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RESPIRATORY ACTIVITY OF ISOLATED CHONDROCYTES WITH A MINIATURIZED OXYGEN ELECTRODE SYSTEM[1]

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Abstract. A technique for the isolation of chondrocytes from the articular cartilage of rabbits was modified and improved to yield 5 to 20 x 10^6 viable cells per preparation. A YSI Model 5331 O_2 sensor was modified so that it could rapidly respond in as little as 1 ml of medium. Mean oxygen uptake of cell samples showed that chondrocytes obtained from mature rabbits (1.33 μl $O_2/10^7$ cells/hr) had a higher oxidative activity than chondrocytes from immature rabbits (0.8 μl $O_2/10^7$ cells/hr). Elevation of the incubation temperature from 25 °C to 35 °C increased the chondrocyte oxygen uptake approximately 20% but incubation at 37 °C tended to decrease oxygen uptake. It is evident that articular chondrocyte cells have a real, but fairly low, temperature sensitive oxidative metabolism.

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It has long been assumed that the energy requirements of cartilage are met primarily through anaerobic pathways (Bywaters 1937, Rosenthal *et al* 1942a, b). During the past decade, interest in the study of articular cartilage has increased because of the development of better fractionation and biochemical methods. Mankin and Orlic (1964) indicated that there were well-developed anaerobic pathways in cartilage cells because of their tolerance to potassium cyanide and the minimal effects of short periods of oxygen deprivation. More recently, Fine and Person (1970) demonstrated the cytochrome activity of chondrocytes and several investigators have suggested the presence of aerobic metabolic activity by the cells. Part of the difficulty in assaying oxidative activity of chondrocytes was the difficulty of obtaining viable intact cells for investigation from the cartilagenous matrix. Kuroda (1964), Kawiak *et al* (1965) and Manning and Bonner (1967) pioneered the development of digestion procedures that yielded viable chondrocytes, and Green (1967) described a technique for the isolation of large numbers of chondrocytes from cartilage sections. Green suggested that further improvements in isolation techniques would make it possible to isolate sufficient cells for metabolic studies *in vitro*. His suggestion led us to the present investigation of improvements in digestion techniques and miniaturization of the YSI oxygen sensor system to allow oxygen uptake studies of as little as 10^6 viable isolated chondrocytes.

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MATERIALS AND METHODS

Male and non-pregnant female New Zealand rabbits, classed as mature (6-10 months old) or immature (6-10 weeks old), were used. Each rabbit was sacrificed by rapid injection of 100-150 cc of air into an ear vein. The leg area was shaved, washed, and prepared with povidone-iodine (Betadine) solution, and draped with sterile towels. The proximal tibia, distal and proximal femoral articular surface, proximal humerus, and glenoid fossa were removed from each animal with sterility maintained in this and all subsequent procedures.

Cartilage shavings were removed from each articular surface and placed into a sterile Petri dish containing Gey's balanced salt solution (GBSS, Microbiological Associates #10-505). When all surfaces had been stripped, the GBSS was decanted and 15 ml of 0.05% testicular hyaluronidase in GBSS was added. The dish was then rocked gently by hand for 4 min at room temperature. The hyaluronidase solution was removed with a sterile syringe and the softened cartilage diced into fragments 1-2 mm square, washed with GBSS, and then placed into the inner compartment of the digestion chamber (figure 1B). After addition of 4 ml

of trypsin solution (0.02% in GBSS), the unit was incubated at 37 °C for 0.5 hr while being magnetically stirred. The trypsin was then removed and 4 ml of sterile collagenase (0.02% in GBSS) was added to the digestion chamber which was then replaced in the incubator and stirred for an additional 90, 120, or 150 min. After the collagenase incubation, the supernatant was removed and centrifuged at 2500xg for 5 min. The cells obtained were resuspended in 1 ml of Delbecco's Modified Eagle Medium (DEM, Microbiological Associates #12–70) and fresh collagenase solution (4 ml) was added to the chamber for an additional 0.5 hr incubation. The cells obtained after this second incubation also were resuspended in 1 ml of DEM. All enzyme preparations were made immediately prior to use by addition of GBSS to previously weighed quantities of the crystalline enzyme. Cell counts and Trypan Blue exclusion tests were performed on sub-samples of both cell suspensions to determine the number of viable cells. Chondrocyte samples were kept on ice until temperature equilibrated for the oxygen electrode studies.

