Detection of glycogen in a glycogen storage disease by ^{13}C nuclear magnetic resonance

Anthony N. Stevens, Richard A. Iles[+], Peter G. Morris* and John R. Griffiths

Department of Biochemistry, St George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, [+]Academic Unit of Metabolism and Endocrinology, Alexandra Wing, London Hospital Medical College, London E1 1BB and **MRC Biomedical NMR Centre, National Institute for Medical Research, London NW7 1AA, England England*

Received 17 November 1982

The livers of *gsd/gsd* rats homozygous for the glycogen storage disease phosphorylase *b* kinase deficiency were observed by ^{13}C NMR using a surface coil. Clear signals were detected from glycogen. The concentration of glycogen as determined by NMR was ~3-times that found in normal strains agreeing well with chemical determinations. Starvation did not significantly reduce the glycogen content of the livers with glycogen storage disease whereas it reduced the signal below detectability in normal rats. Difference spectra of starved normal rats from fed *gsd/gsd* rats gave spectra similar in appearance to that of purified glycogen. Glycogen both in vivo and in vitro is fully visible using ^{13}C NMR.

Surface coil *^{13}C natural abundance* *Phosphorylase kinase deficiency* gsd/gsd *rat* *Liver*

1. INTRODUCTION

Nuclear magnetic resonance studies have yielded much information on the metabolism and localisation of compounds in situ in living animals [1], but few such studies have been performed by ^{13}C NMR. In [2] surface coils were used to obtain spectra from the naturally abundant (~1% of total carbon) ^{13}C in rat organs and human arms in vivo. They were able to obtain a resonance corresponding to liver glycogen after enriching the ^{13}C content of the glycogen by feeding [1-^{13}C]-glucose. No signals were observed from the native glycogen in these experiments, however.

A strain of rats (gsd/gsd) has been described in which liver phosphorylase kinase is absent so that phosphorylase *a* cannot be formed [3,4]. They have ~3-fold higher glycogen levels in their livers than do normal, well-fed rats. Here, we report high resolution natural abundance ^{13}C spectra of normal and gsd/gsd rat liver in situ, obtained, using surface coils, at a frequency of 50.24 MHz. Clear signals from glycogen were obtained both from normal and gsd/gsd rats, and they corresponded in amount to the total glycogen assayed by orthodox methods.

2. MATERIALS AND METHODS

2.1. *Animals*

Rats (200–250 g) homozygous for phosphorylase *b* kinase deficiency (gsd/gsd) were obtained from the Department of Clinical Biochemistry, Medical School, University of Otago (Dunedin) and bred in the Animal House at St George's Hospital Medical School (London). The primary defect in these animals is a deficiency in hepatic phosphorylase *b* kinase [3,4]. Porton Wistar rats bred in the Animal House at the National Institute for Medical Research (London) were used as controls. All rats were maintained on Oxoid 41B animal diet for at least 2 weeks before use.

2.2. *NMR spectra*

Male gsd/gsd or Wistar rats were anaesthetised by intraperitoneal sodium pentobarbital injection,

Published by Elsevier Biomedical Press
00145793/82/0000—0000/$2.75

and secured vertically within a purpose-built, 7 cm diam. NMR probe. By means of an abdominal incision a flat one turn 30 mm diam. radiofrequency (rf) coil was placed on the surface of the liver and within this was placed a two-turn rf coil of 20 mm diam. The larger coil was tuned to 200 MHz and used to decouple the protons, while the smaller coil was used for detection of the ^{13}C signal. To prevent evaporation and to electrically insulate the coil, the liver surface was covered with a thin plastic film. The probe was inserted into the magnet of a wide bore Bruker WM200 spectrometer. ^{13}C spectra were recorded at an irradiation frequency of 50.24 MHz and a sweep width of ± 7000 Hz. Radiofrequency pulses of 20.0 μs pulse width with a recycle time of 1 s were used (longer delay times did not significantly alter the observed signal intensities). Broad band decoupling (3 W effective) was used during data acquisition; it was reduced to a lower level during the remainder of the cycle in order to prevent overheating of the sample while maintaining Nuclear Overhauser Enhancement (NOE). The decoupler irradiation was centered on the CH_2 proton frequency. For all spectra the line broadening was 12 Hz.

