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^{31}P NUCLEAR MAGNETIC RESONANCE OF METABOLIC CHANGES ASSOCIATED WITH CYANIDE INTOXICATION IN THE PERFUSED RAT LIVER

J.M. Salhany,*§ S.J. Stohs,
L.A. Reinke,† G.M. Pieper§ and J.M. Hassing

Departments of Biomedicinal Chemistry and Internal Medicine*
and the Cardiovascular Center,§
University of Nebraska and VA* Medical Centers,
Omaha, Nebraska, 68105

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SUMMARY

Metabolic changes associated with cyanide intoxication were observed for the first time in perfused rat liver using ^{31}P nuclear magnetic resonance (NMR) at 60.7 MHz. Well-oxygenated control livers showed strong ATP peaks and little discernable internal orthophosphate (Pi). Perfusion with 2 mM cyanide eliminated the observable ATP peaks and caused internal Pi to increase. Despite clear evidence for ATP hydrolysis, resonances from cytoplasmic ADP were conspicuously absent. Resumption of perfusion with cyanide-free buffer caused a dramatic return of the ATP peaks with a concomitant fall in internal Pi. These metabolic changes are consistent with reversible binding of cyanide to mitochondrial cytochromes and their observation by ^{31}P NMR indicates the potential of this method for studying metabolism in whole, perfused rat liver under physiologic conditions.

INTRODUCTION

The application of ^{31}P nuclear magnetic resonance (NMR) to intact biological systems has developed rapidly since the pioneering work of Moon and Richards (1) with erythrocytes. Our interest in this application began during studies which showed that the technique could be applied to living cells (2) and has more recently been encouraged by work with aerobic perfused organs such as heart (3-5), kidney (6) and brain (7).

*To whom correspondence should be addressed at the Department of Biomedicinal Chemistry, University of Nebraska Medical Center, Omaha, NE 68105.

†Present Address: Department of Pharmacology, University of North Carolina, Chapel Hill, NC 27514.

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Isolated, perfused rat liver preparations are a widely used, physiologically acceptable system to study hepatic metabolism. Although recent work with isolated hepatocytes has illustrated the utility of ^{31}P NMR for studying intracellular events (8), no published spectra exist showing the feasibility of this method with whole perfused rat liver. One aspect of the ^{31}P NMR method which we sought to test with perfused liver preparations is the ability to induce metabolic changes, and subsequently follow recovery or reversal in the same preparation. Experiments of this type would be most useful in those toxicity studies where the liver is the primary site of metabolism for the toxic substance. In this preliminary report we show that ^{31}P NMR can be used to detect changes in important phosphate metabolites in perfused rat liver. The effects of cyanide intoxication are studied. We show that cyanide inhibits oxidative metabolism, as expected, but that this inhibition can be reversed by subsequent perfusion with cyanide-free buffer.

MATERIALS AND METHODS

Male albino rats of a Sprague-Dawley derived strain (Sasco, Inc., Omaha, NE) weighing 100 gms were anesthetized with sodium pentobarbital (50 mg/kg). A polyethylene cannula was inserted into the portal vein, securely tied and the liver was excised from the animal. The liver was perfused with a modified Krebs-Henseleit bicarbonate (pH 7.4) buffer consisting of 119 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, .0.6 mM Ca EDTA and 25 mM $NaHCO_3$. The perfusate was maintained at 37°C and was continuously gassed with 95% O_2:5% CO_2. Perfusate flow rate was set constant at 17 mls/min with a Gilson peristaltic pump. After cannulation, the liver was placed in a 2.0 cm teflon-stoppered NMR tube. The effluent hepatic flow was evacuated from the tube by a peristaltic pump. However, the liver was surrounded by the buffer during NMR spectra collection.

