transferred once more, and tested for growth on MEM supplemented with asparagine and proline, on MEM supplemented with nonessential amino acids, on unsupplemented F12 (F12-IX, F12-X), and on complete F12. Cells from clones that did not grow in minimal mediums or unsupplemented mediums were further examined.

Of the large number of clones isolated, six were found that did not grow in MEM supplemented with asparagine and proline, in MEM with nonessential amino acids, or in F12 deficient in purines.

Each of the mutants was examined for response to the addition of nutritional supplements. All six grew well when any of the mediums were supplemented with hypoxanthine, while no growth occurred when thymidine was added. However, hypoxanthine could be replaced by other purines or purine derivatives, such as adenosine monophosphate, adenosine triphosphate, and guanosine monophosphate.

The mutants isolated are very stable, and no spontaneous revertants have been found. Since this class of mutants responds to hypoxanthine, the genetic block must occur prior to inosinic acid formation in the purine biosynthetic pathway. Consistent with this is the observation that these mutants are insensitive to HAT medium (hypoxanthine, aminopterin, and thymidine) (*5*), which inhibits growth of mutants lacking the enzyme hypoxanthine guanine pyrophosphorylase. Initial tests for growth of these mutants on purine intermediates suggest that these mutants are blocked between glycinamide ribotide and aminoimidazole carboxamide ribotide (AICR) (Table 1). There are at least six enzymatic steps between glycinamide ribotide and AICR (*6*).

The following observations suggest that these mutants are not identical mutational events. (i) Both 104/2b and 102/2b undergo a few divisions in very low concentrations of hypoxanthine (0.1 μg/ml); (ii) both 17/1 and 102/2b grow more slowly in MEM supplemented with AICR than other isolates; (iii) we have noted complementation as a result of heterokaryon formation between all six mutants under selective conditions. Heterokaryons are formed by mixing two mutant cell lines together at high population densities in minimal medium for 48 hours, and then by plating 10^4 cells into petri dishes containing minimal medium. Only heterokaryons or revertants should form clones under these conditions. The frequency of heterokaryon formation under such conditions is fairly high. This does not appear to be a characteristic of the mutant type, but of the Chinese hamster cell line. We assume that the growth of heterokaryons in mediums lacking hypoxanthine is due to genetic complementation, although we cannot rule out gene dosage effects or other regulatory mechanisms (*7*).

Table 1. Growth of mutants of Chinese hamster ovary cells (CHO/Pro$^-$) in the presence of different purines and purine intermediates. Numbers refer to cultures grown from different clones isolated following mutagenesis and selection. ++, Good growth; +, poor growth; −, no growth. Abbreviations: ATP, adenosine triphosphate; PRPP, phosphoribosyl phosphate; Gly R, glycinamide ribotide; AIC, aminoimidazole 4-carboxamide; AICR, aminoimidazole 4-carboxamide ribotide.

Mutant	Guanidine	Adenine	ATP	Hypoxanthine	PRPP	Gly R	AIC	AICR
130/1b	++	++	++	++	−	−	++	++
17/1	++	++	++	++	−	−	++	+
104/2b	+	+	++	++	−	−	++	++
102/2b	++	++	++	++	−	−	++	+
121/2b	++	++	++	++	−	−	++	++
1c	++	++	++	++	−	−	++	++
CHO-PI	++	++	++	++	++	++	++	++

Other mutants of the purine biosynthetic pathway of mammalian cells have been reported (*8*). The Lesch-Nyhan syndrome of man is associated with hyperuricemia and excess uric acid synthesis. The purine accumulation results from loss of the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT). Littlefield (*5*), using azaguanine resistance as a selective marker, has isolated strains of L cells and human fibroblast deficient in HGPRT; these strains are mutants that occur late in the purine biosynthetic pathway. However, the mutants reported here occur relatively early in the purine biosynthetic pathway and may be utilized to study regulation in mammalian cells. Since these mutants now carry three genetic markers (proline, asparagine, and purine), they should be useful in studying mechanisms of gene recombination in cells of higher animals.

