STUDIES OF METABOLISM IN THE ISOLATED, PERFUSED RAT HEART USING ^{13}C NMR

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1. Introduction

Since 1974 [1] phosphorus-containing metabolites have been studied in a variety of isolated organs, including skeletal muscle [2], heart [3], liver [4] and kidney [5] using ^{31}P nuclear magnetic resonance (NMR). Recently, non-destructive ^{13}C NMR [8] has been used to define pathways of biosynthesis, measure the partition of isotope flux between different pathways, and determine the displacement of certain cellular reactions from equilibrium in yeast suspensions [6], isolated liver cells [7] and perfused mouse livers [8]. Here we report an NMR investigation of the incorporation of ^{13}C into amino acids in isolated rat hearts perfused with sodium [2-^{13}C]acetate. Spectra were obtained sufficiently rapidly to follow isotope fluxes and to observe changes in the steady-state levels of enriched metabolites. Our work suggests that the method can be of general use in metabolic studies of the perfused rat heart.

2. Experimental

Hearts from 280–320 g male Wistar rats (OLAC Ltd) were perfused in the Langendorff mode with recirculation of the coronary effluent as in [9]. Unless otherwise noted, the Krebs-Henseleit bicarbonate buffer [10] contained 5 mM sodium acetate enriched as described with the 95% [2-^{13}C]salt (Prochem. Ltd). Hearts may be perfused in this manner for periods in excess of 3 h without significant changes in their developed aortic pressure (systolic–diastolic) or in their high energy phosphate content as measured by ^{31}P NMR. When required, metabolites were extracted from the freeze-clamped tissue using the procedure in [11].

Proton-decoupled ^{13}C NMR spectra at 45.867 MHz were obtained in the Fourier-transform mode using a solenoidal coil wound with 7 turns of 1.7 mm copper wire mounted inside a decoupler coil made of 1 mm thick copper tubing. The latter coil, tuned to 182.4 MHz, was a horizontal version of the type suggested in [12]. Chemical shifts are expressed relative to tetramethylsilane by using the acetate C-2 resonance at 25.9 ppm as an internal reference.

3. Results

3.1. *Spectra of extracted metabolites*

The spectrum of a perchlorate extract of 2 hearts perfused for 20 min with [2-^{13}C]acetate is shown in fig.1. The resonances were assigned by comparison of the chemical shifts with those of the pure compounds in saline solution (150 mM NaCl and 1 mM Na_2EDTA) at pH 7, by identification of ionisable groups from observations of the effect of changing pH on the NMR spectrum of the extract, and by observation of the effects of specific, enzyme-catalyzed conversions on the extract spectrum. An example of the last method was the identification of the resonances from glutamate by following its conversion to 2-oxoglutarate hydrazone on adding glutamate dehydrogenase (EC 1.4.1.3; see [13]). The unidentified resonances seen in fig.1 do not appear in spectra of the perfused heart.

The symmetry of the succinate molecule causes a label at C-4 of 2-oxoglutarate to be incorporated into the C-2 or C-3 positions with equal probability in the second turn of the tricarboxylic acid cycle. Thus, a ^{13}C nucleus entering on the second turn of the cycle as the methyl carbon of acetyl-CoA has an equal chance of being incorporated adjacent to, or one carbon distant from, a ^{13}C nucleus in 2-oxoglutarate. If isotope incorporation into glutamate is in equilibrium with incorporation into 2-oxoglutarate and the methyl

