studied *in vitro*. Papain treatment, both *in vivo* and *in vitro*, has been demonstrated to have a greater effect on the water-soluble fraction (PP) of the total MPS from this tissue. Small doses of papain, administered *in vivo*, were associated with an increased S^{35} MPS incorporation, evident within 6 hours and present up to 24 hours. These studies suggest that extracellular alterations of MPS in their physiologic state (protein complexes) have affected the biologic processes and matrix integrity of REC.

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Rat Epiphyseal Cartilage: V. Glucose-C^{14} Metabolism as Related to Growth and to Various Anatomical Areas, *in vitro*.* (31747)

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Studies of the processes involved in endochondral bone formation and remodeling have revealed the significance of cellular kinetics (1-3). However, the relation between these functions and glycolysis, recently reviewed (4,5), is not well understood. While studies in this laboratory have been directed towards proteinpolysaccharide metabolism in rat epiphyseal cartilage (REC)(6), the data(7) on the metamorphotic and growth properties of this tissue have suggested that it might afford an opportunity to explore the relationship of intermediary carbohydrate metabolism to various aspects of cellular kinetics and remodeling processes.

Utilizing glucose-C^{14} and REC of constant cellular composition, the alterations in C^{14} incorporation produced by modifications of the incubation media *in vitro* have been reported(8). Studies employing the same *in vitro* technique in varying anatomic areas of cartilage as well as during the formation of the ossification center might apply in correlating different cellular populations and/or their predominant remodeling process to glucose metabolism. The incorporation of glucose-C^{14} into CO_2, lactate, and five tissue fractions (TCA soluble, lipid, alkali extract-

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able, collagen and protein) has been studied in different anatomic areas of REC as well as during its growth (and ossification). Our observations are the subject of this report.

Materials and methods. Preparation of animals and tissue. Sprague-Dawley rats ranging from 3 to 5 weeks old (45 to 150 g) were employed in these studies. The isolation of tissue was similar to our previous description with minor exception(6,8). Both analyses and incubation studies were conducted on the epiphyses obtained from one limb of each animal (one distal femoral and one proximal tibial epiphysis).

In the evaluation of the metabolism of different areas, the entire tibia and femur was separated and dissected free of extraneous tissue. After the bones were scraped clean, a longitudinal slice was applied which separated the bones into approximate halves and exposed the marrow cavity. The marrow was washed out with ice cold saline (0.9% (w/v)), and the bone was divided into epiphysis, metaphysis, and diaphysis by a slice through the epiphyseal-metaphyseal junction, and the proximal and distal end of the shaft proper, respectively. Further to separate the epiphysis, a slice was placed between the epiphyseal plate or physis(7) and the adjoining trabeculae of the secondary ossification center. The remaining articular end of the epiphysis was divided into articular cartilage and secondary ossification center by carefully dissecting the former from the latter. The time lapse from sacrifice of the animal to initiation of incubation in the isolation of intact epiphyses was less than 30 minutes for 2 to 3 animals, while the interval approximated 30 minutes per animal when the individual zones were isolated. Moreover, the intact epiphyses, while washed with saline and sliced into 4 segments(6), were observed to be more contaminated in terms of "vascular" elements (secondary ossification center) than the subareas which were more fragmentary.

Preparation of media and incubation. The media employed was Krebs-Ringer bicarbonate modified to contain one-half the prescribed calcium and magnesium concentration and fortified with glucose (11.1 mM) containing 1.0 μc of uniformly labeled glucose-C^{14} (0.25 mC/mM) per flask. The flasks employed (25 cc) were fitted with plastic cups and contained 3.0 cc of incubation media, but otherwise the method of incubation is similar to that previously described(8).

Analytic methods. After 2 hours of incubation, 0.5 cc of hyamine was added to the center well and media CO_2 was liberated by addition of 0.5 cc of 2 N sulfuric acid followed by intermittent shaking for 30 to 45 minutes. The media and tissue were transferred to a centrifuge tube, centrifuged for 5 minutes (2000 rpm), and the supernatant decanted. Glucose, lactate, and lactate-C^{14} were determined on the neutralized barium hydroxide-zinc sulfate filtrate by methods previously described(8). The tissue remaining after centrifugation was washed quickly with ice cold physiologic saline and homogenized in 7 to 8 volumes of ice cold distilled water in a microhomogenizer (Virtis 45) for 10 minutes at high speed. The homogenate was transferred quantitatively to a centrifuge tube with 1.0 cc aliquots of water and fractionated by a modification of a method employed on cultured limb rudiments(9). The homogenate was adjusted with 60% ice cold TCA added dropwise to a final concentration of 10% and centrifuged. The supernate was decanted and the residue was washed 7 times with 2.0 cc aliquots of 5% TCA. The initial TCA supernatant and the subsequent washes were pooled and this extract represented the *TCA soluble* fraction. The insoluble residue was washed with 2.0 cc of absolute ethanol to remove the TCA and the *LIPID* fraction was obtained by extraction with 95% methanol at 60°C for 30 minutes in 3.0, 3.0, and 2.0 cc volumes successively. The residue was allowed to dry and an *alkali extract* fraction was obtained by extraction with 0.1 N sodium hydroxide at room temperature (22-23°C) for 18 hours followed by 2 repeated washings with the same solution (2.0 cc). The *collagen* fraction represented the hot TCA extract of the residue at 90°C in 3.0 cc volumes of 5% TCA for 30 minutes repeated thrice. The remaining residue (*protein* fraction) was dissolved in 0.5 cc of concentrated formic acid and brought to a suitable volume.

