Studies of cellular metabolism in isolated intact bovine retinas by ^{31}P NMR

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1. INTRODUCTION

^{31}P NMR provides a powerful, non-destructive method for examining the metabolic processes of specific cellular and tissue processes, and has been used to study a wide range of biological organs, including the isolated heart [1], muscle [2], liver [3] and kidney [4]. We now extend these measurements to the eye and report here the in vitro levels of phosphocreatine (PCr), ATP, NAD, sugar phosphates and inorganic phosphate (P_i) of isolated perfused bovine retinas. The results obtained suggest that the technique is of value in assessing the quality of in vitro retinal preparations, and when compared with the values obtained for rabbit, rat and frog retinas by conventional methods [5–8], show both similarities and, in some cases, major differences.

2. MATERIALS AND METHODS

The inherently low sensitivity of NMR requires the use of a considerable amount of tissue, and for this reason experiments were carried out on bovine eyes. These were enucleated immediately after death from cows killed in dim light conditions, and were transported in darkness at 0°C to the laboratory within 20 min. The retinas from 3–6 eyes were isolated and mounted in a 25 mm NMR tube adapted for perfusion at 37°C with an oxygenated (95% O_2:5% CO_2) Ringers solution containing (mM): NaCl (115), $NaHCO_3$ (21), KCl (3.4), $MgCl_2$ (2.4), $CaCl_2$ (2.0), HEPES (5.0) and glucose (28). In separate experiments it was established that in these conditions the retinas maintained physiological activity, as indicated by their electroretinograms, which showed both *a*- and *b*-waves.

Spectra were measured with a Bruker WM200 wide-bore spectrometer operating 81 MHz for ^{31}P. Magnet shimming was accomplished using the proton free-induction decay (FID) from the water in the circulating perfusate: typically a linewidth of 5–10 Hz was achieved. The instrument was operated without a deuterium lock, since its stability was such that no field drift was observed over the timescale of the experiments. Successive blocks of 500 4-k FIDs were accumulated using 45° pulses repeated every 0.8 s, and each block was stored individually, to avoid the possibility of summation over changes in the spectra arising from deterioration of the tissue. In fact, with the perfusion conditions employed, no change was observed between successive blocks. In contrast, anoxic conditions produced major and largely irreversible spectral changes. The use of up to 6 retinas (corresponding to < 0.3 g dry wt) represented a compromise between the need to maintain adequate perfusion of the tissue within the 25 mm NMR tube, and the achievement of a reasonable signal-to-noise ratio/unit time.

3. RESULTS AND DISCUSSION

Fig.1 shows the spectrum obtained following Fourier transformation of 10 000 FIDs (20 blocks, line-broadening of 10 Hz), together with assignments of the major resonances, made by compari-

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son with ^{31}P spectra obtained from other tissues [9,10].

Resonance F, at −16.24 ppm arises exclusively from the β-phosphate of ATP. Resonance D, at −2.54 ppm arises from the γ-phosphate of ATP, but also contains a contribution from the β-phosphate of any ADP present. Similarly, resonance E at −8.34 ppm, while primarily due to the α-phosphates of ATP and ADP, also contains a contribution from NAD/NADH, seen as a partially resolved upfield peak.

The region extending from 1.5–4.0 ppm contains a number of small peaks, the most prominent occurring at 3.0 ppm. Identification of these resonances cannot be made with certainty, though in a variety of tissues it has been shown that they are due to phosphodiesters such as glycerophosphylcholine and serine ethanolamine phosphodiester [11,12]. The spectra also show an underlying broad resonance, extending over a range of > 40 ppm, which arises from relatively immobile phospholipids. It is, of course, possible that sharper resonances from more mobile phospholipids do arise, but are masked by their accidental coincidence with other peaks.

The most prominent feature of the spectrum (resonance A, fig.1), due to the sugar phosphates, appears to have 2 major components. The low field contribution at 6.80 ppm, which accounts for some 2/3rds of the observed intensity, is almost certainly due to glucose 6-phosphate. The high field component probably contains overlapping resonances from a number of sources, such as fructose 1-phosphate and fructose 1,6-bisphosphate. The resonance from P_i occurs at 4.96, a position which corresponds to an internal tissue pH of ~7.13.

With the proviso that coincidence of resonances may give misleading values, it is possible to estimate