2.3. *Glycogen determination*

Tissue samples for glycogen assay were weighed in tubes containing 200 μl 30% (w/v) KOH saturated with Na_2SO_4. They were digested in a boiling water bath for 60 min and the glycogen was isolated by precipitation with ethanol. The glycogen was redissolved in 1 ml H_2O and assayed by an enzymatic method in which glycogen was digested with amyloglucosidase as in [5] and the resulting glucose solution assayed as in [6].

3. RESULTS AND DISCUSSION

Natural abundance ^{13}C spectra of (a) normal liver and (b) gsd/gsd liver in vivo are shown in fig. 1. The Nuclear Overhauser Effect induced by proton decoupling leads to a 3-fold improvement in signal/noise and results in acceptable spectra being obtained over 40 min (2500 scans). Chemical shifts are recorded relative to tetramethylsilane (TMS). The chemical shifts of substances in solution were measured relative to a capillary of dioxan as a secondary standard.

3.1. *Assignment of resonances*

Major ^{13}C resonances have been identified in whole tissue and extracts [2,7,8]. The aliphatic region of the liver spectrum (0–100 ppm) contains a major peak at 30.0 ppm (I in fig. 1) which is almost entirely due to the $(CH_2)_n$ repeating unit of fatty acid chains situated in triglycerides and phospholipids [9,10]. The shoulders on this resonance are thought to be due to the γ and δ carbons of several amino acids and the β carbons of amino acid side chains of proteins capable of free motion [11]. Resonance (H) in fig. 1 at 54.8 ppm

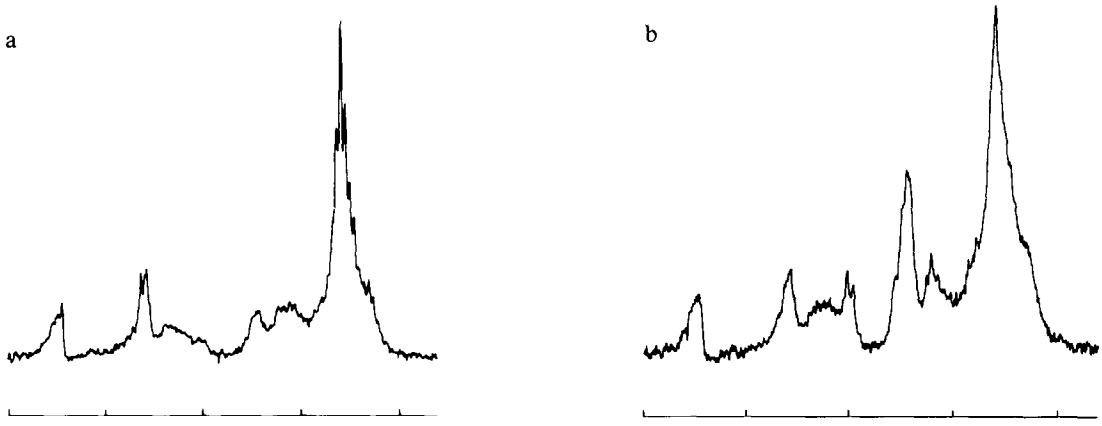

Fig. 1. Proton decoupled 50.24 MHz ^{13}C NMR spectra (2500 scans) of livers from fed rats (a) in vivo normal (b) phosphorylase kinase-deficient (gsd/gsd). Pulses were applied every 1 s. Peak assignments are given in the text. Line broadening of 12 Hz was applied. Chemical shifts are given relative to TMS.

has been identified as the methyl group of choline by measuring the ^{13}C spectrum of a piece of excised liver bathed in a solution of choline. The spectrum (not shown) was similar to that of liver in vivo except that resonance H was much larger. Peaks E–G, only clearly visible in the spectrum of the gsd/gsd liver (fig. 1b) and peak D, have been assigned to glycogen from comparison with a spectrum of pure glycogen (fig. 3b). Peak D represents the C_1 (100.7 ppm), peak E is due to C_4 (76.8 ppm), peak F is due to C_2, C_3, C_5 (72.03 ppm) and peak G is due to C_6 (60.5 ppm) carbons [12]. Peaks E–G will have minor contributions from sugars free in solution; e.g., glucose. The chemical shifts of peaks D–G were measured relative to those of peaks I and H. They correspond closely to those observed in the pure glycogen sample.

