

## Rapid ATP assays in perfused mouse liver by $^{31}\text{P}$ NMR

(hypoxia/ischemia/insulin)

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**ABSTRACT**  $^{31}\text{P}$  NMR was used to continuously monitor ATP and inorganic phosphate levels in perfused mouse liver. Under "optimal" conditions, the time resolution of the technique was approximately 1 min. In the absence of any metabolic perturbations the ATP level remained constant for at least 2 hr and decreased by only approximately 20% in 18 hr. Both ATP and inorganic phosphate levels responded to alterations in the oxygen supply to the liver. The half-time for this response was approximately 1 min, and the response to short periods of hypoxia or ischemia was partially reversible. The addition of insulin caused only a minor decrease in the ATP level but significantly decreased the rate of response of ATP and phosphate levels to hypoxia and ischemia.

The liver performs a number of important metabolic functions, such as the control of the blood glucose level and the processing of nutrients absorbed from the gastrointestinal tract. Most of these functions appear to be localized within a single type of cell, the parenchymal cell, and virtually all of the reactions of intermediary metabolism occur within this cell (1). Because many of these reactions are linked by a common requirement for either adenine or pyridine nucleotide cofactors, an understanding of the integration of these reactions within the parenchymal cell requires a knowledge of the nucleotide cofactor levels. In particular, it requires a knowledge of the response of the nucleotide cofactors to well-defined metabolic perturbations in the liver.

The measurement of nucleotide cofactors in perfused organs poses considerable problems. One approach has been to "freeze-clamp" the sample and use chemical or enzymatic assays (2). Although this technique is relatively sensitive and may be generally applied to many other metabolites, it produces only a static picture of the nucleotide levels. This difficulty may be circumvented by the use of nondestructive spectroscopic techniques. Optical techniques were introduced many years ago to measure reduced pyridine nucleotide levels in perfused liver (3) and were sensitive enough to follow the kinetics of pyridine nucleotide reduction in response to metabolic perturbations such as anoxia.  $^{31}\text{P}$  NMR spectroscopy has recently been used to measure ATP levels in perfused heart (4, 5) and kidney (6). However, even though recent instrumental advances have increased the sensitivity of the technique (7, 8), the signals from perfused organs have been averaged for over 10 min to obtain an adequate signal-to-noise ratio<sup>§</sup> (4, 6). Because the half-time for the response of the ATP concentration to metabolic perturbations in the liver was expected to be of the order of 1 min (3), it was questionable if the sensitivity of the  $^{31}\text{P}$  NMR technique was adequate to give the time resolution necessary to follow these changes. In order to investigate this point we have used  $^{31}\text{P}$  NMR to study the response of ATP levels to normoxic-anoxic transitions in perfused mouse liver. Preliminary results have been reported (10, 11).

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### MATERIALS AND METHODS

Male (nonfasted) Swiss Webster mice were anesthetized with an intraperitoneal injection of pentobarbital (1.5 mg) and the tissue surrounding the liver was excised. While the perfusate was flowing, a small glass tube at the outlet was inserted into the portal vein. The aorta and the vena cava were immediately cut to prevent a buildup of pressure in the liver. The initial Krebs-Henseleit perfusion medium contained 118 mM sodium chloride, 25 mM sodium bicarbonate, 4.7 mM potassium chloride, 1.2 mM potassium phosphate, 1.9 mM calcium chloride, and 1.2 mM magnesium sulfate. The pH of the solution was 7.4. After visual inspection of the liver to determine the absence of blood, the medium was changed to a Krebs-Henseleit medium identical to that described above except for the addition of 4% bovine serum albumin (Sigma, essentially fatty acid free) and 15% deuterium oxide (Stohler, Waltham, MA).

