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Direct observation of lactate and alanine by proton double quantum spectroscopy in rat hearts supplied with [3-^{13}C]pyruvate

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The ^{13}C-fractional enrichments in the lactate and alanine methyl carbon positions were determined by ^{1}H NMR spectroscopy of extracts of rat hearts perfused with various concentrations of [3-^{13}C]pyruvate ± unlabeled glucose or acetate. In general, the ^{13}C-fractional enrichment of the alanine methyl carbon pool paralleled the ^{13}C-fractional enrichment of the acetyl-CoA which entered the TCA cycle (as determined by ^{13}C-isotopomer analysis) while the ^{13}C-fractional enrichment of the lactate methyl carbon was always significantly lower, consistent with a pool of lactate which does not mix with exogeneous [3-^{13}C]pyruvate. This has also been examined in intact, perfused, KCl-arrested rat hearts supplied with [3-^{13}C]pyruvate by proton double quantum metabolite specific spectroscopy (MSS). A comparison of MSS spectra of intact hearts with one pulse spectra of extracts of those same hearts indicates there is a sizeable non-enriched pool of lactate in the intact hearts which is not visible by NMR spectroscopy.

MSS spectroscopy; ^{13}C-fractional enrichment; NMR-invisible lactate; Perfused heart

1. INTRODUCTION

Lactate and alanine, metabolites which play key roles in the heart, are derived from pyruvate via lactate dehydrogenase and alanine aminotransferase, respectively. Although both enzymes are very active in heart tissue, there have been reports which indicate that alanine is in equilibrium with mitochondrial pyruvate while lactate is not. This could reflect subcellular compartmentation of pyruvate or lactate [1-3]. ^{1}H NMR spectra of hearts should be sensitive to the metabolic state of the tissue and the high sensitivity of this nucleus may allow monitoring of metabolite levels with good signal-to-noise over periods of several seconds. However, direct measurement of lactate and alanine is difficult due to the presence of overlapping lipid resonances and the intense water signal also adds a dynamic range problem. Recently, several techniques have been reported for measuring lactate by ^{1}H NMR in perfused organs and in vivo [4–11]. The objective of this work is to extend these techniques to simultaneously measure ^{13}C-enrichment in lactate and alanine in hearts supplied with [3-^{13}C]pyruvate by proton double quantum metabolite specific spectroscopy.

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2. MATERIALS AND METHODS

Standard reagents were obtained from Sigma. [3-^{13}C]Sodium pyruvate enriched to 99.6% was obtained from MSD Isotopes. Hearts isolated from male Sprague-Dawley rats were perfused using the Langendorff technique at a pressure of 70 cmH_2O with standard Krebs-Henseleit bicarbonate-buffered medium containing 10 mM glucose bubbled continuously with 95% O_2/5% CO_2 and maintained at 37°C. The spontaneous heart rate was 240–300 beats/min. After 10 min without recirculation of perfusate, the heart was placed in a 20 mm NMR tube and about 150 ml of perfusate was recirculated. After shimming on the water proton FID, the concentration of KCl was increased to 20 mM to arrest the mechanical activity of the heart before the double quantum experiment was initiated. Double quantum spectra were recorded first in the presence of 10 mM unlabeled pyruvate and again in the presence of 10 mM [3-^{13}C]pyruvate. After completion of the NMR studies, hearts were freeze-clamped, ground to fine powder, extracted into a cold 7% perchloric acid solution, neutralized with 5 N KOH, and freeze-dried. The dry powder was dissolved into deuterated water and the pH adjusted to 7.4.

^{1}H NMR spectra of intact hearts were obtained on a General Electric 9.4 T Omega spectrometer equipped with imaging gradients. A 23 mm GE bird-cage coil was used for this experiment. Metabolite specific spectroscopy (MSS) was used for detection of alanine and lactate [12]. The pulse sequence consists of two 90° pulses to generate zero and multiple quantum coherences, and a third 90° pulse to convert zero quantum and multiple quantum coherences into observable single quantum coherence. The order of coherence (double) was selected by the magnitude of the gradient pulses during the *te*/2 period. The spectral parameters were 4K data points, 4000-Hz spectral width, 2 s predelay time and 128 acquisitions. The transmitter frequency was set at 3.9 ppm, midway between the lactate and alanine methine resonances. A nonphase-shifted 1331 pulse with a 0.5 ms interpulse delay was used as the third 90° pulse to selectively excite both lactate and alanine methine resonances. Proton decoupled ^{13}C NMR spectra and

^{1}H NMR spectra of tissue extracts in D_2O were obtained on a GN 500 spectrometer.