We have not assigned the broad peak C but it may reflect the γ and δ carbons of histidine, tryptophan, phenylalanine and tyrosine [7]. Peak B (128.7 ppm) corresponds to the single and double-bond carbons in fatty acid [9,10] which probably derive mainly from phospholipids. The resonance at 174 ppm (A) corresponds to the carbonyl carbons of proteins [11]. The narrow linewidths of the triglycerides and other lipids indicate rapid molecular motion and have been attributed to signals from fats in the triglycerides and the mobile parts of membranes [8].

3.2. *Quantitation of ^{13}C signals*

The intensity of the glycogen signals in livers of phosphorylase kinase-deficient rats is clearly much greater than that in normal rats, but it is not easy to quantitate. Unfortunately there is no single signal in these spectra which can be assigned to a compound whose concentration has been determined by chemical assay. Thus the method of relating peak areas to the area of the ATP peak and hence to the chemical concentration of ATP, which has been widely used in ^{31}P NMR [1] has no counterpart in ^{13}C work. The intensities of the signals of $(CH_2)_n$ and (C=O) carbons are probably not proportional to their chemical concentrations, since the mobility of fatty acids and other substances containing these groups is very variable. However, if we assume that the signals from $(CH_2)_n$ are of equal intensity in normal and gsd/gsd rat livers and that the same is true of those from (C=O), then we can relate these signals to those of glycogen in the two groups.

Table 1 shows the result of such a comparison. Integration was carried out using standard software supplied by Bruker; it agreed well with manual cutting and weighing of the peaks. The ratios of $(C{=}O):(CH_2)$ are similar in normal and gsd/gsd rats, and so it is possible to compare levels of glycogen in the gsd/gsd rats with the control animals. One must first standardise the glycogen peaks observed by NMR by comparing either the

Table 1

Metabolite ratios as measured chemically and by ^{13}C NMR in fed and 24 h-starved rat livers of a normal and a phosphorylase kinase (gsd/gsd)-deficient strain after normal feeding or 24 h starvation

	NMR					Chemical assay
	C_1 gly:$(CH_2)_n$	$C_{2,3,5}$ gly:$(CH_2)_n$	C_1 gly:(C=O)	$C_{2,3,5}$ gly:(C=O)	(C=O):$(CH_2)_n$	(μmol glycogen.g wet wt^{-1})
Normal (fed)[a]	0.02	0.10	0.14	0.68	0.15	227 ± 18
gsd/gsd (fed)	0.09	0.29	0.58	1.62	0.16	663 ± 43
gsd/gsd (fed): normal (fed)	4.5	2.7	4.14	2.5	1.0	2.92
gsd/gsd (starved)	0.09	0.39	0.58	2.69	0.15	663 ± 36
gsd/gsd (starved): gsd/gsd (fed)	0.9	1.34	1.0	1.55	1.0	1.0

[a] There was no detectable signal from glycogen in livers of starved, normal rats

C_1 gly or $C_{2,3,5}$ gly resonances with the C=O or $(CH_2)_n$ peaks allowing us to obtain a value for glycogen in arbitrary units (table 1, rows 1 and 2). From these values a ratio of the glycogen concentrations in normal and gsd/gsd livers may be obtained (table 1, row 3). While the C_1 peak can be solely attributed to glycogen the C_1 gly:$(CH_2)_n$ ratio and the C_1 gly:C=O ratio are less accurate than the corresponding $C_{2,3,5}$ gly:$(CH_2)_n$ or $C_{2,3,5}$:C= O ratios as the C_1 peak is difficult to measure in the normal strains and so will tend to be underestimated. Comparing ratios derived from peak F ($C_{2,3,5}$ gly) the NMR method gives 2.5- or 2.7-fold more glycogen in gsd/gsd rats whereas chemical assay gives 2.9-fold.

Fig. 2 shows spectra from a normal liver and a gsd/gsd liver in rats that had been starved for 24 h. There is complete absence of resonances assigned to glycogen in normal strain while levels of glycogen remain unchanged in the livers of gsd/gsd rats, in good agreement with chemical determinations.