The liver was placed in a 15-mm NMR tube, which was then sealed with a Teflon plug containing holes for the inlet and outlet tubes. The perfusion fluid was directed into the portal vein and forced out of the NMR tube into the external reservoir, where it was continually bubbled with 95%  $\text{O}_2$ /5%  $\text{CO}_2$ . It was then recirculated into the liver, forming a closed system (see Fig. 1). The total volume of the system was 100 ml.

The NMR tube containing the perfused liver was placed in the magnet of a Bruker WH-360 NMR spectrometer, operating at 145 MHz for phosphorus. The temperature was maintained at  $20^\circ\text{C} \pm 1^\circ\text{C}$ . Because the linewidths of the  $^{31}\text{P}$  NMR signals were of the order of 100 Hz, no proton decoupling was used. A radio-frequency pulse width of 55  $\mu\text{sec}$  was used. The exact precession angle of the spins was not determined. However, the signal-to-noise ratio appeared to approach a limiting value at a pulse width of approximately 100  $\mu\text{sec}$  (the limit for the instrument), so that the precession angle was probably close to  $45^\circ$ .

For the metabolic assays, the freeze-clamped liver was ground to a powder and extracted with 3 vol of 5% perchloric acid. The sample was then neutralized to pH 7.4 with potassium bicarbonate and centrifuged. Assays were performed on the supernatant. Inorganic phosphate was determined by the method of Fiske and Subbarow (12). ATP, ADP, and AMP were quantitatively determined by the stoichiometric oxidation of DPNH; phosphoglycerate phosphokinase and glyceraldehyde phosphate dehydrogenase were used for ATP; pyruvate kinase and lactate dehydrogenase for ADP; and myokinase, pyruvate kinase, and lactate dehydrogenase for AMP. Enzymes were

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§ Freeze-trapping and low-temperature observation of organs by  $^{31}\text{P}$  NMR ["cryo-NMR" (9)] has extended the time range to that of the freezing time (a few seconds). However, "cryo-NMR" suffers the same limitation of other freeze-trapping techniques; i.e., only a single point is obtained.

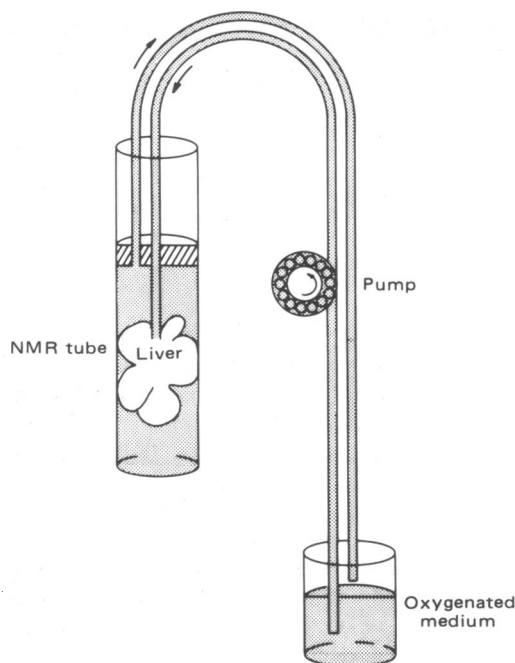


FIG. 1. A schematic illustration of the perfusion system for the  $^{31}\text{P}$  NMR experiments on mouse liver. The perfused liver was placed in a 15-mm diameter NMR tube, which was sealed with a Teflon cap. The perfusion fluid was pumped into the liver from an oxygenated reservoir. After exiting from the liver, it was pumped back into the reservoir where it was reoxygenated. The total volume of the perfusion system was 100 ml. A bubble trap and filter were placed between the pump and the liver.

purchased from Sigma. All other chemicals were analytical reagent grade. Water was purified by passage through "Milli-Q" ion-exchange columns (Millipore).

