Growth studies of subcutaneous rat tumours: comparison of $^{31}$P-NMR spectroscopy, acid extracts and histology

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Summary $^{31}$P-NMR surface coil spectra of three subcutaneously implanted rat tumours (Morris hepatoma 7777, GH3 prolatinoma, Walker carcinosarcoma) and an N-methyl-N-nitrosourea induced rat mammary adenocarcinoma at different stages of growth were obtained and compared with histological sections taken immediately after NMR acquisitions. Metabolite ratios (phosphocreatine (PCr)/$\beta$ nucleoside triphosphate ($\beta$NTP), PCr/PI, $\beta$NTP/PI; see Methods) were calculated from the NMR spectra compared with ratios obtained from acid extracts of tumours of similar size. Measurements of creatine and ADP were also made. Three of the tumours showed positive correlations between increasing tumour size and decreasing metabolite ratios measured both by NMR and in extracts, whereas the Walker carcinosarcoma showed no correlation between size and any parameters measured. Phosphorus metabolite ratios, measured in extracts of skin overlying the tumours, indicated a fall in high energy phosphate when there was histological evidence of skin invasion by the tumour. Surface coil $^{31}$P-NMR spectra of subcutaneously grown or induced tumours in the rat represent a slowly changing steady state as the tumour increases in size. We conclude that increasing numbers of hypoxic tumour cells, rather than large areas of necrotic tissue, contribute largely to the NMR spectrum.

$^{31}$P-NMR spectroscopy has been used to monitor growth and response to therapy in several experimental tumour lines (Ng et al., 1982; Steen et al., 1988; Wehrle et al., 1987; Rodrigues et al., 1988; Tozer et al., 1989). Tumour response to various types of chemotherapy and X-irradiation therapy often involves the reversal of the changes seen during untreated progression; the treated tumour appears more highly energised (i.e. has increased phosphocreatine/$\beta$ nucleoside triphosphate (PCr/$\beta$NTP), PCr/PI or $\beta$NTP/PI; see Methods) than before treatment.

The changes in the $^{31}$P-NMR spectra that occur as animal tumours grow could be due to several causes. Most tumours described in the literature have shown a fall in high energy phosphates with increasing age and size which is usually attributed to increasing hypoxic or necrotic fractions (Ng et al., 1982; Roftstad et al., 1988a). Other factors, such as host cell invasion, oedema, haemorrhage and cyst formation, will also affect the spectrum.

Our recent studies (Stubbs et al., 1988a, 1989) on the contribution of rat skin to surface coil spectra of subcutaneous tumours suggest that this, too, could be important in studies on animals.

If the tumour invades and destroys the skin (and particularly the pannicus muscularis carnosus which forms a major part of rodent skin) the skin contribution to the $^{31}$P-NMR spectrum will decrease with increasing tumour size. In order to elucidate these processes we have embarked on a series of studies of tumour growth.

Here we report the results of NMR studies of a variety of subcutaneously implanted rat tumours in vivo, at different time points in their growth, where we have compared the NMR spectra with histological sections of the tumours and with results obtained by hplc and enzymatic assay of acid extracts of the tumours. The tumours studied included Morris hepatoma 7777, the GH3 prolatinoma, the Walker carcinosarcoma and the NMU-induced mammary adenocarcinoma.

In addition we have freeze-clamped and measured phosphate metabolites in acid extracts of the tissues overlying some of the subcutaneous tumours.

There are several difficulties associated with such a study. First, freeze clamping needs to be done rapidly in order to minimise breakdown of high energy phosphates and because of tumour heterogeneity it is necessary to extract the whole tumour for analysis. Therefore it is only possible to do either NMR and histology or NMR and freeze clamping. This means that comparisons sometimes have to be made between different tumours, albeit of a similar size. Secondly, when making comparisons between NMR and extract data there is only one parameter, PCr/NTP, that is truly comparable since only PCr and NTP are 'free' and therefore quantifiable both by NMR and in extracts (unlike nucleoside diphosphate (NDP) and PI of which significant proportions are known to be bound and therefore NMR invisible in tissues such as muscle, liver and kidney (Gadian, 1982; Iles et al., 1985; Freeman et al., 1983); see also Results section of this paper). For these and other reasons (including lack of the metabolite in question, poor resolution in the NMR spectrum, etc.), we have compared trends in the energy status of the tumours by means of several different ratios in addition to PCr/NTP. It is assumed that, in tumours that contain adequate creatine kinase activity, there will be an equilibrium relationship between cytosolic PCr, creatine (Cr), NTP and NDP so that PCr/Cr, NTP/NDP will reflect changes in PCr/NTP; in tumours that do not contain creatine kinase comparisons are limited to NTP/NDP and NTP/PI.