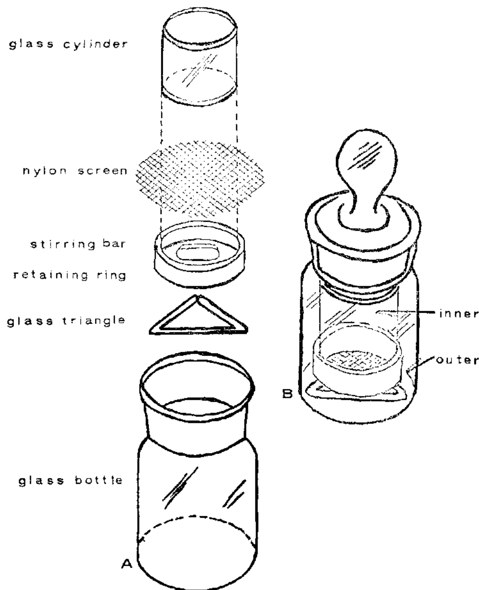

FIGURE 1. A. Exploded diagram of the digestion chamber. B. Assembled 2 compartment digestion vessel.

Digestion Chamber

A 2-compartment digestion chamber was constructed based on designs suggested by Kawiak *et al* (1965) and Green (1967) and patterned after a model seen at the Orthopedic Research Laboratory of the Robert Breck Brigham Hospital in Boston. The unit uses a 25 x 40 mm glass weighing bottle as the outer compartment and an 18 mm (OD) by 12 mm high glass tube to form an inner compartment (fig. 1A, B). The 45 μm pore-size nylon mesh screen is held in position by a nylon retaining ring milled to approximately 19 mm inside diameter and a teflon-coated stirring bar is placed within the glass triangle that supports the inner compartment (fig. 1B). Cartilage shavings were placed into the inner compartment and enzyme solutions were added and removed from the outer compartment with sterile syringes.

Oxygen Uptake Measurements

Cells obtained from the digestion chamber were resuspended in 3 ml of DEM (with L-glutamine, fetal bovine serum and penicillin-streptomycin added) for oxygen uptake measurements with a YSI model 53 Biological O_2 Monitor (Lessler and Brierley 1969). Preliminary experiments using 10^6 chondrocytes/ml in 3 ml of reaction medium indicated that an increase in sensitivity of the instrument was needed. A threefold increase in sensitivity was achieved by milling the oxygen electrode lucite holder to fit into a 12.5 mm (ID) reaction vessel made from standard precision pyrex tubing and modifying the top of the YSI 5301 constant temperature bath to accommodate the small reaction vessels (see fig. 2). This enabled us to work in a stirred volume as small as 1 ml, effectively increasing the response time of the electrode because oxygen depletion was more rapid in the smaller volume. A magnetic stirring bar on a synchronous motor was positioned below the modified water bath and a teflon covered 3/8 x 1/8 inch stirring bar (A. H. Thomas No. 9235–V8) was used to stir the chamber. A Haake Constant Temperature circulating unit was used to control the temperature in the reaction vessels at ±0.02 °C.

The effect of temperature on the respiration of cells obtained from 6 to 8 week old rabbits was studied by testing 2 samples of cells from the same animal simultaneously with 2 separate oxygen electrode systems. System 1 was set initially at 25 °C and System 2 was set at 35 °C. At the end of the initial test period, the temperature of System 1 was raised to 35 °C and System 2 was raised to 37 °C. The samples and oxygen electrode probes were re-equilibrated for 5 minutes at the new temperatures before oxygen measurements were made (Lessler 1972). In this manner, each preparation served as its own control. Cell counts were done on each sample of freshly isolated chondrocytes using a Spencer Bright-line hemocytometer. Estimates of cell viability were obtained by testing subsamples of each suspension for 0.25% Trypan Blue exclusion (McLimans *et al* 1957).