## RESULTS

$^{31}\text{P}$  NMR spectra of perfused mouse liver are shown in Fig. 2. The three peaks for ATP ( $\alpha$ , 9.4 ppm;  $\beta$ , 18.2 ppm; and  $\gamma$ , 4.6 ppm) and a peak for phosphate (-3.2 ppm) are clearly visible. Superimposed on the  $\alpha$  and  $\gamma$  peaks of ATP is a small amount of intensity due to the  $\alpha$  and  $\beta$  peaks of ADP. From the ratio of the signal due to both ATP and ADP (4.6 ppm) to the signal due to ATP alone (18.2 ppm), the apparent ATP/ADP ratio is calculated to be  $\geq 5$ . This is consistent with the value of 7.5 determined from chemical analyses (see Table 1). From the position of the  $\beta$ -ATP peak it can be inferred that ATP is present as the divalent cation complex, most probably the magnesium form. The position of the phosphate peak is consistent with a cellular pH of  $\approx 7.4$ , the same as that of the perfusion medium. However, because the chemical shifts were measured with respect to an external phosphoric acid standard, they are not reliable enough (due to possible susceptibility differences) to determine the exact intracellular pH. This difficulty would be circumvented by adding an internal standard, such as glycerophosphoryletholine, to the perfusion medium.

The widths of the peaks in a  $^{31}\text{P}$  NMR spectrum of a freshly perfused liver were:  $\gamma$ -ATP, 80 Hz;  $\beta$ -ATP, 115 Hz;  $\alpha$ -ATP, 100 Hz; and  $\text{P}_i$ , 100 Hz. In some experiments the width of all three ATP peaks increased as a function of time, so that after an hour of perfusion the widths of all three peaks had approximately doubled. However, in most experiments the widths of the ATP peaks remained roughly constant over this time period. The ATP level was determined from the integral of the  $\beta$ -ATP peak (18.2 ppm). Because of spectral overlap with the

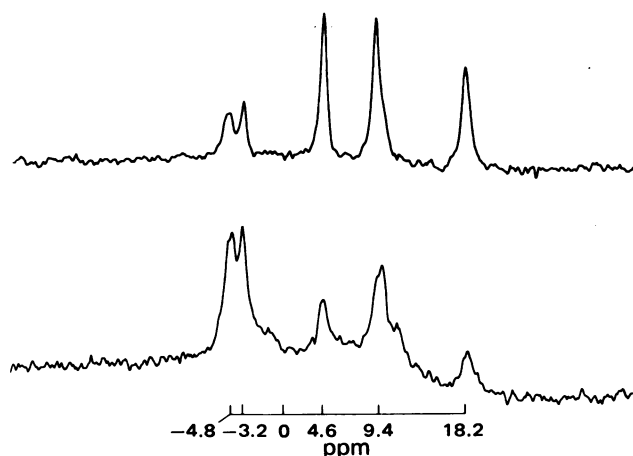


FIG. 2.  $^{31}\text{P}$  NMR spectra from perfused mouse liver. (Upper) Spectrum was accumulated in 6 min with a repetition rate of 30/sec. (Lower) Spectrum was accumulated in 1 hr with a repetition rate of 0.2/sec. The scale at the bottom is in ppm, measured with respect to an external phosphoric acid sample. The assignments are: 18.2 ppm,  $\beta$ -ATP; 9.4 ppm,  $\alpha$ -ATP +  $\alpha$ -ADP; 4.6 ppm,  $\gamma$ -ATP +  $\beta$ -ADP; -3.2 ppm, inorganic phosphate; -4.8 ppm, unassigned (see text). The spectra were run at 20°C and the line-broadening was 20 Hz on each spectrum.

peak at -4.8 ppm, the phosphate level was estimated from the height of the peak at -3.2 ppm.

The peak at -4.8 ppm comes at the expected position for some sugar phosphate monoesters (13) and AMP (14) at this pH. However, the intensity of the peak (roughly equal to the intensity of the phosphate peak under nonsaturating conditions) is inconsistent with the low levels of any single sugar-phosphate monoester or AMP in mouse liver cells (2). This suggests that the peak contains contributions from a number of different sugar-phosphate monoesters and also from AMP.