Preliminary accounts of this work have been presented (Stubbs et al., 1988b, c).

Methods

Tumours

Each batch of tumours (Morris Hepatoma 7777, GH3 prolatinoma, Walker carcinosarcoma; for details of tumours see Stubbs et al., 1989) was grown up from a single inoculum or induced by N-methyl-N-nitrosourea (NMU) injection (mammary adenocarcinoma; see Williams et al., 1981). There were at least 10 in each batch for the prolatinoma, Walker carcinosarcomas and mammary adenocarcinomas, six in one Morris hepatoma 7777 batch and five in another. Tumours were chosen in pairs in increasing sizes; one for the NMR study which was taken for histology after the completion of the NMR collection and one of similar size which was freeze-clamped, acid extracted and assayed for high energy phosphates. Tumour volumes were measured using the formula:

$$V = \pi/6 \ d_1 \cdot d_2 \cdot d_3$$
NMR measurements

The NMR was performed on a 1.8T-TMR 32.200 spectrometer with a 27 cm horizontal bore, from Oxford Research Systems, Abingdon, UK. Spectra were obtained from each batch of tumours with a surface coil (Ackerman et al., 1980), either 1, 1.4 or 2 cm diameter, with a 6, 8 or 10 μs pulse duration respectively. The 90° pulses for these coils at the coil centre were 4, 6 and 6 μs respectively. The repetition time was 3s and 480 scans were collected routinely. Exponential weighting equivalent to 15 Hz line broadening and deconvolution with a function to reduce broad spectral lines was applied before integration of the peaks using the software package supplied with the machine (for further details see Rodrigues et al., 1988). To test the reproducibility of this method we made repeated measurements on two tumours. In the Walker carcinosarcoma the mean ($\pm$ s.d.) BNT/Pi ratio was 0.61±0.05 ($n$ = 7) and in a Morris hepatoma it was 0.47±0.04 ($n$ = 6). However, due to difficulties in baseline definition and overlapping peaks these integrals may not give true chemical concentrations. For this reason the data in this paper are all corrected as ratios of integrals (except in Table IV), which minimises some of the uncertainties.

The ATP signals of tumours co-resonate with those of GTP and the other nucleoside triphosphates. The β peak contains only triphosphate signal so we refer to βNTP in spectral quantitation. In hpc studies of extracts we found that ATP and GTP were the main nucleoside triphosphates so that the main observable spectral features contain NMR data, concentrations are reported as NTP. Similarly NDP is the sum of ADP + GDP in extracts.

pH measurements

pH was calculated from the chemical shift of Pi by the method of Frichard et al. (1983). These measurements were referenced to PCr at 0 p.p.m. when it was present and to αNTP at –7.57 p.p.m. when PCr was negligible. The reproducibility of this measurement was assessed by repeated measurements on a Walker carcinosarcoma that contained both αNTP and PCr. The pH was 7.32±0.1 (mean ± s.d.) when referenced to PCr and 7.41±0.12 when referenced to αNTP. The two means were not significantly different by a paired t test ($P > 0.1$). An estimate of the accuracy of the pH measurement was difficult to assess since there is no other non-invasive method available for comparison.

Acid extracts of tumours and overlying tissues

Skin tissues were rapidly excised from over the tumour with sharp curved scissors and dropped into liquid N₂. The tumour was then excised and freeze clamped in pre-cooled aluminium tongs. The whole procedure took less than 15 s. The frozen tissues were extracted with perchloric acid and neutralised as described in Stubbs et al. (1988a).

Measurements of phosphocreatine, creatine and Pi

PCr and Cr were assayed enzymatically on neutralised perchloric acid extracts by the method described in Bergmeyer (1974) and Pi by the method of Lowry and Lopez (1946) as modified by Chandra Rajan and Klein (1976).

Adenine nucleotides

These were assayed in neutralised extracts by hpc with a hypersil-APS (5 μ) weak anion exchange column and a Waters Instrument. A linear phosphate gradient from 22 mM to 0.7M was used, prepared from Aristar potassium phosphate (Shuttlewood & Griffiths, 1982).