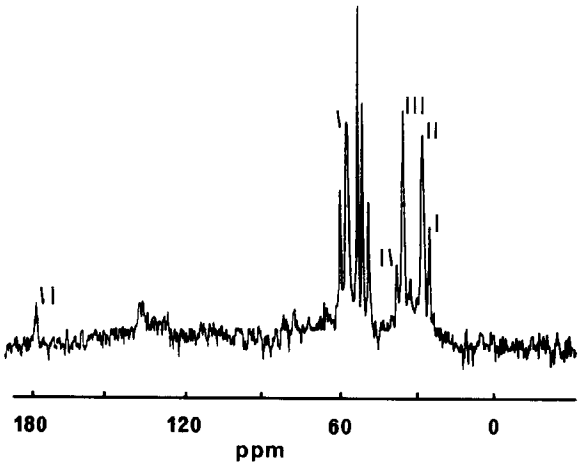

Fig.1. The proton-decoupled 45.867 MHz ^{13}C NMR spectrum of the pooled perchlorate extracts (pH 7) of 2 rat hearts freeze-clamped after 20 min perfusion. The buffer contained 5 mM sodium acetate with 95% of the methyl carbons labelled with ^{13}C. The spectrum represents the sum of 2000 free induction decays accumulated every 2 s with a sweep width of 10 000 Hz and a pulse angle of 60°. The resonances are identified as follows: (I) acetate, C-2; (II) glutamate and glutamine (unresolved), C-3; (III) glutamine, C-4; (IV) glutamate, C-4; (V) aspartate, C-3; (VI) glutamine, aspartate and glutamate (unresolved), C-2; (VII) glutamate and glutamine, C-1.

carbons of acetyl-CoA are fully enriched, then all of the glutamate C-4 carbons should be labelled. In consequence, one-half of the glutamate C-4 carbons will be spin-coupled to ^{13}C nuclei at the C-3 position and appear in the spectrum as a doublet and the remaining C-4 carbons will give rise to a singlet. The ratio of the total observed intensity of the glutamate C-4 doublet to the C-4 singlet is approximately unity (fig.1), suggesting both that the acetyl-CoA pool is effectively fully enriched at C-2 and that isotope incorporation into glutamate is in equilibrium with the labelling pattern of the tricarboxylic acid cycle intermediates. It may therefore be concluded that acetate is the major substrate rather than, for example, glycogen or endogenous triglycerides. This is in agreement with data collected under similar conditions showing that 5 mM acetate provides 80% of the substrate oxidized [14].

^{13}C is not incorporated into alanine, which is present in high concentration [15]. As isotope equilibration between pyruvate and alanine is rapid [16], this suggests that pyruvate remains unlabelled. A similar observation was made after perfusion with [1-^{14}C]-acetate [9]. Considerable malic enzyme (EC 1.1.1.38) activity was reported in the rat heart [17]. This suggests either that the enzyme is strongly inhibited in the perfused heart or that compartmentation prevents equilibration with the isotope-labelled malate pool.

3.2. *Whole tissue spectra*

The natural abundance spectrum of an isolated, perfused rat heart is shown in fig.2a. By comparison with spectra of fatty acids [18] and synthetic lecithins [19], the resonances may be assigned to carbons in relatively mobile fatty acyl esters and phospholipids. While much of the signal originates from the vasculature and fat at the base of the heart, there is a significant contribution from the ventricular muscle.

In the spectrum of a heart perfused with 2-^{13}C-enriched acetate (fig.2b), resonances are observed from all of the glutamate carbons except the terminal carboxyl, which is never labelled under these conditions. A steady-state level of enrichment into glutamate is reached only after 30–40 min perfusion (fig.3). In contrast, the isotope enrichment of the buffer (which changes during the first few minutes because of mixing of labelled and unlabelled buffers in the perfusion lines) reaches its steady-state enrichment within

