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Activity of phosphorylase in total global ischaemia in the rat heart

A phosphorus-31 nuclear-magnetic-resonance study

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1. The uptake and subsequent phosphorylation of deoxyglucose into perfused rat hearts was monitored by ^{31}P n.m.r. 2. The accumulated deoxyglucose 6-phosphate provided (*a*) an independent method for measuring cytosolic pH in the normoxic and ischaemic heart tissue and (*b*) a way of studying the activity of phosphorylase during ischaemia. 3. The cytosolic pH measured from the ^{31}P n.m.r. resonance position of deoxyglucose 6-phosphate is in good agreement under all conditions studied with that obtained previously from the P_i resonances. This eliminates any possible doubts about the use of P_i for measuring intracellular pH. 4. Deoxyglucose 6-phosphate *in vitro* inhibits phosphorylase *b* but not phosphorylase *a*. Its inhibitory effect on glycogenolysis during ischaemia is monitored by measuring tissue acidosis by n.m.r. In the initial stages of ischaemia phosphorylase activity is not inhibited, whereas after about 5 min approx. 50% of the activity is inhibited. These observations are interpreted in terms of the relative contributions of phosphorylase *a* and the AMP-dependent phosphorylase *b* activities during ischaemia.

^{31}P n.m.r. has been used to determine the normoxic intracellular pH of rat hearts perfused *in vitro* (Garlick *et al.*, 1977, 1979; Hollis *et al.*, 1977) and *in vivo* (Grove *et al.*, 1980). So far the pH has been calculated from the position of the intracellular P_i resonance in the spectrum relative to that of phosphocreatine (Garlick *et al.*, 1979). Poole-Wilson (1978) and Gillies & Deamer (1979) have drawn attention to two possible drawbacks of using this P_i resonance. The first was that the sequestering of P_i in mitochondria may mean that the intramitochondrial pH is measured selectively. The second, that membrane-permeability changes in ischaemia may release some of this P_i to give a composite value for mitochondrial, cytoplasmic and interstitial pH.

A number of workers have also studied the onset of acidosis in ischaemia by using this technique (Garlick *et al.*, 1979; Hollis *et al.*, 1977; Salhany *et al.*, 1979). Garlick *et al.* (1979) have shown that there is a direct correlation between the glycogen content of perfused rat hearts and H^+ production in ischaemia. They suggest that the major source of H^+ in ischaemia is the hydrolysis of the ATP produced by anaerobic breakdown of glycogen.

The importance of glycogen in the protection of the anoxic myocardium is well known (Scheurer & Stezoski, 1970). In both anoxia and ischaemia the conversion into the *a* form of phosphorylase *b* is stimulated (Cornblath *et al.*, 1963; Wollenberger *et al.*, 1969). Up to 50% of the enzyme is converted into the more active *a* form in 1 min of ischaemia in the hearts of open-chested dogs (Wollenberger *et al.*, 1969). In the anoxic myocardium, the proportional conversion is insufficient to account for the observed amount of glycogen degradation (Parmeggiani & Morgan, 1962; Cornblath *et al.*, 1963; Morgan & Parmeggiani, 1964). It has been assumed that the rise in the tissue concentrations of AMP and P_i is sufficient partially to activate phosphorylase *b* and so increase the rate of glycogenolysis. However, there is a concomitant increase in the concentration of two potent inhibitors of phosphorylase *b*, namely ADP and glucose 6-phosphate, and a decrease in the concentration of ATP, a less potent inhibitor. The activation state of the enzyme in either the anoxic or the ischaemic myocardium is therefore difficult to predict. Its activity in total global ischaemia has not yet been measured *in vivo* or in the perfused heart.