Histological sections

A section, orthogonal to the plane of the coil, was taken through the centre of the tumour, including the skin, immediately after the NMR acquisition. Sections were cut from these specimens and fixed in 25% formal saline and subsequently cut and stained with Haematoxylin & Eosin.

Results

GH3 prolactinoma

The NMR spectra showed seven clear peaks of which only PCr appeared to change consistently with increasing size of tumours (for representative spectra see Figure 1a–c). In the largest tumour 17.6 cm³; Figure 1c) the PCr resonance had virtually disappeared. Histological sections (see Figure 1d for a tumour of 6.1 cm³) showed little infiltration of the dermis or panniculus carnosus muscle except in the largest tumour (Figure 1e) which also showed myolysis of muscle tissue. The amount of necrotic tissue in the tumours was small; it represented only about 10% in a tumour of 17.6 cm³. There was some evidence of a granulomatous reaction in the subcutis in response to the presence of the implanted tumour cells together with some inflammatory cell infiltration of the surrounding tissues.

Comparisons of NMR spectra with acid extracts of the prolactinomas

PCr/βNTP ratios calculated from both the peak areas in the NMR spectra and in the acid extracts (Table I) decreased significantly with increasing tumour volume (for correlation coefficients see Table III).

Morris hepatoma 7777

Tumours were studied at various times after implantation when they had reached sizes ranging from 3 to 29 cm³. In the NMR spectra of these tumours the PCr peak increased with size (for two representative spectra see Figure 2). No PCr resonance was observed except in the smallest tumour (not shown in Figures).

Histological sections showed that as the tumour grew the cutis became thin and histological evidence of panniculus carnosus muscle, which was present in a tumour of 12.4 cm³ (Figure 2c), had disappeared in a tumour of 21 cm³ (not shown in the Figure). The foci of tumour cell necrosis were much more extensive in the hepatomas than in the prolactinomas (compare Figure 1e with Figure 2c).

Comparison of NMR spectra with acid extracts of hepatoma 7777

Since the acid extracts showed no detectable PCr or Cr at any time there is no directly comparable ratio between the NMR and the acid extract data. However, the significant decrease in βNTP/Pi observed with increasing tumour size (for correlation coefficients see Table III) was mirrored by a fall in the NTP measured in the acid extract from 1.23 mmol g⁻¹ in the smallest tumour to 0.66 mmol g⁻¹ in the largest tumour and also by a fall in the NTP/NDP ratio. pH varied between 7.1 and 7.6 with no apparent correlation to size of tumour.

NMU-induced mammary adenocarcinomas

A distinct PCr resonance was observed in the NMR spectra of three tumours of 1.4, 2.1 and 7.4 cm³. In tumours larger than 7.4 cm³ and in one of 3.9 cm³, the phosphocreatine resonance was negligible (spectra of typical mammary adeno-
carcinomas have been shown in Rodrigues et al. (1988)). Histological sections of the tumours (and overlying skin) showed that the smallest tumour was growing actively with no necrosis. In the larger tumours the subcutis contained a granulomatous reaction. In three of the tumours there was evidence of invasion of the panniculus carnosus muscle by the adenocarcinoma and small areas of necrosis were apparent. There was also a chronic inflammatory response in the dermis. In the largest tumour studied (16 cm) there was ulceration of the overlying epidermis but the large tumour masses were not necrotic.

Comparison of NMR with acid extract data in mammary adenocarcinomas

The PCr/NTP and βNTP/Pi ratios calculated from peak areas of the NMR spectra (Table II) decreased significantly with increasing tumour size. This finding was mirrored by the PCr/NTP ratios in extracts of mammary adenocarcinomas of similar size although significance was not achieved (see Table III). However, the trend of decreasing high energy phosphates with increasing tumour size was evident, especially in the extract PCr/Cr ratio where it was statistically significant (see also Table III). Cr was consistently present in this tumour and pH ranged between 7.0 and 7.5.

