also leads to considerable inactivation of the enzymes(11). Barnett and Seligman(12) have recently introduced 2 more substrates, Indoxyl acetate and Indoxyl butyrate, for the histochemical demonstration of tissue esterase which do not diffuse into lipoids as the azo dye will do in fresh frozen sections. These authors stressed the usefulness of the Coons modification(13) of the Linderstrøm-Lang and Mogensen technic(14) for the preparation of thin frozen sections of various organs well suited for histochemical enzyme studies.

Summary. Esterase activity in the human kidney is not visualized in paraffin sections of acetone fixed tissues with Gomori's technic and only very irregularly with the azo dye method. It can be regularly demonstrated with both methods in fresh frozen sections. In 5 of 7 renal carcinomas esterase was found in the cytoplasm of tumor cells if fresh frozen sections were used but not in paraffin fixed material.

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Calcification VIII. Glycolytic Enzymes and Phosphorylated Intermediates In Preosseous Cartilage.* (19485)

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At present there is direct evidence for the existence of 4 components of the glycolytic cycle in preosseous cartilage, namely, glycogen (1,2), phosphorylase(3), lactic acid(4), and adenosine triphosphate(5). Additional evidence of the glycolytic system in preosseous cartilage comes from the use of glycolytic inhibitors(6). These agents inhibit *in vitro* calcification(6,7), which is restored by phosphorylated intermediates beyond the locus of action of the enzymes inhibited. The suggestion was made from these studies(3,7) that a glycolytic system is present in preosseous cartilage. However, while the use of inhibitors is suggestive, it is not conclusive evidence, since the degree of specificity of the inhibitors is not well established(8).

The purpose of this investigation, therefore, was to seek more direct evidence for the existence of other components of the glycolytic system in preosseous cartilage.

Procedure. Most of the enzymes to be described in cartilage have been demonstrated using the procedures of Racker(9). These depend upon the enzymatic oxidation of reduced diphosphopyridine (DPN) in the spectrophotometer. For this purpose, aqueous extracts of rachitic bone cartilage were employed. 250 to 400 mg of cartilage obtained from the metaphyses of the tibiae of rachitic

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Wistar rats† was crushed and extracted three times with 3 ml of cold distilled water. The extracts were combined and made up to 10 ml. Phosphorylated intermediates in the glycolytic cycle were detected in the following way: rats were injected intraperitoneally with approximately 15 million counts of radioactive phosphorus as inorganic phosphate. They were sacrificed after approximately 24 hours. The tibiae were removed as before and extracted, 3 times with cold 10% trichloracetic acid (TCA). These were spotted on Whatman No. 1 filter paper and chromatographed by downward migration in an ethanol-acetic acid developer (80 ml ethanol, 0.8 ml acetic acid; mixture then brought to pH 3.5 with hydrochloric acid, after which distilled water is added to give a final volume of 100 ml) using a Chromatocab.§ At the end of a 15-hour interval, the chromatograms were removed, dried, and placed in contact with x-ray film for 48-72 hours. The position of the individual compounds which had become radioactive was indicated by film exposure. This procedure is essentially that used by Benson *et al.*, in studies on photosynthesis (10), and by Cohen *et al.*(11). The spots were identified by chromatographing pure compounds under the same conditions and detecting these by chemical means.‡

Results and discussion. The first enzyme investigated was *aldolase*. Goldenberg and Sobel(12) obtained evidence for this enzyme by the colorimetric estimation of the 2,4-dinitrophenylhydrazine derivatives of the carbonyl groups liberated by the enzymatic degradation of hexosediphosphate (HDP)

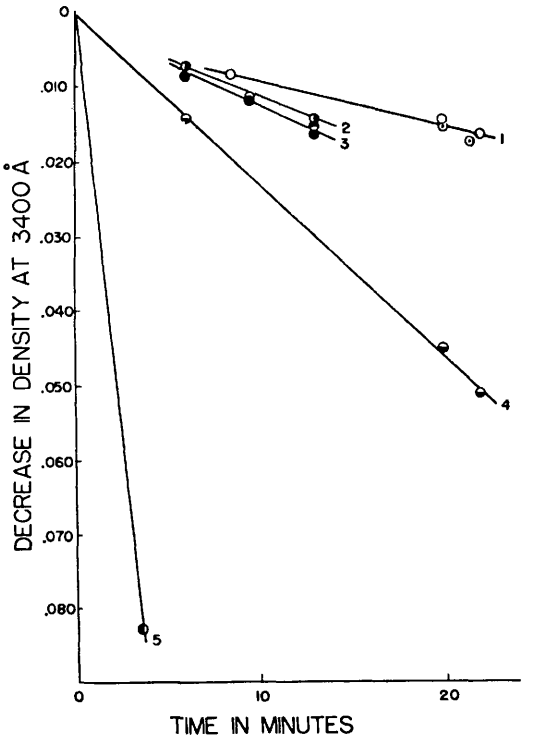

FIG. 1. Oxidation of reduced DPN by extracts of rat cartilage in presence of different substrates. Each cuvette contained .5 ml rat extract, .2 ml .1 M ammonium phosphate buffer pH 7.6, .1 ml .002 M reduced DPN, substrate, and water to 3 ml.

