62. ENZYME SYSTEMS IN ARTICULAR CARTILAGE

By CECILIA LUTWAK-MANN¹

From the Biochemical Laboratory, Cambridge

(Received 25 January 1940)

EARLY research on cartilage was chiefly concerned with the chemical composition of this tissue (mineral content, collagen, chondroitin sulphuric acid). The study of enzymes in cartilage was inaugurated by Robison [1923] who found that cartilage extracts liberate inorganic phosphate from hexosephosphoric esters. Much valuable work has been done since with regard to the phosphatase content of cartilage especially in relation to rickets, but comparatively few attempts have been made to study other enzyme systems in cartilage. Recently Bywaters [1937] carried out a series of experiments on joint tissues of the horse and determined manometrically the respiration and glycolysis quotients of horse cartilage.

In the present study the dephosphorylating enzymes were reinvestigated mainly with the object of learning more about the intermediary and end-products of various substances dephosphorylated by cartilage. Attempts were also made to find out whether there are in articular cartilage any oxido-reduction systems analogous to those known to play an essential part in the respiration and glycolysis of other tissues.

Experimental

Material. Articular cartilage was used throughout this work. Calf carpal joints were obtained from the slaughterhouse as soon as possible after the death of the animal. Only material from normal young animals was used and inflammatory changes or other lesions were met with in not more than a dozen out of over 200 joints examined.

The joints were opened in the laboratory, the synovial fluid drained off and cartilage removed from the joint surface with a sharp knife in the form of thin slices ("shavings") 0.4–0.8 mm. thick. After thorough rinsing with cold sterile water to remove traces of synovial fluid and blood, the slices were used for experiments either (1) directly, a weighed amount being suspended in 3–5 volumes of fluid, or (2) extracts were prepared by incubating cartilage at 37° for 2 days with 5 times its weight of water and a few drops of toluene or octyl alcohol. Liquid air treatment was at first applied to reduce cartilage to a powder. Later, however, it was abandoned as it offered little advantage over the use of finely chopped tissue.

When cartilage slices were used directly without previous maceration in presence of an antiseptic, little time was allowed to elapse between the removal of the tissue from the joint and the onset of the experiment, so as to avoid bacterial contamination. Experiments without a disinfectant were run for not longer than 8 hr. at 37°. Bacterial examinations were made whenever there was any suspicion of the possibility of excessive bacterial growth which might influence the results.

Various fluids were tested for the preparation of cartilage extracts, such as saline, 0.9% KCl, dilute phosphate, glycerol. Simple extraction with water was,

¹ Research Fellow, Newnham College, Cambridge.

however, found to give the most satisfactory and constant results especially because the total volume of the water added could be recovered after incubation, which was not the case with, e.g., dilute phosphate which caused variable and considerable swelling of the cartilage. The aqueous extract is colourless, slightly opalescent and has a pH of 7.6. It preserves its properties for a long time at 0°. A number of such extracts was tested and relatively little variation was found in their content of dry matter (0.2%), total N (5.5 mg./100 ml.), inorganic P (8–10 mg./100 ml.), Mg (0.15 mg./100 ml.) and $\text{NH}_3\text{-N}$ (0.1–1 mg./100 ml.). On the other hand, somewhat more variation was found in their enzymic activity. A second extraction of the cartilage residue was of little value. In all experiments described below the extracts were prepared from fresh tissue but it was possible to use successfully vacuum-dried cartilage for preparing active phosphatase extracts even after keeping several weeks in the dry state.

On several occasions experiments were carried out with synovial fluid and a few were done with synovial mucosa and ligaments. Synovial mucosa was used directly in the form of small scraps, whereas ligaments were cut into little pieces and treated with liquid air, which caused their disintegration into single fibres. These swell enormously and retain most of the added fluid so that it was rather difficult to obtain an extract from ligaments.

Occasionally experiments were carried out with cartilage from bullock joints. The amount of material which can be obtained from the joint surface of a grown animal is about one tenth of that from a young one. In spite of the striking anatomical differences, cartilage from normal adult animals appeared to possess the same enzyme systems as that of growing animals.

