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^{13}C n.m.r. studies of gluconeogenesis in rat liver suspensions and perfused mouse livers

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Our early ^{31}P n.m.r. studies of compartmentation in suspensions of rat liver cells have been extended by following fructose-1-phosphate peaks, known to be in the cytosol, which gave the same pH as the P_i peak previously assigned to the cytosol. Gluconeogenesis has been followed from [^{13}C]glycerol labelled at C1,3 or at C2 and from labelled [3-^{13}C]alanine. With the glycerol substrate it was possible to follow the label into α-glycerophosphate and to determine its distribution in the glucose formed. To a first approximation (i.e. 90%) the glucose label could be followed from its original glycerol position, e.g. [1,3-^{13}C]glycerol to strongly labelled positions 1, 3, 4 and 6 of glucose. Slightly more than 10% of the label was scrambled (i.e. 10% movement of C2 to C1 and *ca.* 10% of C1 was lost, the remainder being unchanged). These are consistent with a flux through the pentose shunt, dominated by the transketolase pathway. With [3-^{13}C]alanine, about 14 resonances are assigned to different carbons of the intermediates β-hydroxybutyrate, acetoacetate, lactate, pyruvate, glutamate, glutamine, asparate, as well as C2-alanine, while another 7 resonances are observed from the different anomeric carbons of glucose. The effects of thyroid hormone treatment of the rats upon numerous *in vivo* rates are clearly observed and will be illustrated.

As shown by the varied papers in this symposium, the study of whole cells and intact tissue by high resolution n.m.r. has recently made rapid advances. In order to illustrate the kinds of studies being carried out in our laboratory, which are reviewed more generally elsewhere (Shulman *et al.* 1979), we discuss in this article our ^{13}C and ^{31}P n.m.r. studies of suspensions of rat liver cells, as well as more recent studies of ^{31}P and ^{13}C n.m.r. of perfused mouse livers. In suspensions of rat liver cells, we have been able to observe well resolved inorganic phosphate (P_i) n.m.r. peaks from the mitochondrial and cytosolic pools (Cohen *et al.* 1978*a*). The positions of P_i n.m.r. peaks depend upon the pH, so that it has been possible to measure the pH of the two compartments separately, and to show that the mitochondrial pH is, in fact, higher than the cytosolic pH, and that the pH difference is destroyed by an uncoupler, FCCP. The cytosolic pH was determined independently from the fructose-1-phosphate n.m.r. peak.

Very recently it has also been shown that ^{13}C-labelled metabolites can be observed by high resolution ^{13}C n.m.r. Glucose metabolism under anaerobic and aerobic conditions has been followed both in *Escherichia coli* (Ugurbil *et al.* 1978) and in yeast (den Hollander *et al.* 1979). In addition to having observed labelling of the end products, we have obtained from the spectra the distribution of the label in intermediates. In this way, it has been possible to measure the departure from equilibrium of certain reactions, the flux partition between different pathways and the dominant pathways of biosynthesis.

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Here, we present some results of a ^{13}C n.m.r. study of the relatively simple gluconeogenic pathway from glycerol in liver cells obtained from euthyroid and hyperthyroid rats (Cohen *et al.* 1979*b*). Glycerol labelled with ^{13}C provides a particularly straightforward illustration of the n.m.r. method in gluconeogenesis and the results obtained allow one to determine the relative fluxes of different pathways. Using [1,3-^{13}C]glycerol and [2-^{13}C]glycerol, we have shown equal labelling in the triose units of glucose and have measured the quantitative flux through the pentose shunt.

A 360 MHz n.m.r. spectrometer was used for these experiments. The liver suspension was prepared by the method of Berry & Friend (1969). To deliver enough oxygen to maintain the concentrated n.m.r. cellular suspensions viable at 25 °C, small quantities of 0.5 M H_2O_2 in normal saline were gently mixed into samples in the n.m.r. probe at regular intervals. A computer program was written by S. Ogawa, that allowed the alternation of periods of H_2O_2 mixing with periods of data accumulation. This program is similar to that described previously for providing controlled bursts of oxygen to suspensions of *E. coli* (Ogawa *et al.* 1978). A typical duty cycle consisted of 3 s of H_2O_2 mixing alternating with 24 s of data accumulation. This method depends upon the catalase activity of the cellular suspension and appears to inflict about the same slight amount of damage to the cells as a similar period of ordinary incubation in which a dilute suspension is gassed with 95 % O_2, 5 % CO_2 and shaken gently.

