but not bacterial systems. In inhibiting sensitive systems, cycloheximide, acetoxycycloheximide, and chloramphenicol display the following common features: (i) protein synthesis is suppressed in intact cells before any effect on RNA synthesis appears; (ii) inhibition of protein synthesis is reversible; (iii) transfer of amino acid from sRNA to polypeptide is inhibited; (iv) the release of nascent polypeptide chains is not accelerated.

Both cycloheximide and its acetoxy derivative are unique in their high specificity for mammalian and yeast systems. They differ from puromycin in their mechanism of action and they are useful alternatives to puromycin when it is desirable to inhibit the synthesis of protein but not of RNA.

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6. Abbreviations: sRNA, soluble RNA or transfer RNA; ATP, adenosine triphosphate; GTP, guanosine triphosphate; polyU, polyuridylic acid; polyAC, random copolymer of adenylic and cytidylic acids; TCA, trichloroacetic acid; S-100 and P-100, supernatant and pellet fractions, respectively, after two centrifugations for two hours at 100,000*g*.
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Bone Cells: Biochemical and Biological Studies after Enzymatic Isolation

Abstract. *Short-term incubation of rat calvaria in buffered crude collagenase permitted the isolation of morphologically intact cells that absorb vital dyes, contain alkaline phosphatase, and multiply in tissue culture. Freshly harvested cells were similar to whole bone segments in aerobic glucose metabolism.*

Because calcified and noncalcified connective tissue interfere with the isolation of bone cells, almost all information concerning the metabolism of bone in vitro has been derived from the use of bone slices and segments in tissue culture or in short-term incubation. Although identifiable bone cells emigrate from bone explants in tissue culture chambers (*1*), the number of cells is inadequate for many metabolic studies. Collagenase preparations have been used to digest tissues and disperse cells effectively (*2*) without altering cell function (*3*). This report describes the dispersion of viable cells from rat bones by crude collagenase and the comparison of glucose metabolism by these cells and by intact bone.

For the isolation of cells, frontal and parietal bones were obtained under aseptic conditions from the calvaria of 17- to 21-day-old rat fetuses and newborn rats and were cleaned of superficial periosteum, dura, and cartilage along the major sutures. Approximately 10 calvaria (100 mg) were fragmented, placed in siliconized 25-ml erlenmeyer flasks containing 4.0 ml of tris-buffered saline (*p*H 7.4), glucose (5 μmole/ml), penicillin-streptomycin [50 units per milliliter (*4*)], and crude collagenase [0.1 to 6.0 mg/ml (*5*)], and shaken 90 times per minute in a Dubnoff incubator at 37°C for periods ranging from 90 to 180 minutes. After incubation, the medium was decanted into siliconized 15-ml culture tubes and centrifuged at 400*g* for 1 to 3 minutes. The pellet of cells that was obtained was washed with medium free of enzymes and centrifuged three times. Cells were finally suspended in the same medium and standardized by counting the cells in a hemocytometer.

Crude collagenase dispersed 20,000 to 50,000 cells/mg of bone from fetal and newborn rat calvaria into the incubation medium. Optimum cell release was obtained by exposure to enzyme concentrations of 1 to 3 mg/ml for 90 minutes. Histologically, collagenase-treated bone showed areas of complete cell removal, suggesting that in those areas affected by the enzyme, there was no selective removal of cell types. Centrifuged pellets contained only intact mononuclear cells. No definite hematopoietic marrow cells were recognized in coronal sections of undigested bone segments or in the cell pellets. As an indication of cell type, alkaline phosphatase activity of isolated cells was estimated histochemically by a modification of the method of Kaplow (*6*), with the use of sodium alpha naphthyl phosphate and Diazo Blue B (*7*). All cells contained detectable cytoplasmic or nuclear alkaline phosphatase, or both, and approximately 50 percent of the cells showed an intense cytoplasmic reaction (Fig. 1). Pronase (*8*), 1.0 mg/ml, produced a similar cell yield, but many cells were damaged or ruptured. Trypsin (Difco), 2.5 to 5.0 percent, without divalent cations in the incubation mixture released an insignificant number of cells.

Viability of harvested cells isolated by collagenase treatment was evaluated by vital dye uptake and multiplication in tissue culture. Over 95 percent of isolated cells appeared to be viable as evidenced by cytoplasmic staining after incubation for 30 minutes at 37°C in buffered 0.01 percent neutral red (Fig. 2). Cells suspended in standard Eagle's No. 2 medium with 20 percent fetal calf serum, glutamine at 0.25 mg/ml, and

Table 1. Glucose metabolism by isolated cells. Data are expressed as percent of glucose in the medium converted to $C^{14}O_2$ or to lactic acid-C^{14} by 10^6 isolated cells in two hours. Each figure represents the mean of four flasks. In repeat experiments relative yields of carbon dioxide and lactic acid from glucose-U-C^{14}, glucose-1-C^{14}, and glucose-6-C^{14} were constant, although small differences in absolute values were noted.