Methods. Phosphate estimations were done by the Fiske and Subbarow method [1925] as modified by Lohmann & Jendrassik [1926]. In experiments with cartilage extracts the method was applied in the ordinary way after removal of proteins with trichloroacetic acid. When cartilage slices were used, the aqueous solution in which they were suspended was poured off, the residue washed twice with a few ml. of water, and the combined washings were precipitated with trichloroacetic acid. This procedure gave better results than if the acid was added to the sample containing cartilage. It was found that a single extraction of sliced cartilage with trichloroacetic acid yielded only about half of the total acid-soluble P-content and further the amount of P varied greatly with the duration of the acid extraction. To exhaust the P-content of cartilage it was necessary to repeat the trichloroacetic acid treatment at least three times, each extraction lasting several hours. On the other hand, it was established in a large number of experiments that the amount of P which diffuses from minced cartilage into an aqueous solution is comparatively small and varies little from one experiment to another. It is realized that P-values obtained by the above procedure are diminished by the retention of a certain amount of P in the cartilage slices. However, the results can be considered satisfactory for the purpose of comparing the extent of dephosphorylation of various esters in a given time at 37°, provided that the amount of tissue and fluid and the molar concentration of the substrates is kept unaltered. The routine composition of the experimental samples was 1 g. tissue, $M/20$ substrate, total volume 5 ml.

Ammonia determinations were carried out by distillation with borate in the Parnas & Heller apparatus [1924] with subsequent nesslerization.

Lactic acid was estimated by the method of Friedemann *et al.* [1927] and occasionally by the colorimetric method of Mendel & Goldscheider [1925]. Proteins were precipitated with tungstic acid and interfering substances removed by means of the copper-lime treatment. Care was taken not to have in any

sample more than 3–4 mg. of substances such as acetate, iodoacetate, pyruvate or salicylate, because it was found that in excess they decrease the yield of lactic acid estimations by 30–40 %.

Bisulphite-binding substances (B.B.S.) were determined by the method of Clift & Cook [1932], glycogen by Pflüger's method, glucose by ferricyanide reduction [Hagedorn & Jensen, 1923], carbohydrates by the orcinol method of Tillmans & Philippi [1929], ascorbic acid by titration with 2:6-dichlorophenol-indophenol [Birch *et al.*, 1933] and glutathione iodometrically.

For manometric measurements, which were carried out in Barcroft manometers with side bulbs and gas outlets, fresh and very finely sliced cartilage was used, not exceeding 0.8 g. wet weight of tissue (78 % water) per 3.5 ml. total volume. A number of experiments was carried out by means of the Thunberg technique with 1 g. tissue per tube and 1.0 ml. *M*/2000 methylene blue in a total volume of 4 ml.

The various phosphoric acid esters and coenzymes used in the experiments were prepared in the Laboratory according to current methods, except hexose-diphosphate, adenylic acid and α -phosphoglycerol, which were commercial preparations. They were all used in the form of their Na or K salts.

Results

In order to gain more insight into the glycolysis and respiration of normal articular cartilage it was thought necessary to analyse in detail the effect of this tissue upon various substances which are known to be closely connected with these processes in other tissues. Experiments will be described below on the behaviour of various phosphorylated and non-phosphorylated components of the glycolytic cycle under the influence of cartilage. A separate section will contain the results obtained from the study of other enzymic oxidations and reductions in cartilage.

1. *Decomposition of adenine derivatives.* Cartilage as well as cartilage extract possesses enzymes which dephosphorylate muscle adenylic acid very rapidly, yeast adenylic acid a little less vigorously and adenylypyrophosphoric acid more slowly still. The liberation of phosphate is accompanied by deamination, for instance 0.9 mg. P and 0.38 mg. $\text{NH}_3\text{-N}$ are found after 3 hr. incubation of 10 mg. muscle adenylic acid with 1 g. cartilage at 37°. In similar conditions 60 % of yeast adenylic acid is decomposed and not more than 20 % of adenosinetriphosphoric acid. It appears that the enzyme responsible for the deamination is more soluble than the phosphatase: if minced cartilage is macerated for a few hours with 2–3 volumes of water and this extract is then discarded, the residue still dephosphorylates, but scarcely deaminates, muscle adenylic acid. Of other substances closely related to those mentioned above, inosinic acid is dephosphorylated and adenosine deaminated by cartilage. Ammonia is also liberated from adenine but only extremely slowly.

