129. THE METABOLISM OF ARTICULAR CARTILAGE

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THIS work is a continuation of that of Bywaters [1937] who showed that cartilage has practically no respiratory or oxidative enzymes but has definite glycolytic and dehydrogenase activities. The object of the present work was to determine the function of the dehydrogenase in the absence of oxidase. After getting indications that it played a part in the glycolytic mechanism, further study of glycolysis was made. While the work was being prepared for publication, a paper by Lutwak-Mann [1940] has appeared dealing with different aspects of cartilage glycolysis, especially the role of possible phosphorylated intermediates and coenzymes.

Methods

Manometric estimations were carried out as described by Dixon [1934] except that for the small O_2 uptakes measured "KOH-papers" were unnecessary and were avoided in order to decrease the blanks. To obtain sufficient material, the carpo-metacarpal joints of freshly slaughtered horses were used for most experiments and except where otherwise stated all data refer to this material. The medium was Ringer solution buffered to pH 7.3 by NaH_2PO_4 - Na_2HPO_4 for O_2 uptake or $NaHCO_3$ - CO_2 for manometric estimation of acid production. Except where other details are given the buffer concentration was 0.2 vol. isotonic solution and glucose was added to give a final concentration of 0.2%. Further details are as given by Bywaters [1937]. Manometric methods were supplemented by chemical estimations of bisulphite-binding substances (BBS) by the method of Cliff & Cook [1932] and lactic acid by that of Friedemann & Graesser [1933].

Metabolic quantities are expressed, for convenience, as $\mu l./g.$ dry tissue, i.e. the Q values given here should be divided by 10^3 for comparison with the usual metabolic quotients for more active tissues.

Results

(1) *Respiration.* Table 1, summarizing the results of a large number of experiments, shows that the respiration of horse articular cartilage is definite though small. The standard deviation indicates there is a 1 in 3 chance of the Q_{O_2} lying between -3 and $-17 \mu l./g./hr.$ and a 21 in 22 chance between $+4$ and -24 . Actually the 80 recorded values ranged from 0 to -39 . These values should perhaps be reduced slightly since controls with the same amount of medium (5 ml.) and glass wool in place of tissue to prevent excessive splashing of this unusually large amount showed a small fall in pressure equivalent to *ca.* $-4 \mu l./g./hr.$ This is presumably due to absorption of the last traces of CO_2 in the vessel. Table 1 also extends the observations of Bywaters [1937] on the increase of O_2 uptake by methylene blue. The optimum effect occurs in the neighbourhood of $10^{-4} M$ but varies with the sample of tissue. Slight inhibition

Table 1. O_2 uptake of articular cartilage and the effect of methylene blue

	Methylene blue concentration ($\times 10^{-5} M$)					
	0	1	3	7	10	100
(a) Horse						
Mean Q_{O_2} ($\mu\text{l./g./hr.}$)	10	42	65	72	88	64
No. of observations	80	7	28	6	23	3
Standard deviation	7	20	37	13	20	7
Mean control (no tissue)	3	—	4	—	5	4
No. of observations	9	—	4	—	5	2
Standard deviation	4	—	5	—	7	4
(b) Rabbit						
Mean Q_{O_2} ($\mu\text{l./g./hr.}$)	150	—	450	—	350	—
No. of observations	4	—	4	—	4	—
Standard deviation	35	—	60	—	60	—

 Table 2. Effect of 2:4-dinitro-o-cresol on O_2 uptake

Exp.	State of cartilage	Q_{O_2} ($\mu\text{l./g./hr.}$)				
		Conc. of 2:4-dinitro-o-cresol ($\times 10^{-5} M$)				
		0	1	3	10	100
15/3	Normal	5	16	18	26	9
22/3	Normal	13	—	—	25	17
1/4	Eroded	5	—	—	6	2
5/4	Normal	8	—	—	21	6

occurs with $10^{-3} M$ but this concentration is not highly toxic as in kidney [Dickens, 1936]. Dinitro-o-cresol also catalyses the O_2 uptake at similar concentrations with normal tissue (Table 2), but the effect is very much smaller. This supports the view that cartilage must respire to a small extent since nitrophenols exert their effect only on tissues with a complete respiratory system [Greville & Stern, 1935]. The rate with high concentrations of methylene blue was not limited by diffusion into the tissue since increase of the usual average thickness of the slices from 0.24 to 0.65 mm. reduced the O_2 uptake only insignificantly from -112 to -102.

Articular cartilage from rabbits had a much greater O_2 uptake than tissue from the horse (Table 1); fibrous cartilage (rabbit meniscus) similarly gave a value -30, increased to -110 and -140 by 3 and $10 \times 10^{-5} M$ methylene blue respectively. This material was, however, less suitable for systematic investigation on account of the number of animals, labour and time involved in preparing sufficient for use in the Warburg apparatus. The micro-respirometer of Heatley *et al.* [1939] would be more suitable for it. The values approach those of Dickens & Weil-Malherbe [1936] for rat costal cartilage, 220-680.

