Rat Epiphyseal Cartilage: III. Metabolism of Glucose-C^{14}, *in vitro*.* (31745)

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(Introduced by Fredrick J. Stare)
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Bone and cartilage have been studied *in vitro* as regards intermediary metabolism(1-3) and matrix production(4,5). While the processes involved in energy production have been demonstrated to be crucial in calcification, endochondral bone formation, and matrix reabsorption(6), the relation between glycolysis and the turnover of matrix components is not completely understood.

The metabolism of glucose is of vital consequence since it represents a major substrate utilized in energy production through the generation of ATP as well as a source of the carbon skeleton utilized in the synthesis of various intermediates(1,3). Cohn and Forscher observed that only 66% of the glucose-C^{14} consumed by epiphyseal-metaphyseal fragments incubated *in vitro* was recovered in endproducts of glycolysis (lactate and pyruvate) and oxidation (CO_2), while the remainder was unaccountable(2). Flanagan and Nichols observed that less than 50% of the glucose-C^{14} consumed by metaphyses appeared in lactate and CO_2, while the remainder was found in collagen and a cellular fraction(7). Studies on limb explants cultured in a radioactive glucose media revealed that the radioactivity was incorporated not only into polysaccharide hexosamine, but also into the purines and pyrimidines of nucleic acids, as well as into protein amino acids(8). The incorporation of glucose into tissue proteins and collagen amino acids in bone fragments has also been demonstrated employing *in vitro* technique(5,7). These studies indicate that while a considerable fraction of the glucose utilized appears in the products of the Embden-Meyerhof and the Krebs tricarboxylic acid cycle, substantial quantities of the carbon skeleton are incorporated into macromolecules. The incorporation of glucose into these tissue constituents is of interest since the utilization of the carbon skeleton for cellular and extracellular components would be quite different in those cells which are primarily concerned with proliferation, matrix formation or matrix resorption.

This report deals with our observations involving the incorporation of glucose-C^{14} by rat epiphyseal cartilage, *in vitro*, into lactate, CO_2 and tissue fractions, as well as the effect of media modifications on these same processes.

Material and methods. Preparation and incubation of tissue. Male Sprague-Dawley weanling rats (40-45 g) (Charles River Lab.) were given Purina Chow and water *ad libitum* until the experiment began. After 4 to 7 days the animals were sacrificed by cervical dislocation. The epiphyses were isolated as described previously(9). The pooled epiphyses from one animal were placed in tared 25 ml flasks, containing 2.5 to 3.0 cc of incubation media, reweighed, and incubated.

Media and incubation. The incubation media employed through these studies consisted basically of Krebs-Ringer bicarbonate modified to contain one-half the prescribed concentration of calcium and magnesium and fortified with 100 or 200 mg glucose/100 ml with added uniformly labeled glucose-C^{14} (0.25 mc/millimole specific activity, New England Nuclear) so that each flask contained 2.0 μc. The media was placed in 25 ml flasks and, after the addition of the tissue, the flasks were covered with serum stoppers and placed in a metabolic shaker at 37°C (75 oscil./min) for 3 hours of incubation following preliminary aeration for 10 minutes with 95% O_2 5% CO_2. In the experiments to test the effect of anaerobism, 95% N_2 5%

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CO_2 was substituted. Iodoacetate, Eagle's Amino Acid mixture (100X), and Eagle's Essential Vitamin mixture (100X), were added directly to the flasks in 30 microliter volumes with water as a control. To test the effect of substrate concentration, carrier glucose was omitted or added in variable concentrations, but the radioactivity was maintained at 2.0 μc/flask.

After 3 hours of incubation, the contents of each flask were transferred to a centrifuge tube and a portion of the media was taken for determination of $C^{14}O_2$ content. CO_2 was liberated by addition of 0.2 volumes of 2 N sulfuric acid to the media in closed flasks containing suspended cups of hyamine (0.5 cc). In the experiments to determine total $C^{14}O_2$ production, cups were fastened to the serum stoppers prior to incubation, and CO_2 released at the end of incubation by addition of acid to the media directly in the unopened flask after addition of hyamine to the suspended cups. The cartilage segments were washed with 5.0 cc of ice cold saline (0.9% (w/v)) containing $\times$ 10^{-3} M iodoacetate initially, followed by 3 rapid washings with 15 cc aliquots of saline. The cartilage segments were immediately frozen and then stored at —20°C.