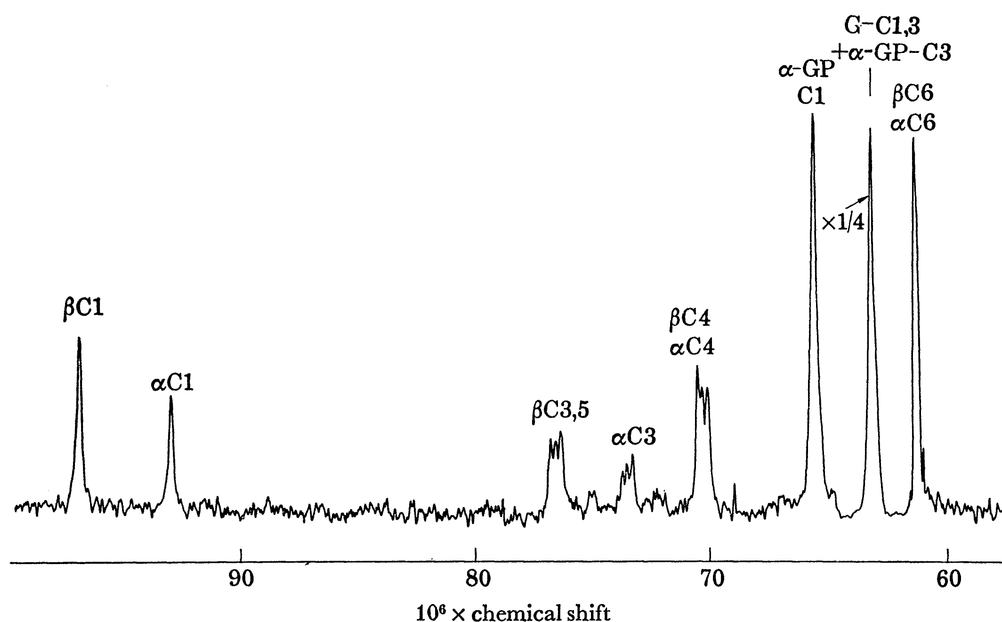

FIGURE 1. ^{13}C n.m.r. spectrum of rat liver cells (at 25 °C) from a normal rat, accumulated between 18 and 35 min after the introduction of 22 mM [1,3-^{13}C]glycerol. Glucose is seen to be labelled strongly at the C1, C3, C4 and C6 positions of the α and β anomers of glucose. Note that the carbon–carbon spin coupling has split the lines from C3 and C4 of glucose. G-C1,3 is the labelled glycerol peak; α-GP is L-glycerol-3-phosphate; the other peaks are from glucose. (Adapted from Shulman *et al.* 1979.)

A typical spectrum of the rat liver cells during gluconeogenesis is shown in figure 1. This ^{13}C n.m.r. spectrum was accumulated between 18 and 35 min after [1,3-^{13}C]glycerol was added as a substrate. In this spectrum one sees n.m.r. peaks from glucose, which is the product as well as from glycerol, the starting material, and from α-glycerophosphate (α-GP) an intermediate. From glycerol there is a peak, labelled G-C1,3, of the 1- and 3-position

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carbon atoms, which had been enriched to 90% in ^{13}C. These carbons give a single peak, since they are identical, and it is superimposed upon the C3 peak of α-GP which, because it is remote from the phosphate, is not shifted from its position in glycerol. One can detect α-GP near to the well resolved α-GP-C1 peak, slightly downfield of the glycerol peaks, where it has been shifted by the attached phosphate group. It is possible to obtain the peak intensity due to the glycerol merely by subtracting the α-GP-C3 contribution from the combined peak of G-C1,3 and α-GP-C3, by assuming that the latter has the same intensity as the α-GP-C1.