The upper spectrum in Fig. 2 was taken under conditions that roughly optimize the signal-to-noise ratio of the ATP peaks. The optimum was found by keeping the length of the radio-frequency pulse fixed at 55  $\mu\text{sec}$  and varying the repetition rate. The optimum repetition rate was approximately 30/sec. This implies that the  $T_1$  relaxation times of the ATP peaks are rather short. Assuming that the radio-frequency pulse gives a precession angle of  $\approx 45^\circ$ , the  $T_1$  relaxation times would be of the order of 0.1 sec.

The optimum repetition rate for the ATP peaks is severely saturating for the phosphate peak. This is seen by a comparison of the upper spectrum in Fig. 2 (taken under optimum condi-

Table 1. Freeze-clamp assays of adenylate nucleotides and inorganic phosphate

	ATP, mM	ADP, mM	AMP, mM	$\text{P}_i$ , mM
Perfusion, 10 min	4.4	0.56	0.15	4.5
	3.8	0.52	0.24	6.1
Perfusion, 6 hr	2.7	0.35	0.54	7.8
Ischemia*	0.65	0.46	2.1	14.0
Ischemia*	0.96	0.80	1.8	13.0

The perfusion flow rate was 5.9 ml/min. Samples were quickly frozen at the times indicated (measured from the start of the perfusion with Krebs-Henseleit medium containing bovine serum albumin). The 10-min sample and the ischemia sample were measured on two different livers. All measurements (except  $\text{P}_i$ ) are the average of three determinations. Concentration was calculated as  $\mu\text{mol/g}$  of wet weight.

\* The ischemia samples were frozen 5 min after the onset of ischemia. Ischemia was induced 10 min after the start of perfusion.

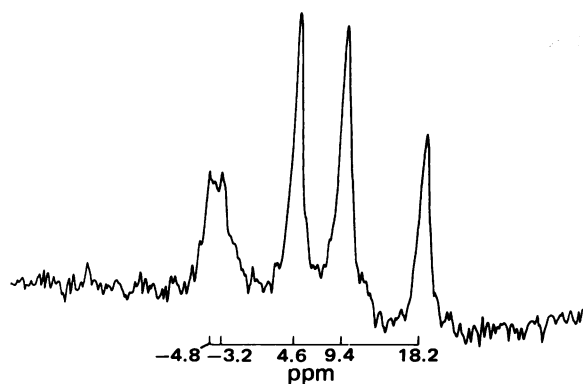


FIG. 3. <sup>31</sup>P NMR spectrum from a perfused mouse liver. The spectrum was accumulated in 75 sec with a pulse repetition rate of 30/sec. A 20-Hz line-broadening was applied to the spectrum. The ppm scale on the bottom was measured relative to an external phosphoric acid sample. The assignments are the same as in the legend for Fig. 2.

tions for the ATP peaks) with the lower spectrum in the same figure (taken under nonsaturating conditions). In the upper spectrum the apparent intensity of the phosphate peak is much less than the intensity of the ATP peaks, whereas the actual phosphate concentration is slightly higher than the ATP concentration (see Table 1). In the lower spectrum, however, the relative intensities of the signals due to ATP and phosphate are consistent with the concentrations of these components determined by freeze-clamp chemical analyses (see Table 1). The broad asymmetric base line is due to the signal from the phospholipids in the liver membranes (15), and the small shoulder on the upfield side of the peak at 9.4 ppm probably arises from DPNH (13).

The nonsaturating conditions require substantially longer accumulation times than the spectra taken under optimum conditions. For example, the upper spectrum in Fig. 2 was accumulated in 6 min, whereas the lower spectrum took an hour to accumulate. Under the optimum conditions, spectra with adequate signal-to-noise may be accumulated in roughly 1 min (Fig. 3). The data in Figs. 5-7 were obtained under the optimum conditions for ATP.