It has long been known that 2-deoxyglucose enters muscle tissue, is phosphorylated and accumulates in the cytoplasm (Kipnis & Cori, 1959). The use of 2-deoxyglucose 6-phosphate to measure pH by ^{31}P n.m.r. should therefore obviate the problems

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raised in using the P_i resonance. Here we report our examination of its use as a pH marker of the myocardial cytoplasm in normoxia and in total global ischaemia. We also describe its effect on isolated enzymes of glycogen metabolism and on the rate of onset and extent of acidosis in ischaemia. These have enabled us to assess the contribution of phosphorylases *a* and *b* to glycogen degradation during total global ischaemia in the isolated perfused rat heart.

Methods

Hearts from 280–320g male Wistar rats (O.L.A.C. Ltd., Shaws Farm, Blackthorn, Bicester, Oxon) were excised and perfused in the Langdendorff mode as described by Garlick *et al.* (1979). The buffer used was Krebs–Henseleit (1932) bicarbonate buffer containing 0.5mM-calcium EDTA and 5mM-D-glucose. In phosphate-free perfusions KH_2PO_4 was replaced by an equivalent concentration of KCl. The use of phosphate-free buffer makes it easier to differentiate in the n.m.r. spectrum between intracellular and any interstitial P_i. Hearts were perfused for 30min without the recirculation of their coronary effluent, followed by 2h of perfusion with a recirculating 100ml of buffer. 2-Deoxy-D-glucose (Sigma) was added to this recirculation buffer (where indicated) to give a final concentration of 1mM. The pump of the recirculation circuit (Gilson Minipuls 2) was adjusted to maintain the coronary flow rate measured after 25min of the initial flow-through perfusion. The pressure trace obtained from a Bell and Howell type 4-327-I transducer, linked to a Lectromed 3552 amplifier and thermal recorder and sampling from a side arm in the aortic perfusion line was monitored throughout the experiment. Under these conditions, hearts maintain excellent viability, as indicated by measurements of their aortic pressure. At the end of the 2h period, the recirculation pump was switched off and spectra were obtained at 4min intervals for a 15min period.

^{31}P n.m.r. spectra were obtained at 73.84MHz by using a 4.3T superconducting magnet (Oxford Instruments Ltd.) interfaced with a Nicolet BNC-12 computer as previously described (Garlick *et al.*, 1979). 45° pulses were applied at a 2s repetition rate throughout.

The standard curves for the variation with pH of the chemical shifts of P_i and 2-deoxyglucose 6-phosphate were obtained by using an aqueous solution of the following: 20mM-phosphocreatine (Sigma), 20mM-2-deoxyglucose 6-phosphate (Sigma), 20mM-KH_2PO_4, 150mM-KCl, 10mM-$MgCl_2$ and 1mM-disodium EDTA. The pH of this solution was adjusted to 6.0, as measured on a Findip 555A pH-meter and combination glass electrode, by the addition of HCl, and its ^{31}P n.m.r. spectrum was recorded at 37°C. The pH was then increased incrementally by the addition of KOH, and a series of spectra were obtained. The chemical shifts for P_i and 2-deoxyglucose 6-phosphate were calculated relative to the phosphocreatine internal standard exactly as described for perfused hearts (Garlick *et al.*, 1979).

The purified *b* form of glycogen phosphorylase (EC 2.4.1.1) was prepared by the method of Fischer & Krebs (1962), dithiothreitol being substituted for cysteine. The effect of 2-deoxyglucose 6-phosphate on the activity of the enzyme was measured in the presence of 70μM-AMP by using the procedure described by Birkett *et al.* (1971).

Phosphorylase kinase (EC 2.7.1.38) was assayed by using a Technicon autoanalyser designed to measure continuously phosphorylase *a* activity in a solution containing purified phosphorylase kinase, 5mM-magnesium ATP and 250μM-phosphorylase *b* at pH8.2 and at 30°C. The solution was continuously drawn off from the sample chamber and mixed with 10mM-EDTA at pH7.0 to quench the phosphorylase kinase reaction. The solution was then mixed with phosphorylase *a* substrate (1% glycogen + 16mM-glucose 1-phosphate at pH7.0) in an incubation coil thermostatically controlled at 30°C. The P_i produced by the phosphorylase reaction was assayed colorimetrically by the procedure described by Hurst (1964), modified for use with the Technicon autoanalyser (Birkett *et al.*, 1971).