Fig. 3a shows the difference spectrum of the spectra of a starved normal rat and a starved gsd/gsd rat (fig. 2a,b). The chemical shifts and linewidths of the resonances are clearly similar to those of pure glycogen in vitro (fig. 3b). Table 1 (bottom row) compares concentrations obtained

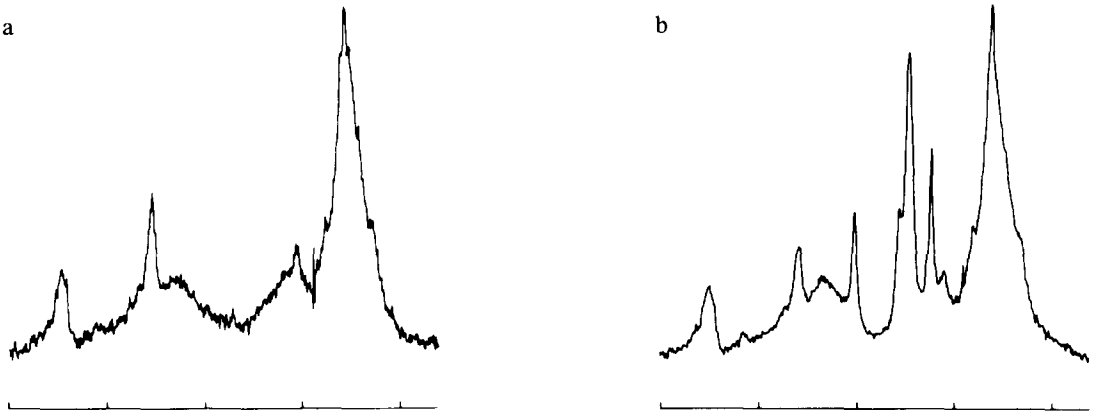

Fig. 2. Proton-decoupled 50.24 MHz ^{13}C NMR spectra of (a) 24 h-starved rat liver in vivo (2500 scans) (a) normal (b) phosphorylase kinase deficient (gsd/gsd). Conditions were as in fig. 1.

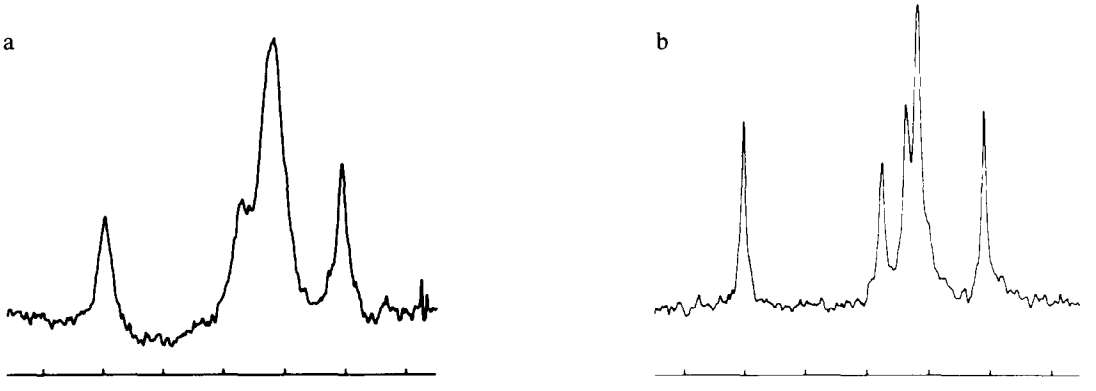

Fig. 3. (a) Difference spectra of fig. 2, spectrum of a 24 h-starved normal rat liver in vivo subtracted from the spectrum of a 24 h-starved gsd/gsd rat liver in vivo. (b) Proton decoupled 50.24 MHz ^{13}C NMR spectra of pure glycogen solution (10 mg/ml) in H_2O (10% D_2O). The spectrum represents 2500 scans; recycle time 1 s; 12 Hz line broadening.

for glycogen by NMR (again in arbitrary units) in starved gsd/gsd rats with those in fed gsd/gsd rats. Once again, there is good agreement with the chemical determination. Degradation of purified glycogen using amyloglucosidase to give glucose was carried out in the NMR machine. The ratio of the total visible carbon atoms present as glucose and as glycogen was 1.03 indicating that all glucose molecules present in the glycogen structure are observed by NMR.