In addition to these intensities it is also possible to see resolved glucose peaks and to assign them to particular carbons. The carbons of glucose are observed to be labelled as expected from considerations of the well established pathway from glycerol; namely, one sees intense peaks from the 1-,3-,4- and 6-position carbon atoms and practically nothing from C2 and C5. In addition, by comparing the integrated intensities of the peaks, we have seen that the ratio of C4 to C3 intensities is 1.00 ± 0.03, showing that the triose pool, containing glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, is at equilibrium. The possible absence of equilibrium in this pool has been a subject of intensive study during the past twenty years. While some studies have shown equilibrium, others have not. However, it must be noted that a variety of liver preparations and conditions have been used for these experiments, making comparisons difficult, and we only wish to note here that the n.m.r. measurements of these parameters can be easily made under different conditions.

The 17 min data accumulation shown in figure 1 is one of six spectra taken over a 2 h period. At the end of these experiments the cells showed the same ability to exclude trypan blue dye as at the beginning, and so the data accumulation time could be extended by using more substrate. It has been possible to measure the concentrations of these metabolites as a function of time and to see how they are affected by hormonal treatment. We have studied the effects of treatment with the thyroid hormone triiodothyronine, comparing gluconeogenesis in liver cells from normal and from hyperthyroid rats. We see that, in the latter, the rate of glycerol consumption is increased twofold and the corresponding rate of glucose formation is increased by the same factor. The relative concentrations of α-GP vary somewhat during the experiment, but, on the average, they are two and one half times lower in cells from hyperthyroid rats. We have interpreted these results (Cohen *et al.* 1979*b*) in terms of an increased activity of mitochondrial α-GP dehydrogenase in the hyperthyroid rat cells, an increase which had been noticed during *in vitro* experiments.

N.m.r. spectra of fasted hyperthyroid rats also showed some alanine and lactate, indicating that glycolysis was occurring under conditions favouring gluconeogenesis, whereas the normal rats showed neither product. In the presence of NH_4^+, numerous other metabolites were observed, including glutamate, aspartate and ketone bodies.

It has been possible to obtain equally good ^{13}C n.m.r. spectra from perfused liver (Cohen *et al.* 1979*a*). To fit an intact perfused liver into a 15 mm diameter n.m.r. tube, we have used mouse livers from 24 h fasted mice weighing *ca.* 30 g. The livers were perfused with a Krebs bicarbonate buffer, containing 3% dialysed BSA. The perfusion fluid was recirculated through a stolastic oxygenator, where it was equilibrated to pH 7.4 with 95% O_2, 5% CO_2. Information is obtained about the gluconeogenic pathway from [3-^{13}C]alanine in the spectra shown in figure 2. These results, recorded in collaboration with A. MacLaughlan, have just been published (Cohen *et al.* 1979*a*). The bottom spectrum shows the n.m.r. peaks from the

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natural abundance of ^{13}C in the unlabelled liver. They have been assigned to fatty acids and triglycerides on the basis of their chemical shifts and the high levels of these compounds in mouse livers. The middle spectrum was accumulated between 150 and 180 min after addition of [3-^{13}C]alanine to the perfusate. New peaks from glucose and from several intermediates are observed. The glucose peaks are distributed among all six carbons; table 1 shows the relative amounts of ^{13}C enrichment at the different sites. Note that in this experiment the label has not proceeded simply from the alanine to the product, because in that event the [3-^{13}C] alanine would have labelled only the C1 and C6 of glucose. Although these are the most strongly labelled carbon atoms, note that the C2 and C5 are almost as strongly labelled, while the C3 and C4 are less strongly enriched. Similar results were observed for [3-^{13}C]alanine, used as a substrate for rat liver cell suspensions (Cohen *et al.* 1978*b*). It has been possible to show that the scrambling to the 2- and 5-positions occurs in the perfused mouse liver when

