Evidence for a negative Pasteur effect in articular cartilage

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Uptake of external glucose and production of lactate were measured in freshly-excised bovine articular cartilage under O_2 concentrations ranging from 21 % (air) to zero (N_2-bubbled). Anoxia (O_2 concentration < 1 % in the gas phase) severely inhibited both glucose uptake and lactate production. The decrease in lactate formation correlated closely with the decrease in glucose uptake, in a mole ratio of 2:1. This reduction in the rate of glycolysis in anoxic conditions is seen as evidence of a negative Pasteur effect in bovine articular cartilage. Anoxia also suppressed glycolysis in articular cartilage from horse, pig and sheep. Inhibitors acting on the glycolytic pathway (2-deoxy-D-glucose, iodoacetamide or fluoride) strongly decreased aerobic lactate production and ATP concentration, consistent with the belief that articular cartilage obtains its principal supply of ATP from substrate-level phosphorylation in glycolysis. Azide or cyanide lowered the ATP concentration in aerobic cartilage to approximately the same extent as did anoxia but, because glycolysis (lactate production) was also inhibited by these treatments, the importance of any mitochondrial ATP production could not be assessed. A negative Pasteur effect would make chondrocytes particularly liable to suffer a shortage of energy under anoxic conditions. Incorporation of [35S]sulphate into proteoglycan was severely curtailed by treatments, such as anoxia, which decreased the intracellular concentration of ATP.

INTRODUCTION

Articular cartilage is avascular and communicates with the rest of the body only by limited processes of diffusion of solutes to and from blood vessels in the underlying bone and through contact with the synovial fluid which washes across the articular surface intermittently, propelled by the movement of the joint. Because of the long diffusion pathways involved, provision of glucose and especially O_2 to the cartilage may be particularly critical. Cells deep within human articular cartilage may be as much as 3 mm from a nutrient artery [1]. Calculations show that the relatively few cells (chondrocytes) that reside in a unit volume of cartilage form a sufficient sink to deplete the O_2 concentration from 6–10 % at the synovial surface to 1–6 % [2], or perhaps almost to zero [3], in the deepest layers [2]. Yet cartilage has a clear requirement for O_2. For example in culture, cartilage or isolated chondrocytes need approx. 20 % O_2 in the gas phase for optimum growth [3–7].

Even so, the principal function of O_2 in this context remains unclear. We still do not know to what extent O_2 is involved in the fundamental processes of respiratory metabolism and energy production. The Embden–Meyerhof–Parnas (EMP) pathway of glycolysis is strongly active in articular cartilage, and lactate is a major end-product of respiratory metabolism [8,9]. On a per-cell basis, the amounts of lactate formed are comparable with those produced by tissues with a good blood supply, such as liver or kidney (Table 1 in [10]). However, the per-cell consumption of O_2 by cartilage is only 2–5 % of that of liver or kidney (Table 1 in [10]), even when measured in air-saturated medium instead of the 6–10 % O_2 concentration typical of synovial fluid [2]. This striking combination of rapid aerobic lactate synthesis with low O_2 consumption has led to the view that, in cartilage, ATP is generated predominantly by substrate-level phosphorylation in the glycolytic pathway [1], in which O_2 is not, of course, involved.

One would predict, therefore, that the energy status of chondrocytes should be largely insensitive to O_2 supply. Calculations based on rates of O_2 uptake and lactate production by cartilage (Table 1 in [10]) suggest that oxidative phosphorylation, if it occurs, could account for no more than one-quarter of the total ATP production; a useful contribution but not a dominant one. Moreover, any reduction in ATP yield from this source during anoxia might be offset if a classical Pasteur effect were present, as has been claimed [11].

In reality, however, there is very little empirical information on the effect of O_2 concentration on energy production either in intact cartilage or in isolated chondrocytes. Such information that is available comes mostly from long-term experiments [3–7] in which the outcome may be influenced by adaptation of the cartilage to the culture conditions (often including a new O_2 concentration) over a period of several days. To avoid this complication we have studied the effects on glucose uptake, lactate production and matrix synthesis over a short period, usually 4.5 h. Changes due to adaptation should thus be minimized, and in this respect at least the cartilage should remain closer to its condition in the joint.

EXPERIMENTAL

Incubation medium

The medium used for washing and incubation was based closely on Dulbecco's modified Eagle's medium (DMEM), but without amino acids, vitamins or Phenol Red. The medium contained the same concentration of mineral salts as DMEM [12], with the exception of $Fe(NO_3)_3$, which was omitted. Glucose (1 g·l⁻¹ or in some cases, 0.3 g·l⁻¹) and 25 mM Hepes buffer were also included in the medium. Bicarbonate was not added because it is not required for internal pH regulation by chondrocytes [13]. After adjusting the pH to 7.40 with NaOH, the calculated sodium concentration was 114.5 mM; routinely at the start of an experiment a further 30 mM of NaCl was added to bring the total sodium concentration to approx. 145 mM and the osmolarity to approx. 280 mosmol. The medium was filter-sterilized and stored deep-frozen until immediately before use.

Preparation of cartilage

Feet of cow, sheep, pig and horse were collected from local abattoirs within 2 h of the animals being slaughtered. Cartilage was dissected from the metacarpal-phalangeal and metatarsal-phalangeal joints, and washed with 5–6 changes of medium (see above). Three human femoral heads were obtained after surgery for femoral neck fracture.