^{31}P NMR measurements were performed with a Nicolet Instrument Corporation (Mountain View, CA) NTC 150 spectrometer operated at 60.7 MHz. The spectrometer operates in the Fourier transform mode. Each spectrum presented represents the accumulation of 1500 60° pulses (12 μ sec) with a pulse delay of 250 msec and an acquisition time of about 1 second. The spectra were collected using broad band (150 MHz) proton decoupling with about 6 watts of decoupling power. The spectra are presented in ppm relative to 85% external phosphoric acid. Total collection time for each spectrum was about 31 min. The liver was perfused about 30 min prior to collection of control spectra. Upon collection of control spectra, 40 μ moles/min of KCN in Krebs-Henseleit bicarbonate buffer were introduced into the perfusate via a sidearm so that the concentration of KCN at the liver was 2 mM. KCN perfusion was allowed to occur for 12 min. prior to collection of the second 31 min. spectrum. Following this, the same liver was perfused with KCN-free buffer for an additional 10 min. after which the last 31 min spectrum was collected.

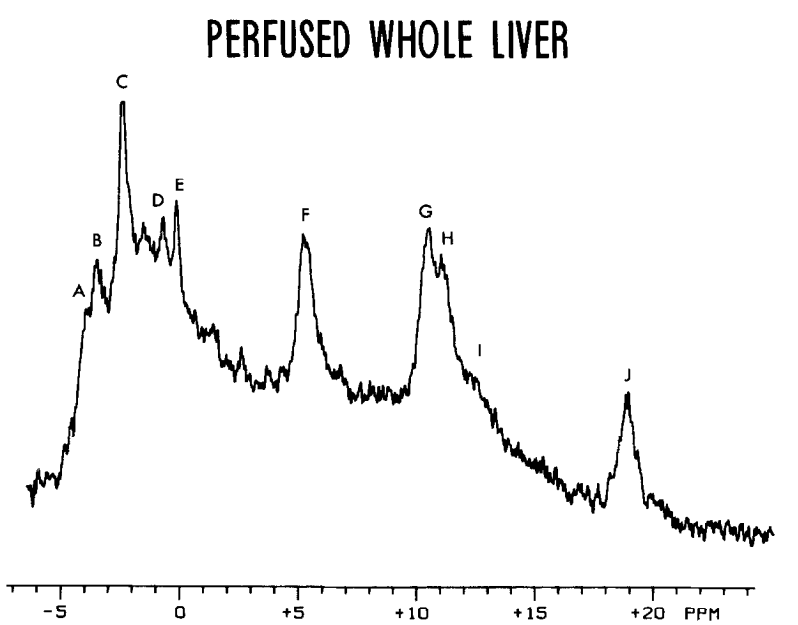

Figure 1. ^{31}P NMR spectrum of a perfused rat liver at 60.7 MHz. The spectrum is a Fourier transform of 1500 pulses of 60° free induction decays of 1 second duration obtained with a Nicolet NTC 150 spectrometer. The spectrum is plotted in ppm relative to external phosphoric acid (85%). Peak identifications and other conditions are given in the text.

RESULTS AND DISCUSSION

Figure 1 shows the ^{31}P NMR spectrum of a perfused rat liver. The significant peaks in the spectrum are lettered A through J. Peak C was identified as external Pi at pH 7.4. This identification was confirmed by running a spectrum on the same buffer. The remaining peaks correspond to those seen in hepatocyte preparations (8). However, unlike the hepatocyte spectra, the perfused liver results show strong ATP peaks (peaks F, G and J) and little observable internal Pi indicating that this liver preparation is viable and well-oxygenated. Peak A consists of several sugar phosphate compounds. Peak B (-3.5 ppm) is at the position of phosphorylcholine (9). Peaks D and E resonate in the positions of glycero-3-phosphorylethanolamine (-1 ppm) and glycero-3-phosphorylcholine (-0.5 ppm), respectively (8). Peak F, G and J are the γ(5.5 ppm), α(10.1 ppm) and β(18.9 ppm) peaks of Mg-ATP,

respectively. The assignment of these peaks is made on the assumption that there is little or no contribution from ADP to the 5.5 ppm and 10.1 ppm peaks (see below). Peaks H and I are in the P,P'-diesterified pyrophosphate region. Peak H is at the NAD^+ position (10).