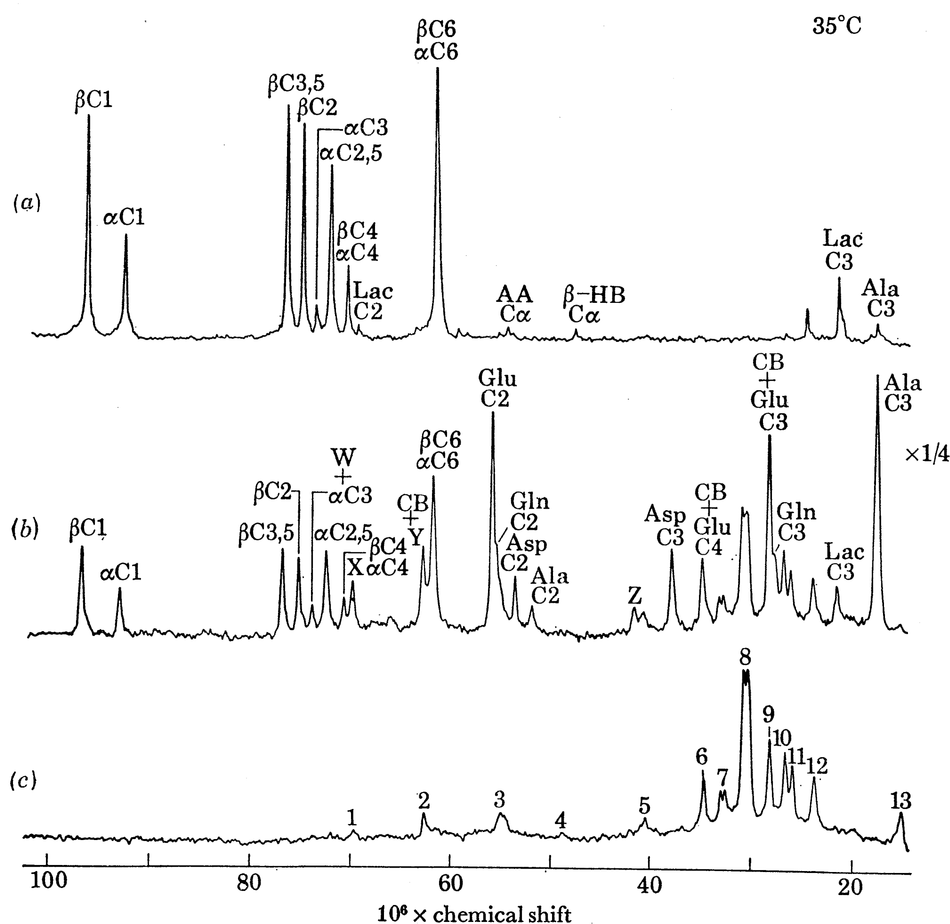

FIGURE 2. ^{13}C n.m.r. spectra taken from a sequence of spectra of a perfused mouse liver at 35 °C. Before the substrate was added, spectrum (*c*), which shows the ^{13}C natural abundance background of this liver, was accumulated. The substrate, 10 mM [3-^{13}C]alanine and 20 mM unlabelled ethanol, was then added at 0 min and again at 120 min, and a series of ^{13}C n.m.r. spectra were taken. Spectrum (*b*) was measured during the period 150–180 min. Spectrum (*c*) is the ^{13}C n.m.r. spectrum of the perfusate and was measured immediately after the perfusion was terminated at 240 min. The pulse repetition rate was 0.5 s for spectra (*b*) and (*c*), and 2 s for spectrum (*a*). (From Cohen *et al.* 1979*a*.) Numbered peaks are from natural abundance ^{13}C in fatty acids and triglycerides; these peaks are labelled CB in (*b*). Peaks W, X, Y and Z have not been identified. Glu, glutamate; Gln, glutamine; Ala, alanine; Asp, aspartate; Lac, lactate; AA, acetoacetate; β-HB, β-hydroxybutyrate. The other assigned peaks are from the α- and β-anomers of glucose.

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oxaloacetate is reversibly converted to fumarate, a path that is consistent with the numerous peaks of intermediates that are assigned in the middle spectrum of figure 2. The details of the pathways and the competition between alanine and ethanol into acetyl CoA and hence into glutamate are discussed by Cohen *et al.* (1979*a*). We show in the top spectrum of figure 2 the ^{13}C spectrum of the perfusate. There, we see that glucose has been moved to the perfusate, but that the other peaks seen in the middle spectrum of figure 2 come from intracellular metabolites. It is clear that ^{13}C n.m.r. is a convenient way to follow the concentrations of these metabolites.

Table 1. Relative ^{13}C intensities at the carbon positions in glucose

	C1	C2	C3	C4	C5	C6
percentage relative to C6	85	70	1.6	14	72	100

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