Procedure

In our standard procedure for measuring glucose uptake, lactate production or sulphate incorporation, cartilage (40–80 mg dry weight per replicate) was incubated for 4.5 h at 37 °C in 2 ml of medium (with additions according to experimental needs) in a polypropylene tube (15 mm × 95 mm) closed with a gas-tight neoprene septum. Where required, either air, O_2-free N_2 or the appropriate mixture of O_2 in N_2 was bubbled continuously through the incubation mixture via an extended 21G needle. Emission to the atmosphere was through a 25G needle. There was negligible loss of liquid volume during the 4.5 h period. When sulphate incorporation was to be determined, [^{35}S]sulphate was added to the medium to give a specific activity of approx. 150 MBq·mmol^{-1} of sulphate, the exact value was found by scintillation spectrophotometry of 10 μl standards. In certain experiments, metabolic inhibitors or amino acids were included in the medium during incubation. To avoid spurious effects, a constant sodium ion concentration was maintained within an experiment. For example, if 10 mM NaN_3 was added, then 10 mM NaCl was added to the non-azide controls. At the end of the incubation period, the sealed tubes containing the cartilage were cooled rapidly in melting ice and then frozen at -20 °C.

Measurement of [^{35}S], glucose and lactate

After removing the supernatant for glucose and lactate analyses (see below), the cartilage was washed by mechanical agitation for 5–6 periods of 1 h each in 2.5 ml of a solution containing 150 mM NaCl and 5 mM Na_2SO_4, at 5 °C. Tests on cartilage incubated with [^{35}S]sulphate for 4.5 h showed that the release of radioactivity into the medium after the third wash was negligible. The cartilage was dried at 65 °C for 3 days, weighed, transferred to scintillation vials and digested overnight with 2 ml of papain solution at 65 °C [14]. ^{35}S was measured by scintillation spectroscopy after the addition of 3 ml of Liquiscint, a water-miscible, gel-forming scintillant.

Glucose uptake was calculated from glucose depletion of the medium, which initially contained 0.3 g glucose·l^{-1}. Samples (20 μl) were taken at the beginning and end of the incubation period and the glucose concentration was measured by a glucose oxidase colorimetric procedure with dianisidine as the chromophore (Sigma). Lactate was quantified using a clinical lactate analyser (Yellow Springs Instruments, Model 23L), employing membrane-immobilized L-lactate oxidase. To avoid making assumptions about linearity with time, values of glucose uptake, lactate production and [^{35}S]sulphate incorporation are reported as μmol (or nmol)/4.5 h per g dry weight of cartilage.

Extraction and estimation of ATP in cartilage

To the incubation mixture containing the cartilage, 2 ml of 20 % (v/v) $HClO_4$ was added, the tube was cooled on ice for 1 h, the pH was adjusted to 6.0–6.5 with 40 % (w/v) KOH and solid $KHCO_3$, and the sample was cooled on ice for a further period of 1 h to allow the $KClO_4$ to precipitate. The ATP concentration of the supernatant was measured by the luciferin–luciferase method (Bio-Orbit). Controls showed that, at the concentrations used, the metabolic inhibitors had a negligible effect on ATP concentration.

RESULTS

General aspects of experiments with articular cartilage

When measured under standardized conditions, the rates of the processes being studied often differed widely between supposedly similar cartilage samples obtained on separate occasions from animals of the same species and approximately the same calendar age. Thus in identical experiments on metacarpal-phalangeal cartilage taken from separate weekly deliveries of fresh bovine tissue, the highest and lowest rates of aerobic glucose uptake, [^{35}S]sulphate incorporation and lactate production differed 3.7-fold, 4.4-fold and almost 5.5-fold respectively. By contrast, the coefficient of variation (S.D./mean) of the 4–5 replicates of a treatment within an experiment was usually quite small; typically 8 % for aerobic lactate production, 11 % for aerobic glucose uptake, and 12 % for aerobic [^{35}S]sulphate incorporation (median values from 60, 22 and 59 experiments respectively). True replication of a given treatment could be achieved only by repeating the experiment many times. For this reason, the results of an entire set of experiments are given wherever possible, and, except in the Tables and where otherwise stated, the term S.D. is used to denote the standard deviation of the group of mean values obtained from these independent experiments.

### Glucose uptake and lactate production in relation to oxygen supply

The consumption of external glucose and the formation of lactate provided a convenient index of glycolytic metabolism in cartilage (see critique in Discussion).

Bovine articular cartilage took up (mean ± S.D.) 22 ± 5 μmol of glucose/4.5 h per g dry weight from aerobic medium containing 0.3 g of glucose·l^{-1} (Table 1). Anoxia adversely affected

Table 1   Glucose uptake and lactate production by bovine articular cartilage under aerobic or anoxic conditions

Bovine articular cartilage from 1.5–2 year old animals was incubated at 37 °C for 4.5 h in medium containing 0.3 g of glucose·l^{-1}, under aerobic or anoxic conditions. Each line of the Table shows the uptake of glucose and concurrent production of lactate within the same experiment. The results are of 12 separate experiments, and are the means (± S.D.) of six replicates per group unless otherwise stated (in parentheses). *Experiments in which the glucose uptake under anoxic conditions was not significantly different from zero ($P > 0.1$).