3. RESULTS

3.1. *Perfused heart extracts*

Rat hearts were perfused with various combinations of substrates for 30 min and freeze-clamped. The frozen tissues were extracted in cold perchloric acid and a high resolution ^{1}H spectrum was run on each extract to determine the ^{13}C fractional enrichment in the lactate methyl and alanine methyl carbons. The results are presented as [^{13}C]lactate/total lactate and [^{13}C]alanine/total alanine ratios in Table I. High resolution ^{13}C NMR spectra were also obtained on these same extract samples and the glutamate multiplets were deconvoluted and that data applied to a steady-state isotopomer analysis [13]. The fractional contribution made by [3-^{13}C]pyruvate to the acetyl-CoA which entered the TCA cycle in each heart is also tabulated.

There are two interesting and consistent observations in this data. First, one might expect the ^{13}C-fractional enrichments in lactate and alanine to be roughly equal since both metabolites are derived from [3-^{13}C]pyruvate in a single step. However, this was not observed for any of the conditions examined; instead, the alanine pool always had a higher ^{13}C-fractional enrichment than the lactate pool. The second observation which can be made concerning the data in Table I is that the fraction of acetyl-CoA derived from [3-^{13}C]pyruvate (in the absence of unlabeled acetate) as determined by ^{13}C-isotopomer analyses is, within experimental error, the same as the ^{13}C-fractional enrichment observed in the alanine pool. This indicates that alanine can be used as an index of ^{13}C-fractional enrichment of pyruvate entering the TCA cycle under these conditions. This same conclusion was reached earlier by Peuhkurinen et al. [1,2] based upon ^{14}C-radiolabeling experiments in perfused rat hearts. Taken together, these data indicate that virtually *all* of the acetyl-CoA which enters the TCA cycle in a pyruvate perfused heart is, in fact, derived from

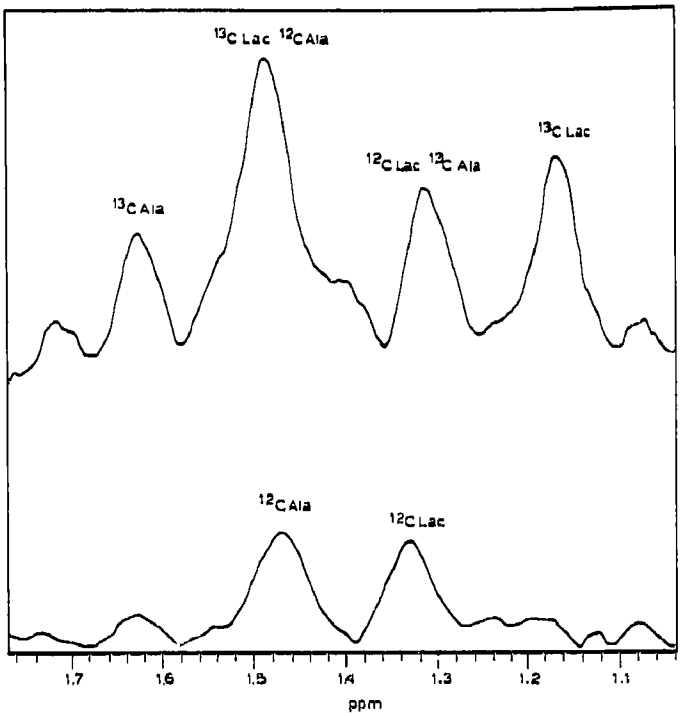

Fig. 1. ^{1}H MSS spectra of the alanine and lactate methyl resonances of an intact rat heart. (Bottom) Heart perfused with 10 mM unlabeled glucose plus 10 mM unlabeled pyruvate and (top) same heart perfused with 10 mM unlabeled glucose plus 10 mM [3-^{13}C]pyruvate.

pyruvate (the sum of exogeneous [^{13}C]pyruvate plus unlabeled pyruvate derived from endogeneous glycolytic sources).