Curve 1: No substrate ○; no substrate, .2 ml 7×10^{-4} M ATP (dot in circle). Curve 2: .1 ml .25 M glucose, .2 ml 7×10^{-4} M ATP (solid right side circle). Curve 3: .1 ml .0058 M glucose-6-phosphate, .2 ml 7×10^{-4} M ATP (solid top half circle); .1 ml .0058 M fructose-6-phosphate, .2 ml 7×10^{-4} M ATP ●. Curve 4: .2 ml 1.6×10^{-4} M HDP (solid bottom half circle). Curve 5: .2 ml .035 M pyruvate (solid left side circle).

(13). Evidence for aldolase and α-glycerophosphate dehydrogenase was obtained by incubating the aqueous extract with HDP, reduced DPN, and ammonium phosphate buffer, pH 7.6, in the quartz cells placed in the spectrophotometer set at 3400 Å. If HDP is split to dihydroxyacetone phosphate and 3-phosphoglyceraldehyde, oxidation of reduced DPN can only occur via the conversion of dihydroxyacetone phosphate to α-glycerophosphate. The results of a typical experiment are shown in Fig. 1 (curves 1 and 4). There is a small decrease in density in the control cuvette (curve 1), which contains everything but HDP, and a

† The authors are indebted to Miss Mona Oser and to Dr. B. Oser of Food Research Laboratories, Long Island City, for generously providing many of the rachitic rats used in these studies. We wish to thank Dr. Harry Goldenberg of the Department of Biochemistry, Jewish Hospital of Brooklyn for many helpful suggestions.

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‡ Pure phosphorylated compounds for use in enzyme experiments as well as chromatographic runs were obtained from the Schwarz Laboratories, New York City, and the Ernst Bischoff Co., Ivoryton, Conn. Phosphopyruvate, as the silver salt was a gift from Dr. Gerhardt Schmidt.

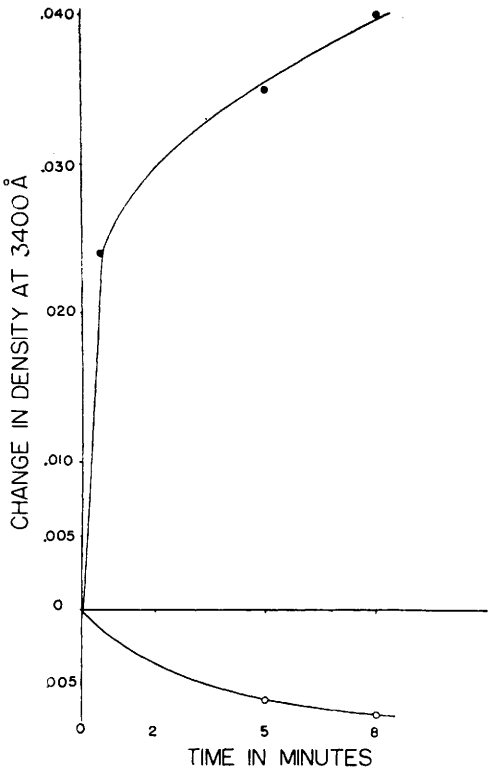

FIG. 2. Reduction of oxidized DPN by extracts of rat cartilage in presence and absence of α-glycerophosphate. Each cuvette contained .5 ml rat extract; .1 ml 3×10^{-4} M oxidized DPN; .2 ml .1 M pyrophosphate buffer pH 8.8; .3 ml 30% α-glycerophosphate and water to 3 ml. Lower curve, no substrate; upper curve with substrate.

rather rapid change in the presence of the substrate. These results point to the presence of aldolase and α-glycerophosphate dehydrogenase. Direct evidence for the presence of α-glycerophosphate dehydrogenase was obtained by starting with α-glycerophosphate and measuring the reduction of oxidized DPN in the spectrophotometer. The results of such an experiment are shown in Fig. 2.