Fractionation of cartilage. The incubated tissue was homogenized in 25 cc of water in a Virtis-45 homogenizer operating at maximum speed (45,000 rpm) for 30 minutes. The *homogenate* was centrifuged at 34,800 *g* for 10 minutes which resulted in the separation of a fibrous residue and a *supernatant* layer that contained the proteinpolysaccharide (PP). The *supernatant* was adjusted to 75% (v/v) ethanol and after 30 minutes at 4°C, a gelatinous precipitate was isolated by centrifugation and discarded. Potassium acetate solution (20% w/v) was added to the decanted *supernatant* to give a final concentration of 1% and, after incubation overnight, a crude PP was isolated by centrifugation. The crude PP was dissolved and reprecipitated 3 times to yield the *PP fraction.* The residue remaining after centrifugation (34,800 *g*) was washed with several volumes of water followed by 3 washes with 95% ethanol and finally dried with ether. The dried residue was dissolved in 10 cc of distilled water and autoclaved at 15 psi for 3 hours. An extract was removed after centrifugation and the residue was resuspended in water (10 cc) and reautoclaved. The combined extracts were filtered to produce a clear *collagen* fraction. Portions of the *homogenate (H), supernatant (S) (34,800 g),* reprecipitated *PP* and *collagen (C)* fractions were removed for radioactivity determinations. With appropriate calculations this fractionation allowed separation of cartilage total radioactivity into four fractions: water-soluble intermediates (S-PP), PP, collagen (C) and residue (H-(S+C)).

Radioactivity determination. Radioactivity was determined by liquid scintillation counting of 1.0 cc portions of the various fractions in naphthalene-dioxane-PPO-POPOP with an efficiency of 65.4%. Quenching was corrected by the addition of internal standards.

Analytical methods. Glucose was determined by the glucose oxidase method (Worthington), while lactate and lactate-C^{14} were determined by the method of Barker and Summerson(10) and Shaw and Stadie(11), respectively.

Results. Preliminary studies revealed that approximately 60%-70% of the glucose-C^{14} metabolized by rat epiphyseal cartilage was recovered in lactate and CO_2 which is in agreement with previous observations(2). In addition, these studies demonstrated that a significant fraction of the radioactivity could be recovered in the incubated cartilage fragments. Since the two major matrix macromolecules (proteinpolysaccharide and collagen) were of interest, further differentiation of this radioactivity in the tissue was attempted by high speed homogenation and centrifugation(9). This method of fractionation led to the recovery of the major portion of the tissue acid mucopolysaccharides (approx. 70% of cartilage uronic acid) in the 34,800 *g* supernatant, while almost all of the collagen (approx. 95% of cartilage hydroxyproline) remained in the residue and could be extracted by repeated gelatinization. The major portion of cartilage C^{14} was found in the non-proteinpolysaccharide fraction of the 34,800 *g* supernatant and probably represents the labeled intermediates of intracellular origin through

TABLE I

Mucopolysaccharide fraction	Mucopolysaccharide: Uronic acid, μmoles	Mucopolysaccharide: Total radioactivity, CPM
Proteinpolysaccharide (PP)	2.04 ± .14	9,617 ± 989
PP-light (75,000 *g* soluble)	1.81 ± .12	8,666 ±1,101
PP-heavy (75,000 *g* insoluble)*	.23	952
Cartilage residual polysaccharides	1.09 ± .09	1,853 ± 136

* Calculated from difference between PP and PP-light (by uronic acid and radioactivity measurements).

Cartilage segments were incubated as described and after homogenization separated into a supernatant and residue by centrifugation at 34,800 *g*. PP was isolated from the supernatant, reprecipitated and, after solubilization in 0.15 M KCl, was separated into PP-light and PP-heavy by centrifugation at 75,000 *g*. The residual polysaccharides were isolated from the 34,800 *g* residue by papain digestion (60°C, 18 hr) and alkali extraction (0.2 N NaOH, 4°C, 18 hr). The data represent the mean of 5 flasks and are expressed per 100 mg wet cartilage.

which glucose is metabolized. Consistent with this observation is the fact that this water-soluble radioactive fraction, while not removed by brief washing of the intact cartilage segments with 0.9% (w/v) saline, could be progressively leached out by repeated washings and, in addition, was almost entirely soluble in trichloroacetic acid. The remaining radioactivity was distributed into proteinpolysaccharide (PP) and the water insoluble residue.