The methods employed in determination

of wet weight, dry weight, ash weight, and DNA have been reported previously(6). On the incubated tissue, DNA determinations were made directly on the alkali extract(10). Thymus DNA with determined phosphorus content was used as a standard and the results are expressed in DNA-phosphorus equivalents. For radioactivity determinations, aliquots of lactate-C^{14}, the tissue fractions, and the entire CO_2 adsorbents were counted in Bray's solution(8) with correction of quenching by the addition of internal standards in a liquid scintillation counter.

Results. Initial studies on the incorporation of glucose-C^{14} into cartilage fractions were oriented towards proteinpolysaccharide (PP)(8). However, the design of this study suggested that the separation of cartilage radioactivity into TCA soluble, lipid, alkali extractable, collagen, and protein fractions might have greater application. Phosphorus determinations on these fractions revealed that virtually all the tissue phosphorus was extracted in the first 3 fractions with the major fraction being inorganic and TCA soluble. At least 7 washes were necessary to remove the entire inorganic phosphorus. Attempts to separate this tissue into more specific chemical fractions met with difficulties imposed by the major matrix macromolecules (PP and collagen). Both of these components in their newly synthesized form (labeled) are TCA soluble while the TCA insoluble residue contained these same components in a complex form (insoluble collagen, alkali extractable acid mucopolysaccharides (AMPS)) in addition to lipids, nucleic acids (N.A.), and other proteins. While a lipid fraction could be easily extracted with 95% methanol(9) attempts to remove nucleic acids alone(neutral 10% saline at 100°) and collagen (hot TCA) were unsuccessful due to the presence of variable quantities of AMPS in the saline, hot TCA, and residual fractions. These inherent difficulties were somewhat resolved by extracting both N.A. and AMPS in alkali and leaving the collagen fraction similar in composition to that reported in bone(10). While this method of fractionation presents a considerable simplification of the utilization of glucose-C^{14} by tissue, it does extend the separation beyond the "cellular" and "collagen" fractions employed previously by Nichols (10). Studies on the rate of C^{14} incorporation into these fractions over 3 hours of incubation were performed. The rate of uptake of C^{14} into the TCA insoluble fraction was not uniform as would be expected from the complex handling of the labeled carbon skeleton prior to its incorporation into these chemically different moieties. Comparison of the incorporation of C^{14} into CO_2 and the lipid fraction revealed a marked similarity and might be indicative of a common pathway such as one involving acetyl-Co-A. The radioactivity contained in the TCA soluble fraction was observed to decrease in proportion to the total cartilage radioactivity and may be consistent with the presence of precursors involved in the synthesis of the TCA insoluble contained constituents(8,10).

TABLE I

Body wt (g)	50.2	58.8	72.3	96.2	134.0
Age in days	23	25	28	32	37
Cartilage					
Wet wt (mg)	133.6	141.9	161.6	201.2	217.4
Dry wt "	35.7	38.7	48.7	63.8	73.9
Ash wt "	11.5	12.8	16.8	22.8	28.7
Water content*	.733	.729	.698	.671	.660
Organic*	.181	.188	.197	.210	.208
Ash content*	.086	.090	.105	.119	.132

* g/g wet cartilage.

The epiphyses isolated from one limb of animals of different ages were analyzed for the above parameters as described. Results represent the means observed on a minimum of 4 animals (epiphyses from 8 limbs composed of distal femoral and proximal tibial epiphyses).

