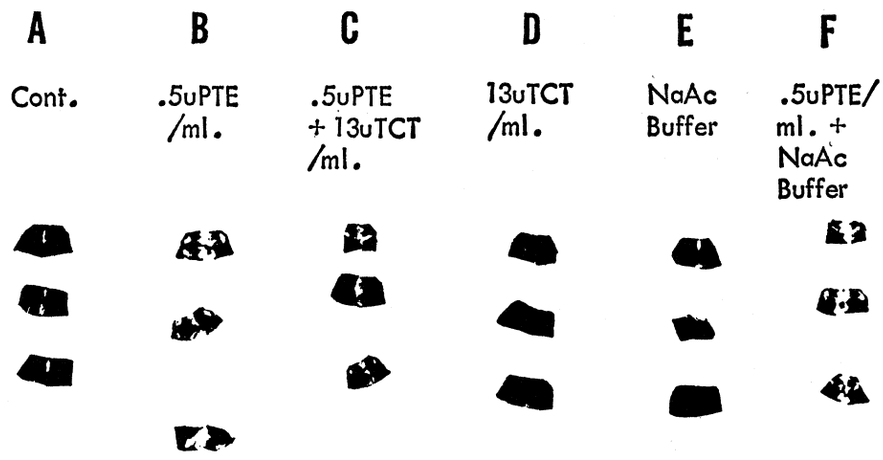

Fig. 1. Inhibition by thyrocalcitonin of bone resorption induced by parathyroid hormone.

The effect of lower concentrations of added thyrocalcitonin was observed in a similar experiment. As shown in Fig. 2*B*, addition of 5 units of thyrocalcitonin per milliliter of medium was effective in partially counteracting the resorptive action of 0.5 unit of parathyroid hormone (Fig. 2*A*). A lesser effect was obtained with 0.5 or 0.05 unit of thyrocalcitonin (Fig. 2, *C* and *D*).

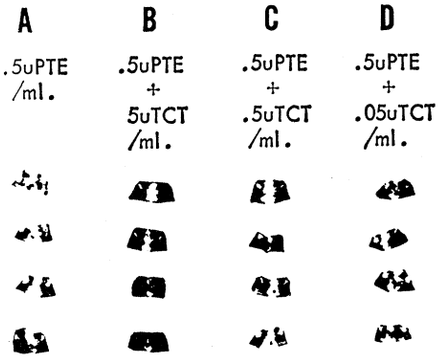

Fig. 2. Effect of decreasing concentrations of thyrocalcitonin as an inhibitor of bone resorption.

Thus, thyrocalcitonin added in vitro to tissue culture of bone can counteract the resorption of bone stimulated by parathyroid extract. Bone resorption obtained with the commercial extract is qualitatively similar to that obtained with a highly purified preparation (*5*). Quantitatively, the preparations were comparable if the enhancing effect of cysteine in the assay in vivo was taken into account.

Since thyrocalcitonin lowers the blood calcium in parathyroidectomized rats, it is likely that the effect shown in tissue culture is not due specifically to inactivation of parathyroid hormone or to competitive inhibition of parathyroid hormone at its specific site of action. Along this line, some experiments indicate that thyrocalcitonin counteracts resorption of the bone induced by vitamin A in tissue culture as well, thereby supporting the conclusion that the action of thyrocalcitonin is independent of parathyroid hormone and that it blocks resorption at a site which is basic to the entire mechanism, regardless of the particular agent inducing bone resorption (*5*). Whether other fractions of thyroid extracts or extracts of other tissues can also inhibit bone resorption in our tissue culture system is not yet known.

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