The end-product of decomposition of all these compounds by calf cartilage is hypoxanthine. This was shown both by the chemical isolation of the characteristic hypoxanthine silver-picrate derivative as well as by the use of a purified milk xanthine oxidase preparation.

The fact that it is possible to demonstrate the presence of hypoxanthine after several hours' incubation of adenine derivatives or hypoxanthine itself with cartilage at 37°, makes it rather improbable that normal (calf) cartilage is capable of metabolizing this purine to any great extent. Nevertheless, it should be recorded that on a few occasions it was possible to observe a slight but definite

oxidation of hypoxanthine by cartilage slices (not however, by extracts) both in aerobic and anaerobic conditions (see also p. 525).

The effect of cartilage upon more complex adenine derivatives such as cozymase and Warburg's coenzyme was also examined. The experiments were arranged in the following way. The coenzyme solutions were incubated with cartilage at 37° and after a certain time their presence was tested for by a suitable enzyme system such as the lactic dehydrogenase (for cozymase) and the hexose-monophosphate dehydrogenase (for Warburg's coenzyme). As controls, coenzyme solutions were put up with boiled tissue. It was found that while a short incubation with cartilage leaves these substances unaffected, they are no longer able to display their coenzyme activity if acted upon by cartilage for a longer time. It may be added here that boiled juice (Kochsaft) from cartilage neither activates nor interferes with dehydrogenase activity.

While adenine derivatives are obviously unstable in presence of cartilage, guanylic acid, guanosine and guanine remain unchanged. It was also found that no substance other than adenine derivatives is deaminated by cartilage although several amino-acids as well as glutamine, asparagine, creatine, guanidine and glucosamine were tested.

2. *Decomposition of phosphorylated sugar derivatives.* The following phosphoric acid esters were examined and all found to undergo dephosphorylation in presence of calf cartilage, but to a varying extent: fructose-1:6-diphosphate (Harden & Young ester), fructose-6-monophosphate (Neuberg ester), glucose-1-monophosphate (Cori ester), 3-phosphoglycerate and α -phosphoglycerol. At pH 7.6 Cori ester is dephosphorylated most rapidly, next follows Neuberg ester, then Harden & Young ester; in standard conditions at 37° and after 4 hr. the whole P of the first, 50 % of the second and 20 % of the last ester, being liberated. Phosphoglyceric acid yields inorganic P slowly unless suitable acceptors are provided (see below) and α -phosphoglycerol gives off only about 20 % of its P-content after several hours of incubation with calf cartilage.

Cori ester. A ferricyanide-reducing sugar is formed on dephosphorylation of this ester. A small proportion of the sugar formed is converted into lactic acid. The dephosphorylation of Cori ester by cartilage is inhibited by $M/20$ NaF but not completely. It may be mentioned here that generally it was found difficult to obtain NaF-effects in cartilage, even using comparatively high concentrations of the inhibitor and a long time of incubation. It is probable that this is due to the high content in this tissue of Ca and Mg.

Experiments were also arranged to test whether there is an uptake of inorganic phosphate by glucose and glycogen in presence of NaF in cartilage. However, only insignificant results were obtained even after prolonged incubations.

Neuberg ester. The dephosphorylation of this substance is usually accompanied by the production of small amounts of lactic acid. The reaction is not sensitive to NaF.

Harden & Young ester. Whereas the dephosphorylation of the two esters quoted above is independent of pH in the range 6.5–8.0, hexosediphosphate remains practically untouched unless the pH is above 7.4. Since the pH of cartilage is at least 7.6 and that of synovial fluid varies between 7.8 and 8.2, one can assume that suitable conditions exist normally for a rapid breakdown of this substance.