Recent reports(12,13) stressing the physiologic significance of the various pools of acid mucopolysaccharides prompted further studies on the incorporation of C^{14} into these groups of compounds in an isolated experiment (Table I). Analysis of these PP fractions revealed nitrogen/hexosamine molar ratios of: total PP, 6.9; PP-light, 3.9; PP-heavy, 8.8. Although the residual cartilage polysaccharides remaining after extraction of PP represented 34% of the tissue uronic acid, its radioactivity was significantly less. Labeled PP was further studied by column chromatography on Dowex-50(5) and the presence of 40% of the radioactivity in the hexosamine eluate suggests that the radioactivity found in PP is predominantly on the mucopolysaccharide moiety.

Application of these methods afforded analysis of glucose metabolism not only in relation to lactate and CO_2 production, but also in relation to two major cartilage fractions, the water soluble cellular intermediates and the macromolecules. The results obtained in 2 experiments are presented in Table II. The high rate of aerobic glycolysis is evident with 60% of the glucose label appearing in lactate, while less than 6% was incorporated into $C^{14}O_2$. Of the remainder, 20% was recovered in the cartilage with the predominant fraction as cellular intermediates. The macromolecules contained the remaining tissue radioactivity which represented 7% of the consumed glucose-C^{14}. This method of fractionation was employed throughout the subsequent study.

Effect of time. In Fig. 1 the rate of C^{14} incorporation into lactate, CO_2, and cartilage is presented. As glucose consumption increased, there were parallel changes in the other fractions, although their rates were of different magnitudes. The incorporation of radioactivity in both PP and collagen over 3 hours of incubation is presented in Fig. 2A, which is in accord with previous reports em-

TABLE II

	Glucose-C^{14} incorporation, μmoles/3 hr*	%†	%‡
Lactate	2.320 ± .093	(59.6)	
Total CO_2	.230 ± .017	(5.9)	
Cartilage	.791 ± .022	(20.3)	
Water-soluble intermediates	.516 ± .029		(65.2)
Proteinpolysaccharide	.092 ± .006		(11.4)
Collagen	.079 ± .008		(10.0)
Residue	.102 ± .014		(13.2)
Glucose consumption	3.895 ± .264		
Recovery	3.341	(85.7%)	

* Expressed per 100 mg wet cartilage incubated.
† % of glucose metabolized (consumption).
‡ % of total glucose incorporated into cartilage.

Cartilage segments were incubated as described in modified KRB with glucose-C^{14} (200 mg %-11.1 mM). The incorporated radioactivity was determined as described under *Methods*. Data presented are from 2 experiments each containing 3 flasks.

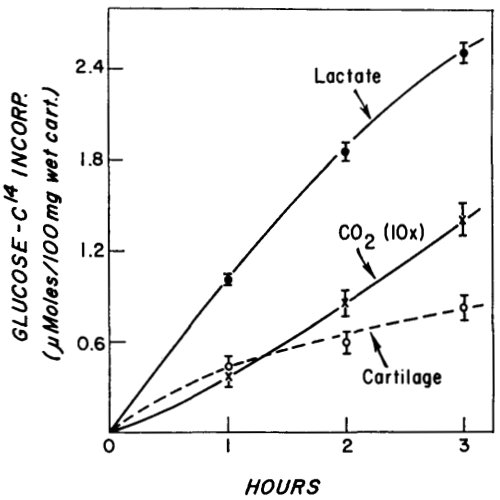


FIG. 1. Metabolism of glucose-C^{14} as related to incubation time, *in vitro*. Cartilage segments from 9 animals were incubated in an equal no. of flasks containing 3.0 cc of modified KRB with added glucose-C^{14} (11.1 mM, 2 μc) for 1, 2, and 3 hr. The points represent mean incorporation into each fraction and 1 S.D. Data for $C^{14}O_2$ have been expressed at 10 times the observed value for convenience.

ploying more specific indicators(5). The progressive increase in radioactivity in the two fractions was associated with a similar increase in the percent of the total radioactivity occupied by the macromolecular fractions (Fig. 2B). This is consistent with the fact that the water soluble intermediates represent radioactive precursors which are gradually incorporated into these macromolecules.