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2-Thiophenecarboxylic Acid: Inhibitor of Bone Resorption in Tissue Culture

Abstract. *In tissue culture 2-thiophenecarboxylic acid inhibited both spontaneous resorption of bone and that induced by parathyroid hormone. Its action was to inhibit calcium transfer out of bone.*

Fang first discovered the hypoglycemic and antilipolytic actions of 2-thiophenecarboxylic acid (2-TCA) in rats made diabetic with alloxan (*1*). 2-Thiophenecarboxylic acid also has strong hypocalcemic and hypophosphatemic actions in intact and thyroparathyroidectomized rats (*2*), and bone appears to be a principal site of these actions (*3*). We now present further evidence, derived from experiments with mouse calvaria in culture, showing that 2-TCA inhibits metabolically mediated calcium transfer out of bone and thus

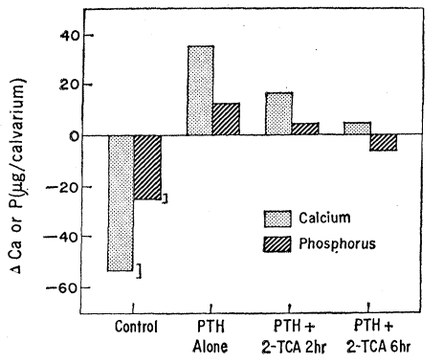

Fig. 1. Inhibitory effect of 2-thiophenecarboxylic acid on PTH-induced transfer of mineral from bone to culture medium. The bars give cumulative mean changes (Δ) of calcium and phosphorus in medium per calvarium in a group of four bones. Negative values indicate minerals taken up by bone from medium, and positive values indicate minerals transferred from bone to medium. Media were changed every day for 3 days. See the text for experimental procedure. The brackets give the standard errors.

inhibits the process of bone resorption.

We used calvaria of Swiss albino mice of the Webster strain, aged 4 to 6 days. The frontal and parietal bones were removed aseptically and cultured by two different procedures, designated A and B. In culture system A, the bone was attached to a cover slip with a mixture of chicken plasma and chicken embryo extract (2 : 1), inserted into the well of a Leighton tube, and covered with 2 ml of medium. The tubes were gassed with a mixture of oxygen and nitrogen (1 : 1), stoppered, and placed horizontally in a rotating rack in an incubator at 37°C. Control medium was composed of Gey's balanced salt solution and heated (56°C, 30 minutes) horse serum (1 : 4) containing 100 units of penicillin and 100 μg of streptomycin per milliliter. In this control medium, a net uptake of calcium into bone was observed. When 0.5 unit of parathyroid hormone (PTH) (Parathyroid Injection, Lilly) was added per milliliter of medium, bone resorption was observed microscopically (*4*), and there was an increase in calcium and phosphorus in the culture medium (*5*) (Fig. 1) as well as disappearance of bone mineral (Fig. 2A). If bones were cultured in medium containing PTH (0.5 unit/ml) and 2-TCA (1 mg/ml) for either 2 or 6 hours and then were cultured for 3 days in medium containing PTH alone, the PTH-stimulated movement of mineral from bone to medium was greatly reduced (Fig. 1). A similar inhibitory effect of 2-TCA on mineral transfer was also seen when bones were cultured continuously for 7 days in medium containing both PTH and concentrations of 2-TCA varying from 0.1 to 0.25 mg/ml. At the end of 7 days of culture the calvaria were fixed in 10 percent neutral formalin and stained by the Von Kossa reaction for identification of mineralized tissue (*4*). The results show inhibition of PTH-induced resorption of bone related to dosage of 2-TCA (Fig. 2A).

Table 1. Inhibitory effect of 2-thiophenecarboxylic acid (2-TCA) on release of ^{45}Ca from paired half calvaria. Total culture time was 3 days. Each group contained three to seven pairs. The percentage of release inhibited was calculated by the formula $[(C-T)/C] \times 100$, where C and T represent radioactivity in the medium of control and treated calvaria, respectively. The maximum theoretical inhibition is 65.0 ± 5.0 percent. The mean and standard error of the mean are given.