(2) *Dehydrogenase activity.* Since respiration is normally small, the optimal O_2 uptake in the presence of methylene blue is a measure of the activity of complete dehydrogenase systems (i.e. enzyme-coenzyme-diaphorase) in the tissue. To get some idea of the substrates used, the effects of glucose, lactate and pyruvate were tested (Table 3) after a period of substrate deprivation, which supplied a control figure for the same sample of tissue when corrected for the slight spontaneous fall with a similar sample of untreated tissue. Glucose and lactate had no effect and pyruvate was slightly inhibitory in the experiment quoted. In other experiments it appeared that lactate was most efficient in maintaining O_2 uptake at its initial level, while the fall in the presence of pyruvate might be as much as 60%. It is evident that the tissue itself provides ample substrate for maximum dehydrogenase activity. Lactic dehydrogenase seems to be involved

Table 3. *Effects of substrates and other reagents on O₂ uptake with or without methylene blue*

Exp.	Reagent	Conc. 10 ⁻³ M	Dye conc. 10 ⁻⁵ M	Q _{O₂} (μl./g./hr.)		
				Control	With reagent	% change
*8/6	Pyruvate	25	0	36	40	+11
	"	25	3	109	94	-14
	l(+)-Lactate	25	3	109	111	+2
	Glucose	12.5	3	120	119	-1
†12/8	Ca ⁺⁺	2	3	83	84	+1
	"	2	10	112	117	+5
†18/1	F ⁻	2	3	36	38	+6
	"	6	3	37	44	+33
	"	12	3	37	50	+35
†22/1	"	2	10	68	69	+1
	"	6	10	45	46	+2
	"	12	10	51	43	-16
27/1	HCN	1	7	50	64	+28
19/2	"	10	10	68	91	+34
22/2	n-Heptyl alcohol	ca. 12 (sat.)	10	108	17	-84
†24/2	"	ca. 12 (sat.)	10	86	6	-93
30/4	o-Iodoxybenzoate	1	0	31	29	-7
	"	1	3	125	121	-3
4/5	"	10	0	7	8	+14
	"	10	3	85	55	-35

* No glucose present.

† Using Ca⁺⁺-free Ringer.

‡ Cartilage showing erosion and fibrillation.

on account of the pyruvate inhibition [Green & Brosteaux, 1936]. Bywaters [1937] showed that the rate of lactate production either with or without added glucose was adequate to account for the O₂ uptake now observed.

The effect of various reagents on the process is also shown in Table 3. No constant inhibition was produced by fluoride, and with a methylene blue concentration barely sufficient to induce maximal O₂ uptake a small but definite rise was observed. The table also shows that the necessary omission of Ca⁺⁺ from the Ringer in these experiments does not affect the values. The effect of HCN is of greatest interest since the value was increased by about a third, suggesting the removal of a ketonic oxidation product as cyanohydrin [Wendel, 1934; Green & Brosteaux, 1936]. The precautions of Krebs [1935] to prevent loss of HCN to the absorbing KOH were adopted. As with *n*-heptyl alcohol which was a powerful inhibitor, it was only possible to determine controls on separate samples of tissue in different vessels, owing to the volatility of the reagents. The results are therefore less accurate than with other reagents on account of the difficulty of getting identical samples with such large quantities of tissue. The effect of *o*-iodoxybenzoate was tested since its use in the treatment of arthritis suggested the possibility of a specific effect on cartilage metabolism. It caused some inhibition in the 1st hr. in the experiment quoted but was effective only at high concentration. Other experiments showed that the inhibition increased to about 50% in the 2nd hr. with 10 mM.

Further evidence for the production of pyruvate was the positive nitroprusside reaction [Simon & Piaux, 1924] in the vessel contents, after the removal of methylene blue with kaolin, only at the end of a long experiment (5 hr.). The

BBS formation, determined also after the removal of methylene blue, was inadequate to account for the O_2 uptake (Table 4, exp. 19/12) according to equation (1) which is discussed later. The production of CO_2 aerobically in the presence of methylene blue was always observed, though the accuracy was much less than is usual in the determination by the phosphate method of Dickens & Simer [1930] owing to the large blank, determinable with an error as large as