Walker carcinosarcoma

Two batches of tumours (a total of nine) ranging from 2.5 to 27.5 cm were examined by NMR. Figure 5a shows the NMR spectrum and Figure 5b the histological section of a tumour (6.2 cm) in the range studied (2.6–20.4 cm). Histological sections showed evidence of a granulomatous reaction in the subcutis in response to the presence of implanted tumour cells with some inflammatory cell infiltration of the surrounding tissues (Figure 3b). In the largest tumour there were large areas of cellular necrosis. There were, however, no differences in the histological structures of the actively growing tumour aggregates.

Table I High energy phosphates and Cr concentration in GH3 prolactinomas of different sizes

| NMR measurements | Acid extracts * |
|------------------|-----------------|
| Size of tumour (cm³) | PCr/βNTP | βNTP/Pi | Size of tumour (cm³) | PCr/NTP | Cr conc. (μmol g⁻¹ wet wt) |
| 0.77              | 1.42          | 1.10      | 0.77              | 1.21      | 3.35 |
| 2.9               | 0.96          | 1.38      | 2.9               | 0.52      | 2.19 |
| 6.1               | 0.50          | 1.30      | 6.1               | 0.79      | 2.26 |
| 10.7              | 0.83          | 0.90      | 10.7              | 0.34      | 2.89 |
| 17.6              | 0.46          | 0.62      | –                 | –         | –         |

*Metabolite range (μmol g⁻¹ wet wt) PCr, 0.12–1.35; NTP, 0.35–1.12. 1hphl distinguishes between the different triphosphates of which ATP is the biggest contributor and GTP the next (the others are <5%). For this reason NTP in the tumour extracts has been calculated from ATP + GTP in order to make comparisons with NMR data.

Figure 1 31P-NMR spectra and histology of GH3 prolactinoma. Peak assignments as follows: (1) β phosphate of NTP; (2) α phosphate of NTP; α phosphate of NDP, NAD; (3) γ phosphate of NTP, β phosphate of NDP; (4) phosphocreatine; (5) phosphodiester; (6) P; (7) phosphomonoester. a is a spectrum from a tumour of 0.77 cm³, b and d from a tumour of 6.1 cm³ and c and e from a tumour of 17.6 cm³. The relative position of the coil is marked with open circles. The photographs are from the sections marked on the histological drawings and are ×11. The key to the histological drawing is as follows: A, epidermis/dermis; C, panniculus carnosus muscle; D, actively growing tumour tissue; E, necrotic tumour tissue; F, subcutis showing inflammatory cell infiltration; G, granulomatous reaction in subcutis.
Because of the lack of correlation between size and any parameter measured in the Walker carcinosarcomas we studied a single tumour throughout its growth by NMR alone. This study showed that βNTP/Pi values, calculated from the NMR spectra of a tumour that increased from 1.8 to 29.5 cm³ over a period of 6 days, also did not correlate with increasing size (P>0.1).

**General comparisons between NMR and extract data**

Pi measured in extracts of Walker carcinosarcomas and mammary adenocarcinomas was consistently higher than that measured by NMR (Table IV). This finding has been made in a number of normal tissues and is thought to be due to binding or compartmentalisation of Pi in vivo causing it to be NMR invisible (Ross et al., 1984; Iles et al., 1985) although in contrast Corbett et al. (1987) found more Pi in vivo than in vitro in a human melanoma grown in nude mice.

In tumours containing PCr it was possible to calculate ‘free’ ADP (assuming equilibrium in the creatine kinase reaction) from the extract measurements of PCr, Cr, ATP H⁺ (calculated from the pH measured in the NMR spectrum) and the Kₑq for creatine kinase (Veech et al., 1979). The results shown in Table IV suggest that 10–20% of the total tumour ADP is ‘free’, a value similar in order of magnitude to that observed in kidney and liver (Freeman et al., 1983; Iles et al., 1985).

**Phosphate metabolite ratios in tissue overlying subcutaneously implanted tumours**

In three of the tumour types and at three or four points in the growth studies, skin tissue overlying the tumours was rapidly frozen. The data from the extracts suggest that there is some correlation between PCr/Cr and PCr/NTP ratios in the skin and the degree of skin invasion by the tumour. In the Walker carcinosarcoma and mammary adenocarcinomas, where histological evidence of skin invasion was observed more often than not, the PCr/Cr ratios were 0.44 ± 0.15 and 0.41 ± 0.12 respectively and the PCr/NTP ratios were 0.80 ± 0.14 and 1.98 ± 0.43 respectively, considerably lower than in normal skin (1.17 and 3.6; Stubbs et al., 1988) whereas in the prolactinomas where skin invasion was observed only in one tumour of 17 cm³, the PCr/Cr and PCr/NTP were higher: 1.54 ± 0.17 and 5.0 ± 1.3 respectively, both slightly higher than the normal skin range. The changes in the NTP/NDP ratios were not as clear cut but a change in this ratio would be expected to be less marked than the change in PCr/NTP due to the equilibrium position of the creatine kinase reaction (McGilvery & Murray, 1974).