The effect of perfusion with 2 mM KCN is shown in Figure 2. A complete elimination of all observable ATP peaks occurred. The appearance of a strong internal Pi line with external Pi (peak C) as a downfield shoulder occurred simultaneously with hydrolysis of ATP. Note the complete absence of ADP peaks. Internal pH was estimated from internal Pi to be about 6.8. Since we could not observe a significant internal Pi peak in the control spectrum under our perfusion conditions, it is difficult to decide if acidosis did or did not develop during KCN perfusion. Some qualitative indication that intracellular acidosis did develop comes from the observed upfield shift in the sugar phosphate peak (peak A) (Fig. 2). In control, peak A exists as a downfield shoulder of the phosphorylcholine peak (peak B). With KCN perfusion, this shoulder is less discernable suggesting an upfield shift since peak B does not shift with pH (9).

In order to show that these changes resulted from inhibition of mitochondrial electron transport through cyanide binding to cytochromes, we attempted to reverse the process by perfusing the same liver with cyanide-free buffer. Since cyanide binds reversibly to cytochromes, cyanide-free perfusion might be expected to restore electron transport and ATP production. The last spectrum in Figure 2 shows the result of this process. The strong internal Pi peak is now no longer observable while external Pi (peak C) is observable in the orthophosphate region. The disappearance of a predominant internal Pi peak occurred concomitant with the reappearance of ATP (peaks F, G and J) to about 40% of the original ATP intensities. This shift would seem to be a consequence of cyanide release from mitochondrial cytochromes since liver oxygenation was invariant. It is also important to note that the sugar phosphate peak (peak A) is now well separated from peak B indicating a return

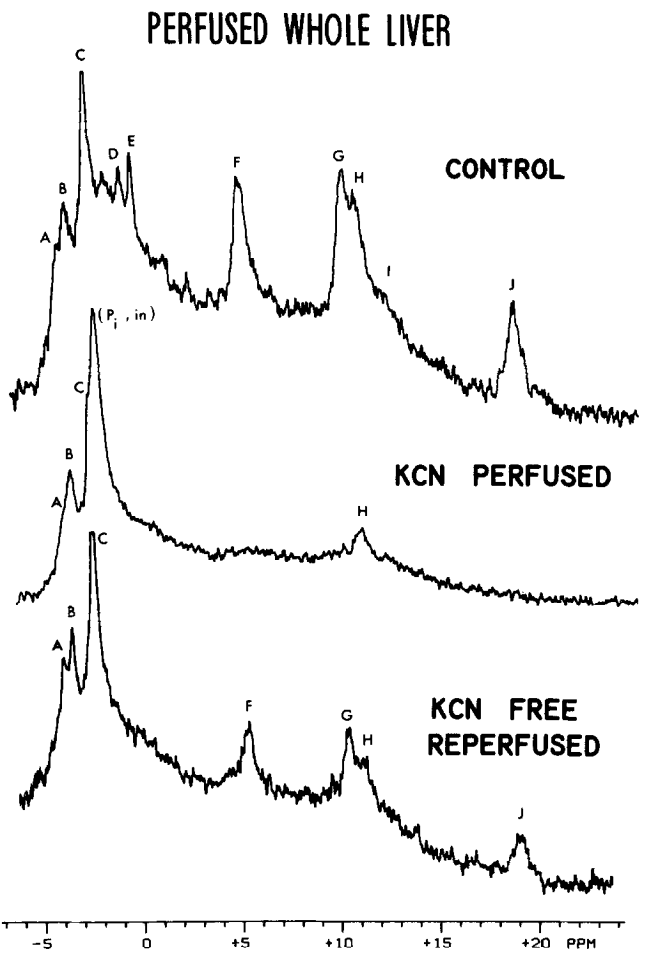

Figure 2. ^{31}P NMR spectra showing the effect of KCN (2 mM) perfusion (10 min prior to and during spectral collection of 31 min) followed by perfusion with KCN-free buffer. The NMR conditions were the same for each spectrum shown as stated in Figure 1. Additional details are given in the text.