 Table 4. *Pyruvate metabolism and lactate formation*

Exp.	Conditions		Metabo- lite	Initial μl.	Final μl.	μl./g./hr.
19/12	Glucose, 5 hr.	O ₂	CO ₂	42	88	+ 39
	Tissue, 230 mg. dry wt.	MeB, 10 ⁻⁴ M	O ₂	—	—	- 54
			BBS	32	75	+ 36
11/1	Pyruvate, 4 hr.	N ₂	CO ₂	64	80	+ 14
	Tissue, 275 mg. dry wt.		BBS	109	99	- 9
			Volatile BBS	2	2	0
4/1	Glucose, 4 hr.	N ₂	CO ₂	57	79	+ 20
	Tissue, 270 mg. dry wt.	MeB, 10 ⁻⁴ M	Lactate	60	204	+ 133
			O ₂	77	65	- 11
		O ₂	—	—	- 22	
			Lactate	42	234	+ 178
		O ₂	CO ₂	57	98	+ 38
			MeB, 10 ⁻⁴ M	O ₂	—	—
	Lactate	120	388	+ 248		

$\pm 15\%$. This was unavoidable owing to the difficulty of washing CO_2 completely from the large samples of tissue, although that in the medium was kept as low as possible by reducing the phosphate concentration to $M/60$. The CO_2 formed cannot arise by simple decarboxylation of pyruvate, since there was no anaerobic formation of volatile BBS (Table 4, exp. 11/1; solution made acid to Congo red, added acetaldehyde giving 98% recovery). In this experiment the total BBS disappearance was small and CO_2 formation insignificant. It therefore seems most likely that CO_2 formed aerobically arises by oxidative decarboxylation of pyruvate, perhaps catalysed by methylene blue. Such oxidative decarboxylations are of widespread occurrence in animal tissues and bacteria with O_2 [Long, 1938; Barron & Lyman, 1939], dyes [Lipmann, 1937] or even a molecule of the same or another ketonic acid [Krebs & Johnson, 1937; Krebs, 1937; Lipmann, 1939] as oxidant. If O_2 is the ultimate oxidant the oxidation of lactate may occur in accordance with the scheme (quantitative data from Table 4, exp. 19/12):

Net formation of pyruvate = 30μ l./g./hr. (calc.)
 Formation of BBS = 36μ l./g./hr. (obs.)

The O_2 uptake and loss of pyruvate in reaction (2) were calculated from the CO_2 formed and the remainder of the observed O_2 uptake was assumed to be due to the formation of pyruvate from lactate according to equation (1). The net calculated pyruvate formation agreed very well with the BBS found experi-

mentally. Direct confirmation of this hypothesis by determination of volatile acid was not possible on account of the difficulties in determining the small amount expected (e.g. about 4 ml. 0.5 mM per vessel in the exp. quoted). It seems unlikely that much of the BBS was methylglyoxal although this was formed from hexosediphosphate by calf cartilage [Lutwak-Mann, 1940], since it would require even more complete oxidation of some other substrate to account for the O_2 -uptake, presumably with greater evolution of CO_2 .

(3) *Anaerobic glycolysis*. It had been observed that lactate production (determined chemically) was greater aerobically than anaerobically when the O_2 -uptake was above the average, and that it was further increased by methylene blue (Table 4, exp. 4/1). A decrease had been expected according to the hypothesis that lactate was oxidized to pyruvate under these conditions. Bywaters [1937] had found only irregular differences between aerobic and anaerobic glycolysis but the lactic acid formed was equivalent to the CO_2 liberated from $NaHCO_3$ buffer. The effect of pyruvate on glycolysis was therefore studied manometrically (Fig. 1). There was little effect in the absence of glucose, but in

Fig. 1.

Fig. 1. *The effect of pyruvate on glycolysis*. Compounds added at 0 min. to give final concentration: G = glucose, 10 mM; P = pyruvate, 2 mM; P' = pyruvate, 0.2 mM. 60 min. preliminary anaerobiosis.

Fig. 2.

Fig. 2. *The effect of fluoride on glycolysis and its pyruvate catalysis*. Compounds added at 0 min. (after 60 min. preliminary anaerobiosis) to give final concentration: G = glucose, 12.5 mM; P = pyruvate, 1 mM; F = fluoride, 7 mM.

the presence of that substrate, after a period of anaerobiosis without it, the induction period of 30–50 min. normally needed to reach the maximum rate was completely abolished by pyruvate at as low a concentration as 0.2 mM. The effect was catalytic since (a) 0.2 mM pyruvate, *ca.* 75 μ l./g. tissue, produced more than 130 μ l./g. extra CO_2 , (b) higher pyruvate concentration was slightly inhibitory, (c) pyruvate without glucose produced little extra CO_2 . Pyruvate is apparently a normal intermediate in cartilage glycolysis, but is not normally present in sufficient amount for maximal glycolysis in the presence of added substrate. Since Bywaters [1937] found that the glycolysis is less sensitive to fluoride in the absence than in the presence of added glucose, the effects of fluoride were studied

further (Fig. 2). Pyruvate catalysis was largely abolished and the results of Bywaters were confirmed.