Effect of anaerobism, glucose lack and iodoacetate. The effect of these alterations on the incorporation of C^{14} into these various cartilage fractions is presented in Fig. 3. Although these 3 experimental conditions demonstrated quite different changes in the incorporation of C^{14} into the cartilage fractions, they suggested an association between the per cent of utilized glucose incorporated into CO_2 and the ability of cartilage to incorporate glucose label into the macromolecules. The depression of oxidation by anaerobism and iodoacetate was accompanied by a similar depression of C^{14} uptake into PP, collagen, and residue. In the absence of substrate, the increase in per cent of glucose oxidized paralleled the increase in per cent of glucose incorporated into the macromolecules.

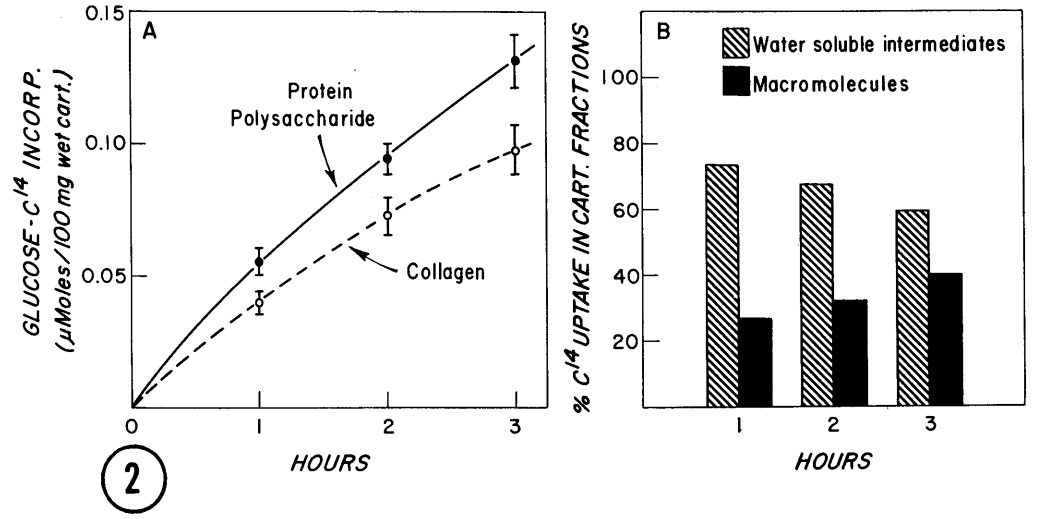
Oxygen depressed glycolysis (—26.9%) while glucose consumption was slightly increased. $C^{14}O_2$ production was virtually completely inhibited (97%) under nitrogen and was associated with highly significant differences in C^{14} incorporation into PP (—48.7%) and collagen (—73.1%). Only 29.5% of the cartilage radioactivity was found in the macromolecules in the absence of oxygen in comparison with 46.3% in the controls.

Absence of substrate (carrier glucose omitted) resulted in proportionally less glucose-C^{14} uptake in all fractions. However, qualitative differences were noted when the data were compared in terms of per cent glucose incorporated into these fractions. In the absence of glucose, the per cent of C^{14} incorporated into CO_2 increased significantly (+105%) and was associated with a 61.5% increase in the per cent of cartilage radioactivity found in the macromolecules in comparison with the controls.

Iodoacetate at a media concentration of 10^{-3} M inhibited the incorporation of radioactivity into CO_2 (—92%) while lactate production was virtually absent. C^{14} incorporation into cartilage was significantly decreased (—74%) and was almost entirely confined to the water soluble intermediates with a 98% inhibition of uptake into PP and collagen.

Effect of glucose concentration. Glucose consumption increased with increasing substrate concentrations and at concentrations 2 and 4 times the concentration reported previously as being maximal(14) (Table III). Lactate production and the per cent of glucose incorporated into lactate paralleled this increase. The inhibition of $C^{14}O_2$ production by increasing glucose concentrations and glycolysis was associated with a similar decrease in per cent of cartilage radioactivity found in the macromolecules.