Glucose uptake (μmol of glucose/4.5 h per g dry weight of cartilage)		Lactate production (μmol of lactate/4.5 h per g dry weight of cartilage)	
Aerobic	Anoxic	Aerobic	Anoxic
14.4 ± 4.2	2.6 ± 3.9*	35.9 ± 2.3	12.7 ± 1.0
17.3 ± 2.1	2.9 ± 4.3*	45.4 ± 4.2	14.4 ± 2.7
17.8 ± 2.5	0.0 ± 1.3*	37.4 ± 4.3	8.1 ± 0.6
18.7 ± 1.4 (4)	2.1 ± 1.6* (4)	41.0 ± 2.0 (4)	8.2 ± 1.3 (4)
20.5 ± 3.3	3.1 ± 1.2	48.0 ± 2.2	13.9 ± 2.3
21.8 ± 1.5 (4)	5.7 ± 2.6* (4)	45.5 ± 3.1 (4)	11.4 ± 1.1 (4)
21.8 ± 1.1	7.9 ± 2.3 (5)	43.1 ± 6.0	20.1 ± 3.4 (5)
22.2 ± 3.4	3.7 ± 2.8*	47.5 ± 4.4	12.1 ± 1.4
23.1 ± 4.5	1.9 ± 3.3*	54.6 ± 2.5	19.1 ± 3.4
29.1 ± 2.4	15.4 ± 3.1	53.8 ± 3.2	28.3 ± 2.4
31.0 ± 5.6	4.9 ± 2.1	65.4 ± 9.3	18.9 ± 3.6
31.2 ± 2.4	10.5 ± 5.1	62.9 ± 4.9	18.8 ± 2.3
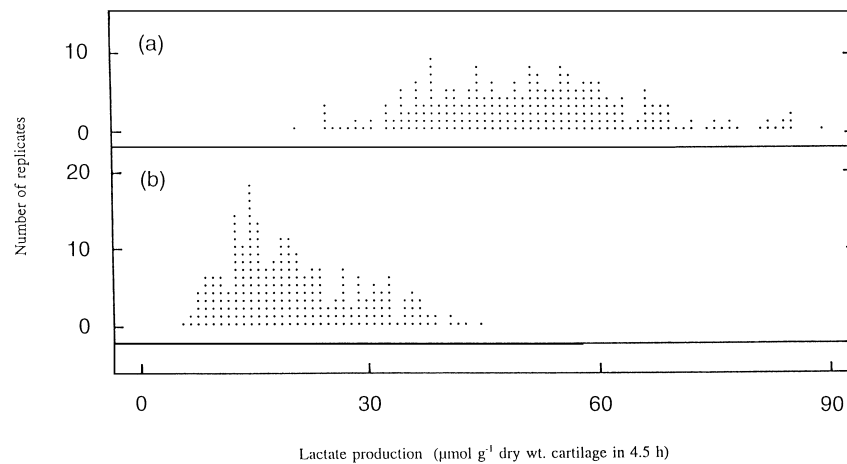
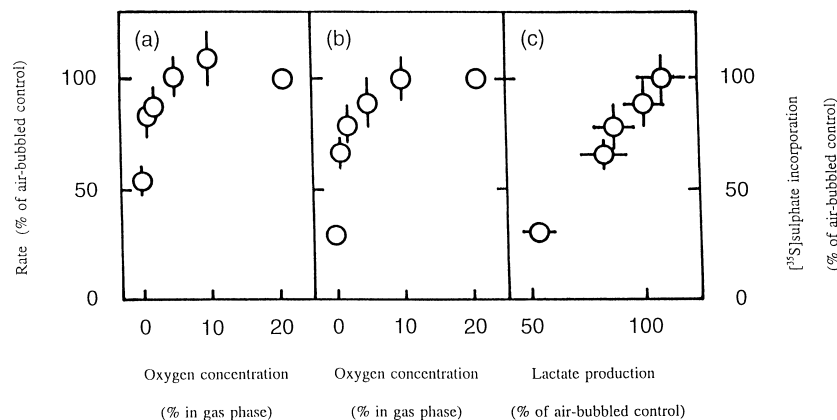**Figure 1   Lactate production by bovine articular cartilage under (a) aerobic conditions or (b) anoxic conditions**

The cartilage was incubated for 4.5 h in medium containing 1 g of glucose·l⁻¹. The Figure shows the combined results of 48 experiments, comprising a total of 247 aerobic and 238 anoxic replicates. Each dot represents a replicate in one of those experiments. The median values for aerobic and anoxic conditions were, respectively, approx. 51 and 18 μmol lactate produced/4.5 h per g dry weight of cartilage.

glucose uptake, which was decreased (mean$\pm$S.D.) to 5 ± 4 μmol/4.5 h per g dry weight of cartilage (Table 1). Indeed, the results of seven of the experiments show that anoxic glucose uptake did not differ significantly from zero ($P > 0.1$). Anoxia also depressed lactate production from (mean$\pm$S.D.) 48 ± 9 (aerobic) to 16 ± 6 μmol lactate/4.5 h per g dry weight of cartilage (Table 1).

Most measurements of lactate production were made in medium containing 1 g of glucose·l⁻¹ (Experimental section), and the larger body of data obtained in this way supported the findings described above. Figure 1 shows the complete set of data for lactate production by bovine articular cartilage incubated in this medium, from 1.5–2 year-old animals. Based on 48 independent experiments, the mean rate ($\pm$S.D.) of aerobic lactate production (Figure 1a) was 52 ± 13 μmol/4.5 h per g, and of anoxic lactate production (Figure 1b) was 21 ± 8 μmol/4.5 h per g dry weight of cartilage. Log-transformation of the data to

take account of its non-Gaussian distribution (cf. Figure 1b) gave mean values of 51 μmol (aerobic) and 19 μmol (anoxic) of lactate/4.5 h per g dry weight of cartilage. On average, anoxic lactate production was $39\pm10\%$ ($\pm$S.D.) (median 39%) of the aerobic rate measured in the same experiment (Figure 1). Lactate production was depressed by bubbling N_2 through the medium to the same extent as that found in anoxia imposed under static conditions in Thunberg tubes. Continuous bubbling with air did not affect lactate production when compared with static aerobic controls; the mean air-bubbled rate was $94\pm9\%$ ($\pm$S.D.) of the controls ($P > 0.5$, data from eight experiments).

Lactate production began to decrease markedly when the O_2 concentration in the gas phase was reduced to 5%. Analysis of variance showed that the inhibition reached statistical significance ($P < 0.05$) in 2% O_2, and was highly significant ($P < 0.001$) in anoxic conditions (Figure 2a). Note that the comparison here is with air-bubbled controls.