Adenosine, adenylic acid and pyruvate in small amounts were each tested as possible acceptors for the phosphate given off by fructosediphosphate but no significant results were obtained. Addition of Mg is without effect upon the

dephosphorylation of Harden & Young ester by whole cartilage which is itself rich in Mg. Cartilage extracts contain also a fair amount of Mg, practically all of which can be removed by dialysis. Even then, however, only slight acceleration is seen on addition of Mg. Similar observations with regard to the effect of Mg on the rate of dephosphorylation by cartilage were also made in the case of adenosine triphosphate and inorganic pyrophosphate (see below).

Iodoacetate, salicylate, bile salts and cinchophen (phenylcinchoninic acid) were also tested but appeared to have no marked effect upon the dephosphorylation of hexosediphosphate.

When the Harden & Young ester was acted on by aqueous toluene cartilage extracts, as the source of enzymes, it was observed that an orange hydrazone is formed in the trichloroacetic acid filtrate on addition of 2:4-dinitrophenylhydrazine. The hydrazone is insoluble in acid and carbonate and dissolves in alcoholic KOH with a deep blue-violet colour. Its M.P. is 298° which is that of the hydrazone of methylglyoxal. No such hydrazone is formed from hexosediphosphate after incubation with boiled cartilage. It was found later that formation of methylglyoxal from hexosediphosphate occurs also in fresh cartilage in absence of toluene so that it may be regarded as a normal intermediary of hexosediphosphate breakdown.

Quantitative estimations of the methylglyoxal formed were carried out by the method of Clift & Cook [1932]. Cartilage itself has no bisulphite-binding substances. It is impossible to determine exactly the relation between the amount of phosphate liberated from hexosediphosphate and the methylglyoxal formed, because the latter undergoes a further change in cartilage as will be reported below. When whole cartilage is used slightly more methylglyoxal is found in aerobic than in anaerobic conditions. Toluene treatment also increases the yield to some extent. Still more triose is found in presence of iodoacetate, whereas addition of GSH usually decreases the amount of bisulphite-binding substances (Table 1).

Table 1. *Decomposition of Harden & Young ester*

3.1 mg. Harden-Young ester (H.Y.)-P. 2 g. cartilage, 10 ml. veronal buffer pH 7.8, 4 ml. synovial fluid. Incub. 8 hr. 37°.

Additions		mg. P liberated	mg. B.B.S. (calc. as methylglyoxal)	mg. lactic acid	2:4-Dinitro- phenylhydra- zone
Cartilage					
0		0.08	0	0.52	—
H.Y.	in air	2.10	0.90	3.52	+
„	+ M/20 NaF	0.15	0	0.12	—
„	+ cozymase	2.70	0.50	5.04	+
„	+ toluene	2.50	1.10	3.40	++
„	+ GSH	Not estimated	0.40	4.52	±
„	+ M/50 iodoacetate	2.00	1.45	3.52	++
„	anaerobic	2.30	0.60	3.90	+
Synovial fluid					
0		0.02	0	2.30	—
H.Y. in air		0.15	0.66	4.80	+

Small amounts of lactic acid are found to be formed from hexosediphosphate in presence of cozymase. The reaction is considerably increased by the addition of pyruvate (Table 3).

Phosphoglyceric acid. As already stated inorganic phosphate is liberated from phosphoglyceric acid only very slowly. However, when small amounts of creatine and adenylic acid are added, the rate of dephosphorylation is markedly

accelerated. Creatine alone has a similar but much smaller effect. Pyruvic acid can be demonstrated as the product of dephosphorylation, both as a bisulphite-binding substance and by precipitation with 2:4-dinitrophenylhydrazine. NaF inhibits the reaction largely but not completely (Table 2).

Table 2. *Decomposition of phosphoglyceric acid*

1.5 mg. phosphoglyceric acid (pgl.)-P. 1 g. cartilage, 5 ml. veronal buffer pH 7.8. Incub. 12 hr. 37°.