2-TCA (μg/ml)	^{45}Ca release inhibited (%)
5	10.7 ± 1.1
10	8.2 ± 0.4
50	27.1 ± 3.9
150	46.5 ± 3.4
250	44.2 ± 3.8
350	47.4 ± 3.0

In culture system B, half calvaria were used; bones were first labeled with ^{45}Ca by injection of neonatal mice with 12.5 μc of isotope 2 days after birth; the bones were used 4 days later. Each calvarium was divided in half; one half served as an experimental bone, the other as the control in a paired system. The bones were placed on a stainless steel grid in a petri dish and cultured in a synthetic medium containing 5 percent heated rabbit serum by means of a minor modification of the method of Reynolds and Dingle (*6*). The bones were incubated at 37°C after being gassed with a mixture of air and CO_2 (19 : 1). The release of ^{45}Ca from bones was followed by analyses of small samples (25 μl) of the medium for radioactivity by means of liquid scintillation spectrometry. During incubation, untreated control bones release labeled calcium as a result of spontaneous resorption. At the termination of the experiment each half calvarium was dissolved in 0.5 ml of formic acid

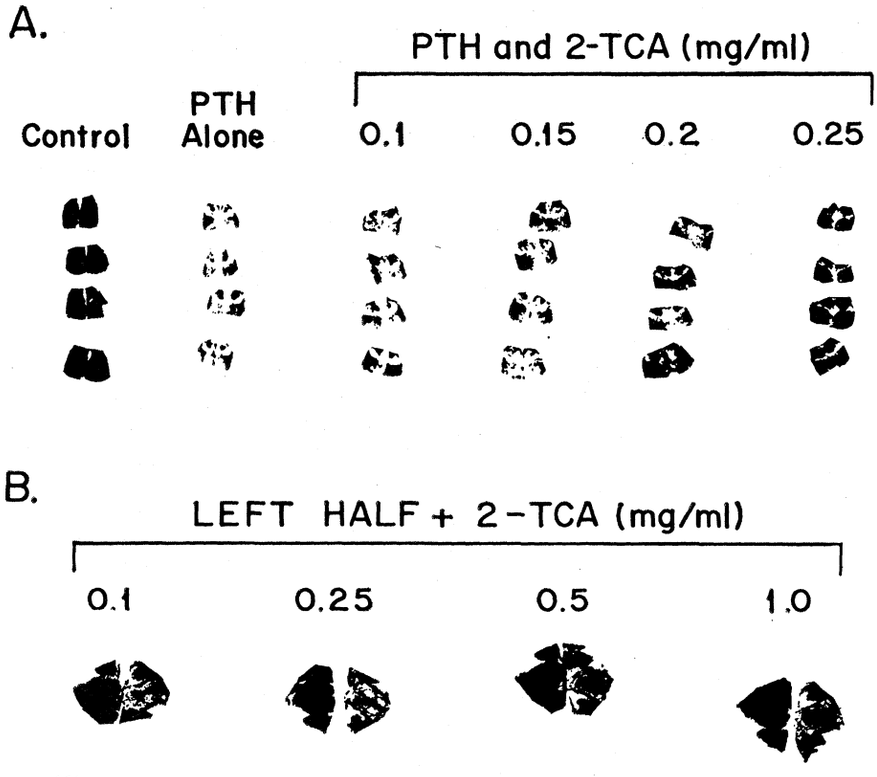

Fig. 2. Inhibition by 2-thiophenecarboxylic acid (2-TCA) of bone mineral loss from bones induced by parathyroid hormone (PTH, 0.5 unit/ml) (A) and of spontaneous bone resorption (B). In (B) the right half calvarium of each pair was the control, and the left was treated with 2-TCA at the concentrations shown (0.1 to 1.0 mg/ml). The figure shows samples stained by the Von Kossa reaction.

(98 percent) and analyzed for the remaining radioisotope. A summation of the radioactivity found in the medium and in the bone insured that pairs of bones had comparable total radioactivity (*6*). The inhibitory effect of 2-TCA on ^{45}Ca release from experimental half calvaria was calculated as the percentage of ^{45}Ca release inhibited by means of the formula $[(C - T)/C] \times 100$, where C represents ^{45}Ca in medium from the control half calvarium and T represents counts in medium from the half calvarium treated with 2-TCA. It has been demonstrated (*6*) that approximately 35 ± 5 percent of the total release of ^{45}Ca in this system is due to physicochemical exchange between bone and medium and is unaffected by hormones and vitamins. Therefore, the maximum inhibitory effect on a metabolically mediated process can be only 65 ± 5 percent of the total release of ^{45}Ca from the control bones. The results in Table 1 show that at doses as low as 5 to 10 μg/ml, 2-TCA significantly inhibited the cell-mediated ^{45}Ca transfer out of labeled bone.