When the flow rate was maintained at 2.9 ml/min, the ATP level remained constant for the first 2 hr of perfusion (Fig. 4).

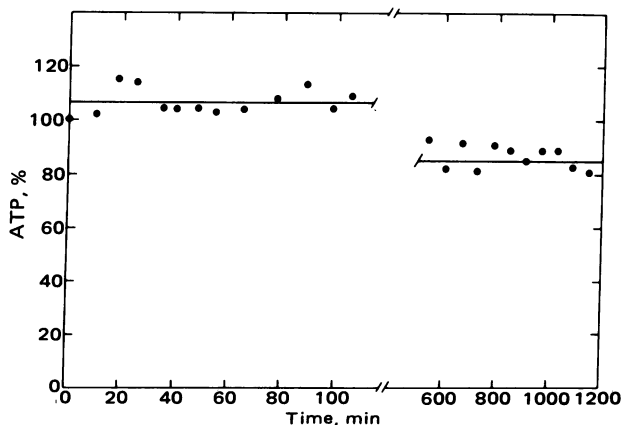


FIG. 4. Time dependence of the ATP level in perfused mouse liver. ATP was determined from the area of the  $\beta$ -ATP peak (18.2 ppm, see Fig. 2) in the <sup>31</sup>P NMR spectra and is expressed as a percentage of the initial value ( $\approx 4.1$  mM, see Table 1). Note that the time scale changes by a factor of 100 after 100 min. The experiment was performed at 20°C.

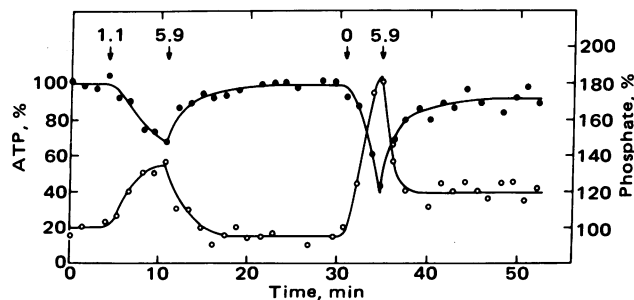


FIG. 5. Time dependence of ATP (●) and phosphate (○) levels in a perfused mouse liver as a function of oxygen supply. The oxygen supply was varied by varying the perfusion rate. The numbers above the curves refer to the perfusion rate (ml/min). ATP and phosphate levels are expressed as percentages of their initial values ( $\approx 4.1$  mM and  $\approx 5.5$  mM, respectively; see Table 1). Other conditions were the same as for Fig. 3.

After 8 hr of perfusion the ATP level had fallen to 80% of its original value, but it then remained constant for at least 10 more hours. The height of the phosphate peak remained constant (to within the experimental error of  $\pm 10\%$ ) during this time period.

The oxygen tension in the perfusion medium was reduced by decreasing the flow rate. Decreasing the flow rate from 5.9 ml/min to 2.9 ml/min had no effect on the ATP or phosphate levels. However, decreasing the flow rate from 5.9 ml/min to 1.1 ml/min did significantly affect both the ATP and phosphate levels, as shown in Fig. 5. After 5 min at the reduced flow rate the ATP level had fallen by  $\approx 30\%$  and the phosphate level had risen by  $\approx 40\%$ . After the flow rate was returned to its original value (5.9 ml/min), the ATP and phosphate levels returned almost to their original values in about 5 min and then remained constant. The half-time for both of these changes was approximately 1 min.