Figure 2   Effect of oxygen concentration on (a) lactate production, (b) [³⁵S]sulphate incorporation, by bovine articular cartilage; and (c) relationship between lactate production and [³⁵S]sulphate incorporation in the same sample of cartilage

The cartilage was incubated for 4.5 h in medium containing 1 g of glucose·l⁻¹ and [³⁵S]sulphate, bubbled with the O_2 concentrations indicated. Each experiment included all six O_2 concentrations, except in one case where the 5% and 10% concentrations were omitted. Each symbol is the mean ($\pm$S.E.M.) of 5–6 experiments. Figure 2(c) is based on the data from Figures 2(a) and 2(b).
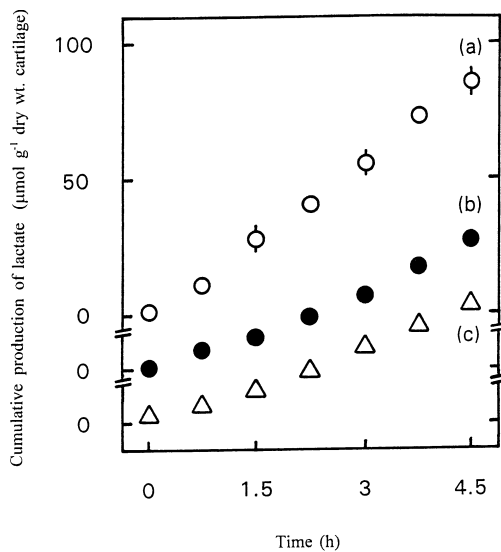
Figure 3   Time-course of lactate production by bovine articular cartilage under aerobic conditions (○), anoxic conditions (●) or anoxic conditions and cycloheximide (△)

The cartilage was incubated in medium containing 1 g of glucose·l⁻¹, with 0.4 mM cycloheximide added in (**c**). Samples (100 μl) were taken at 45 min intervals and frozen at −20 °C for lactate analysis later. Bars show the S.D. of three replicates within the same experiment. In (**b**) and (**c**), the vertical scale has been displaced downwards for clarity. Total amounts (±S.D.) of lactate produced/4.5 h per g dry weight of cartilage were: (**a**) 86±4, (**b**) 47±3, and (**c**) 43±4 μmol; $n = 3$ replicates. From fitted polynomials, the initial rate of lactate production was 7.4 μmol/h per g dry weight for anoxic cartilage compared with 13.8 μmol/h per g dry weight for cartilage incubated in air.

Table 2   Effect of glucose supply under aerobic or anoxic conditions on lactate production by bovine articular cartilage

Bovine articular cartilage from 1.5–2 year old animals was incubated at 37 °C for 4.5 h in medium containing 1 g of glucose·l⁻¹ or in the same medium without glucose, under aerobic or anoxic conditions. The results of seven separate experiments are presented. Results appearing on the same line were obtained in the same experiment, and are the means (±S.D.) of four replicates per group unless otherwise stated (in parentheses).

Lactate production (μmol of lactate/4.5 h per g dry weight of cartilage)

Anoxic		Aerobic	
No glucose	With glucose	No glucose	With glucose
7.7±0.9	10.7±3.2	20.5±0.7	46.2±8.7
10.7±2.6 (5)	21.9±3.1 (5)	17.1±1.8 (5)	55.6±1.3 (5)
12.1±1.2	12.8±2.8	22.7±2.3	48.9±1.8
12.1±0.3	31.0±3.2	23.4±1.7	56.8±1.1
14.1±1.4	19.9±1.9	20.9±1.5	51.1±1.6
	37.2±2.4	38.2±4.2 (5)	77.2±6.2 (5)
		30.5±1.9	60.6±1.9

Reduced lactate production under anoxic conditions was immediately obvious (Figure 3b), when compared with the rate of production under aerobic conditions (Figure 3a), and was unaffected by the addition of 0.4 mM cycloheximide (Figure 3c), which has been shown to reduce protein synthesis by cultured bovine articular cartilage to a negligible level within 20 min [15].

Testing for reversibility, we exposed bovine articular cartilage to successive 4.5 h periods of air or anoxia. During the first 4.5 h

Table 3   Effect of medium composition on lactate production by bovine articular cartilage under aerobic or anoxic conditions

Bovine articular cartilage from 1.5–2 year old animals was incubated at 37 °C for 4.5 h under aerobic or anoxic conditions. The results shown are from six separate experiments and are the means (±S.D.) of five replicates per group unless otherwise stated (in parentheses). The standard medium is described in the Experimental section. In experiments where DMEM was used, Hepes (25 mM) was added so that it was similar in this respect to the standard medium. Within the group of six experiments, the median rates of lactate production in the standard medium alone were 75 μmol of lactate and 41μmol of lactate/4.5 per g dry weight of cartilage for aerobic and anoxic conditions respectively. *Amino acids added at the same concentrations as found in DMEM [12]. †Comparison of media within the same experiment.

Composition of incubation medium	Lactate production (μmol of lactate/4.5 h per g dry weight of cartilage)	
	Aerobic	Anoxic
Standard medium plus amino acids* and L-glutamine (4 mM)	77±5†	29±4
	94±10	51±9
	106±18	61±10
Standard medium plus L-glutamine (20 mM)	88±7 (4)	42±3 (4)
DMEM plus L-glutamine (4 mM)	104±5†	98±8
	123±4 (6)	111±8 (6)
	125±15	133±20

under anoxia, lactate production was reduced to 35–44% of its aerobic rate, as expected (the results quoted are from two separate experiments). The rate was further reduced during a second 4.5 h period of anoxia, to 25–35% of the aerobic rate during this period, but in cartilage that was returned to aerobic conditions during the second 4.5 h period, lactate production rose to 52–65% of the corresponding aerobic rate. This was approximately double the rate found in anoxic cartilage during the second 4.5 h period. The inhibitory effect of anoxia on lactate production could, therefore, be partially reversed.

Effect of medium composition on lactate production

The presence of external glucose tended to increase lactate production by cartilage, even in anoxic conditions. In five experiments, the mean (±S.D.) anoxic lactate production in glucose-free medium was 11±2 μmol/4.5 h per g (Table 2, column 1), and the addition of 1 g of glucose·l⁻¹ to the medium increased the rate to 19±8 μmol lactate/4.5 h per g dry weight of cartilage (Table 2, column 2). In three of these experiments, the stimulation due to external glucose was significant ($P < 0.01$). The effect of external glucose was somewhat greater under aerobic conditions. In seven experiments, the mean (±S.D.) aerobic lactate production in glucose-free medium was 25±7 μmol/4.5 h per g (Table 2, column 3), and this increased to 57±10 μmol lactate/4.5 h per g dry weight of cartilage when 1 g of glucose·l⁻¹ was included in the medium (Table 2, column 4).