**Discussion**

The first tumour to be studied by NMR spectroscopy in vivo, the Walker carcinosarcoma, gave spectra that sometimes showed PCr (Griffiths & Stevens, 1981) and sometimes did.

**Table II** High energy phosphates and Cr concentration in NMU-induced mammary adenocarcinomas of different sizes

| NMR measurements | Acid extracts * |
|-------------------|-----------------|
| Size of tumour (cm³) | PCr/βNTP | βNTP/Pi | Size of tumour (cm³) | PCr/NTP | Cr conc. (µmol g⁻¹ wet wt) |
| 1.37 | 1.09 | 2.14 | | | |
| 2.07 | 1.50 | 2.48 | | 1.84 | 1.35 | 2.91 |
| 3.90 | 0 | 1.11 | | 4.32 | 1.47 | 3.93 |
| 7.45 | 1.14 | 0.80 | | 7.15 | 0.20 | 2.57 |
| 9.95 | 0 | 0.38 | | 8.89 | 0.20 | 3.75 |
| 16.4 | 0 | 0.75 | | 16.4 | 0.57 | 3.29 |

*Metabolite range (µmol g⁻¹ wet wt) PCr, 0.21–1.85; NTP, 0.93–2.13.
not (Griffiths et al., 1981). PCr was found in spectra of mouse tumours (Ng et al., 1982) and in human xenografts grown in mice (Griffiths & Iles, 1982). Furthermore, Ng et al. (1982) showed that the PCr peak diminished during tumour growth and that it became larger after chemotherapy.

The tumours in the present study fell into three classes with respect to their PCr content. At one extreme the Morris hepatoma 7777 showed no evidence of PCr or Cr in acid extracts; the PCr signals observed in vivo in some of these tumours (Stubbs et al., 1988a, 1989) were presumably caused by skin contamination. The lack of PCr is probably because hepatomas, like the hepatocyte from which they arise, do not express creatine kinase (Shatton et al., 1979).

In contrast, the GH3 prolactinomas always contained PCr and Cr in extracts and almost always showed a PCr signal in vivo (Table 1). The mammary adenocarcinomas also contained Cr and varying amounts of PCr in all extracts, but here three of the four largest tumours in a parallel series showed negligible PCr signal in vivo (Table II).

The Walker carcinosarcomas fell into a third category. All those studied by acid extraction contained PCr and Cr but only about 50% of the NMR spectra showed signals in vivo, explaining our earlier observations (Griffiths & Stevens, 1981; Griffiths et al., 1982).

### Table III Correlations between tumour volume and high energy/low energy phosphate ratios in four tumour types

| Tumour type | PCr/βNTP | βNTP/Pi | PCr/NTP | PCr/Cr | NTP/Pi/NDP |
|-------------|----------|---------|---------|--------|------------|
| Prolactinoma | 0.75 (5) | 0.85 (5) | 0.74 (4) | 0.78 (4) | 0.76 (4) |
| Hepatoma     | <0.02    | <0.01   | <0.05   | <0.02  | <0.05      |
| Mammary     | 0.63 (6) | 0.75 (5) | 0.59 (4) | 0.98 (4) | 0.57 (4)   |
| Walker      | <0.05    | <0.01   | <0.01   | <0.01  | <0.01      |
| P           | >0.1     | >0.1    | >0.1    | >0.1   | >0.1       |

The data are the correlation coefficients achieved when comparing the ratios with tumour size. The closer the coefficient is to 1 the greater the correlation between the decrease in high energy/low energy phosphate ratio compared to the increase in tumour size.