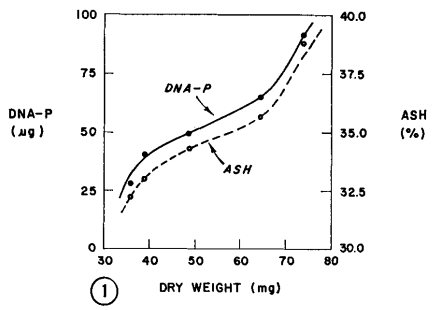
The rapidly changing character of REC (7) with growth necessitated preliminary evaluation of a suitable reference for the expression of metabolic data. In Table I the increase in wet weight, dry weight, and ash weight occurring with growth is presented, in addition to the water/organic ash content ratios. Analysis of DNA-P on the same samples revealed no definite correlation between cellularity (DNA-P) and either dry or wet weight. However, comparison of the increase in DNA-P and ash during the process of growth (Fig. 1) suggested an association between these 2 functions. Furthermore, in fractionating the total DNA-P found in the

TABLE II

	Glucose-C^{14} to							
	Lactate	CO_2	TCA soluble	Lipids	Alkali extractable	Collagen	Residue	$C^{14}O_2$/Lactate-C^{14} ratio
	μmoles per 100 μg DNA-P							
A. Epiphyses (25 day)	2.800 ±.178	.230 ±.016	.662 ±.045	.044 ±.004	.120 ±.006	.070 ±.008	.009 ±.001	0.073
B. Epiphyses (32 day)	2.265 ±.321	.235 ±.023	.367 ±.045	.033 ±.002	.118 ±.006	.038 ±.003	.009 ±.000	0.102

The tissue was isolated from 2 groups of rats (A. 25 days old, B. 32 days old) and incubated as described for 2 hr. The data represent the means plus 1 S.D. of 5 to 6 flasks containing epiphyses isolated from one limb of individual animals. The incorporation has been determined on the basis of radioactivity found in individual fractions. The mean recovery of glucose consumed was 88%.

three areas of REC of 32 day old animals (Table III), 65% of the cellularity was observed in the secondary ossification center. The formation of this highly vascularized area has been reported to be an integral component in the development of the ossification center (11) and our data suggest that an element of cellular kinetics (multiplication) is intimately involved with ossification within this growth period, although at present the relation between DNA-P and the specific cell content remains poorly defined. The compositional data are consistent with the gradual formation and growth of the ossification center with a relative increase in the osseous fraction of this tissue.



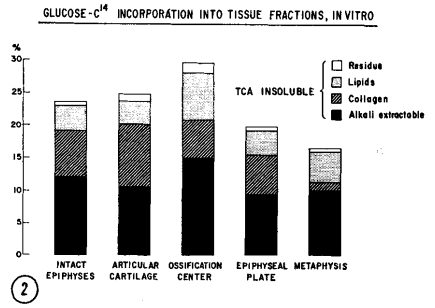


FIG. 1. Growth of rat epiphyseal cartilage is expressed as dry weight(mg). The relationship of ash weight (%) to DNA-P (uG) is depicted.

FIG. 2. Patterns of glucose-C^{14} incorporation into various tissue fractions of rat epiphyseal cartilage are illustrated.

To evaluate the effect of these changes on the incorporation of glucose-C^{14} into lactate, CO_2, and tissue fractions, various tissues of different ages and types were incubated, *in vitro*. Epiphyseal fragments with a higher ash content and presumably a larger ossification center (and osseous cellular population) demonstrated increased oxidative glycolysis of the labeled glucose. The ratio of $C^{14}O_2$/lactate-C^{14} was observed to be greater when REC of 39 day old animals was compared to that of 24 day old animals and when the femoral epiphysis was compared to the tibial epiphysis. In Table II the results noted in two age groups are presented. While there is a consistent decrease in C^{14} incorporation into lactate, this depression was related to the increase in the content of incubated DNA-P. The ratio of C^{14} into CO_2/lactate was statistically significant with 5.6% of the glucose consumed oxidized in group A as opposed to 10.1% in group B. The increased oxidation was paralleled by a similar increase in the percent of radioactivity found in the TCA insoluble fractions. A similar experiment in a third group of REC obtained from animals 40 days old demonstrated an equal depression in these parameters proportional to the increased content of DNA-P, but failed to demonstrate a sequential increase in the oxidation of labeled glucose.

The difference in these same parameters in various areas of incubated long bone is pre-

TABLE III

Area	DNA-P, μg/flask	Glucose-C^{14} to Lactate, %*	Glucose-C^{14} to CO_2, %*	$C^{14}O_2$/Lactate-C^{14} ratio
Articular	24.6 ± 4.2	60.0 ± 2.9 (3.72)	7.67 ± .79 (.46)	.124
Ossification center	69.8 ± 6.6	57.9 ± 1.7 (.69)	13.39 ±1.39 (.16)	.232
Epiphyseal plate	17.1 ± 1.6	58.1 ± 2.0 (5.21)	5.68 ± .18 (.51)	.098
Metaphysis	52.4 ± 6.8	54.2 ± 1.9 (1.86)	11.20 ± .99 (.38)	.204