RESULTS

The technique of sequential hyaluronidase, trypsin, and collagenase digestion was found to free viable chondrocytes from the matrix of the cartilage specimens. Initial experiments with the oxygen electrode system indicated that approximately 2 x 10^6 cells/ml were required for good oxygen uptake measure-

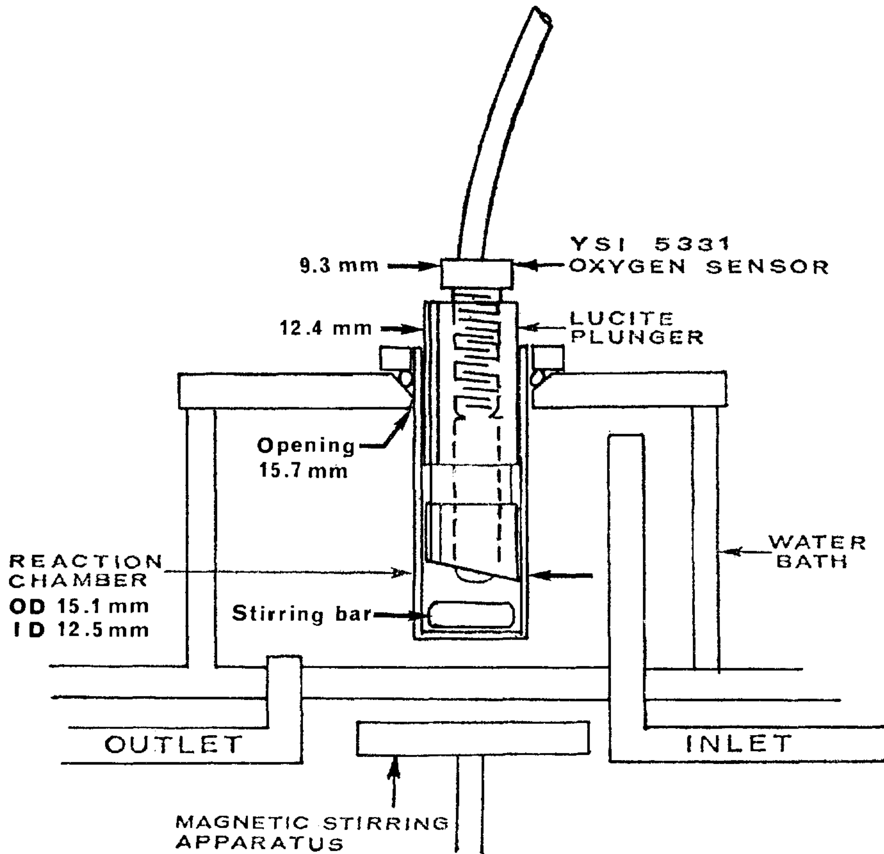

FIGURE 2. Dimentions of the miniaturized reaction vessel and YSI model 5331 oxygen sensor holder in a modified constant temperature water bath..

ments. For this reason, several modifications were made in the isolation techniques described by Green (1967). The initial collagenase digestion period was lengthened from 60 to 120 min and the collagenase solution, after the initial digestion period, was replaced with fresh collagenase solution for a second 30 min digestion period. Phase contrast microscopic studies of the partly digested material remaining on the nylon screen of the inner compartment of the digestion chamber (see fig. 1) revealed that many cells remained trapped in the mesh or were loosely embedded in matrix at the end of the collagenase digestion. When the inner compartment was rinsed with GBSS following removal of the last enzyme solution, many additional cells were added to the suspension of cells obtained during the enzyme digestion.

These improvements in technique made it possible to obtain samples of 5 to 20 x 10^6 chondrocytes from each preparation (see table 1). Yields of viable cartilage cells were progressively increased by lengthening the digestion period. Cartilage obtained from immature rabbits yielded larger numbers of chondrocytes than that of mature animals. Respiratory studies (table 2), however, indicated that cells obtained from immature rabbit cartilage had a significantly ($P<0.01$) lower mean oxygen uptake (0.81 ± 0.14 μl $O_2/10^7$ cells/hr) as compared to chondrocytes obtained from mature cartilage (1.33 ± 0.16 μl $O_2/10^7$ cells/hr).

When free of the intracellular matrix, chondrocytes examined under the microscope appeared to have a spherical shape. Highly refractive cytoplasmic vacuoles were prominent as well as a clearly defined

TABLE 1
Cell yields from sequential enzymatic digestion of rabbit articular cartilage.

Prep.	Age	Total Cells (x 10^6) At End of Digestion Period		
		90 min	120 min	150 min
1	Mature	2.7	5.0	—
2	Immature	3.0	5.0	—
3	Mature	—	6.0	—
4	Immature	—	8.5	17.5
5	Immature	—	6.0	15.0
6	Immature	—	3.5	20.5
	Mean±SD	2.9±0.2	5.7±1.6	17.7=2.2

nucleus with a large nucleolus (see figs. 3 and 4).