Anoxic conditions decreased glycolysis in cartilage incubated in medium containing a mixture of amino acids (identical to those found in DMEM), or with a higher concentration (20 mM) of L-glutamine alone (Table 3). However, when cartilage was incubated in DMEM, lactate production was notably rapid in aerobic conditions and continued at a high rate under anoxia (Table 3).

Table 4 Effect of metabolic inhibitors on lactate production and [^{35}S]sulphate incorporation by bovine articular cartilage

Bovine articular cartilage from 1.5–2 year old animals was incubated at 37 °C for 4.5 h in medium containing 1 g of glucose·l^{-1} and [^{35}S]sulphate (see the Experimental section), with inhibitors as indicated. The medium was aerobic unless otherwise stated. Each value is the result of a single experiment, except as indicated: * is the median of 5 experiments (mean ± S.D., 45 ± 30 %); † is the mean (± S.D.) of 4 experiments (median value, 11 %); ‡ are the means (± S.D.) of the same 14 experiments; § is the median of 4 experiments (mean ± S.D., 201 ± 21 %). n.d., not determined.

Inhibitor		Lactate production (% of control without inhibitor)	Incorporation of [^{35}S]sulphate (% of control without inhibitor)
2-Deoxyglucose	0.05 M	42, 53	49, 51
Iodoacetamide	0.01 M	33*	12 ± 8†
NaF	0.01 M	38, 45	25
Sodium oxamate	0.01 M	52, 53, 55	48, 50, 66
NaCN	0.01 M	n.d.	66, 82
NaN$_3$	0.01 M	70 ± 15‡	21 ± 6‡
Anoxia		33 ± 7‡	20 ± 9‡
DNP	1×10^{-5} M	128	100
	3×10^{-5} M	157, 169, 177	82, 86
	1×10^{-4} M	173§	72, 77, 94
	3×10^{-4} M	101	35
CCCP	1×10^{-6} M	127	89
	3×10^{-6} M	149, 164	66, 78
	1×10^{-5} M	85, 116, 176	26, 52, 92
	3×10^{-5} M	68	12

Table 5 Effect of metabolic inhibitors on ATP concentration in bovine articular cartilage

Bovine articular cartilage from 1.5–2 year old animals was incubated at 37 °C for 3 h in 2 ml of medium containing 1 g of glucose·l^{-1}, with metabolic inhibitors as indicated. Except in the anoxic experiment, the medium was aerobic (in contact with air). At the end of the incubation period, ATP was extracted and measured as described in the Experimental section. The results of three separate experiments are presented. The values appearing in the same column were obtained in the same experiment and are the means (± S.D) of three replicates per group unless otherwise stated (in parentheses). *The ATP concentration was too low for accurate measurement. †A small modification to the apparatus enabled the perchloric acid to be added without exposing the incubation medium to air, hence this second anoxic experiment may be more reliable than the first.

Inhibitor		Concentration of ATP (nmol of ATP per g dry weight of cartilage)		
Control		235 ± 8	200 ± 21	217 ± 28
2-Deoxyglucose	0.02 M		73 ± 5	
Iodoacetamide	0.01 M	approx. 0*		
NaF	0.01 M		24 ± 15	
NaN$_3$	0.01 M	152 ± 67	153 ± 16	
NaCN	0.01 M			159 ± 10 (4)
Anoxia		218 ± 13		146 ± 10†
DNP	3×10^{-5} M	253 ± 16		
DNP	1×10^{-4} M	205 ± 24		

Metabolic inhibitors, lactate production, and the concentration of ATP

We examined the response of bovine articular cartilage to three different groups of well-known metabolic inhibitors.

The first group, consisting of 2-deoxy-D-glucose, iodo-acetamide, and NaF, was intended to block specific steps in the glycolytic pathway by inhibiting the enzymes glucose-6-phosphate isomerase [16], glyceraldehyde-3-phosphate dehydrogenase [17] and enolase [18] respectively. Oxamate, a competitive inhibitor of lactate dehydrogenase [19], was also used. Treatment of cartilage with substances in this group severely depressed both lactate formation (Table 4) and ATP concentration (Table 5). The effect of oxamate on ATP level was not tested.

The second group of inhibitors comprised NaCN and NaN$_3$, which primarily act on the terminal oxidase (complex IV) of mitochondrial electron transport [20]. The presence of NaCN in the incubation medium interfered with the lactate assay and its influence on lactate formation could not be determined easily. NaN$_3$ did not interfere with the assay procedure. Anoxia inhibited lactate production to a greater extent than NaN$_3$ at the concentration tested (Table 4) but NaCN, NaN$_3$ or anoxia lowered the concentration of ATP in cartilage to a similar extent (Table 5).

The protonophores dinitrophenol (DNP) and carbonyl cyanide m-chlorophenylhydrazone (CCCP) comprised the third group of inhibitors. Either compound stimulated aerobic lactate formation when present at low concentration (Table 4). As expected, CCCP was at least ten times as potent as DNP [21]. Based on the results of one experiment only, DNP at $(3–10) \times 10^{-5}$ M seemed to cause little change in ATP concentration (Table 5).

Sulphate incorporation in relation to O$_2$ supply and metabolic inhibitors

Conversion of [^{35}S]sulphate into an insoluble form which was not exchangeable with external sulphate gave a measure of proteoglycan synthesis, a physiologically important activity of chondrocytes [10].