Effect of vitamins and amino acids. The effects of added vitamins or amino acids were evaluated in another group of experiments (Table IV). No significant changes were observed in lactate or CO_2 production, while some alterations in C^{14} incorporation into cartilage PP were observed. The addition of either amino acids or vitamins was associated with a significant decrease in C^{14} incorpora-



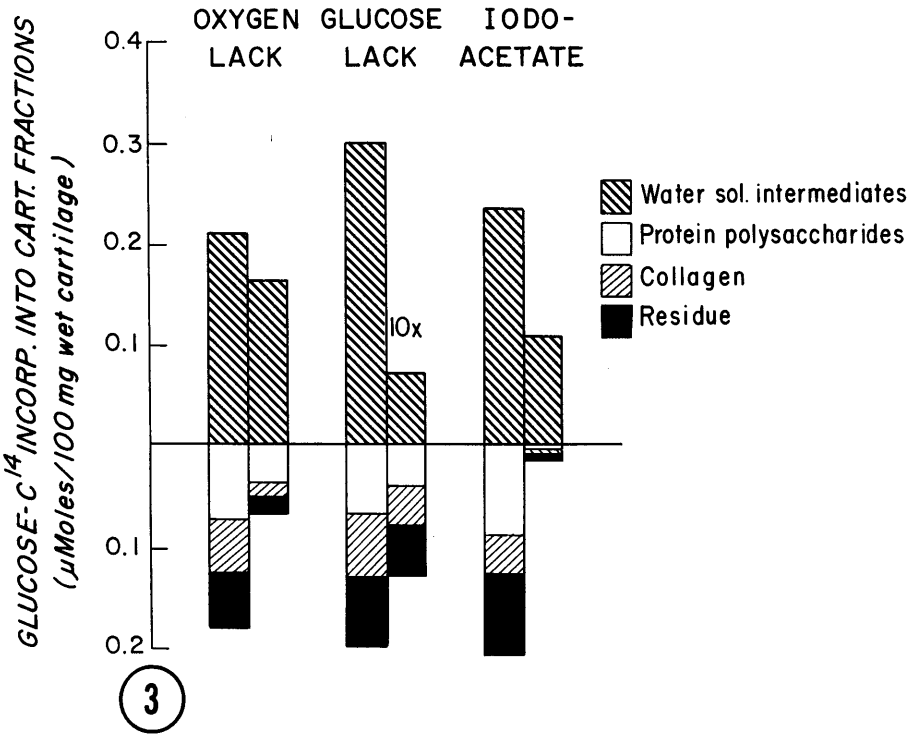


FIG. 2. Incorporation of C^{14} into cartilage as related to time of incubation, *in vitro*. A. Incorporation of C^{14} into PP and collagen. B. Comparison of percent of radioactivity incorporated into water-soluble intermediates fraction and macromolecules (PP, collagen, and residue). Data have been derived from the same study employed in presentation of FIG. 1.

FIG. 3. Effect of anaerobism, glucose lack, and iodoacetate on incorporation of glucose-C^{14} into cartilage, *in vitro*. Cartilage segments from each of 2 animals were divided into each of 2 paired flasks containing 2.5 cc of modified KRB and glucose-C^{14} (5.6 mM, 2 μc) and incubated for 3 hr. Data have been derived from 3 paired flasks testing each condition individually (*i.e.*, substitution of 95% N_2 5% CO_2), emission of carrier glucose (0.2 mM glucose, and addition of iodoacetate (1.0 mM). Column on left represent control observations while that on its immediate and adjacent right represent experimental observations. Data derived from the flasks in which carrier was omitted have been expressed as 10 times the amount observed for convenience.

TABLE III

Media glucose, mM	Glucose-C^{14} incorporated into: Lactate	CO_2	Water-soluble intermediates	Protein-polysaccharide	Collagen	Residue
			μmoles/3 hr			
11.1	2.134 (61.5)	.138 (4.0)	.524 (15.1)	.080 (2.3)	.075 (2.2)	.098 (2.7)
5.6	1.650 (63.5)	.128 (4.9)*	.299 (11.5)*	.066 (2.5)	.066 (2.5)	.068 (2.6)
2.8	1.058 (64.6)	.097 (5.9)†	.141 (9.8)†	.042 (2.8)*	.040 (2.5)	.063 (4.0)*

Figures in parentheses represent percent of glucose metabolized (consumption) found in each fraction.

Significance: * $p < 0.05$, † $p < 0.01$.