To study further the inhibitory effect of 2-TCA on mineral loss from bone in culture system B, we cultured unlabeled half calvaria separately with or without 2-TCA. At the end of a 3-day incubation, they were stained by the Von Kossa reaction. The results (Fig. 2B) provide further evidence that 2-TCA inhibited directly the spontaneous resorption of bone in tissue culture.

Microscopic observations of the living culture and histological examination of bone sections showed that 2-TCA, at the dosages and time intervals used here, did not produce any cytotoxic effects, as judged by fibroblastic outgrowth and bone cell morphology.

The hypocalcemic effects of 2-TCA are very similar to those produced by the polypeptide hormone calcitonin (*2, 3*). Our findings demonstrate further the close similarity between the direct actions of 2-TCA and calcitonin on bone in tissue culture (*7*). However, 2-TCA is a simple compound and, therefore, may provide a useful, new pharmacological tool for studies of bone metabolism and calcium transfer.

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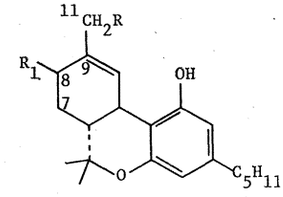
Activity of Δ^8- and Δ^9-Tetrahydrocannabinol and Related Compounds in the Mouse

Abstract. *The 11-hydroxy metabolites of Δ^8- and Δ^9-tetrahydrocannabinol are more active than the parent compounds when administered to mice by either the intravenous or intracerebral route. Both Δ^8- and Δ^9-tetrahydrocannabinol are rapidly and extensively metabolized by the liver and not by the brain. The hypothesis that the 11-hydroxy metabolites may be the active form of tetrahydrocannabinol is discussed.*

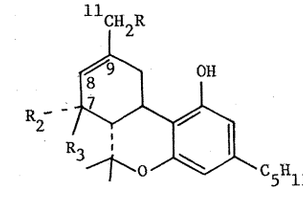
Δ^9-Tetrahydrocannabinol (Δ^9THC) is considered responsible for most of the psychotomimetic effects of hashish and marihuana (*1–3*). *trans*-Δ^9-Tetrahydrocannabinol (Δ^9THC) is the major natural THC, although small amounts of Δ^8THC also occur. The isolation and structural elucidation of two metabolites of Δ^9THC, 11-hydroxy-Δ^9THC and 8,11-dihydroxy-Δ^9THC, have recently been reported (*4*). The former metabolite has also been independently isolated (*5*). Others have reported on the isolation of 11-hydroxy-Δ^8THC (*2, 6*). These findings have been confirmed and the isolation of two additional metabolites of Δ^8THC, 7α- and 7β,11-dihydroxy-Δ^8THC has been reported (*7*). The structures of these compounds are shown below.

The biological activity of the above compounds plus other available cannabinoids, including cannabidiol (CBD), cannabinol (CBN), and $\Delta^{9(11)}$THC (*8*) was compared to that of Δ^9THC in the mouse after intracerebral and intravenous administration (*9*). Although the potency of the compounds varied depending on structure and route of administration (Table 1), similar neurological and behavioral responses were found for all of the substances provided that high concentrations of the less potent compounds were used.

The general overt behavioral pattern is characterized by three progressive stages: (i) irritability; (ii) decrease in spontaneous activity with hypersensitivity to auditory and tactile stimuli; (iii) marked depression of spontaneous activity, decreased awareness, loss of muscle coordination, reduced sensorimotor responses, and a crouched posture and gait similar to that produced by the narcotic analgesics (*10*). Provoked freezing or immobility, a property which is also induced by the narcotic analgesics, was much more pronounced with the Δ^8THC compounds. Hypothermia and decreased respiration rate also occur, and then death by respiratory arrest. When 11-hydroxy-Δ^9THC was administered intracerebrally, measurable behavioral alterations were observed at doses of



Δ^9THC, R = R_1 = H
11-hydroxy-Δ^9THC, R = OH, R_1 = H
8,11-dihydroxy-Δ^9THC, R = R_1 = OH



Δ^8THC, R = R_2 = R_3 = H
11-hydroxy-Δ^8THC, R = OH;
R_2 = R_3 = H
7α,11-dihydroxy-Δ^8THC, R = R_2 = OH;
R_3 = H
7β,11-dihydroxy-Δ^8THC, R = OH;
R_2 = H; R_3 = OH