Tissue samples were prepared for glycogen determination by freeze-clamping hearts perfused for 2h in the presence and absence of 1mM-2-deoxyglucose. The samples were extracted in KOH and ethanol (Hammermeister *et al.*, 1964) and their glycogen content measured by the phenol/H_2SO_4 method (Montgomery, 1957).

Values are quoted as means ± S.D. for the number of observations given throughout the present paper.

The errors we quote for our pH determination are statistical errors, representing the precision with which we measure pH, rather than representing absolute values for intracellular pH. All our measurements rely on a standard calibration performed on dilute aqueous solutions and so are not entirely free from systematic errors.

Results

Normoxic phosphorylation

The uptake and phosphorylation of 2-deoxyglucose was monitored over a 2h period by ^{31}P n.m.r. (Fig. 1). The 2-deoxyglucose 6-phosphate accumulated gives a single, sharp resonance ($\nu_{\frac{1}{2}}$ is between 25 and 30Hz when the line broadening is 8Hz) at a convenient part of the spectrum ~3p.p.m. downfield of the P_i resonance.

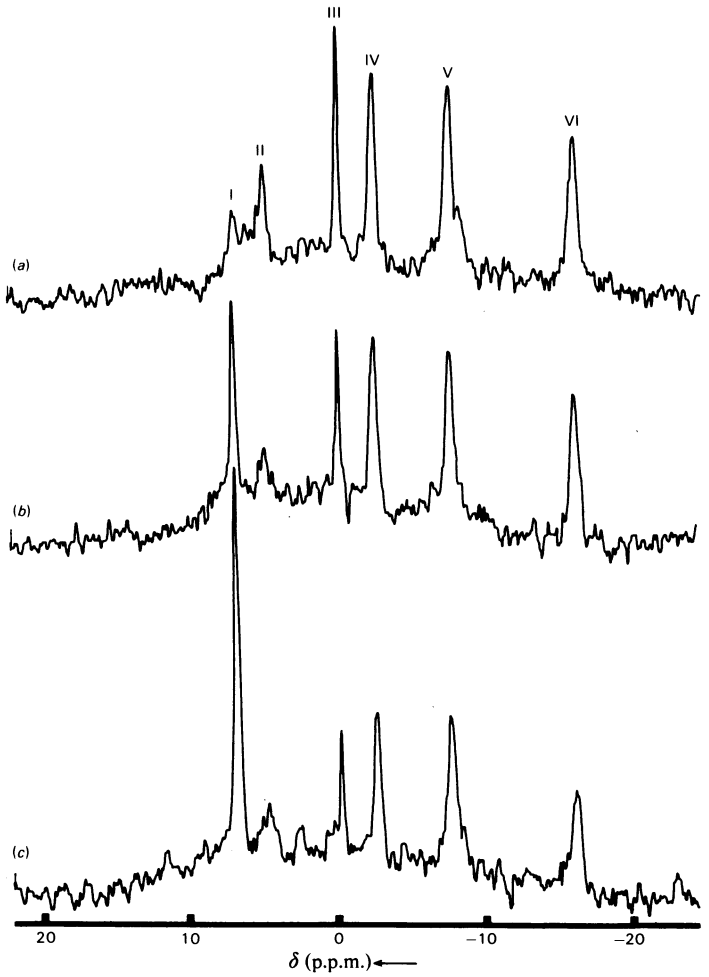

Fig. 1. *^{31}P n.m.r. spectrum of perfused rat heart in the presence of 2-deoxyglucose*
Each spectrum represents the accumulation of 300 transients at 2 s intervals after 15 min (*a*), 50 min (*b*) and 102 min (*c*) of recirculation perfusion with phosphate-free buffer containing 1 mM-2-deoxyglucose and 5 mM-D-glucose. Assignments: I, 2-deoxyglucose 6-phosphate; II, P_i; III, phosphocreatine; IV, V and VI, MgATP. Chemical shifts (δ) are expressed relative to phosphocreatine.