### Table IV Total and free ADP and Pi in tumours

| Tumour type | Total ADP | Free ADP | P value |
|-------------|-----------|----------|---------|
| Mammary     | 0.58 ± 0.14 | 0.061 ± 0.013 | <0.02 |
| Prolactinoma | 0.30 ± 0.1 | 0.056 ± 0.013 | <0.02 |
| Mammary     | 5.51 ± 1.4 | 1.76 ± 0.46 | <0.05 |
| Walker      | 5.10 ± 0.29 | 2.90 ± 0.50 | <0.01 |

The results are expressed as pmol g⁻¹ wet wt and are mean ± s.e.m. P values compare total versus free metabolites. Total ADP and Pi were measured in tumour extracts as described in the text. Free ADP was calculated from ATP, PCr, and Cr measured in tumour extracts, pH measured from the NMR spectra, and the Keq for creatine kinase according to Veach et al. (1979). Free Pi was calculated from the βNTP/Pi ratios measured from the NMR spectra making the simplifying assumption that NTP measured in extracts was equal in concentration to the βNTP integral in the NMR spectra.

Figure 3 ³¹P-NMR spectrum and histology of a Walker Sarcoma. Details as Figure 1. a and b from a tumour 6.2 cm³.
surface coils produce (Ackerman et al., 1980) it was not possible to relate the skin extract data directly to the NMR spectra. Precise localisation and quantitation techniques are needed for this.

What does the $^{31}$P-NMR spectrum of a subcutaneously implanted tumour tell us about the status of the tumour?

In three of the tumours studied here (mammary adenocarcinoma, GH3 prolactinoma and Morris Hepatoma 7777), there was a tendency for the high energy/low energy phospho-rates to decline as the tumours enlarged (Table III). Thus these tumours show a decline in high energy phosphates ($\beta$NTP and PCr) relative to Pi with increasing tumour size (see Glisson et al. (1989) for review) and PCr falls more rapidly than $\beta$NTP. However, in the Walker carcinosarcoma there was no correlation between high energy/low energy phosphate ratios and size. Rofstad et al. (1988a) also found one tumour, a human ovarian xenograft grown in athymic mice, amongst four they studied, in which spectral parameters did not correlate with an increase in tumour volume.

No correlations between increasing tumour size and pH were observed in any of the tumours. Such correlations have previously been made in some tumours (Ng et al., 1982; Adams et al., 1985; Rofstad et al., 1988a, 1988b) but not in others (Rofstad et al., 1988a; Ross et al., 1988; Steen et al., 1988; Rodrigues et al., 1988).

The fall in PCr usually seen in enlarging tumours has often been attributed to PCr being used to synthesise ATP in cells that were becoming hypoxic as a result of impaired tumour circulation (Evanochko et al., 1984; Remy et al., 1987; Rofstad et al., 1988a) and is said to compensate for the 'inefficiency' of the glycolytic pathway as a source of ATP (Rofstad et al., 1988; Evanochko et al., 1984; Remy et al., 1987; Glickson et al., 1989).

$$\text{ADP} + \text{Cr} \rightarrow \text{ATP} + \text{Cr}\text{kinase}$$

In practice, however, the amount of PCr in a tumour is far too small (typically $1-2\mu$mol g$^{-1}$ wet weight) to make a significant contribution to the ATP supply. Consider that tumour ATP turnover is probably about $6\mu$mol min$^{-1}$ g$^{-1}$; conversion of all the PCr would supply ATP for only 10–20 s, whereas the fall in PCr concentration actually takes place over a period of days, or even weeks.

What we observe in the $^{31}$P spectrum of a tumour is a slowly changing steady state in which the concentrations of the metabolites vary so as to maintain the maximum possible rate of ATP synthesis despite a steady fall in cellular oxygen concentration.

Do the $^{31}$P-NMR changes that we, and others, have observed in growing tumours reflect events in all the cells of a tumour? Or do they occur only in the necrotic cells, with the remainder being unaffected? This question has some practical importance: does $^{31}$P-NMR tell us about the proportion of the tumour that has become necrotic or does it tell us about the metabolism of viable but hypoxic cells?

We can answer these questions for tumours such as the GH3 prolactinoma and the NMU-induced mammary tumour in the present study, and also for the MOPC 104E mouse myeloma, described by Ng et al. (1982). In all these tumours the PCr signal is lost before there is a significant fall in the NTP peaks. This is clearly inconsistent with the hypothesis that the changes occur only in cells that have become necrotic, since necrotic cells have no NTP. If the tumour consisted of normal cells with both PCr and NTP and necrotic cells with neither PCr nor NTP one would observe a synchronous fall in the PCr and NTP signals as the necrotic fraction increased in the growing tumour. This was clearly not the case in the NMU-induced mammary tumour or the GH3 prolactinoma (see PCr/NTP ratios in Tables I and II), and in the larger NMU-induced mammary tumours PCr was undetectable despite strong NTP signals. Significant NTP resonances have been reported (Ross et al., 1988) even when there was 80–90% necrosis present in a C6 glioma, supporting the suggestion that the NMR signal comes from the viable cells.

