pig chromosomes. Significantly, very few guinea pig chromosomes were visible in these preparations and the human chromosome content of the hybrids was somewhat less than that of the HeLa cell line.

Two principal interpretations of these data were considered. (i) The genetic information derived from C4-deficient guinea pig cells may have been capable of initiating C4 synthesis by cells (HeLa) in which this function had not previously been expressed. This would indicate that the genetic abnormality responsible for C4 deficiency is probably not a result of repression of gene function, but rather is a consequence of an inability to respond to an initiator signal, that is, perhaps due to a structural gene abnormality. (ii) Alternatively, it is possible that fusion of guinea pig and HeLa cells led to the loss of a chromosome bearing information for the repression of C4 synthesis, but not loss of the chromosome bearing the C4 gene. A loss of repressor might then permit expression of human C4 gene function. It is not yet possible to distinguish between these two alternatives.

Levy and Ladda (*12*) have obtained indirect evidence that genetic information required for the production of C5 could be incorporated into cells obtained from C5-deficient mice. These cells, hybrids of C5-deficient cells with chicken erythrocytes, when administered to the deficient mice, led to a temporary restoration of hemolytic complement activity in the serums of the recipients. Unfortunately, the gene product (C5) was not directly identified, nor was it possible to determine whether genetic information derived from the deficient or the intact cell (chicken erythrocyte) was directing the synthesis of C5. Our experiments permit identification of the gene product (C4), as well as the functional and antigenic species-specific characteristics of the C4. A definition of the precise genetic lesion responsible for inherited deficiencies of plasma proteins requires specific identification of the gene product. Ultimately, correction of the genetic abnormality would then be possible.

HARVEY R. COLTEN
ROBERTSON PARKMAN
Immunology Division, Department of Medicine, Children's Hospital Medical Center, and *Department of Pediatrics, Harvard Medical School, Boston, Massachusetts 02115*

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Carbonic Anhydrase and Bone Remodeling: Sulfonamide Inhibition of Bone Resorption in Organ Culture

Abstract. *Five sulfonamide inhibitors of carbonic anhydrase inhibited parathyroid hormone–induced resorption of bone in organ culture. The relative activities of the sulfonamides as resorption inhibitors were such as to suggest the presence of a functional carbonic anhydrase system in bone linked to the mechanism of bone resorption.*

The enzyme carbonic anhydrase (CA) is widely distributed in nature. In mammals it serves two general physiological roles. The first is in respiration, where it functions in erythrocytes to catalyze the uptake and release of carbon dioxide. The other function is in various secretory organs (for example, kidney, stomach, pancreas, ciliary process), where it is involved with the

Table 1. The effect of sulfonamide inhibitors of carbonic anhydrase on PTH-induced resorption in organ cultures of bone. Resorption was assessed by measuring the transfer of ^{45}Ca from bone to medium. Percentage inhibition was calculated by using the formula $(P - I/Pc) \times 100$, where P and I represent the radioactivity found in culture mediums treated with PTH (P) and PTH plus inhibitor (I), respectively. The denominator (Pc) estimates the isotope found in the medium of PTH-treated cultures due to cell-mediated activity corrected for physicochemical exchange and crystal dissolution as discussed previously (*8*). The mean and standard error of the mean of four to eight observations are given in column three.

Inhibitor	Molar concentration	Percent inhibition of PTH-induced resorption
Sulfanilamide	1×10^{-2}	68 ± 4
	1×10^{-3}	35 ± 3
	1×10^{-4}	1 ± 8
Acetazolamide	4.5×10^{-4}	98 ± 1
	1×10^{-4}	92 ± 3
	4.5×10^{-5}	59 ± 2
	1×10^{-5}	17 ± 5
	4.5×10^{-6}	0
Methazolamide	1×10^{-3}	96 ± 2
	1×10^{-4}	87 ± 4
	1×10^{-5}	29 ± 4
	1×10^{-6}	9 ± 4
Benzolamide	1×10^{-3}	93 ± 2
	1×10^{-4}	49 ± 6
	1×10^{-5}	6 ± 1
Ethoxzolamide	1×10^{-5}	78 ± 3
	2.5×10^{-6}	34 ± 2
	1×10^{-6}	5 ± 3
	1×10^{-7}	12 ± 1

Table 2. Comparison of the bone resorption inhibitory activity of sulfonamides with their carbonic anhydrase inhibitory activity in the kidney and eye. Concentrations for 50 percent inhibition of resorption were calculated from data in Table 1 and corrected for tissue binding to estimate free inhibitor (*1*). Data for eye and kidney were taken from Maren (*1*), and activities relative to SAA were calculated as discussed above (*9*).

Sulfonamide	Molar concentration of inhibitor for 50 percent inhibition of resorption		Activity relative to SAA for 50 percent physiological effect		
	Total inhibitor	Free inhibitor	Bone	Eye	Kidney
Ethoxzolamide	4.2×10^{-6}	2.10×10^{-7}	12,850	8700	6700
Methazolamide	2.3×10^{-5}	1.04×10^{-5}	260	145	182
Sulfanilamide	3.0×10^{-3}	2.70×10^{-3}	1	1	1

transfer or accumulation of hydrogen and bicarbonate ion (*1*).