The effects of perfusion with 2-deoxyglucose in normal and phosphate-free buffer on the concentration of ATP, phosphocreatine and intracellular phosphate were as follows: in the absence of buffer phosphate, the concentration of ATP was maintained at the expense of both phosphocreatine and P_i. In the presence of buffer phosphate, both ATP and phosphocreatine were maintained, whereas the concentration of P_i again fell. The magnitude of the decline in phosphocreatine is shown in Fig. 2. These effects do not appear to alter the aortic pressure measurement of heart viability. The variation with time of the intracellular concentration of 2-deoxyglucose 6-phosphate is shown in Fig. 3. The time course is essentially linear over the 90 min of perfusion.

Measurement of cytosolic pH

Fig. 4 shows the variation with pH of the chemical shifts of P_i and 2-deoxyglucose 6-phosphate in solution. From these data the pK_a values and chemical shifts of the acidic and basic forms of the two compounds were fitted to the equation:

$$\text{pH} = \text{p}K_a - \log\left(\frac{\text{chemical shift} - x}{y - \text{chemical shift}}\right)$$

where x is the chemical shift for free base, and y is

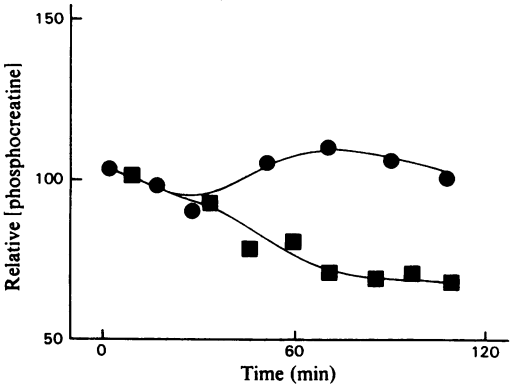

Fig. 2. *Effect of buffer phosphate on the concentration of phosphocreatine during normoxic perfusion with 2-deoxyglucose*

The variation in the concentration of phosphocreatine in the presence (●) and absence (■) of buffer phosphate is illustrated. Data from one representative heart is shown in each of the two cases. Spectra were obtained from the accumulation of 300 transients, and individual data points represent the heights of the appropriate peaks from these spectra.

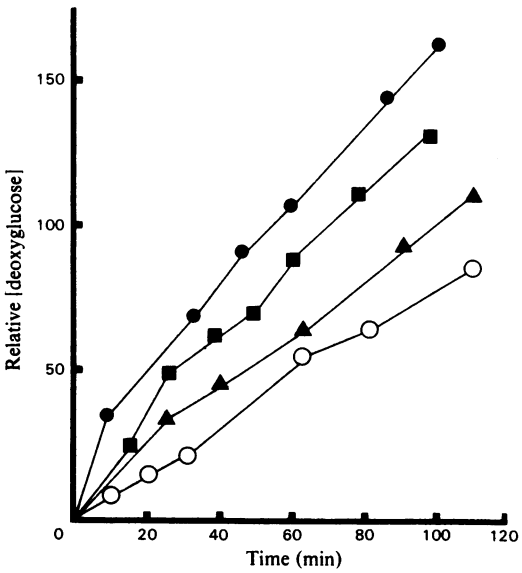

Fig. 3. *Normoxic accumulation of 2-deoxyglucose 6-phosphate*

The variation with time of the intracellular concentration of 2-deoxyglucose 6-phosphate of four hearts perfused in the absence of buffer phosphate is shown. The individual data points represent the heights of peaks taken from ^{31}P n.m.r. spectra obtained after the accumulation of 300 transients.

the chemical shift for free acid. The best fit to the data was obtained with pK_a values of 6.72 and 6.16 for P_i and 2-deoxyglucose 6-phosphate respectively.