It is not possible to demonstrate triosephosphate dehydrogenase in the presence of α-glycerophosphate dehydrogenase spectrophotometrically. If cartilage contains triosephosphate dehydrogenase, its presence can be detected in the following way: Using HDP as a source of 3-phosphoglyceraldehyde and oxidized DPN, the activity of the enzyme would lead to the formation of phosphoglyceric acid and reduced DPN. By adding an excess of pyruvate and lactic dehydrogenase, lactic acid would accumulate and this could be measured directly. The results of such an experiment are shown in Table I. The formation of lactic acid does not take place to any great extent in the absence of substrate, not in the presence of 0.0033 M iodoacetate. This first group of experiments has proven that aldolase, α-glycerophosphate dehydrogenase, and triosephosphate dehydrogenase are present in extracts of cartilage.

The presence of enolase in these extracts can be checked by using 2-phosphoglyceric acid as a substrate and reduced DPN. Results of such an experiment are shown in Fig. 3 along with the same system in the presence of fluoride. Phosphoglyceric acid in this case would be converted to phosphoenolpyruvic acid and then to pyruvic acid. Pyruvate in turn would be reduced to lactate, and reduced DPN oxidized, thus accounting for the change in density at 3400 Å. More direct evidence of the presence of enolase may be obtained by measuring the formation of phosphoenolpyruvate from 2-phosphopyruvate directly. This can be measured since the appearance of phosphoenolpyruvic acid is accompanied by an increase in density at 2400 Å (Fig. 4)(14). In the initial formulation of the enolase experiment described above, the assumption was made that a lactic dehydrogenase is present. This can also be proven directly by using

TABLE I. Triosephosphate Dehydrogenase Activity of Rat Cartilage.

Test system contains:	ml
$KHCO_3$ (.4 M)	.2
HDP (.15 M)	.1
Na arsenate (.15 M)	.1
Na pyruvate (.54 M)	.1
Lactic dehydrogenase (1 mg 50-70 fraction from rabbit muscle)	.1
Na F (1 M)	.1
Cysteine (.09 M)	.1
Cartilage extract	.5
Water	to 1.7

At end of 1 hr, add 1.5 ml 10% TCA; centrifuge; 1 ml used for lactic acid determination.

μg of lactic acid formed:	
(1) Complete system	55.6
(2) Complete system in presence of .0033 M iodoacetate	27.9
(3) Complete system without HDP	24

pyruvate as a sole substrate in the presence of reduced DPN. That this enzyme is present is shown by the data in Fig. 1 (curve 5).

We have thus far demonstrated several of the enzymes in glycolysis: aldolase, α-glycerophosphate dehydrogenase, triose phosphate dehydrogenase, enolase, and lactic dehydrogenase. The question then arose as to the presence of enzymes which convert glucose to hexose diphosphate. Fructose-6-phosphate and ATP, when added to tissue extracts, lead to the oxidation of reduced DPN. This means that hexosediphosphate is formed and that a phosphohexokinase is present (Fig. 1). Phosphohexoisomerase activity was demonstrated by the fact that glucose-6-phosphate in the presence of ATP leads to the oxidation of reduced DPN (Fig. 1). Finally, hexokinase activity is indicated by the addition of glucose and ATP as sole substrates and the subsequent oxidation of reduced DPN (Fig. 1, curve 2).

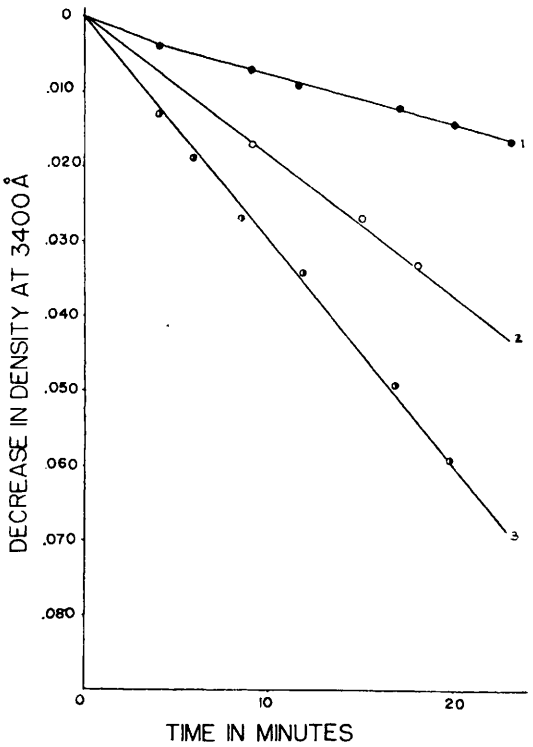

FIG. 3. Oxidation of reduced DPN by extracts of rat cartilage in presence of phosphoglyceric acid and fluoride. Each cuvette contained, except where otherwise indicated, .5 ml rat extract; .2 ml 8.5 × 10^{-4} M ADP; .2 ml .016 M phosphoglyceric acid; .1 ml .002 M reduced DPN; .2 ml .1 M ammonium phosphate buffer pH 7.6; .3 ml .1 M sodium fluoride; water to 3 ml. Curve 1: No fluoride, no substrate ●. Curve 2: With fluoride and substrate ○. Curve 3: No fluoride, with substrate (solid right side circle).