The effects of other inhibitors were also studied (Table 5). Iodoacetate caused 50% inhibition at 0.017 mM and almost complete inhibition at mM. Inhibition was also almost complete with *n*-heptyl alcohol and *o*-iodoxybenzoate, but at

Table 5. *Effect of inhibitors on glycolysis*

Exp.	Inhibitor	Conc. 10 ⁻³ M	Q _G ^N (μl./g./hr.)		
			Control	With inhibitor	% change
*25/4	Iodoacetate (5-35 min.)	1	33	10	- 70
	„ (45-115 min.)	1	14	0	- 100
27/4	„	1	128	11	- 91
	„	0.1	79	11	- 86
	„	0.017	130	62	- 52
25/5	<i>o</i> -Iodoxybenzoate	1	155	140	- 10
20/7	„	10	164	18	- 89
	Washed tissue from above	0	158	9	- 94
30/8	<i>n</i> -Heptyl alcohol	ca. 12 (sat.)	222	14	- 93

* No added substrate.

higher concentrations. With the latter "retention" was shown to be negligible by tipping in 20 mg. 50 mM lactic acid at the end of the experiment. The effect was not reversed by washing the tissue (600 mg. wet wt. in 25 ml. glucose-NaHCO₃-Ringer) and resuspending in fresh fluid. It seems likely to be due to irreversible inactivation of some enzyme, probably not the lactic dehydrogenase since the redox-dye-catalysed O₂ uptake was less affected (Table 3, exp. 4/5).

DISCUSSION

Although the above evidence is insufficient to establish the Embden-Meyerhof-Parnas scheme as a glycolytic mechanism for cartilage, it does suggest, especially in conjunction with the work of Lutwak-Mann [1940], the possibility of such a process. Pyruvate catalysis in particular suggests that it takes part in the oxido-reduction:

The dehydrogenase of cartilage for which Bywaters [1937] could suggest no function in the absence of oxidase evidently plays a part in this reaction, lactic and triosephosphate dehydrogenases being linked through a coenzyme. Iodoacetate inhibits at this stage, while fluoride prevents the rearrangement of phosphoglycerate to phosphopyruvate [Lohmann & Meyerhof, 1934] giving a fresh supply of pyruvate by dephosphorylation. In spite of the different sensitivities of autoglycolysis and glucolysis to pyruvate catalysis and fluoride inhibition, it is unnecessary to postulate separate mechanisms as did Bywaters [1937], since pyruvate concentration is evidently the limiting factor in attaining maximum glycolysis (cf. Dickens & Greville [1933] on rat testis and Jensen rat sarcoma). Fluoride inhibition is not complete except at high concentration and an inhibition of pyruvate re-formation insufficient to affect greatly the rate of autoglycolysis may yet affect maximal glucolysis, requiring a greater amount of catalyst. Lutwak-Mann [1940], working with calf cartilage, concluded that, in addition to the oxido-reduction mechanism, lactic acid could also be produced by

the action of cartilage glyoxalase, methylglyoxal being a normal intermediate in the breakdown of hexosediphosphate by the tissue. Since lactic acid production from hexosediphosphate was unaffected by 20 mM iodoacetate in her experiments, this mechanism cannot apply to old horse cartilage where almost complete inhibition of glycolysis was always observed with 1 mM iodoacetate both with and without added glucose. It seems likely that the glyoxalase mechanism is not specifically associated with cartilage metabolism but rather with the metabolism of all young and embryonic tissues, though glucolytic and glyoxalase enzymes react independently in chick embryos [Needham & Lehmann, 1937; Needham & Nowiński, 1937].

SUMMARY

1. The O_2 uptake of horse articular cartilage, normally *ca.* $10 \mu\text{l./g./hr.}$, is increased optimally by $3\text{--}10 \times 10^{-5} M$ methylene blue to 50–150 or by $10^{-4} M$ 2:4-dinitro-*o*-cresol to 20.
2. The cartilage from a joint showing arthritic changes failed to respond to dinitro-*o*-cresol.
3. The O_2 uptake of rabbit articular cartilage is similarly increased by $3 \times 10^{-5} M$ methylene blue from 150 to $450 \mu\text{l./g./hr.}$ and that of rabbit fibrous cartilage from the meniscus by $10^{-4} M$ methylene blue from 30 to 140.
4. The dye-catalysed O_2 uptake of horse cartilage is inhibited strongly by *n*-heptyl alcohol and partially by *o*-iodoxybenzoate. It is increased by HCN, sometimes increased by fluoride and unaffected by Ca^{++} .
5. The oxidation leads to the formation of pyruvate, but this is oxidized further with liberation of CO_2 .
6. Pyruvate catalyses the glycolysis of cartilage and this catalysis is inhibited by fluoride.
7. The anaerobic glycolysis is also strongly inhibited by iodoacetate, iodoxybenzoate and *n*-heptyl alcohol.

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