When unlabeled acetate was present with [3-^{13}C]pyruvate as an equimolar mixture, the contribution made by [3-^{13}C]pyruvate to the acetyl-CoA entering the TCA cycle dropped to 45% (due to competition of acetate as a source of acetyl-CoA) while the ^{13}C-fractional enrichment in alanine remained near 86%. Although the ^{13}C-isotopomer analysis indicates that 45% of all acetyl-CoA entering the TCA cycle under these conditions is derived from [3-^{13}C]pyruvate, one might choose to use the alanine ^{13}C-fractional enrichment data to correct

Table I

The ratios of [^{13}C]lactate to total lactate and [^{13}C]alanine to total alanine in ^{1}H NMR spectra of the extracts of perfused rat hearts. Data are presented as the mean ± SD[a]

Substrate mixture	[^{13}C]Lactate/total lactate	[^{13}C]Alanine/total alanine	% Acetyl-CoA[b] (from [3-^{13}C]pyruvate)
2.5 mM [3-^{13}C]pyruvate (n = 3)	44 ± 8%	82 ± 2%	87 ± 2%
2.5 mM [3-^{13}C]pyruvate + 2.5 mM acetate (n = 3)	46 ± 4%	86 ± 2%	45 ± 3%
2.5 mM [3-^{13}C]pyruvate + 10 mM glucose (n = 3)	44 ± 7%	82 ± 2%	90 ± 11%
5.0 mM [3-^{13}C]pyruvate + 10 mM glucose (n = 3)	62 ± 5%	91 ± 2%	97 ± 2%
10 mM [3-^{13}C]pyruvate + 10 mM glucose (n = 3)	51 ± 3%	89 ± 5%	89 ± 8%

[a] Each entry in column 1 is significantly different ($P < 0.05$) from the corresponding entries in columns 2 and 3 (except for the hearts perfused with acetate). The entries in columns 2 and 3 (again, except for the acetate column) are not significantly different ($P < 0.05$). Comparisons were made by an analysis of variance with a Bonferroni correction for multiple comparisons [18].

[b] Determined by a ^{13}C-isotopomer analysis of glutamate as measured in high resolution ^{13}C NMR spectra of heart extracts.

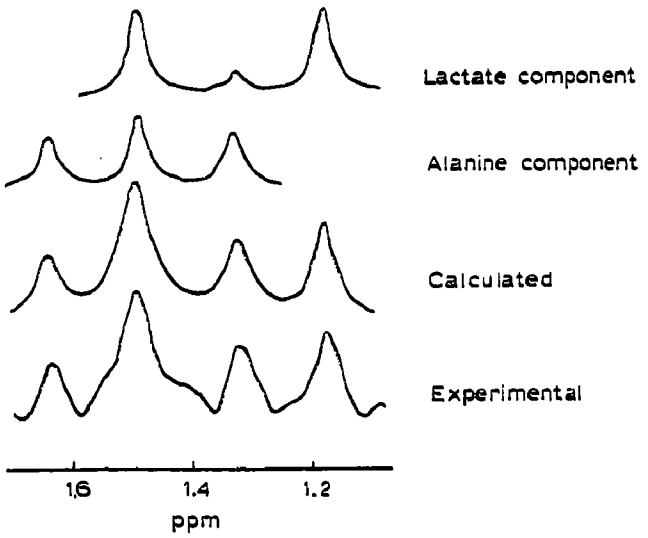

Fig. 2. ^1H MSS spectrum of the same heart shown in Fig. 1 along with the deconvoluted ^{12}C and ^{13}C components in the alanine and lactate methyl resonances.

this value for dilution with [^{12}C]pyruvate. This would yield a value of 52% (45/0.86) for the total acetyl-CoA derived from pyruvate (labeled plus unlabeled).