	Additions	mg. P liberated	mg. B.B.S. (calc. as pyruvate)	2:4-Dinitro- phenylhydra- zone
Pgl.	0	0.03	0	—
„	+ M/20 NaF	0.15	0.18	Trace
„	+ 0.2 mg. adenylic acid	0.08	Trace	—
„	+ 5.0 mg. creatine	0.57	1.80	+ +
„	+ 5 mg. creatine	0.22	0.38	+

α -Phosphoglycerol. This ester is slowly dephosphorylated by normal calf cartilage but only at a pH higher than 7.6. No bisulphite-binding or hydrazone-forming substances could be detected as intermediary products of breakdown. There is no increase in lactic acid from this substance.

Pyrophosphoric acid. It may be of some interest to add that inorganic pyrophosphate is fairly rapidly converted by cartilage into orthophosphate. Addition of Mg has little effect upon the rate of this reaction.

Although it is evident that cartilage possesses enzymes which act upon a number of phosphoric acid esters, it appears to lack the substances which it attacks so readily. Trichloroacetic acid extracts were prepared from fresh cartilage and phosphate estimations were made after acid hydrolysis (*N* HCl, 100°) at 0, 7 and 30 min., 2 and 5 hr. as well as of the total acid-soluble P after ashing. However, there was no increase in phosphate content after acid hydrolysis or incineration.

That there are no substrates present in cartilage which can easily yield inorganic phosphate is suggested by the fact that the amount of phosphate which appears in aqueous cartilage extracts even on prolonged autolysis of the tissue at 37° is comparatively small (about 10 mg./100 ml.). Addition of toluene somewhat increases the amount of phosphate diffusing.

It seems also that cartilage lacks substances which would give rise to rapid ammonia formation, since not more than 0.01 mg. $\text{NH}_3\text{-N}$ per g. is found after several days' autolysis.

For comparison with cartilage other joint tissues were examined with regard to their behaviour towards some of the phosphorylated compounds. Synovial fluid shows a content of 6–8 mg./100 ml. acid-soluble P and no acid-hydrolysable fractions are found. It does, however, decompose adenylic acid both by dephosphorylation and deamination, though more slowly than cartilage does. Here too, hypoxanthine is the end product. Hexosediphosphate undergoes dephosphorylation in synovial fluid with the formation of a bisulphite-binding substance and an orange 2:4-dinitrophenylhydrazone (Table 1). On the other hand, ligaments and synovial membrane, while showing a certain anatomical differentiation in their acid-hydrolysable P-fractions, appear to be inactive towards the substances mentioned above.

3. *Non-phosphorylated sugar-derivatives.* Before describing the results obtained with sugars a few remarks should be made with regard to the behaviour of lactic acid itself in cartilage. Fresh and well-rinsed cartilage shows a fairly high

content of lactic acid, 20–40 mg./100 g. (wet weight), which remains unchanged on incubation. A brief aqueous extraction of sliced cartilage brings practically the whole of the lactic acid into the fluid and on repeated extraction of the residue no more is obtained. Thus it would appear that washed cartilage has no ability to produce lactic acid. The lactic acid found in fresh cartilage is probably due to a post-mortem diffusion from blood and also perhaps from synovial fluid which was found to contain between 50 and 70 mg./100 ml. lactic acid. Other joint tissues, tendons and mucosa also contain some 20–30 mg. lactic acid/100 g. (wet weight).

Lactate is quite stable when added to cartilage alone but small amounts of it disappear in presence of cozymase, and at the same time traces of pyruvic acid can be detected (Table 3).

Glycogen. It has been known for a long time that cartilage contains a certain amount of glycogen. This might of course form a source of lactic acid under suitable conditions. The following experiment suggests that cartilage glycogen undergoes little change even during prolonged autolysis. 40 g. fresh articular cartilage were divided into two equal lots; in one of these glycogen was estimated at once, and the other was allowed to stand for a week at 20° with a few volumes of water and some toluene. Then the supernatant fluid was removed and the glycogen estimation carried out on the residue. After the acid hydrolysis of glycogen 130 mg. glucose/100 ml. were found in the fresh and 98 mg. glucose/100 ml. in the autolysed sample. The glycogen isolated from cartilage gives a strong red colour with iodine, it is precipitated with alcohol from acid solutions and rapidly fermented by Lebedew juice.