The rate of external sulphate incorporation varied widely between one sample of cartilage and another. Under aerobic conditions, bovine articular cartilage from 1.5–2 year old animals incorporated approx. 89 nmol of external sulphate/4.5 h per g dry weight of cartilage (median value of 22 experiments), with mean rates in individual experiments ranging from 50–137 nmol of external sulphate/4.5 h per g dry weight of cartilage. Under anoxia, the median incorporation, in 23 experiments, was approx. 15 nmol of external sulphate/4.5 h per g dry weight of cartilage, which was approx. 17 % of the corresponding aerobic rate. Compared with air-bubbled controls, [^{35}S]sulphate incorporation was very significantly inhibited ($P < 0.001$) in O$_2$ concentrations of 2 % or less (Figure 2b).

Inhibitors acting principally upon the glycolytic pathway, namely 2-deoxyglucose, iodoacetamide, NaF and oxamate, decreased [^{35}S]sulphate incorporation markedly (Table 4). NaN$_3$ (10 mM) in aerobic medium produced a similar effect to that observed in anoxic conditions. The results found with CCCP and DNP were somewhat variable, but either compound reduced [^{35}S]sulphate incorporation at concentrations which stimulated lactate production (Table 4).

Response to O$_2$ supply: comparison of species and age

To investigate whether the pattern of results seen above is a general one or is confined to bovine cartilage, we studied the influence of O$_2$ supply on glucose uptake, lactate production and [^{35}S]sulphate incorporation in articular cartilage from the functionally equivalent joints of cow, horse, sheep and pig. The tissue was taken from animals of widely differing ages wherever possible.

Expressed on a dry weight basis, the rates of all of these processes tended to be greater in cartilage from young animals than from older animals of the same species, reflecting, in part, the greater cellularity of the juvenile material [22,23]. Despite

Table 6   Glucose uptake and lactate production by articular cartilage from animals of various species and ages

Articular cartilage was incubated at 37 °C for 4.5 h in medium containing 1 g of glucose l^{-1}, (or *containing 0.3 g of glucose·l^{-1}), under aerobic or anoxic conditions. The values appearing on the same line were obtained in the same experiment and are the means ($\pm$ S.D.) of six replicates per group unless otherwise stated (in parentheses). † Denotes the results (means $\pm$ S.D.) of 12 experiments (Table 1).

Species	Age	Glucose uptake (μmol/4.5 h per g dry weight of cartilage)		Lactate production (μmol/4.5 h per g dry weight of cartilage)	
		Aerobic	Anoxic	Aerobic	Anoxic
Cow	Newborn			170 $\pm$ 20	100 $\pm$ 10
Cow	1 month	37 $\pm$ 9	2 $\pm$ 4 (5)	68 $\pm$ 11 (10)	19 $\pm$ 6
Cow	1.5–2 years	22 $\pm$ 5*†	5 $\pm$ 4*†	48 $\pm$ 9*†	16 $\pm$ 6*†
Cow	16 years	14 $\pm$ 5*	4 $\pm$ 5*	29 $\pm$ 7*	9 $\pm$ 1*
Horse	18 years	14 $\pm$ 2*	4 $\pm$ 2*	31 $\pm$ 2*	14 $\pm$ 1*
Horse	30 years	9 $\pm$ 4*	3 $\pm$ 4*	34 $\pm$ 5 (5)	21 $\pm$ 3 (5)
Sheep	3 months			110 $\pm$ 5	45 $\pm$ 7
Sheep	3 months	74 $\pm$ 6	57 $\pm$ 9 (5)	100 $\pm$ 5 (11)	75 $\pm$ 9 (11)
Sheep	7–8 years	25 $\pm$ 6*	15 $\pm$ 5*	66 $\pm$ 6*	26 $\pm$ 3*
Pig	4 months	61 $\pm$ 7	36 $\pm$ 9	110 $\pm$ 10	60 $\pm$ 7
Pig	4 months	70 $\pm$ 12	52 $\pm$ 11	120 $\pm$ 20 (11)	120 $\pm$ 20 (11)
Human	80 years	3 $\pm$ 2* (4)	4 $\pm$ 1* (4)	11 $\pm$ 2* (4)	11 $\pm$ 2* (4)
Human	90 years			22 $\pm$ 10 (8)	18 $\pm$ 10 (8)
Human	93 years			15 $\pm$ 5 (4)	12 $\pm$ 4 (4)

considerable variability between experiments, the same trends appeared to persist across the range of animal species, namely greater rates of glucose uptake and of lactate production under aerobic conditions than under anoxia (Table 6). The results for [^{35}S]sulphate incorporation were similar (results not shown).

Three experiments on cartilage removed from human femoral heads showed that lactate production per unit dry weight was low, and the effect of anoxia was not convincing (Table 6).

DISCUSSION

Factors affecting glycolysis in articular cartilage

Some caution is necessary when attempting to estimate the rate of glycolysis from the uptake of external glucose, because glucose taken up may accumulate in the cells during the experimental period, or an existing intracellular glucose pool may be depleted. Glucose may also be drawn off the pathway for other purposes, for example as glucose-1-phosphate for UDP-glucose synthesis, or carbon may enter from other sources, notably from glycogen breakdown. Measurements of lactate production might tend to underestimate glycolytic flux because pyruvate has other possible fates besides reduction to lactate.

Even so, these caveats are unlikely to have a bearing on the interpretation of the results in this case. The changes in glucose consumption and in lactate production agreed closely, and both results pointed to the same conclusion: that in articular cartilage treated with a simple salts-plus-glucose medium, glycolysis operated rapidly under aerobic conditions (Table 1; Figures 1a, 2a), but was strongly inhibited by anoxia. Thus in cartilage from bovine feet, glycolysis was reduced to 40 % of its aerobic rate under anoxic conditions, based on lactate production (Table 1; Figure 1b); or to 25 % of the aerobic rate if glucose uptake was the criterion (Table 1). Krebs [24] first used the expression 'negative Pasteur effect' to describe a situation in which carbohydrate breakdown is suppressed under anoxia, and we follow

his terminology here. Other more recent authors have preferred 'the reverse Pasteur effect' [25,26].