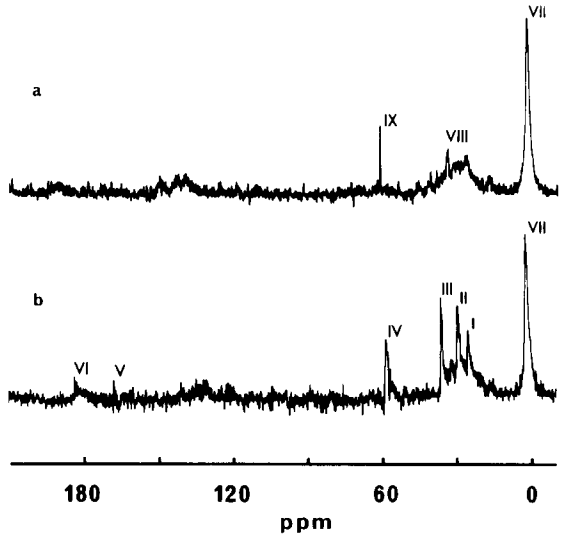

Fig.2. The proton-decoupled 45.867 MHz ^{13}C NMR spectra of an isolated perfused rat heart before (a) and after (b) incorporation of isotope from 5 mM sodium acetate with 50% of the methyl carbons labelled with ^{13}C. Each spectrum represents 2000 free induction decays accumulated with a sweep width of 10 000 Hz every 0.75 s and a pulse angle of 60°. Resonances are identified as follows. (I) acetate, C-2; (II) glutamate, C-3; (III) glutamate, C-4; (IV) glutamate, C-5; (V) possible carbamino adduct; (VI) glutamate C-1; (VII) external standard; (VIII) un-enriched endogenous triglyceride $-CH_2-$, (IX) unenriched $-N(CH_3)_3^+$ groups.

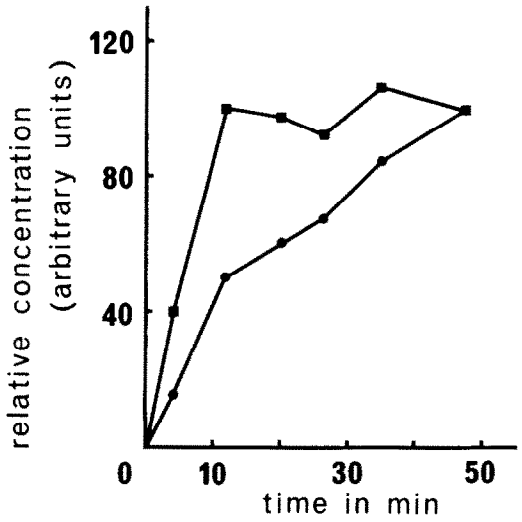

Fig.3. Time-course of incorporation of ^{13}C into glutamate C-3 (•) and C-4 (▪) carbons in an isolated, perfused rat heart. The buffer contained 5 mM sodium acetate with 50% of the methyl carbons labelled with ^{13}C. Each point was determined from a spectrum accumulated in 8 min.

5–10 min. The rate of incorporation of label into glutamate may be interpreted as a measure of flux through glutamate–oxaloacetate transaminase (EC 2.6.1.1) which is thought to be the major pathway for interconversion of 2-oxoglutarate and glutamate carbon skeletons. The steady-state enrichment at glutamate C-3 is reached more slowly than at C-4 because of isotopescrambling between C-2 and C-3 of succinate.

The sharp resonance at 163 ppm is absent in the spectra of extracts and may arise from a species unstable to the extraction procedure. We would not expect a signal in this region from any metabolite which might incorporate ^{13}C under these conditions. However, the chemical shift does correlate well with the range of values reported for carbamino derivatives of peptides and proteins [20]. The formation of carbamino derivatives of haemoglobin, which are thought to have a regulatory function, has previously been demonstrated by ^{13}C NMR in intact erythrocytes [21].

3.3. *Substrate dependence of glutamate and aspartate levels*

When acetate is the sole exogenous substrate or when fatty acids are oxidized, oxaloacetate levels decrease relative to those observed during perfusion with glucose [14]. This is accompanied by a fall in the aspartate concentration [22]. Fig.4a shows that, under the perfusion conditions used, relatively little label is incorporated into aspartate. The ratio of extractable, labelled aspartate to glutamate is also low (see fig.1). By lowering the concentration of acetate and increasing that of glucose in the perfusion medium, the equilibrium is shifted to increase this ratio (fig.4b). The dynamic character of this equilibrium is demonstrated on returning to the original perfusion conditions (fig.4c), when the aspartate to glutamate ratio increases again.