We can conclude, for these tumours at least, that the $^{31}$P-NMR data tell us about the metabolic changes that occur in viable cells, and thus in cells that may be the target of therapy.

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References

ACKERMAN, J.J.H., GROVE, T.H., WONG, G.G., GADIAN, D. & RADDA, G.K. (1980). Mapping of metabolites in whole animals by 31P NMR using surface coils. Nature, 283, 167.

ADAMS, D.A., DENARDO, G.L., DENARDO, S.J., CONBOY, C.B. & BRADBURY, E.M. (1985). $^{31}$P NMR analysis of metabolic status in KHHJ tumours. Magn. Reson. Med., 2, 419.

BERGMeyer, H.U. (ed.) (1974) Methods of Enzymatic Analysis, 2nd edn. p. 1777. Verlag Chemie: Weinheim.

CHANDRA RAJAN, J. & KLEIN, L. (1976). Determination of inorganic phosphorus in the presence of organic phosphorus and high concentrations of proteins. Anal. Biochem., 72, 407.

CORBETT, R.T., NUNNALLY, R.L., GIOVANA, A., B.C. & ANTICH, P.P. (1987). Characterization of the 31P nuclear magnetic resonance spectrum from human melanoma tumors implanted in nude mice. Cancer Res., 47, 5065.

Evanochko, W.T., NG, T.C. & Glickson, J.D. (1984). Application of in vivo NMR spectroscopy to cancer. Magn. Reson. Med., 1, 508.

FREEMAN, D., BARTLETT, S., RADDA, G. & ROSS, B.D. (1983). Energetics of sodium transport in the kidney. Biochem. Biophys. Acta, 762, 325.

GADIAN, D.G. (1982) Nuclear Magnetic Resonance and its Application to Living Systems. Clarendon Press: Oxford.

Glickson, J.D., Evanochko, W.T., SAKAI, T.T. & NG, T.C. (1989). In vivo NMR spectroscopy of tumors. In NMR Spectroscopy of Cells and Organisms, Vol. 1, Gupta R.K. (ed.). CRC Press: Boca Raton.

GRIFFITHS, J.R., BHUWALLA, Z., COOMBES, R.C. & 10 others (1987). Monitoring cancer therapy by NMR spectroscopy. Ann. N.Y. Acad. Sci., 508, 183.

GRIFFITHS, J.R. & ILES, R.A. (1982). NMR studies of tumours. BioSci. Rep., 2, 719.

GRIFFITHS, J.R. & STEVENS, A.N. (1981). Topical magnetic resonance studies of tumours. Biochem. Soc. Trans., 9, 283F.

GRIFFITHS, J.R., STEVENS, A.N., ILES, R.A., GORDON, R.E. & SHAW, D. (1981). $^{31}$P-NMR investigation of solid tumours in the living rat. BioSci. Rep., 1, 319.

ILES, R.A., STEVENS, A.N., GRIFFITHS, J.R. & MORRIS, P.G. (1985). Phosphorylation of liver by 31P NMR spectroscopy, and its implications for metabolic control. Biochem. J. 229, 141.

LOWRY, O.H. & LOPEZ, J.A. (1946). The determination of inorganic phosphate in the presence of labile phosphate esters. J. Biol. Chem., 162, 421.

MCGILVERY, R.W. & MURRAY, T.W. (1974). Calculated equilibria of phosphocreatine and adenosine phosphates during utilization of high energy phosphate by muscle. J. Biol. Chem., 249, 5845.

NG, T.C., EVANOCHKO, W.T., HIRAMOTO, R.N. & 6 others (1982). 31P NMR spectroscopy of in vivo tumors. J. Magn. Reson., 49, 271.

PRICHRAD, J.W., ALGER, J.R., BEHAR, K.L., DETROFF, O.A.C. & SHULMAN, R.G. (1983). Cerebral metabolic studies in vivo by $^{31}$P-NMR. Proc. Natl Acad. Sci U.S.A., 80, 2748.