The estimation of the carbohydrate content in a cartilage extract (1 week old) shows 14 mg. sugar/100 ml., of which however only one third is dialysable. Some of the carbohydrate found in the extract may be due to small amounts of glycogen and chondroitin sulphuric acid and the rest to diastatic breakdown products of glycogen; for it was found that cartilage, as well as synovial fluid, possesses weak diastase activity towards starch and glycogen.

When, however, added glycogen is incubated with whole cartilage or cartilage extract in the presence of cozymase and adenylic acid small quantities of lactic acid are produced in almost all experiments (Table 3). Nicotinic acid amide cannot replace cozymase. In a few instances in addition to lactic acid small amounts of a bisulphite-binding substance could be detected and a slight precipitate of yellow 2:4-dinitrophenylhydrazone was formed, which was soluble with a red colour in KOH and probably due to pyruvic acid.

Table 3. *Lactic acid formation in cartilage*

10 mg. substrate. 2 g. cartilage. pH 7.6. Incub. 8 hr. 37°.

Additions	mg. lactic acid	B.B.S. in ml. M/100 I ₂	2:4-Dinitro- phenylhydra- zone
0	0.38	0	—
Glycogen	0.78	0	—
Glycogen + cozymase + adenylic acid	1.98	0.22	Trace
Glucose	4.18	0	—
Galactose	0.90	0	—
Cori ester	1.16	0	—
Neuberg ester	1.00	0	—
Harden & Young ester + pyruvate + cozymase	5.28	19.2	+++
Pyruvate	0.58	22.3	+++
Pyruvate + cozymase	1.08	19.8	+++
Lactate	10.10	0	—
Lactate + cozymase	8.98	0.38	+

Hexoses. With other non-phosphorylated sugars, glucose, galactose, fructose, mannose and glucosamine, only small positive results were obtained even in presence of cozymase. There were however a few experiments with glucose where a significant increase in the lactic acid content took place (Table 3). No bisulphite-binding substances or hydrazone formation were observed.

Trioses. Of the three trioses examined, *dl*-glyceraldehyde, dihydroxyacetone and methylglyoxal, the first two remain practically unchanged under the influence of cartilage, whereas methylglyoxal disappears fairly rapidly both in aerobic and anaerobic conditions (Table 4). The reaction is thermolabile. The disappearance of methylglyoxal is due to its conversion into lactic acid. The course of the reaction can be followed by manometric measurements of CO_2 -liberation from bicarbonate in equilibrium with 5% CO_2 in N_2 . Acid production in cartilage alone is small and varies between 30 and 50 $\mu\text{l.}$ per g. in 4 hr.

The enzyme glyoxalase is known to convert methylglyoxal into lactic acid in presence of traces of GSH [Lohmann, 1932]. In cartilage (and in synovial fluid) the nitroprusside test is negative and yet the reaction proceeds quite well without added GSH. Addition of GSH to whole cartilage only accelerates the rate of the reaction to a moderate extent, but when cartilage extract is used as the source of enzyme the effect is more pronounced. In synovial fluid there is also a slow acid production from methylglyoxal. The effect of iodoacetate, to which the glyoxalase of other tissues is very sensitive, is comparatively small in cartilage (Table 4).

From a series of chemical estimations of the methylglyoxal used up and lactic acid formed, as well as from manometric data, it follows that somewhat more methylglyoxal disappears than is found as lactic acid, especially in anaerobic conditions. This would suggest that some other substance besides lactic acid is formed in the course of the reaction, but so far no direct evidence has been obtained (Table 4). Phenylglyoxal also gives rise to production of acid in cartilage (presumably mandelic acid). It may be mentioned that the ability of cartilage to act on methylglyoxal varies and in one case it was found completely inactive both aerobically and anaerobically.

Table 4. *Behaviour of trioses in cartilage*

5 mg. substrate. 1.5 g. cartilage. Incub. 3 hr. 37°.