of cytoplasmic pH to control values with the resumption of electron transport. The apparent relative alkalinization of the cytoplasmic domain indicated by the movement of the titratable sugar phosphate peak (peak A) (assumed to be cytoplasmic), would be consistent with the view that protons are pumped into mitochondria from the cytoplasm during ATP synthesis (11). There does not appear to be a significant return of the compounds associated with peaks D and E.

When cyanide inhibits mitochondrial electron flow, ATP synthesis should be inhibited and the observable ATP should hydrolize to ADP and Pi. Since internal Pi increases with cyanide and decreases with cyanide-free perfusion and since resynthesis of ATP occurs, ADP's virtual absence in the middle spectrum of Figure 2 is conspicuous. We suggest that ADP may be within the mitochondria under these conditions and broadened either by paramagnetic effects or by binding to a mitochondrial component (12).

The results just presented clearly show that ^{31}P NMR can provide considerable detail about metabolic events occurring in the whole rat liver during inhibition of liver mitochondrial electron transport by cyanide. Changes one might have expected to occur, are vividly illustrated in the spectra before, during and after the toxic insult. The distinct advantage of being able to follow high energy phosphate metabolism during an hepatotoxic event should prove useful in characterizing the effects of other hepatotoxic substances (e.g. ethanol) and drugs (e.g. nitroprusside, which is degraded to cyanide (13)) metabolized by the liver.

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REFERENCES

1. Moon, R.B. and Richards, J.H. (1973) J. Biol. Chem. 248: 7276-7278.
2. Salhany, J.M., Yamane, T., Shulman, R.G. and Ogawa, S. (1975) Proc. Natl. Acad. Sci. U.S.A. 72: 4966-4970.
3. Garlick, P.B., Radda, G.K., Seeley, P.J. and Chance, B. (1977) Biochem. Biophys. Res. Commun. 74: 1256-1262.
4. Hollis, D.P., Nunnally, R.L., Jacobus, W.E. and Taylor, G.J., IV. (1977) Biochem. Biophys. Res. Commun. 75: 1086-1091.
5. Salhany, J.M., Pieper, G.M., Wu, S., Todd, G.L., Clayton, F.C. and Eliot, R.S. (1979) J. Molec. Cell. Cardiol. (in press).
6. Sehr, P.A., Radda, G.K., Bore, P.J. and Sells, R.A. (1977) Biochem. Biophys. Res. Commun. 77: 195-202.
7. Chance, B., Nakase, Y., Bond, M., Leigh, J.S., Jr. and McDonald, G. (1978) Proc. Natl. Acad. Sci. U.S.A. 75: 4925-4929.

8. Cohen, S.M., Ogawa, S., Rottenberg, H., Glynn, P., Yamane, T., Brown, T.R., Shulman, R.G. and Williamson, J.R. (1978) Nature 273: 554-556.
9. Navon, G., Ogawa, S., Shulman, R.G. and Yamane, T. (1977) Proc. Natl. Acad. Sci. U.S.A. 74: 87-91.
10. Navon, G., Navon, R., Shulman, R.G. and Yamane, T. (1978) Proc. Natl. Acad. Sci. U.S.A. 75: 891-895.
11. Mitchell, O. and Moyle, J. (1969) Eur. J. Biochem. 7: 471-484.
12. Ogawa, S., Rottenberg, H., Brown, T.R., Shulman, R.G., Castillo, C.L. and Glynn, P. (1978) Proc. Natl. Acad. Sci. U.S.A. 75: 1796-1800.
13. Cottrell, J.E., Casthely, P., Bordie, J.D., Patel, K., Klein, A. and Turndorf, H. (1978) New Engl. J. Med. 298: 809-811.