A negative (or reverse) Pasteur effect has been detected in animal materials as diverse as intestinal mucosa [27] and the blood-parasite, *Leishmania* [25] when tested under experimental conditions similar to ours. However, in its response to low ambient O$_2$, articular cartilage contrasts strongly with the fibrocartilaginous intervertebral disc [28], where both glucose uptake and lactate production increase under anoxia.

A few scattered results in the literature support our findings for fresh cartilage incubated in simple media (Krebs–Ringer solution or similar). For example, under these conditions, freshly-excised equine or bovine articular cartilage produced lactate at the same rate, or more slowly, in N$_2$ compared with in air [22,29], or in 100 % O$_2$ [30]. This phenomenon may be obscured when complex media are used (Table 3). Frequently cited evidence of a classical Pasteur effect in cartilage comes from two studies on rabbit articular chondrocytes or cartilage slices grown in DMEM or a similar medium for 6–7 days before the experiment [3,7]. Treatments which counteract the effect of anoxia will be described more fully in a separate communication.

The role of internal versus external carbon sources as substrates for glycolysis

Our results reinforce the consensus [1] that lactate is the principal end-product of external glucose metabolism by articular cartilage under aerobic conditions. In the results of 12 experiments shown in Table 1, the mean mole ratio of aerobic lactate production/aerobic glucose uptake was 2.19 $\pm$ 0.23 ($\pm$ S.D.) (median 2.12; range 1.85–2.62), which was not significantly different ($P > 0.4$) from the mole ratio of 2.0 predicted from the EMP pathway. Evidently, when aerobic cartilage is supplied with external glucose very little carbon from endogenous sources is metabolized through glycolysis. Moreover, it follows that even with O$_2$ (air) available (Table 1, columns 1 and 3), a negligible fraction of the reductant generated by the EMP pathway is used in any reaction other than the reduction of pyruvate. This, together with the notably low O$_2$ consumption per cell in freshly-isolated cartilage (Table 1 in [10]), places limits on the possible role of oxidative phosphorylation as a source of energy in this tissue.

The decrease in external glucose consumption almost exactly matched the decrease in lactate production in anoxic conditions. In the results of 12 experiments shown in Table 1, the mole ratio of the decrease in lactate production to the decrease in glucose uptake was 1.90 $\pm$ 0.19 (mean $\pm$ S.D.), which was not significantly differently from 2.0 ($P > 0.5$). So does anoxia suppress glycolysis merely by inhibiting transport of glucose across the cell membrane? This mechanism appears unlikely because anoxic conditions depressed lactate production by bovine articular cartilage deprived of an external glucose supply and was therefore entirely dependent on internal carbon sources (Table 2, columns 1 and 3). Transport of external glucose was clearly not involved under these circumstances. Table 2 (columns 1 and 3) shows that bovine articular cartilage contains a reserve of carbon, probably in the form of glycogen [11], which enables lactate production to continue for several hours without a supply of external glucose.

Indeed, even with external glucose present (Table 2, column 2), anoxic cartilage was still drawing, to a large extent, on internal carbon sources because more lactate was produced than could be accounted for by the depletion of glucose from the external solution. The mean mole ratio of lactate production/glucose uptake under anoxic conditions was approx. 3.9 (median 3.8) (Table 1, omitting the third line of data).
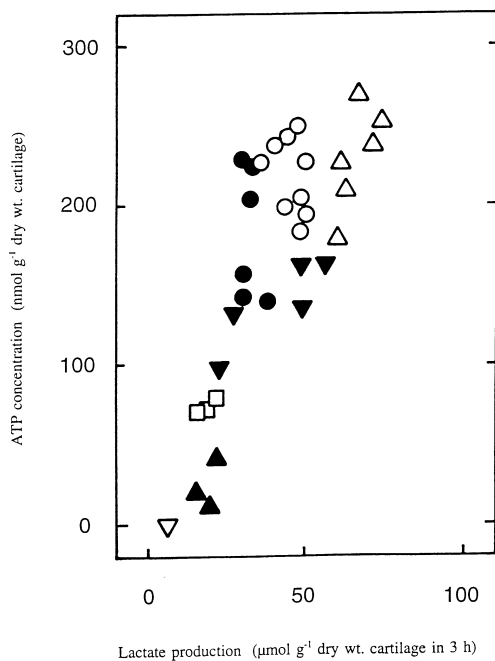
Figure 4   Effect of metabolic inhibitors on ATP concentration and lactate production by bovine articular cartilage

The Figure shows the final concentration of ATP in, and the cumulative production of lactate by articular cartilage during a 3 h incubation in medium containing 1 g of glucose·l^{-1}, with the following treatments: air, (○); anoxia, (●); 10 mM NaN$_3$, (▼); 0.03 mM or 0.1 mM DNP, (△); 20 mM 2-deoxyglucose, (□); 10 mM NaF, (▲); 10 mM iodoacetamide, (▽).

Glycolysis, ATP concentration and [^{35}S]sulphate incorporation

The luciferin–luciferase method shows the concentration of ATP in the tissue at the moment when metabolism is stopped by the addition of perchloric acid. Under aerobic conditions, bovine articular cartilage contained approx. 220 nmol ATP per g dry weight (Table 5). Cartilage of this kind has a fresh weight/dry weight ratio of approx. 3.7, and if chondrocytes occupy 2% of its volume, the concentration of ATP within the chondrocytes would be approx. 3 mM, which is close to the range found in other cell types.