Exclusion of 0.25% Trypan Blue was used as a criterion of cell viability. Freshly suspended samples of isolated chondroyctes excluded the dye in more than 95% of the cells, but after prolonged exposure to the dye, cytolysis was observed and many more stained cells became evident. If cells were held overnight in sterile growth medium at 4 °C, straining occurred in approximately 50% of the cells.

Fresh suspensions of chondrocytes consume small but measureable quantities of oxygen. The micro-modification of the YSI 5331 oxygen sensor enabled us to measure this activity in 1 ml of medium and was capable of measuring the oxygen uptake of as few as 2.5 x 10^6 isolated chondrocytes. Because of its greater cellularity, cartilage from 6 to 8 week old rabbits was used for the majority of the studies. The respiratory activity of these cells, though less than that of cells from mature animals, was well within the range of measurement for the miniaturized O_2-electrode system. The mean oxygen uptake of chondrocytes increased by approximately 30% when the temperature was raised from 25 °C to 35 °C (fig. 5). Elevating the incubation temperature to 37 °C, however, caused a decrease in the observed oxygen uptake to about the level observed at 25 °C.

DISCUSSION

Techniques for the isolation of chondro-

TABLE 2
*Oxygen consumption of chondrocyte suspensions in DEM.**

Rabbit No.	Age	Incubation Temp. °C	No. Cells/μl (x 10^6)	Oxygen Uptake (μl Oxygen/10^7 cells/hr)
1	Mature	37	2.5	1.2
	Mature	37	2.5	1.6
2	Immature	37	5.0	1.0
3	Mature	25	6	1.2
4	Immature	25	8	0.75
	Immature	35	9	0.91
	Immature	37	9	0.61
5	Immature	25	9	0.60
	Immature	35	6	0.86
	Immature	37	6	0.86
6	Immature	25	10	0.84
	Immature	35	10	0.90
	Immature	35	10	1.03
	Immature	37	10	0.61

*Measurements in Delbecco's Modified Eagle Medium (DEM).

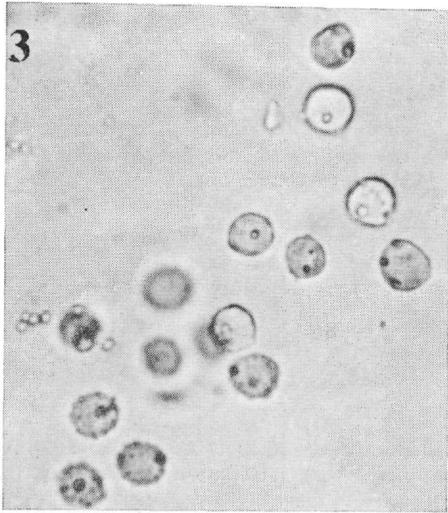

FIGURE 3. Wet mount preparation of matrix free chondrocytes (x100). The cells have assumed a spherical configuration with dark intracytoplasmic vacuoles. The nucleus is not seen well at this magnification.

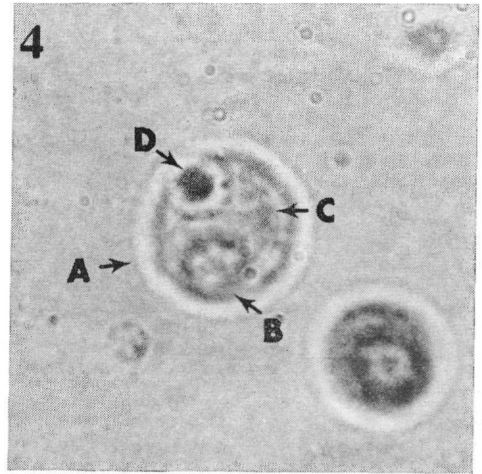

FIGURE 4. Oil-immersion phase-contrast image of living chondroyctes (x800). A=plasma membrane, B=nucleus and nucleolus, C=coarsely granular cytoplasm, D=vacuole.

cytes as previously reported (Kuroda 1964 and Kawiak *et al* 1965) gave cell yields that were too low to permit direct measurement of oxygen uptake with a standard YSI Oxygen Monitor. Green's (1967) introduction of the technique of sequential hyaluronidase, trypsin and collagenase digestion allowed him to get more than 1 x 10^6 cells from pooled articular cartilage surfaces. In our work, the combination of fresh enzyme solution and prolongation of the digestion time greatly increased the yield of viable chondryocytes (see table 1). Microscopic analysis of the residue remaining in the digestion vessel after removal of the last enzyme solution showed the presence of substantial numbers of chondrocytes loosely caught in the remaining softened matrix and enmeshed in the nylon gauze. When we added a final rinse to the technique, we were able to free many of these enmeshed cells.