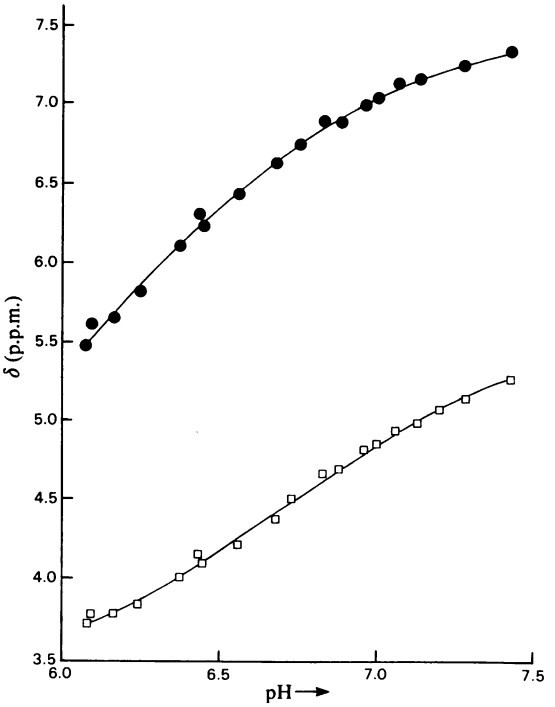

Fig. 4. *Variation of the chemical shifts of P_i (□) and 2-deoxyglucose 6-phosphate (●) with pH*

A solution containing 20mM-P_i, 20mM-2-deoxyglucose 6-phosphate, 20mM-phosphocreatine, 10mM-$MgCl_2$ and 150mM-KCl was titrated at 37°C by addition of HCl and KOH. Chemical shifts are expressed relative to the phosphocreatine. The lines are fitted to the equations in the text by using the following parameters: for P_i, $pK_a = 6.72$, chemical shift of acidic form, 3.27; of the basic form = 5.69; for 2-deoxyglucose 6-phosphate, $pK_a = 6.16$, chemical shift of acidic form, 3.80; of the basic form, 7.52.

The internal pH can be calculated from the chemical shifts of P_i or deoxyglucose 6-phosphate by using these calibration curves.

After 2h of recirculating perfusion in the presence of deoxyglucose, the pH values measured from the P_i and 2-deoxyglucose 6-phosphate resonances were both 7.01 ± 0.04 (six hearts). After a similar period of perfusion in the absence of deoxyglucose, the pH measured from the P_i resonance was 7.06 ± 0.01 (4), a significant increase of 0.05 pH units ($0.01 < P < 0.02$). The extra H^+ in the former situation probably arise from the phosphorylation of the intracellular deoxyglucose:

$$\text{Deoxyglucose} + MgATP^{2-} \rightarrow$$
$$\text{2-deoxyglucose 6-phosphate}^{2-} + MgADP^- + H^+$$

If it is assumed that the buffering capacity of the heart is 35 μequiv./pH unit (Garlick *et al.*, 1979) and that the adenine nucleotides are effectively entirely bound to Mg^{2+}, and allowing for the incomplete

dissociation of the acidic form of 2-deoxyglucose 6-phosphate at neutral pH, it can be shown that the phosphorylation of only 2.5μmol of deoxyglucose·(g wet wt.)$^{-1}$ would be required to produce the observed pH change. Although the resonances are partially saturated under the conditions of spectral accumulation (i.e. 2s repetition rate), we can estimate, by comparing the ATP and 2-deoxyglucose 6-phosphate resonances, that about 4μmol of 2-deoxyglucose 6-phosphate·(g wet wt.)$^{-1}$ has been accumulated after 2h.

Table 1 shows the effect of various lengths of total global ischaemia on the myocardial pH after incubation for 2h with deoxyglucose. The pH decreases to 6.4 by the end of the ischaemic period as measured by both the P_i and 2-deoxyglucose 6-phosphate resonances. There is never any significant difference between the pH values obtained from the two resonances at any point during the ischaemic period.