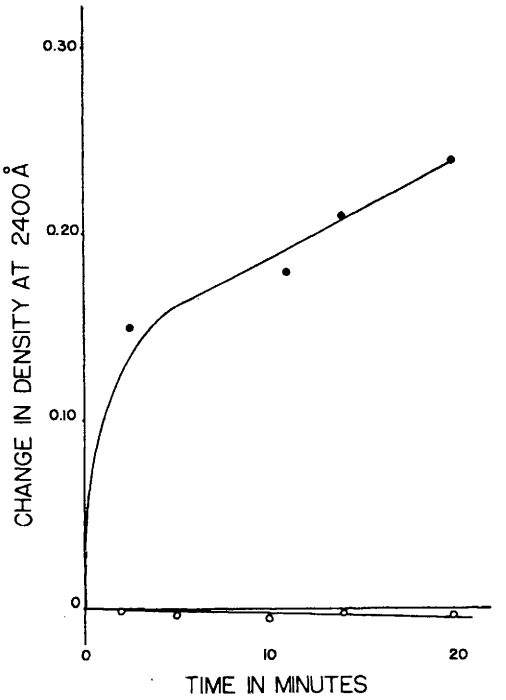

FIG. 4. Enolase activity of extracts prepared from rat cartilage. Except where otherwise indicated, each cuvette contained .4 ml rat extract; .2 ml .025 M phosphoglyceric acid; .2 ml .1 M phosphate buffer pH 7.4; .03 ml .1 M $MgSO_4$; water to 3 ml. Lower curve no substrate; upper curve with substrate.

If cartilage possesses a glycolytic system, one should also expect to find those of the phosphorylated intermediates which accumulate to some extent. We have been able to demonstrate some of the intermediates, as

TABLE II. Rf Values for Pure Compounds and Compounds Present in Trichloracetic Acid Filtrates of Epiphyseal Cartilage.

Compound	Rf for pure compound	Rf for tissue extract
Hexose diphosphate	.17	.163 (5)
Phosphoglyceric acid	.21	.224 (5)
Glucose-1-phosphate	.30	.299 (9)
Fructose-6-phosphate	.40	.405 (5)
Inorganic orthophosphate	.44	.439 (9)
Phosphopyruvic acid	.62	.614 (11)

Figures in parentheses indicate number of determinations used to obtain average value for Rf.

shown in Table II, after injecting radioactive phosphorus and preparing TCA filtrates.

Summary. 1. Most of the enzymes of glycolysis present in muscle and yeast appear to be present in epiphyseal cartilage. 2. Several of the phosphorylated intermediates of the cycle have been identified using chromatographic procedure.

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Further Studies on Relationship Between Human Serum Cholinesterase and Serum Albumin.* (19486)

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Of the two types of cholinesterase which have been described for the human, one, the specific or "true" cholinesterase, has received most of the emphasis in research because of its important role in the transmission of nerve impulses. The other, the cholinesterase of serum, the so-called "pseudo-cholinesterase", has received less attention because its specific function as an enzyme, as yet, has not been defined. Our own investigations, however, have made use of the latter enzyme as an indicator of albumin metabolism. We have reported(1) a close correlation between human serum cholinesterase and serum albumin levels, confirming the earlier work of Faber(2). In a series of 294 patients with a variety of diseases, we were able to demonstrate in all cases but those with albuminuria, that the serum albumin and the serum cholinesterase levels paralleled each other in a statistically significant manner.

A number of possibilities exist to explain the correlative relationship between the serum albumin and the serum cholinesterase, some of which are: 1) Albumin could act as a precursor to serum cholinesterase, and an albumin deficiency could cause the cholinesterase to decrease, as implied by Kunkel and Ward(3). 2) The decrease could be due primarily to a deficiency in protein intake, resulting in a general decrease of synthesis of all proteins both in serum and in the body organs. 3) The possibility exists that both the albumin and the serum cholinesterase are produced independently of each other, but by similar synthesizing mechanisms.

Methods. Patients were given concentrated normal human serum albumin, intravenously, containing 25 g of protein in 100 cc of neutral buffered diluent. *Protein determinations* were made by the biuret method of Weichselbaum(4) with two modifications: for the precipitation of globulins a solution of 26.8% sodium sulfate was used rather than 23% solution (giving lower albumin values than the customary Howe method which in-

* The albumin used in this study was provided by the National Blood Program of the American Red Cross.