REMY, C., ALBRAND, J.P., BENABID, A.L. & 4 others (1987). In vivo 31P NMR studies of $T_1$ and $T_2$ relaxation times in rat brain and rat brain tumours implanted in nude mice. Magn. Reson. Med., 4, 144.

RODRIGUES, L.M., MIDWOOD, C.J., COOMBES, R.C., STEVENS, A.N., STUBBS, M. & GRIFFITHS, J.R. (1988). 31P Nuclear magnetic resonance spectroscopy studies of the response of rat mammary tumours to endocrine therapy. Cancer Res., 48, 89.
ROFSTAD, E.K., DE MUTH, P. & SUTHERLAND, R.M. (1988a). 31P NMR spectroscopy measurements of human ovarian carcinoma xenografts: relationship to tumour volume, growth rate necrotic fraction and differentiation status. Radiother. Oncol., 12, 315.

ROFSTAD, E.K., HOWELL, R.L., DEMUTH, P., CECKLER, T.L. & SUTHERLAND, R.M. (1988b) 31-P NMR spectroscopy in vivo of two murine tumor lines with widely different fractions of radio-biologically hypoxic cells. Int. J. Radiat. Biol., 54, 635.

ROSS, B.D., FREEMAN, D.M. & CHAN, L. (1984). Phosphorus metabolites by NMR. Adv. Exp. Med. Biol., 178, 455.

ROSS, B.D., HIGGINS, R.J., BOGGAN, J.E., KNITTEL, B. & GARWOOD, M. (1988). 31-P NMR spectroscopy of the in vivo metabolism of an intracerebral glioma in the rat. Magn. Resonance Med., 6, 403.

SHATTON, J.B., MORRIS, H.P. & WEINHOUSE, S. (1979). Creatine kinase activity and isozyme composition in normal tissues and neoplasms of rats and mice. Cancer Res., 39, 492.

SHUTTLEWOOD, R. & GRIFFITHS, J.R. (1982). The purine nucleotide profile in mouse, chicken and human dystrophic muscle: an abnormal ratio of inosine plus adenine nucleotides to guanine nucleotides. Clin. Sci., 62, 113.

STEEN, R.G., TAMARGO, R.J., MCGOVERN, K.A. & 4 others (1988). In vivo 31P NMR spectroscopy of subcutaneous 9L gliosarcoma: effects of tumour growth and treatment with 1,3-bis (2-chloro-ethyl)-1-nitrosourea on tumour bioenergetics and histology. Cancer Res., 48, 676.

STUBBS, M., RODRIGUES, L.M. & GRIFFITHS, J.R. (1988b). Comparison of 31-P spectra of animal tumours with histology. Second European Congress of NMR in Medicine and Biology, Berlin, p. 93.

STUBBS, M., RODRIGUES, L.M. & GRIFFITHS, J.R. (1988c). Correlation of 31-P-NMR spectra with acid extracts and histology in a growth study of some animal tumours. Proc. 7th Ann. Meeting Soc. Magn. Resonance Med., 1, 407.

STUBBS, M., RODRIGUES, L.M. & GRIFFITHS, J.R. (1989). Potential artefacts from overlying tissues in 31-P NMR spectra of subcutaneously implanted rat tumours. NMR in Biomed., 1, 165.

STUBBS, M., VANSTAPEL, F., RODRIGUES, L.M. & GRIFFITHS, J.R. (1988a). Phosphate metabolites in rat skin NMR Biomed., 1, 50.

TOZER, G.M., BHUJWALLA, Z.M., GRIFFITHS, J.R. & MAXWELL, R.J. (1989). Phosphorus-31 magnetic resonance spectroscopy and blood perfusion of the RIF-1 tumor following X-irradiation Int. J. Radiat. Oncol. Biol. Phys., 16, 155.

VEECH, R.L., LAWSON, J.W.R., CORNELL, N.W. & KREBS, H.A. (1979). Cytosolic phosphorylation potential. J. Biol. Chem., 254, 6538.

WILLIAMS, J.C., GUSTERSON, B., HUMPHREYS, J. & 4 others (1981). N-methyl-N-nitrosourea-induced rat mammary tumors. Hormone responsiveness but lack of spontaneous metastasis. J. Natl Cancer Inst., 66, 147.