Additions		mg. triose used up	mg. lactic acid formed	$\mu\text{l. CO}_2$ -prod. (measured manometr.)
Glyceraldehyde	in 5% CO_2 -95% N_2	0.08	0.40	35
Dihydroxyacetone	"	0.10	0.28	30
Methylglyoxal	"	4.00	3.80	860
"	+ GSH	4.40	4.80	990
"	+ <i>M</i> /50 iodoacetate	2.00	1.50	310
"	in air	3.15	2.90	Not measured

Pyruvic acid. Very little pyruvic acid disappears when added alone to cartilage. In presence of cozymase a small proportion is converted into lactic acid. The lactic acid formation increases on addition of hexosediphosphate (Table 3). Cocarboxylase appears to have no effect whatever upon the breakdown of pyruvate in cartilage. Incidentally it was found that after prolonged incubation with cartilage, cocarboxylase loses much of its coenzyme activity as tested by the method of Lohmann & Schuster [1937].

Yet another substance tested in this series of experiments was *d*-glucurone, which however appeared to remain unchanged after incubation with cartilage.

Oxidation and reduction systems in cartilage. There is very little O_2 -uptake when fresh finely-minced cartilage is tested manometrically (1–2 μ l. per g. per hr.). Addition of synovial fluid is without effect, but carriers like methylene blue and pyocyanine slightly increase the reaction. In anaerobic conditions there is a slow, thermolabile reduction of methylene blue, 1.0 ml. $M/2000$ being reduced by 1 g. of fresh cartilage at 37° in 1–2 hr. In a few instances when cartilage slices were left for a day or two in phosphate buffer without an antiseptic the time for the methylene blue reduction fell to 20 min. but this acceleration was found to be due to bacterial infection. No O_2 -uptake or methylene blue reduction was obtained with cartilage extracts.

The following substances were examined as substrates for aerobic and anaerobic oxidation by cartilage.

Succinate	Pyruvate	Glutamate
Lactate	Glucose	Alanine
α -Phosphoglycerol	Glucosamine	Adrenaline
Malate	Aldehyde	3:4-Dihydroxyphenylalanine
Citrate	Alcohol	Catechol
Acetate	Hypoxanthine	
Formate	Urate	

The necessary coenzymes and carriers were added and the experiments were generally arranged so as to afford for every reaction optimum conditions as known from the study of other tissues. Very small positive results were found with lactate in presence of cozymase, glucose in presence of coenzyme II, and hypoxanthine, but otherwise negative results were obtained.

When fresh cartilage is used for the aerobic oxidation of dihydroxyphenylalanine there is no O_2 -uptake and the solution of the amino-acid in contact with the tissue remains colourless, while the control solution without tissue takes up a certain amount of O_2 and becomes dark. When instead of fresh cartilage toluene-treated cartilage is used for the experiment, there is a slight uptake of O_2 and the tissue and the solution of dihydroxyphenylalanine both become dark. Adrenaline, but not catechol, shows a similar behaviour.

On spectroscopic examination of a thick layer of powdered cartilage no cytochrome spectrum is visible and only a faint trace of reduced cytochrome *c* bands can be seen on addition of hydrosulphite. Similar observations were made with ligaments and synovial fluid.

Ascorbic acid was estimated in fresh cartilage, synovial fluid and synovial mucosa but negative results were obtained. Glutathione appears to be absent from cartilage and synovial fluid, judging by the negative nitroprusside test, but in some parts of the synovial mucosa the test is slightly positive.

On examination for heavy metals in cartilage, 0.5 mg. Fe was found per 100 g. vacuum-dried cartilage. Mn and Cu are absent.

DISCUSSION

The present study was carried out in an attempt to elucidate the mechanism of glycolysis and oxidation in cartilage. The work of Robison and his collaborators has contributed much to our knowledge of the action of bone phosphatase on various phosphorylated glycolysis products, but little information was available with regard to lactic acid production in normal articular cartilage from these or any other substances.

The study of fermentation in yeast and muscle has established its dependence on certain catalysts such as the adenine nucleotides and cozymase. It appears

that these substances are relatively unstable when acted upon by calf cartilage. It may be assumed, however, that conditions exist for their resynthesis, e.g. by the interaction of adenylic acid with phosphoglyceric acid (p. 522). An interesting point in the behaviour of the adenine derivatives in cartilage is their breakdown to hypoxanthine, in contrast to their behaviour in muscle, where the process does not proceed beyond inosinic acid.