The oxygen tension was further reduced by stopping the perfusion flow (i.e., ischemia). After a lag period of about 1 min, the ATP level began to fall rapidly and the phosphate level began to rise rapidly. The initial rate of change of these two components in response to ischemia was approximately 3 times the initial rate found in response to a reduction of the flow rate to 1.1 ml/min. After 5 min of ischemia, the ATP level had fallen almost to zero while the phosphate level had doubled (Fig. 6). When the ATP level had fallen to zero, some ADP ( $\approx 20\%$  of the original ATP level) still remained. The ADP then decreased to zero with a half-time of roughly 2 min. If the flow rate was increased to its original value (5.9 ml/min) after only 3 min of ischemia, the ATP recovered almost back to its original value

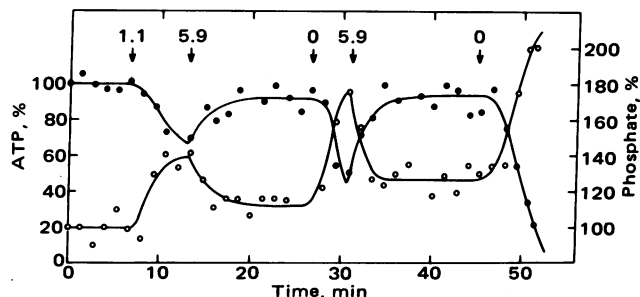


FIG. 6. Time dependence of ATP (●) and phosphate (○) levels in a perfused mouse liver as a function of oxygen supply. The ATP and phosphate levels are expressed as percentages of their initial values ( $\approx 4.1$  mM and  $\approx 5.5$  mM, respectively; see Table 1). Other details are given in the legend for Fig. 5.

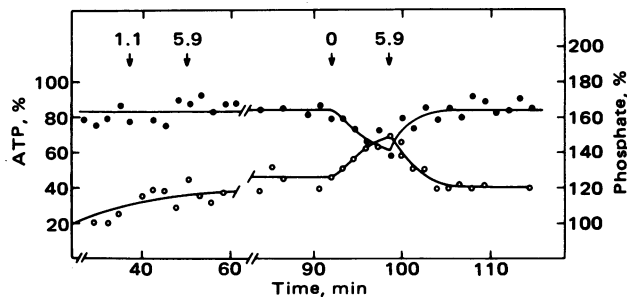


FIG. 7. Time dependence of ATP (●) and phosphate (○) levels in perfused mouse liver in the presence of glucose (2 mg/ml) and insulin (25 microunits/ml). The oxygen supply was varied by varying the perfusion rate. Glucose was added at 10 min and insulin was added at 20 min. The numbers above the curves refer to the perfusion rate (ml/min). The ATP and phosphate levels are expressed as percentages of their initial values ( $\approx 4.1$  mM and  $\approx 5.5$  mM, respectively; see Table 1). Other conditions were the same as for Fig. 5.

and the phosphate level fell to within 20% of its previous value (Figs. 5 and 6). Both of these recoveries took place within 5 min, with half-times of approximately 1 min.

In the absence of insulin the addition of glucose (2 mg/ml) had no effect on either the ATP or the phosphate level. The addition of insulin (25 microunits/ml) in the presence of glucose (2 mg/ml) caused an abrupt decrease of the ATP level by  $\approx 15\%$  and an abrupt increase in the peak at  $-4.8$  ppm (sugar phosphate) by  $\approx 20\%$ . Although the phosphate level did not immediately change upon the addition of insulin, it slowly increased over a period of about 40 min to a level approximately 25% above its original value.

When the flow rate was decreased from 5.9 ml/min to 1.1 ml/min in the presence of insulin and glucose, neither the ATP nor the phosphate level was significantly altered (Fig. 7). When the flow was completely stopped (ischemia), the ATP and phosphate levels changed only slowly. The rate of change of the ATP and the phosphate levels in response to ischemia was approximately a factor of 3 slower than in the absence of insulin. After 5 min of ischemia the ATP level fell to about 75% of its previous level, whereas in the absence of insulin it had fallen to about 20% (see Fig. 6). Also, after 5 min of ischemia the phosphate level rose by only about 25%, whereas in the absence of insulin it had doubled (see Fig. 6). When the flow rate was returned to 5.9 ml/min, the ATP and phosphate levels returned to their previous values with half-times of approximately 2 min.