Dulce *et al.* (*2*) have postulated that CA could aid in the mineralization and demineralization of bone in chickens by controlling the local secretion of hydrogen ions. Attempts to implicate a role for this enzyme in mammalian bone by Maren and his group have led them to the conclusion that although it can be measured in epiphyseal areas of bone it is not involved in bone remodeling and is probably present only in cells destined to become hemopoietic (*3*). However, studies by Waite and his colleagues (*4*) have demonstrated a *hypo* calcemic response to the CA inhibitor acetazolamide in nephrectomized rats, as well as an inhibition of the *hyper* calcemia induced by parathyroid hormone (PTH) or dibutyryl-3′,5′-cyclic adenosine monophosphate. It is also of interest that acetazolamide has been shown to inhibit the *hypo* calcemic response to calcitonin in rats (*5*). Finally, Cuervo *et al.* (*6*) in an elegant ultramicroanalysis of calcifying sites in cartilage of young rats have reported significant levels of CA activity in the secondary spongiosa of the metaphysis. Our interest in mechanisms of bone resorption led us to examine the role of CA by using organ cultures of bone, and we now present evidence that a series of aromatic sulfonamides known to inhibit CA activity in vitro as well as in vivo can inhibit bone resorption induced by PTH in organ cultures of newborn mouse calvaria.

An organ culture system for studying inhibitors of bone resorption has been modified from that reported previously (*7, 8*). Swiss albino mice were labeled with ^{45}Ca by injection with 10 μc of isotope 1 day after birth. Four days later the calvaria (frontal and parietal bones) were removed aseptically and affixed to a glass cover slip with a mixture of chicken plasma and chicken embryo extract (2 : 1), placed in the well of a Leighton culture tube, and fed with 2 ml of culture medium. The tubes were flushed with a gas mixture of oxygen and nitrogen (1 : 1), stoppered, and incubated at 37°C in a rotating culture drum. Cultures were treated for 48 hours with medium containing Gey's balanced salt solution (30 percent) and heated (56°C, 30 minutes) horse serum (70 percent) to which was added 0.1 unit of PTH (Parathyroid Injection, Lilly) per milliliter. This 48-hour period allowed for the achievement of maximal stimulation of resorption by PTH as well as for "washout" of nonincorporated ^{45}Ca from the bones. The culture media were then harvested, samples were taken for liquid scintillation spectrometry, and the cultures were refed media as described above containing identical amounts of PTH but in addition were supplemented with varying concentrations of a series of sulfonamides (sulfanilamide, acetazolamide, ethoxzolamide, methazolamide, benzolamide) known to possess CA inhibitory activity. The cultures were gassed and incubated as before for an additional 48 hours, at which time samples of the medium were taken and the bones were removed, placed in 0.5 ml of formic acid (98 percent) and analyzed for ^{45}Ca by liquid scintillation spectrometry.

The results of these experiments are presented in Table 1. Clearly, all compounds inhibit the release of ^{45}Ca stimulated by 0.1 unit of PTH, and a log-dose response relationship for each compound can be demonstrated. The relative effectiveness of these compounds in inhibiting resorption can be assessed by first calculating the molar concentration which produces a 50 percent inhibition of the PTH-induced resorption ($C.I._{50}$) and then expressing these values as activity ratios relative to sulfanilamide (*9*). These calculations are presented in Table 2 and compared with data published for the effect of sulfonamides on inhibition of CA activity in the eye and kidney (*1*). The concentration of free inhibitor was estimated by correcting for tissue binding by using information reported by Maren (*1*). There is a marked similarity between our results on inhibition of bone resorption and those of inhibition of CA activity in the eye and kidney which suggest the presence of a functional CA system in bone. However, it is also possible that these results could be due to some other system, as yet unidentified, which is inhibited by sulfonamides and which is part of the mechanism of bone resorption.

Some preliminary experiments were also conducted to assess the morphological appearance of bones treated with acetazolamide in an organ culture system which demonstrates osteoid formation (*7*). Light microscopic observations indicated no obvious differences between treated and control cultures with respect to proliferation of bone-forming cells or degree of osteoid formation. It is therefore unlikely that the resorption inhibition reported with acetazolamide is due to toxicity or to a decrease in formation.

We have presented evidence that certain carbonic anhydrase inhibitors can inhibit bone resorption in vitro. A comparison of the relative effectiveness of the CA inhibitors on bone resorption with the inhibition of CA in the eye and kidney suggests that carbonic anhydrase is present in these bones and somehow is involved in the mechanism of bone resorption. This suggestion is supported by recent evidence (*6*) for the presence of measurable enzyme activity in rat bone as well as work demonstrating inhibition of bone resorption in rats (*4*).