The results obtained with various substrates show that lactic acid formation is possible in cartilage (Table 3). They also suggest two mechanisms: one, which appears to involve the presence of cozymase, is the oxido-reduction between hexosediphosphate and pyruvate, in a manner similar to the glycolytic cycle in muscle, and another is the conversion of methylglyoxal into lactic acid. Cozymase must be added in experiments with glycogen and hexosediphosphate to enable the reaction to proceed, but the reaction with methylglyoxal requires no addition of GSH to cartilage although it is definitely accelerated by traces of GSH. Methylglyoxal seems to represent a normal breakdown product of hexosediphosphate, but even with the lactic acid it accounts for not more than 30–40% of the carbohydrate dephosphorylated. According to rotation measurements carried out by Martland & Robison [1929] the main product of decomposition of hexosediphosphate by bone phosphatase is a mixture of aldoses and ketoses and not fructose itself.

This enquiry into the oxidative systems proves that normal cartilage is rather poor in cytochrome and reducing substances such as ascorbic acid and glutathione, although there is obviously enough of the latter to evoke the glyoxalase activity in cartilage. These findings agree with the inability of cartilage to oxidize any of the many substances tested to an appreciable extent.

SUMMARY

An attempt was made to study in detail enzyme systems in cartilage concerned with glycolysis and other oxido-reductions. Cartilage from young normal animals (calf) was used in the form of slices or extracts. For comparison some experiments were carried out with other joint tissues, synovial fluid, synovial membrane and ligaments.

Adenosinetriphosphoric, muscle adenylic, yeast adenylic and inosinic acids and adenosine are attacked in cartilage by dephosphorylation and/or deamination. Hypoxanthine is the end-product of these reactions. Cozymase and Warburg's coenzyme lose their catalytic activities after prolonged incubation with cartilage. Cocarboxylase also is unstable under the influence of cartilage.

The ability of cartilage to produce lactic acid from various phosphorylated and non-phosphorylated carbohydrates was tested. The small positive results obtained with some of the substances investigated suggest two mechanisms of lactic acid production in cartilage, one of which depends upon the presence of cozymase while the other is catalysed by glyoxalase. As intermediary products methylglyoxal is formed from hexosediphosphoric acid and pyruvic acid from phosphoglyceric acid.

Articular cartilage appears to be almost completely unable to oxidize substances such as succinate, lactate, aldehyde and several other substances which are rapidly oxidized in other tissues.

This work was carried out with the assistance of a grant from the Empire Rheumatism Council. The Co-operative Society and Messrs Warrington & Son, of Cambridge, have very generously supplied the experimental slaughterhouse material.

The author is indebted to Dr Stephenson for help in the bacteriological examinations and to Dr Drury for encouragement and advice and finally to Prof Sir F. G. Hopkins for his kind interest in this work.

REFERENCES

- Birch, Harris & Ray (1933). *Biochem. J.* **27**, 590.
- Bywaters (1937). *Path. Bact.* **44**, 247.
- Clift & Cook (1932). *Biochem. J.* **26**, 1788.
- Fiske & Subbarow (1925). *J. biol. Chem.* **66**, 375.
- Friedemann, Cotonio & Shaffer (1927). *J. biol. Chem.* **73**, 335.
- Hagedorn & Jensen (1923). *Biochem. Z.* **135**, 46.
- Lohmann (1932). *Biochem. Z.* **254**, 332.
- & Jendrassik (1926). *Biochem. Z.* **178**, 418.
- & Schuster (1937). *Biochem. Z.* **294**, 188.
- Martland & Robison (1929). *Biochem. J.* **23**, 237.
- Mendel & Goldscheider (1925). *Biochem. Z.* **164**, 164.
- Parnas & Heller (1924). *Biochem. Z.* **152**, 5.
- Robison (1923). *Biochem. J.* **17**, 286.
- Tillmans & Philippi (1929). *Biochem. Z.* **215**, 36.