## DISCUSSION

Nondestructive spectroscopic techniques have the potential to follow continuously the response of nucleotide cofactors to perturbations in the metabolic state of a perfused liver. The time resolution of these techniques is determined by the signal-to-noise ratio of the corresponding spectra. Whereas optical methods for the determination of pyridine nucleotides have an adequate signal-to-noise ratio (3), the  $^{31}\text{P}$  NMR method for the determination of adenine nucleotides does not, and signal averaging must be used.

In pulsed NMR experiments the transient radio-frequency response of the sample to a short radio-frequency pulse (i.e., the radio-frequency "fluorescence") is averaged in a computer. The Fourier transform is performed on the averaged signal to obtain the usual NMR spectrum (16). There are essentially two different ways in which transients may be averaged. The first is to accumulate transients as rapidly as possible in order to obtain the optimum signal-to-noise ratio. The maximum rate at which

transients may be accumulated is determined by the  $T_1$  relaxation time<sup>†</sup> of the phosphorus nuclei. The optimum signal-to-noise ratio is approached when the delay time between transients is approximately one-third of the  $T_1$  relaxation time. Because different phosphorylated metabolites generally have different  $T_1$  values, the signal-to-noise ratio cannot be simultaneously optimized for all the signals in the spectrum. If the conditions are optimized for one component, the relative intensity of the signals for the other components will depend on their respective  $T_1$  relaxation times. Under these conditions, the relative concentrations of phosphorylated metabolites cannot be directly determined from the relative intensities of the corresponding signals in the  $^{31}\text{P}$  NMR spectrum.<sup>‡</sup>

The second way to accumulate transients is under nonsaturating conditions, which require that the delay time between transients is at least 3 times longer than the longest  $T_1$  relaxation time in the sample. Under these conditions the relative intensity of signals from different components is not affected by differences in the  $T_1$  values of these components. However, the signal-to-noise ratio is substantially lower than that obtained under "optimum" conditions.

In most  $^{31}\text{P}$  NMR studies of metabolism in perfused organs it has been considered important to use nonsaturating conditions (4, 6). Long delays (of the order of a few seconds) were used between transients, and at least 10 min or more of averaging was required to obtain an adequate signal-to-noise ratio on the ATP signal. When  $^{31}\text{P}$  NMR spectra of perfused mouse liver are accumulated under nonsaturating conditions, a total accumulation time of approximately 30 min is required to obtain an adequate signal-to-noise ratio for ATP signal (lower spectrum of Fig. 2). Because the half-time for the response of adenine nucleotides to metabolic perturbations in the liver is expected to be of the order of 1 min (3), the signal-to-noise ratio of spectra taken under nonsaturating conditions is clearly not adequate to follow this response. However, if spectra are taken under roughly optimum conditions for the ATP signals (upper spectrum in Fig. 2) the signal-to-noise ratio on the ATP signal is significantly increased.

By use of the "optimum" conditions for ATP, spectra with adequate signal-to-noise ratio may be accumulated in approximately 1 min (see Fig. 3). That this is fast enough to follow the time course of ATP and phosphate levels in response to metabolic perturbations such as a reduction in oxygen supply or ischemia is shown in Figs. 5 and 6. Because we are primarily concerned with the kinetic response of each component, the partial saturation of the signals is not a problem. In the data presented here these components are given as percentages of their initial levels. These could be converted into concentrations by using initial concentrations determined from the freeze-clamp experiments (see Table 1), although this would not affect the analysis of the data.

When the oxygen supply is reduced by decreasing the perfusion rate, both the ATP and phosphate levels respond with a half-time of approximately 1 min. The pyridine nucleotide change has been found to have a half-time of 60 sec at  $37^\circ\text{C}$  (see figures 3 and 8 of ref. 3). Thus the  $^{31}\text{P}$  NMR has an adequate response time to follow the kinetics of the metabolic changes in the perfused liver at  $20^\circ\text{C}$ . If the liver is made ischemic, the

<sup>†</sup>  $T_1$  is the exponential time constant for the relaxation of the component of the magnetic moment that is parallel to the magnetic field.