The ability to visualise glycogen by ^{13}C NMR, especially in the gsd/gsd strain, indicates that although the glycogen complex is large there must be considerable motion within the structure. Liver glycogen is a polydisperse high-M_r (up to 10^9) compound. Electron microscopy has shown glycogen to be composed of single spheres (particles) and clusters of spheres (particles) [13]. The latter are held together by covalent bonds and are large and irregular in shape. Associated with the particulate glycogen are a number of enzymes: glycogen synthetase, phosphorylase, phosphorylase phosphatase and glycogen synthetase phosphatase [14,15] forming an enzyme–glycogen complex.

Electron micrographs of livers from gsd/gsd rats show that glycogen is evenly distributed throughout the cytoplasm, and not associated with organelles. The similarity of the NMR and chemical assay data on the two strains (table 1) indicates that the glycogen must be in a normal environment in gsd/gsd livers. This is surprising, since the glycogen synthesising and degrading enzymes are present in normal amounts [3] so that the ratio of enzymes:glycogen complex would be expected to be lower than normal.

4. CONCLUSIONS

Elevated glycogen levels can be observed in the livers of phosphorylase kinase-deficient gsd/gsd rats; the degree of elevation is similar to that measured by chemical assay. Starvation did not significantly reduce the glycogen content of the livers with glycogen storage disease whereas it reduced the signal below detectability in normal rats. Glycogen storage diseases have been demonstrated indirectly in skeletal muscle, using ^{31}P NMR by the absence of a normal acidification on exercise [16,17]. These results suggest that human glycogen storage diseases, and particularly those of the liver, could be diagnosed by ^{13}C NMR when whole-body high resolution instruments become available.

ACKNOWLEDGEMENTS

We would like to acknowledge the Medical Research Council for financial support and we thank Dr R. Malthus for the kind donation of breeding pairs of gsd/gsd rats and the MRC Laboratory Animal Centre for importing and initially breeding the rats. A.N.S. is a Science and Engineering Research Council student.

REFERENCES

[1] Iles, R.A., Stevens, A.N. and Griffiths, J.R. (1982) Prog. NMR Spectrosc. 15, 49–200.
[2] Alger, J.R., Sillerud, L.O., Behar, K.L., Gillies, R.J., Shulman, R.G., Gordon, R.E., Shaw, D. and Hanley, P.E. (1981) Science 214, 660–662.
[3] Malthus, R., Clark, D.G., Watts, C. and Sneyd, J.G.T. (1980) Biochem. J. 188, 99–106.
[4] Watts, C. and Malthus, R.S. (1980) Eur. J. Biochem. 108, 73–77.
[5] Hue, L., Bontemps, F. and Hers, H.G. (1975) Biochem. J. 152, 105–114.
[6] Trinder, P. (1969) Ann. Clin. Biochem. 6, 24–27.
[7] Doyle, D.D., Chalovich, J.M. and Barany, M. (1981) FEBS Lett. 131, 147–150.
[8] Cohen, S.M., Shulman, R.G. and McLaughlin, A.C. (1979) Proc. Natl. Acad. Sci. USA 76, 4808–4812.
[9] Godici, P.E. and Landsberger, F.R. (1974) Biochemistry 13, 362–368.
[10] Wedmid, Y., Lais, A.C., Dyck, P.J. and Baumann, W.J. (1980) Biochem. Biophys. Res. Commun. 97, 139–147.
[11] Gurd, F.R.N. and Keim, P. (1973) Methods Enzymol. 27, 836–911.
[12] Dais, P. and Perlin, A.S. (1982) Carbohydrate Res. 100, 103–116.
[13] Geddes, R., Harvey, J.D. and Wills, P.R. (1977) Biochem. J. 163, 201–209.
[14] Stalmans, W. (1976) Curr. Top. Cell. Regul. 11, 51–97.
[15] Krisman, C.R. and Barengo, R. (1975) Eur. J. Biochem. 52, 117–123.
[16] Ross, B.D., Radda, G.K., Gadian, D.G., Rocker, G., Esiri, M. and Falconer-Smith, J. (1981) New Eng. J. Med. 304, 1338–1342.
[17] Edwards, R.H.T., Dawson, M.J., Wilkie, D.K., Gordon, R.E. and Shaw, D. (1982) Lancet *i*, 725–732.