Effects on glycogen metabolism

After 2h of recirculation perfusion in the presence and absence of 2-deoxyglucose, hearts were subjected to total global ischaemia while a series of spectra were taken at 4min intervals. The changes in their intracellular pH and ATP content during this period are shown in Fig. 5. During ischaemia, the pH of these hearts decreases more slowly than that of control hearts, reaching a value of 6.40 ± 0.09 (five hearts) after 15min compared with 6.13 ± 0.12 (four hearts) in the absence of deoxyglucose. Perfusion with deoxyglucose also increases the rate at which ATP depletion occurs in ischaemia.

The amount of glycogen broken down in ischaemia is directly proportional to the amount of ATP produced by anaerobic glycolysis. Garlick *et al.* (1979) have shown that the amount of H^+ production in total global ischaemia is also proportional to the amount of glycolytic ATP production. The extent of ischaemic acidosis is thus an indication of the amount of glycogen breakdown. These data therefore show that there is less glycogen breakdown in the presence of deoxyglucose than in its absence.

The total glycogen content of a second series of hearts perfused with and without deoxyglucose was determined after freeze-clamping. The glycogen concentration was found to be 1.0 ± 0.4mg·(g wet wt.)$^{-1}$ (four hearts) in the absence of deoxyglucose

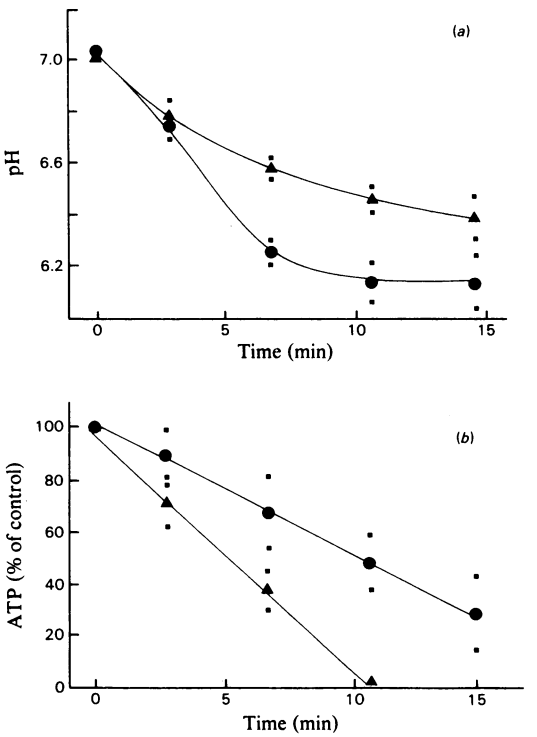

Fig. 5. *The fall in pH (a) and ATP (b) during total global ischaemia*

^{31}P n.m.r. spectra were taken every 4min during total global ischaemia by accumulating 100 scans. The ATP is expressed as the ratio of the β-ATP signal to that obtained before total global ischaemia. The pH is calculated from the chemical shift of the P_i resonance relative to phosphocreatine, by using the calibration curve of Fig. 4. Symbols: ▲, results (mean ± S.D.) for five hearts preperfused with deoxyglucose; ●, for four hearts preperfused without deoxyglucose (control).

Table 1. *Comparison of the decrease in pH in total global ischaemia measured by the P_i and the 2-deoxyglucose 6-phosphate resonances*

After 1min of total global ischaemia, n.m.r. spectra were collected every 4min. A total of 100 transients at a 2s repetition rate were accumulated in each spectrum. The pH values were calculated using the calibration curves of Fig. 4. The values quoted are means ± S.D. for six hearts in each case.

			pH			
Method	Normoxic	Mean time after cessation of flow (min) ...	2.6	6.6	10.6	14.6
P_i	7.01 ± 0.04		6.79 ± 0.08	6.59 ± 0.05	6.47 ± 0.05	6.40 ± 0.09
Deoxyglucose phosphate	7.01 ± 0.04		6.82 ± 0.04	6.59 ± 0.05	6.44 ± 0.04	6.37 ± 0.08