<sup>‡</sup> If the  $T_1$  relaxation times of all the components were accurately known, the relative intensities of the signals could be corrected for this effect. However, in these experiments it is difficult to measure  $T_1$  values precisely. Other factors (i.e., radio-frequency inhomogeneity) also complicate this correction.

initial rate of change of the ATP and phosphate levels is roughly 3 times faster than when the oxygen supply is reduced. The changes observed after 5 min of ischemia are consistent with the freeze-clamp assays under similar conditions (see Table 1). If the flow rate is increased to its normal value when the ATP level has fallen to one-half of its initial level (after 3 min of ischemia), the changes in ATP and phosphate levels are approximately reversible. The half-time for the recovery from this short period of ischemia is again of the order of 1 min.

A comparison of the data in Figs. 5 and 6 shows that the response of different livers to reduction in the oxygen tension and to ischemia is similar, but not identical. The improved signal-to-noise ratio in the data in Fig. 5, as compared to the data in Fig. 6, may be ascribed to the slightly larger liver used in that experiment.

The  $T_1$  relaxation time of the ATP phosphate groups is of the order of 100 msec, which is an order of magnitude shorter than the  $T_1$  relaxation time of free ATP in solution. The reason for this may be the relatively high concentration of paramagnetic cations (such as manganese, nickel, and cobalt) in the liver (17). If so, the effect of these paramagnetic cations is beneficial; the reduced  $T_1$  relaxation time allows the more rapid accumulation of data, increasing the time resolution of the technique.

The usefulness of the  $^{31}\text{P}$  NMR technique in investigating alterations in the metabolic response of the liver is illustrated in Fig. 7. The presence of insulin and glucose presumably activates glycolysis, which decreases the rate of response of ATP and phosphate levels to ischemia to at least one-third. Also, the activation of glycolysis maintains ATP and phosphate levels constant, in spite of the reduction in oxygen tension produced by a decreased flow rate (1.1 ml/min). Because the insulin increases the rate of glycogenesis (18), the increased glycolytic flux may simply be due to an increase in the amount of glycogen in the liver.

The long-term stability of adenine nucleotide levels in the perfused liver (see Fig. 4) is important because the reasonable degree of reversibility in the changes of the ATP and phosphate levels in the liver, coupled with the nondestructive nature of the  $^{31}\text{P}$  NMR technique, allows a number of experiments (and their respective controls) to be done on the same liver. One factor in this long-term stability may be the hydrodynamic details of the perfusion system (see Fig. 1). The perfusion fluid is pumped into the liver via the portal vein and is allowed to escape from the liver into an open reservoir placed roughly 30 cm below the liver. The resulting negative pressure between the outflow and inflow of the perfusion system thus mimics the "in vivo" pressure differential between the vena cava and the portal vein in the blood circulation through the liver (19).

Apart from the advantage of long-term stability, the perfused liver has another important advantage over suspensions of isolated liver cells (20) for  $^{31}\text{P}$  NMR determinations of adenine nucleotides. In the cellular suspensions it is difficult to simultaneously satisfy the need for adequate signal-to-noise ratio (which requires concentrated suspensions) and adequate oxygenation (which requires dilute suspensions, especially at physiological temperatures). In the perfused liver, however,

the solid matrix of intact cells provides the maximum  $^{31}\text{P}$  NMR sensitivity, with adequate oxygenation being provided through the sinusoidal capillary bed of the intact organ (21, 22). The perfused liver thus combines the physiological advantage of an intact organ with the practical advantages of good  $^{31}\text{P}$  NMR sensitivity for adenine nucleotides, adequate oxygenation, and long-term stability.

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