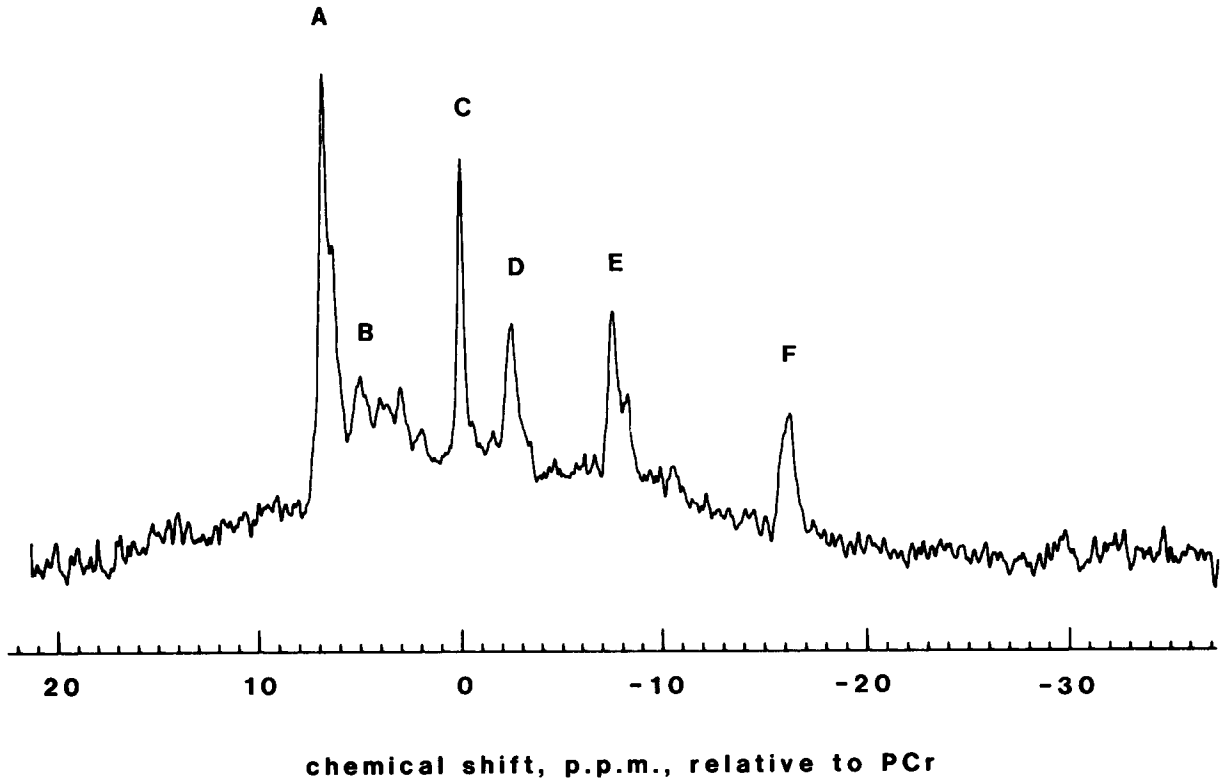

Fig.1. ^{31}P NMR spectrum of 3 bovine retinas perfused with an oxygenated medium (pH 7.4) at 25 ml/min. The spectrum represents the accumulation of 10 000 FIDs repeated at 0.8 s intervals: peak A arises from sugar phosphates; B from P_i; C from PCr; D from γ ATP and β ADP; E from α ATP, α ADP and NAD; F from β ATP.

the relative concentrations of the substances giving rise to peaks in the spectrum of fig.1. Relative to [ATP] = 1 these are: hexose phosphates 3.53; PCr 1.31; NAD/NADH 0.39; ADP 0; P_i 0.83. Since it was necessary to use short interpulse delays in order to obtain adequate signal-to-noise ratios, these values will be subject to differential T_1 relaxation effects. However, if corrections based on the T_1-values measured in other tissues are applied, the relative values are not significantly changed.

Comparison of these values with other estimates of retinal levels shows both similarities and marked differences. Thus the PCr concentration is comparable with the values quoted in [7], and this is also the case for NAD/NADH. In contrast, the hexose phosphates are present in much greater abundance than has been found by biochemical assays [6,7], and ADP appears to be almost completely absent in our preparation (cf. [7]). It should be stressed that the present method only detects 'free' molecules, and any bound ADP, such as occurs in skeletal muscles, is not observed. The level of P_i seen in the present preparation is also much lower than that previously reported for retinal tissue. However, it is possible that this may be due in part to 'washout' of the phosphate into the phosphate-free perfusion medium [13].

Anoxic conditions caused considerable changes to the spectra of the retinas. Initially there was a mild reduction of phosphocreatine, and this was increased as the anoxia was continued. Over 15 min the level of phosphocreatine fell by $\geq$ 50%. During this time there was also a marked increase in the size of the P_i peak. No substantial change occurred in the ATP resonances. The changes ceased when oxygenation was recommenced, but no substantial reversal towards the original levels was observed, in contrast to the behaviour seen in the heart [1].

When retinas are exposed to light, the concentrations of several phosphorus containing substances within the photoreceptors and other cells are reported to undergo changes [7,14]. Of these, changes in phosphocreatine and ATP should be readily observable by the ^{31}P-NMR method, though as yet, with the partially light-adapted tissues and perfusion media (cf. [7]) we have been using, they have not been seen. Of the particular interest, because of possible roles in transduction and adaptation, are cGMP and cAMP. However, the levels at which these are present in the retina [14] are such that their observation would require signal averaging over several hours to achieve the ATP signal-to-noise ratio of fig.1. Nevertheless, if larger quantities of tissue can be kept viable within the confines of the NMR-magnet, their study remains an exciting possibility for the future.

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