3.2. *Intact heart spectra*

To examine this question further, we have been exploring ways one might test whether there are significant alanine and lactate pools in intact hearts which do not mix with exogeneously added [3-^{13}C]pyruvate. Our first attempt to monitor the ^{13}C-fractional enrichments in these two pools in the intact heart involved the use of ^1H metabolite specific spectroscopy (MSS) [12]. Since this technique is quite sensitive to heart motions, we were required to arrest the heart with 20 mM KCl. A proton double quantum spectrum of an intact Langendorff-perfused rat heart arrested with 20 mM KCl and perfused with 10 mM unlabeled glucose plus 10 mM unlabeled pyruvate is shown in Fig. 1. The methyl resonances of alanine and lactate could not be detected in these hearts by proton MSS before pyruvate was added to the perfusate, indicating that the concentrations of these two metabolites clearly increase upon addition of pyruvate. After switching the perfusate to 10 mM [3-^{13}C]pyruvate, the proton resonances from lactate and alanine due to J_{CH} coupling (the hydrogens coupled to labeled carbons) appear to high frequency of the central alanine resonance (representing one-half of the [3-^{13}C]alanine proton resonance) and to low frequency of the central lactate resonance (representing one-half of the [3-^{13}C]lactate proton resonance). A comparison of MSS versus one pulse experiments on phantom samples showed that the MSS parameters used in these experiments gave quantitative results. Interestingly, the resonance due to J_{CH} in the lactate methyl resonance is more prominent than that due to J_{CH} in alanine. ^{13}C-fractional enrichment data were obtained from these spectra by deconvolution of the MSS spectra into separate alanine and lactate components, as illustrated in Fig. 2 and the quantitative resonance area ratios for the 5 hearts are summarized in Table II. ^1H NMR spectra of extracts of the same hearts were also recorded and the quantitative fractional enrichment data is also presented in Table II. The data indicate that the ^{13}C-fractional enrichments of the alanine and lactate methyl carbons is not significantly different in extracts of KCl-arrested hearts. A comparison of this result with the MSS results (Table II) indicates that ^{13}C-fractional enrichment in the lactate methyl carbon is significantly higher ($P < 0.05$) in intact hearts than in extracts of those same hearts, while the ^{13}C-fractional enrichment in the alanine methyl carbon is the same in intact hearts versus extracts, within experimental error.

Table II

The ratios of [^{13}C]lactate to total lactate and [^{13}C]alanine to total alanine in ^1H NMR spectra from the intact rat heart arrested with KCl and the extracts of the same hearts. Data are presented as the mean ± SD (n = 5)

	[^{13}C]Lactate / Total alanine	[^{13}C]Alanine / Total lactate	Fraction of acetyl-CoA derived from [3-^{13}C]pyruvate
Intact hearts	85 ± 9%	56 ± 2%	–
Extracts	67 ± 5%	61 ± 8%	70 ± 5%

4. DISCUSSION

We have used ^1H spectroscopy to investigate the ^{13}C-fractional enrichments of the alanine and lactate methyl carbons in heart extracts and in intact hearts perfused with [3-^{13}C]pyruvate. In all cases except for extracts of perfused hearts that were arrested with KCl, the ^{13}C-fractional enrichment in the alanine methyl carbon was significantly higher than that observed in the lactate methyl carbon. Similar observations have previously been reported by our group [14] and others [15] from ^1H spectra of heart extracts. Peuhkurinen et al. [2] have also reported that the ^{14}C-fractional enrichment differs in the alanine and lactate pools in rat hearts perfused with [3-^{14}C]pyruvate and concluded that the higher ^{14}C specific activity found in alanine is due to two different lactate pools, one which equilibrates with exogeneous [3-^{14}C]pyruvate and another which does not. These authors also conclude that the ^{14}C specific activity observed in alanine accurately reflects the ^{14}C specific activity of pyruvate which enters the TCA cycle. Both conclusions are entirely consistent with the ^{13}C data presented in Table I. Nevertheless, it should be pointed out that it is impossible to differentiate between two non-exchanging pools of pyruvate (or lactate) vs. two

or more cell populations which assimilate the labeled pyruvate differently based upon data such as this alone.