* % of total radioactivity recovered.
( ) μMoles glucose incorporated per 100 μg DNA-P.
Tissue was isolated and incubated as described for 2 hr. Data are the mean plus 1 S.D. of 4 flasks containing the tissue isolated from the respective areas of long bone from 4 individual animals.

sented in Table III. The quantitative differences imposed by the relation of data to DNA-P are evident with glucose consumption and utilization of cartilagenous tissue (articular and physis) being 3-5 times as active as the metaphysis and the secondary ossification center. While these observations are related to the quite different DNA-P contents of these areas, the qualitative differences in aerobic glycolysis are apparent. The oxidative ability was significantly different in both tissues of osseous nature (metaphysis and ossification center) as evidenced by the percent of glucose recovered in $C^{14}O_2$ and the CO_2/lactate ratio when compared to the cartilagenous tissue (articular and epiphyseal plate).

The different patterns of C^{14} incorporation into the various tissue fractions are presented in Fig. 2. Consistent with previous observations on the intact incubated epiphyses the 3 individual areas also demonstrated a positive relation between the percent of C^{14} incorporation into CO_2 and the percent of tissue C^{14} incorporated into the TCA insoluble fractions. Comparison of the secondary ossification center with metaphyseal segments, however, demonstrated a quantitative difference even though the qualitative pattern of tissue C^{14} incorporation and aerobic glycolysis were not significantly different and the compositional data (dry/wet weight) were similar. The pattern in both articular and epiphyseal plate is grossly similar although a significant increase was noted in the collagen radioactivity of +16% in the former. Comparison of the secondary ossification center with the cartilagenous areas demonstrated additional differences characterized by proportionally greater incorporation of radioactivity into the lipid and alkali extractable fractions and lesser incorporation into bone collagen. The pattern remained essentially similar in both types of osseous tissue with the exception of collagen radioactivity.

Discussion and conclusions. The qualitative differences in glucose-C^{14} metabolism noted in these experiments parallel the known histologic and physiologic differences in the tissue preparations which were incubated(7). The relatively greater oxidation observed in ossifying REC, the secondary ossification center, and the metaphysis are in agreement with the data suggested by histochemical measurements of these pathways in the osteocytic and chondrocytic cellular series(12). The association between the labeled CO_2/lactate ratio and incorporation of the label into the TCA insoluble constituents suggests that the oxidation of glucose bears a relationship to cellular and matrix increases during this active growth period in intact as well as in isolated areas of REC. On the other hand the marked resorption known to occur in the metaphysis was accompanied by a high rate of oxidative glycolysis with a comparatively smaller C^{14} incorporation into cells and matrix. The variations in these parameters suggest that the pathways in the metabolism of glucose (lactate and CO_2) are a function of not only variable cellular populations but also predominant remodeling processes. These data would be consistent with recent speculations that the different metabolic patterns observed in these tissues represent different cell groups(5).

The quantitative differences observed indicate the necessity of considering cellular populations and kinetics in the interpretation of

metabolic data. While the marked differences noted in bone (represented by the secondary ossification center and metaphysis) when compared to cartilage (epiphysis and articular) may be real (*i.e.*, a uniformly lower rate in bone), a more plausible explanation might be found in the presence of a cellular group of vascular nature which is not as metabolically active, but which may represent a source of the osteocytic series. The data as presented suggest inherent difficulties in the direct application of quantitative data on glycolysis to specific processes such as accretion or resorption, especially when cellularity is affected and when histologic evidence indicates alterations in the rates of cell formation, differentiation, or modulation.

The incorporation of radioactivity into the cartilage fractions is of some significance since it indicates metabolic differences in the various areas of REC and metaphysis, although this coarse fractionation only allows speculation. The lower rate of incorporation into collagen in the metaphysis is consistent with the high rate of resorption present in this area(7). The difference in collagen radioactivity noted between epiphyseal and articular areas may be expected in view of the dissimilar collagen present in these two areas (7). The presence of comparatively greater radioactivity in the lipid and alkali fraction of both the metaphysis and the ossification center may be indicative of the relatively greater cellular change observed in these areas as evidenced by the rapid increase in DNA-P observed. It is hoped that with the use of specific precursors and with refinement of the fractionation procedures, further studies into cellular and matrix components may be undertaken employing this tissue preparation.

Summary. 1. Dissection of the various anatomic areas of rat epiphyseal cartilage and incubation of each area with C^{14} glucose has been performed. 2. The metabolism of each area was studied and it was shown that the secondary ossification center and the metaphyseal area were similar in their rates of glycolysis as were the epiphyseal plate and articular areas. 3. Further fractionation of the cartilagenous and osseous areas was performed and the TCA insoluble portion was subdivided into a residue fraction, lipid fraction, collagen fraction, and alkali extractable fraction. 4. The results are discussed in view of the morphologic and aging characteristics of this type of tissue.

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