If CA is present in bone cells it could be expected to augment the rate of output of hydrogen ion. Hitherto mechanisms involving citrate or lactate production, or both, have been postulated to account for the concentrations of hydrogen ion necessary for bone demineralization as well as lysosomal enzyme action on bone matrix (*10–13*). Heersche (*11*) has measured lactate production in organ culture experiments and has shown that although calcitonin treatment completely inhibited bone demineralization, the lactate production was either partially inhibited or not at all affected. Calculations of citrate accumulations in similar systems

(*10*) also have not accounted for the degree of demineralization reported. Carbonic anhydrase, on the other hand, has been shown to be intimately involved with hydrogen ion secretion in other organ systems (*1*), and if it is present in bone, as the data presented above suggest, then it may function as a mechanism for hydrogen ion secretion in areas undergoing bone resorption. In each of the secretory systems which involve CA the fundamental process of "OH-separation" is linked with another specific process involving the transport of a cation or another anion (*14*). It will be worthwhile to examine the relationship of CA activity to the influx and efflux of calcium in bone cells in view of recent suggestions regarding the role of ionic calcium in hormonal regulatory mechanisms mediated by cyclic adenosine monophosphate (*15*).

CEDRIC MINKIN
JOAN M. JENNINGS
Department of Periodontology, Harvard School of Dental Medicine, Boston, Massachusetts 02115

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Reversion of Murine Sarcoma Virus Transformed Mouse Cells: Variants without a Rescuable Sarcoma Virus

Abstract. *Murine sarcoma virus transformed mouse 3T3 cells, which are negative for murine leukemia virus and which yield sarcoma virus after superinfection with murine leukemia virus, spontaneously give rise to flat variants from which murine sarcoma virus can no longer be rescued. The revertants support leukemia virus growth and show an enhanced sensitivity to murine sarcoma superinfection and, like normal cells, do not release RNA-dependent DNA polymerase activity. Because revertants could be obtained with high frequency from progeny of single transformed cells, each cell that contains the sarcoma virus genome seems to have the capacity to suppress or eliminate an RNA tumor virus native to its species of origin.*

Murine sarcoma virus (MSV) can both transform and replicate in mouse cells in the presence of murine leukemia virus (MuLV), but can only transform them in its absence (*1–3*). Mouse cells that have been infected and morphologically altered by MSV in the absence of MuLV have been isolated and are readily detected by both their altered morphology and the presence of a rescuable MSV genome (*2, 3*). Some of these MSV-transformed cell lines originating from soft agar colonies have been named sarcoma-positive leukemia-negative (S+L−) cells (*2*). These S+L− cells contain no infectious MSV or MuLV but release small amounts of "C"-type particles and some but not all MuLV group-specific antigens (*4*). Superinfection of S+L− cells with MuLV leads to a rescue of MSV and replication of MuLV and serves as a simple, rapid assay for MuLV (*5*). During extended cultivation of single colonies of S+L− cells in liquid medium, we observed the appearance of flat variant cells which, when isolated, had many properties of untransformed 3T3 cells and from which MSV could no longer be rescued by MuLV. We now describe the morphology, frequency of occurrence, and some of the special features of these revertant cells. This seems to be the first observation that cells transformed by an oncornavirus native to the species can revert in their morphology and function to resemble normal cells.

Table 1. Frequency of occurrence of flat, low density, S+L− cell-derived clones which were negative for rescue of MSV. The designation of the cell lines refers to clonally derived cells from single agar colonies. The derived cells were grown in liquid medium and the subline numbers refer to clonal isolates tested in different laboratories over an 18-month test period. Rescue of the MSV genome was tested by passing the clone to obtain several dishes with 10^5 cells per 20-cm² dish. These were infected with MuLV (1 to 3 FIU per cell) and followed for characteristic changes. Supernatants of infected cultures were verified by the focus assay for the presence of free MSV in 3T3 cells with optimum helper MuLV. The cell density was determined at or beyond confluence, but the medium was not changed. Not every S+L− or revertant subline was tested. The values represent an average of at least two representative determinations; NT, not tested.

Cloned subline	Clones tested	Clones positive for MSV rescue	Maximal density (No./cm²)	
			S+L− cells	Sarcoma-negative cells
		EC-10 cell line		
FG-10	40	11/40	1.9×10^5	3.2×10^4
FG-10T	11	5/11	2.8×10^5	3.5×10^4
FG-10M	4	4/4	$0.7–2.4 \times 10^5$	
FG-10-0	19	15/19	2.2×10^5	4.9×10^4
Flat variant of FG-10T	7	0/7		4.2×10^4
		3-19 cell line		
3-19-7	25	25/25	2.4×10^5	
3-19-2T	10	10/10	2.7×10^5	
3-19-6T	21	20/21	2.7×10^5	4.4×10^4
2-43-3H	14	14/14	NT	NT
3-197-3	239	238/239	2.4×10^5	1.5×10^4
Flat variant "R9" of 3-19-6T	9	0/9		4.3×10^4
Flat variant "D" of 3-197-3	27	0/27		3.0×10^4