The articular cartilage of immature animals has been reported to have a higher cell population than cartilage of mature animals (Rosenthal *et al* 1942a, b; Manning and Bonner 1967). The results obtained in our study support those findings. Cell yields of 3 to 4 times that of mature animals were obtained from

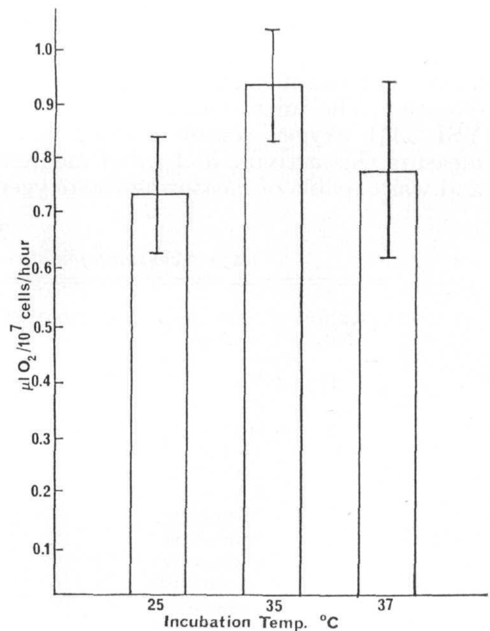

FIGURE 5. Oxygen uptake of chondrocytes from immature rabbit cartilage incubated at 25 °, 35 ° or 37 °C. Each bar represents the mean of 4 determinations ± SD.

the cartilage of immature rabbits, therefore 6 to 8 week old animals were used to provide the chondrocytes for most of our studies.

The total number of cells we were able to free after 150 min of incubation was usually more than twice the number obtained at 120 min. The prolonged digestion apparently did not affect the proportion of viable cells obtained because more than 95% of the chondrocytes showed dye exclusion immediately after a 2½ hr exposure to the collagenase solution. The possibility that prolonged digestion alters cell metabolism does exist, but no reports of the *normal respiratory activity* of chondrocytes could be found in the literature. It is assumed that any cell damage incurred during digestion would decrease both the oxygen consumption and likelihood of obtaining meaningful measurements. We found, however, that digestion times of up to 3 hr affected the oxygen uptake of the chondrocytes very little, but prolonged digestion would probably cause some cell damage.

The YSI Model 53 Biological Oxygen Monitor, conventionally used in studies of oxidative metabolism, could not accurately measure the small volumes of oxygen used by 10^6 chondrocytes in 3 to 4 ml suspensions. To achieve the required increase in sensitivity, we had to redesign the unit to operate with 1 ml of medium. This was based on the knowledge that the current flow in an oxygen sensor of the Clark-type is proportional to the partial pressure of oxygen in the solution in contact with the oxygen sensor membrane, and that changes in current flow reflect changes in the quantity of oxygen dissolved in the medium contained in the closed reaction chamber. A given number of cells would consume a constant quantity of oxygen regardless of the volume of medium in which the cells were suspended, but as the medium volume is halved, the total oxygen content is proportionately decreased and the oxygen sensor records a more rapid removal of oxygen from the medium. By reducing the reaction chamber volume from 3 ml to 1 ml, the initial oxygen content is reduced two thirds. Thus, the reduction of the chamber volume from 3 ml to 1 ml in effect gave a threefold increase in sensitivity of the oxygen sensor.

Chondrocytes obtained from mature rabbits showed, on the average, a higher rate of oxygen consumption than cells obtained from immature rabbits. This finding is in contrast to studies previously reported by Rosenthal *et al* (1941, 1942a, b) who reported that the metabolic activity of cartilage decreases with its age. It should be noted that Rosenthal's studies applied primarily to measurements of anaerbic metabolism. Our findings, that respiration in mature cartilage was at least as great as that in immature cartilage, coincide with the currently evolving views of the dynamic turnover of cartilage matrix expressed by Mankin (1970) and others. Our study is significant in that it presents a means for the further physiological study of articular cartilage chondrocytes and may lead to a better understanding of their metablic activity.

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