Cartilage segments from 9 animals were divided randomly into a similar number of flasks containing 2.5 cc of modified KRB with 2.0 μc of glucose-C^{14} per flask but with different glucose concentrations. The data represent the mean observation of 3 flasks testing each concentration and expressed per 100 mg wet cartilage incubated. The probability (p) is based on the changes observed from a glucose concentration of 11.1 mM.

TABLE IV

Flask	No.	Glucose-C^{14} incorporated into: Water-soluble intermediates	Protein-polysaccharide	Collagen	Residue
			μmoles/3 hr†		
Control	5	.474 (13.9)	.089 (2.6)	.054 (1.6)	.065 (1.9)
Amino acids	3	.469 (14.2)	.057 (1.7)*	.055 (1.7)	.080 (2.4)*
Vitamins	3	.470 (13.8)	.064 (1.9)*	.056 (1.7)	.089 (2.6)*

Figures in parentheses represent percent of glucose metabolized (consumption) found in each fraction.

* Significance ($p < .05$).

† Expressed per 100 mg wet cartilage incubated.

Cartilage segments were placed in flasks containing 3.0 cc of incubation media fortified with glucose-C^{14} (2.0 μc/flask) (11.1 mM). Thirty μl of amino acid (100×) or vitamin (100×) mixture or distilled water (control) were added to the appropriate flasks and after incubation, the incorporation of radioactivity into the various fractions was determined.

tion into PP of 36.0% and 28.1%.

Discussion. These studies utilizing rat cartilage indicate that glucose is not only metabolized through the Embden-Meyerhof pathway (with the production of lactate) and the tricarboxylic acid cycle (with the generation of CO_2 and ATP), but it is also significantly utilized for synthesis of tissue macromolecules. Quite prominent in this macromolecular fraction was proteinpolysaccharide, which was water soluble and represented the predominant polysaccharide fraction labeled. The difference noted in the specific activity of PP in comparison to the residual polysaccharides is consistent with previous reports on labeling of these fractions, *in vivo* (13).

The fractionation employed in this study suggests that the radioactivity incorporated into the cartilage fragments is composed of two major fractions: a water soluble fraction composed of intermediates in the pathways mentioned above and the macromolecules. It is apparent that this tissue has the ability to utilize the glucose carbon skeleton in the synthesis of amino acids and the complex hexoses involved in the synthesis of protein-polysaccharide and collagen. Since the synthesis of these complex macromolecules necessitates the regulation of biologically useful energy in the form of energy-rich phosphate bonds, interference with oxidation (anaerobism, iodoacetate) led to inhibition of glucose incorporation. While oxidative processes were essential, it would appear that anaerobic glycolysis was capable of supporting this activity, presumably through its ATP generation. Suggestive of the importance of the Krebs tricarboxylic cycle in these processes is the effect of glucose concentration. Although net incorporation of glucose was increased by additional substrate, this increased glycolysis was associated with reciprocal changes in oxi-

dation and in the incorporation of glucose by these high energy requiring biosynthetic processes.

The alterations induced by modification of the media and atmosphere on glucose incorporation by this tissue, *in vitro,* are consistent with the marked effect of the microenvironment on cell function noted previously (15). The influence of the microenvironment may, in part, be mediated at a biochemical level through a modification of glycolytic pathways in energy production and its utilization. The primitive (Stadie) nature of these cells provides a suitable basic mechanism for regulation by the microenvironment(16). However, although aerobic glycolysis and oxidative phosphorylation appear essential in these biosynthetic mechanisms, the known diversity of these processes suggests a considerable complexity in the function of these pathways beyond ATP synthesis and the production of the glucose-derived carbon fragments.

Summary. The metabolism of glucose-C^{14} by rat epiphyseal cartilage incubated *in vitro* has been investigated. While 65% of the metabolized carbon label was recovered in lactate and CO_2, 20% was observed incorporated into the surviving cartilage fragments after 3 hours of incubation. The cartilage radioactivity was further divided into a water-soluble cellular intermediate fraction and the tissue macromolecules composed of proteinpolysaccharide and collagen. The effect of media alterations (anaerobism, iodoacetate, glucose concentration, amino acids, and vitamins) on glycolysis and the incorporation of radioactivity into these cartilage fractions were studied. The relation between glycolysis and the incorporation of glucose into cartilage and the macromolecules was discussed.

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