Except in cartilage treated with DNP or CCCP, the tissue concentration of ATP was proportional to the rate of glycolysis, measured by lactate production (Fig. 4). Deoxyglucose, iodoacetate and NaF are recognized inhibitors of glycolysis (lactate production) in equine and bovine articular cartilage [22,29,30] and in chondrosarcoma cells [31,32]. In the present experiments, 2-deoxyglucose, iodoacetamide and NaF reduced the tissue concentration of ATP by the same proportion as the decrease in the glycolytic flux (Figure 4). These results are consistent with the general belief [10] that substrate-level phosphorylation in glycolysis is a major means of ATP generation in cartilage, although the data are not sufficient to prove that substrate-level phosphorylation is the only source. Even so, because glycolysis is clearly central to energy production in this tissue, the existence of a negative Pasteur effect *in vivo* could make articular chondrocytes particularly vulnerable to any reduction in O$_2$ supply, even a temporary or localized one such as might occur when the joint is static.

There was a clear relationship between glycolysis and [^{35}S]sulphate incorporation (Figure 2c; Table 4), except in cartilage treated with CCCP or DNP. The link is presumably through the

supply of ATP. NaN$_3$ (10 mM) or anoxia inhibited [^{35}S]sulphate incorporation to the same extent (Table 4), in accord with their broadly comparable effects on the tissue concentration of ATP (Table 5). In another study [33], proteoglycan synthesis by cultured bovine articular cartilage was correlated with the intracellular concentration of ATP, which was varied by partial denaturation of glyceraldehyde-3-phosphate dehydrogenase. However, in chondrosarcoma cells cultured in DMEM, neither NaN$_3$ (5 mM) nor a N$_2$ atmosphere affected the intracellular concentration of ATP [32].

Are chondrocyte mitochondria involved in the response to O$_2$?

Anoxia or NaN$_3$ decreased the glycolytic flux to lactate profoundly under our experimental conditions (negative Pasteur effect). While recognizing the hazard of inferring the rate of ATP synthesis from tissue concentrations, we consider that the loss of ATP production by substrate-level phosphorylation would, itself, go far towards explaining the reduction in intracellular ATP concentration (Table 5), and in [^{35}S]sulphate incorporation (Table 4), without the need to invoke large-scale mitochondrial synthesis of ATP. Certainly in chondrocytes the flow of electrons through any putative mitochondrial electron transport chain must be small, because: (a) cartilage is characterized by a low O$_2$ uptake rate per cell (Table 1 in [10]); (b) the mole ratio of lactate production/glucose uptake indicates that negligible reductant from glycolysis is directed into any process except pyruvate reduction in aerobic articular cartilage (see above); and (c) blocking lactate dehydrogenase with oxamate lowers [^{35}S]-sulphate incorporation (Table 4), suggesting, by analogy with other glycolytic inhibitors, a substantial decrease in glycolytic flux. This, in turn, would imply that articular cartilage mitochondria are incapable of re-oxidizing the bulk of the cytosolic reductant generated in aerobic glycolysis. Indeed, the cytochrome complement of articular chondrocytes *in situ* may be incomplete [34,35].

Even so, there is a hint that the mitochondrial electron transport chain in chondrocytes may not be entirely inactive. Protonophores such as DNP, dinitro-*o*-cresol or CCCP stimulate O$_2$ uptake and aerobic glycolysis in articular cartilage as they do in other more widely studied tissues [36–40]. Thus Hills [29] noted that O$_2$ uptake by horse articular cartilage was stimulated 2–5-fold by 10^{-4} M dinitro-*o*-cresol, and there are tentative data from bovine articular cartilage suggesting that 5×10^{-5} M DNP more than doubles O$_2$ uptake (Dr E. M. Bartels, personal communication). Low concentrations of CCCP or DNP promoted aerobic glycolysis (lactate production) in bovine articular cartilage (Table 4), as reported previously with 10^{-4} M DNP [30], but there was no increase *pro rata* in the standing concentration of ATP (Table 5). With $(3–10) \times 10^{-5}$ M DNP, a concentration of approx. 365 nmol ATP per g dry weight of cartilage would have been predicted from the enhanced glycolytic rate (Tables 4, 5). These responses of articular cartilage to protonophores might be seen as evidence for mitochondrial electron transport being coupled to the vectorial transport of protons across the inner mitochondrial membrane under aerobic, control conditions. The resultant $\Delta\bar{\mu}_{H^+}$ is, of course, an essential pre-requisite for ATP synthesis by the mitchondrial F$_1$-ATPase, though not proof that such synthesis occurs.

Concluding remarks

The influence of O$_2$ on glycolysis itself remains to be explained. The classical, positive Pasteur effect depends principally upon the regulatory properties of 6-phosphofructo-1-kinase [41]

and its partner in the substrate cycle, fructose-1,6-bisphospha-tase. Stimulation of aerobic glycolysis by DNP or CCCP in a tissue that relies heavily on oxidative phosphorylation would be attributed to: (a) decreased inhibition of 6-phosphofructo-1-kinase because of lower ATP concentrations; (b) greater acti-vation of 6-phosphofructo-1-kinase due to higher concentrations of AMP and fructose 2,6-bisphosphate; (c) greater inhibition of fructose 1,6-bisphosphatase due to the higher AMP level. So, by collapsing $\Delta\bar{\mu}_{H^+}$, DNP or CCCP produce what is really a classical Pasteur effect, in air.

Yet there is a paradox here. If, in spite of some doubts (see Introduction, and above), oxidative phosphorylation makes an important contribution to ATP generation in articular cartilage, why does anoxia not stimulate glycolysis (the classical Pasteur effect) in this tissue, as do DNP or CCCP? Conversely, if mitochondria are not involved and ATP is generated solely by substrate-level phosphorylation in the EMP pathway (which ends in lactate and is self-contained in oxidation–reduction terms), why is the rate of glycolysis sensitive to ambient O_2 at all? We do not know. The glycolytic pathway in articular cartilage is enzymically capable of simulating a classical Pasteur effect, as the DNP and CCCP treatments show (Table 4), but anoxia produces exactly the opposite response: glycolysis is suppressed. The decrease in glycolytic rate is abrupt and is unaffected by cycloheximide (Figure 3), suggesting that it is mediated by existing enzymes rather than by new proteins. The regulatory mechanism underlying the negative Pasteur effect in articular cartilage requires further investigation.

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