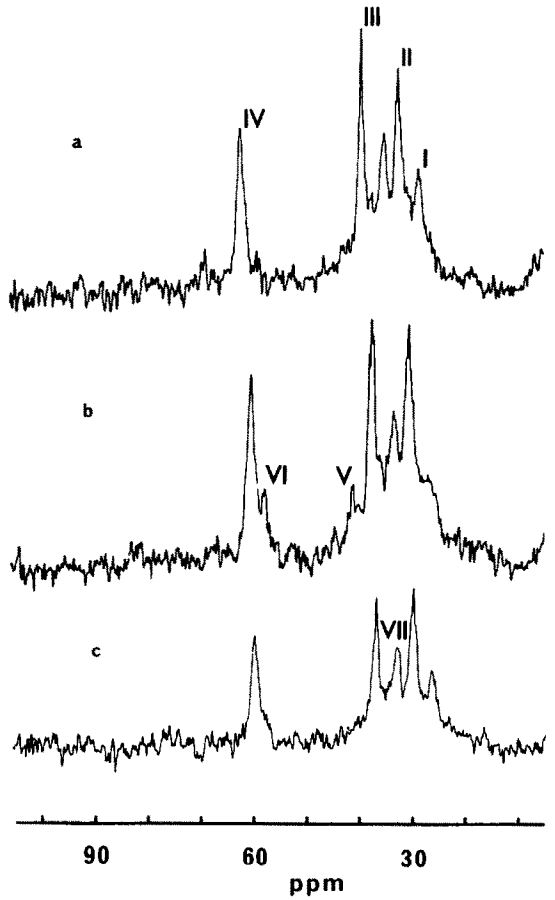

Fig.4. The 45.867 MHz ^{13}C NMR spectra of an isolated, perfused rat heart showing a shift in the relative isotope enrichment of glutamate and aspartate with a change of substrate. In (a) the buffer contained 5 mM sodium acetate with 50% of the methyl carbons labelled with ^{13}C. Spectrum (b) was collected 15 min after the same heart began to be perfused with buffer containing 11 mM glucose and 1.25 mM sodium acetate with 95% of the methyl carbons labelled with ^{13}C. Spectrum (c) was collected 15 min after the heart was again perfused with the buffer used during accumulation of spectrum (a). Each spectrum was accumulated as described for fig.2. The resonances are identified as follows: (I) acetate, C-4; (II) glutamate, C-3; (III) glutamate, C-4; (IV) glutamate, C-2; (V) aspartate, C-3; (VI) aspartate, C-2; (VII) unenriched endogenous triglyceride $-CH_2-$.

4. Discussion

The primary purpose of this work was to study intracellular fluxes and equilibria in a dynamic and non-invasive manner. Compared to isotopic labelling techniques NMR directly measures the position and relative distributions of the label within a given molecule. Thus comparison of the proportions of singlet and doublet C-4 signals in glutamate after equilibrium is reached, demonstrates that exogenous acetate provides all (or nearly all) of the carbon skeleton oxidized in the heart perfused with acetate.

The slower rate of ^{13}C incorporation into C-3 compared with C-4 of glutamate is a measure of the relative rates of cycling in the citric acid cycle and glutamate production. As it also depends on the pool sizes of the citric acid cycle intermediates, detailed analysis is not yet possible.

The transformation catalyzed by malic enzyme provides a mechanism for distribution of label from tricarboxylic acid cycle intermediates to pyruvate and hence to alanine. Although considerable malic enzyme activity was found in rat heart [17], we find no detectable incorporation of label into alanine even after 2 h. We propose that the malic enzyme is kinetically controlled to feed carbon skeletons effectively unidirectionally into the carboxylic acid cycle. Alternatively, the enzyme is inhibited in vivo or is unable to equilibrate the isotopically labelled malate pool because of compartmentation.

The slow incorporation of label into glutamate observed in this work shows that the flux through the glutamate–oxaloacetate transaminase is slower under our conditions (perfusion with acetate) than when label (^{14}C) was incorporated from acetate into hearts pre-perfused with glucose and insulin [14].

Changes in aspartate levels (and labelling) compared to those of glutamate with different substrates provide a way of investigating the rates of transport of reducing equivalents between the mitochondria and the cytosol [23].

Finally, the detection of the unstable carbamino haemoglobin intermediate in vivo is of interest in relation to its postulated regulatory functions.

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