Substrate		
Glucose-U-C^{14}	Glucose-1-C^{14}	Glucose-6-C^{14}
	$C^{14}O_2$	
0.12 ± 0.02	0.20 ± 0.02	0.07 ± 0.01
	Lactic acid-C^{14}	
3.30 ± 0.18	2.56 ± 0.17	3.67 ± 0.26

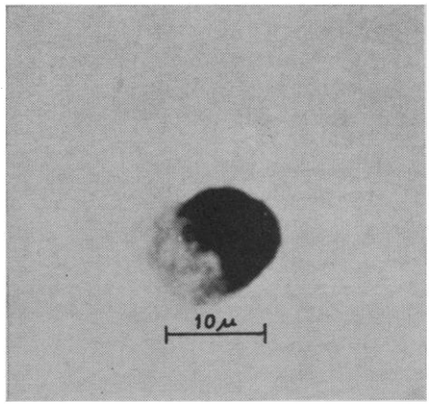

Fig. 1. Cytoplasmic alkaline phosphatase reaction of an isolated cell.

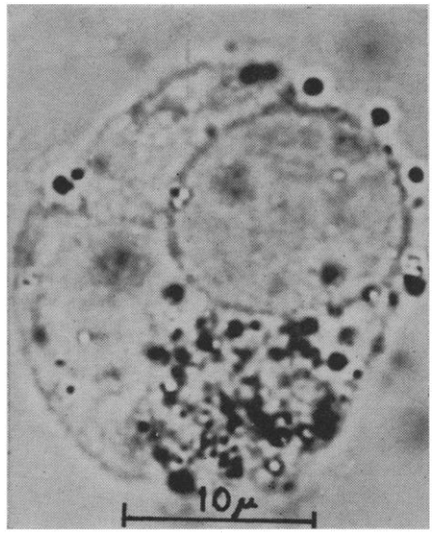

Fig. 2. Particles of neutral red within cytoplasm of an isolated cell.

penicillin-streptomycin at 50 units per milliliter, were incubated at 37°C in falcon plastic tissue culture vials. Cultures were examined daily and fed with newly prepared medium every 3 to 5 days. Subcultures were achieved by trypsinization of monolayers, and cell counts were done at each transfer. Tissue culture multiplication from cell isolates was apparent within 24 hours, and repeated subculturing was required as monolayers rapidly developed and separated from the bottom of the vial. Cell counts (after trypsinization) disclosed a threefold increase in cell population per week. No evidence of bone formation has been noted after one month in Eagle's medium. While round and stellate-shaped cells were noted in primary cultures, all cells assumed a stellate appearance after subculturing.

Isolated cells were characterized further by studies of glucose metabolism. Washed cells were suspended in Krebs-Ringer bicarbonate buffer, *p*H 7.4, with either 95 percent oxygen–5 percent carbon dioxide or air as the gas phase. An inoculum of known cell number was added to each 25-ml polyethylene flask containing buffer, glucose at 1 to 3 μmole/ml, and uniformly labeled glucose-C^{14}, glucose-1-C^{14}, or glucose-6-C^{14}, 0.5 to 1.0 mc/ml (*9*). After 2 to 4 hours' incubation with gentle shaking at 37°C, evolved $C^{14}O_2$ was trapped in hyamine hydroxide and counted in a liquid scintillation counter (*3*). After the addition of carrier lactic acid, radioactive lactic acid was isolated by ascending thin layer chromatography on cellulose plates (800 μ thick) developed with the upper phase of an ethyl acetate:water:formic acid (60:35:5) solvent system. Lactic acid spots were detected with bromcresol green spray and exposure to ammonium hydroxide vapor. Spots were then removed from the plates, placed in 10 ml of Bray's solution (*10*), and counted as above. Internal standards were added to account for quenching.

Inocula of 10^6 cells converted approximately 0.2 percent of medium glucose to carbon dioxide and 3.0 percent to lactic acid within two hours. While the release of $C^{14}O_2$ from glucose-1-C^{14} was three times that from glucose-6-C^{14}, there was 30 percent more lactic acid than $C^{14}O_2$ from glucose-6-C^{14} (Table 1). Substitution of air for 95 percent oxygen–5 percent carbon dioxide produced a 50 percent reduction in oxidation of glucose to carbon dioxide with a concomitant increase in lactic acid production. Oxidation was slightly less in Krebs phosphate buffer. Studies of glucose metabolism of cleaned, intact calvaria gave similar results, in agreement with the observations of Cohn and Forscher, which indicated that lactic acid is a principal end product of aerobic glucose metabolism by bone slices in vitro and qualitatively demonstrated significant hexose monophosphate shunt activity (*11*). Thus the impression gained by histologic examination, that cells isolated by collagenase treatment are representative of the entire cell population in the parent bone, is fortified by the similarity in the metabolism of glucose between whole bone segments and isolated cells.

Although we have demonstrated cell viability, it has not been possible to prevent dedifferentiation or overgrowth of fibroblasts in tissue culture, or both, and no bone formation has been noted. The presence of considerable alkaline phosphatase in the cytoplasm of many cells provides some evidence that these cells were osteoblasts or osteocytes (*12*).

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N^6-Benzyladenine: Inhibitor of Respiratory Kinases

Abstract. N^6-*benzyladenine, an active phytokinin, inhibits the respiration of many explants. The chemical also delays senescence and extends the postharvest life of many green or leafy vegetables. Evidence is presented that these phenomena may be linked to a competitive inhibition of the glycolytic kinases by* N^6-*benzyladenine.*

Treatment with N^6-benzyladenine delays the breakdown of certain leafy or green vegetables after they have been harvested (*1*). There is a greater retention of chlorophyll and carotene, less loss in dry and fresh weights, and an apparent delay in the onset of senescence. This has been attributed to an inhibition of the overall respiratory rates of the explants as measured by the uptake of oxygen and the evolution of carbon dioxide (*2*). Phosphorylation of hexoses in excised leaves and heads of broccoli is also greatly in-