In an effort to investigate these two possibilities in more detail, we have used MSS ^{1}H spectroscopy to monitor the ^{12}C and ^{13}C populations in the alanine and lactate pools in intact KCl-arrested hearts. Unfortunately, the ^{13}C specific activity of the lactate and alanine pools was not significantly different (compare 67% vs. 61%; Table II) in extracts of KCl-arrested hearts. This indicates that the physiological consequences of membrane depolarization alters not only the ^{13}C specific activities of lactate and alanine but also changes the fraction of acetyl-CoA that is derived from pyruvate (compare 67%, 61%, and 70% in Table II with 51%, 89%, and 89% in Table I). Nevertheless, the MSS ^{1}H spectra of intact KCL-arrested hearts show quite clearly that significantly more ^{13}C was detected in the lactate methyl carbon pool of intact hearts than observed in extract spectra of those same hearts. This suggests that a pool of [^{12}C]lactate exists which is not visible by NMR in the intact heart but which does contribute to the total acetyl-CoA pool. This invisible lactate pool would be released during perchloric acid extraction and hence dilute the specific activity of total lactate found in extracts. Assuming that [^{13}C]lactate is fully visible in the intact heart, one can estimate that the NMR invisible [^{12}C]lactate pool represents about 27% [(85/67)−1] of the total lactate in these hearts. Similar results have been reported for hypoxic or ischemic rat brain [16] and blood plasma [17] where about 28% and 33% of the total lactate, respectively, is reported to be NMR-invisible.

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REFERENCES

[1] Peuhkurinen, K.J., Hiltunen, J.K. and Hassinen, I.E. (1983) Biochem. J. 210, 193–198.
[2] Peuhkurinen, K.J. and Hassinen, I.E. (1982) Biochem. J. 202, 67–76.
[3] Wolfe, R.R., Jahoor, F. and Miyoshi, H. (1988) Am. J. Physiol. 254, E532–E535.
[4] William, S.R., Gadian, D.G. and Proctor, E. (1986) J. Magn. Reson. 66, 562–567.
[5] Keller, A.M., Sorce, D.J., Sciacca, R.R., Barr, M.L. and Cannon, P.J. (1988) Magn. Reson. Med. 7, 65–78.
[6] McKinnon, G.C. and Boesiger, P. (1988) Magn. Reson. Med. 8, 355–361.
[7] Meyer, R.A. (1987) Magn. Reson. Med. 4, 297–301.
[8] Von Kienlin, M., Albrand, J.P., Authier, B., Blondet, P., Lolito, S. and Decorps, M. (1987) J. Magn. Reson. 75, 371–377.
[9] Ugurbil, K., Petein, M., Maidan, R., Michurski, S., Cohn, J.N. and From, A.H. (1984) FEBS Lett. 167, 73–78.
[10] Reddy, R., Subramanian, V.H. and Leigh, J.S. (1990) SMRM 9th Annual Meeting, New York, NY, Abstr. 447.
[11] Rothman, D.L., Behar, K.L., Hetherington, H.P., Den Hollander, J.A., Bendall, M.R., Petroff, O.A.C. and Shulman, R.G. (1985) Proc. Natl. Acad. Sci. USA 82, 1633–1637.
[12] Hurd, R.E. and Freeman, D.M. (1989) Proc. Natl. Acad. Sci. USA 86, 4402–4406.
[13] Malloy, C.R., Sherry, A.D. and Jeffrey, F.M.H. (1990) Am. J. Physiol. 259, H987–H995.
[14] Sherry, A.D., Malloy, C.R. and Jeffrey, F.M.H. (1988) 13th International Conference on Magnetic Resonance in Biological Systems, Madison, WI, Abstr. P3-2.
[15] Lewandowski, E.D., Johnston, D.L. and Roberts, R. (1991) Circ. Res. 68, 578–587.
[16] Chang, L.-H., Pereira, B.M., Weinstein, P.R., Keniry, M.A., Murphy-Boesch, J., Litt, L. and James, T.L. (1987) Magn. Reson. Med. 4, 575–581.
[17] Bell, J.D., Brown, J.C.C., Kubal, G. and Sadler, P.J. (1988) FEBS Lett. 235, 81–86.
[18] Primer of Biostatistics, by S.A. Glantz, McGraw-Hill, New York, 2nd edn., 1987.