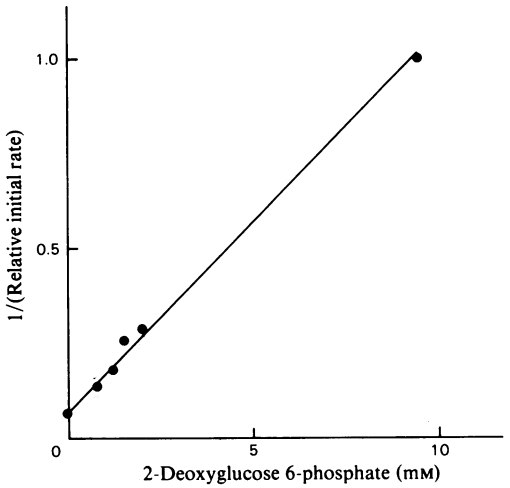

Fig. 6. *Dixon plot of the inhibition of AMP-induced activity of phosphorylase b by 2-deoxyglucose 6-phosphate*

The activity of phosphorylase *b* in the presence of 70 μM-AMP was assayed by the method of Birkett *et al.* (1971) (see the Methods section), and was titrated by addition of 2-deoxyglucose 6-phosphate.

Table 2. *Effect of 2-deoxyglucose 6-phosphate on the activity of phosphorylase kinase*

The proportional conversion of phosphorylase *b* into phosphorylase *a* in 1 min was assayed by continuously monitoring the phosphorylase *a* activity produced in a solution containing 250 μM-phosphorylase *b*, 5 mM-MgATP and purified phosphorylase kinase at pH 8.2 and 30°C.

[2-Deoxyglucose 6-phosphate] (mM)	Relative amount of phosphorylase *a*
0	1.0
6	0.9
12	1.1
18	0.9
24	0.8

and 4.3 ± 0.6 mg·(g wet wt.)$^{-1}$ (nine hearts) in its presence, a significant increase ($P < 0.005$). As the S.D. of three measurements on each heart was relatively high (sometimes 30% of the mean), the mean values for each heart were ranked and a non-parametric statistical test applied. This produced a probability of between 0.002 and 0.001 that deoxyglucose had no effect.

The results show that hearts perfused in the presence of deoxyglucose store more glycogen than normal, but that less is available for use in total global ischaemia. We therefore examined the effects of 2-deoxyglucose 6-phosphate on certain isolated enzymes of glycogen metabolism.

Fig. 6 is a Dixon plot of the effect of 2-deoxyglucose 6-phosphate on phosphorylase *b*. The results show an inhibition of the AMP-induced activity by the deoxy sugar at a saturating concentration of glucose 1-phosphate. The K_i for 2-deoxyglucose 6-phosphate is 0.45 ± 0.1 mM, which compares with a K_d value for glucose 6-phosphate of 0.03 mM (Griffiths *et al.*, 1976).

Table 2 shows the relative amount of conversion of phosphorylase *b* into the *a* form in the first minute of incubation with various concentrations of 2-deoxyglucose 6-phosphate. The proportion of phosphorylase *a* appears to be independent of the concentration of the sugar phosphate at the concentrations used.

Phosphorylase *a* activity is not affected by 2-deoxyglucose 6-phosphate.

Discussion

Normoxic phosphorylations

The effects of 2-deoxyglucose and buffer phosphate on the intracellular concentrations of P_i, phosphocreatine and ATP can be explained with reference to the creatine kinase and hexokinase reactions and to oxidative phosphorylation. The phosphorylation of deoxyglucose produces ADP, which is rephosphorylated by the mitochondria at the expense of intracellular P_i. When perfused in phosphate-free buffer, the intracellular P_i cannot be replaced from the external pool and so the ATP concentration is maintained at the expense of phosphocreatine by the creatine kinase equilibrium. In the presence of buffer phosphate, the falling intracellular P_i concentration can be replenished by the entry of P_i and the ATP concentration maintained by oxidative phosphorylation.

The rate of phosphorylation by hexokinase can only be equated to the rate of sugar transport if there is no accumulation of free sugar inside the cell. ^{31}P n.m.r. is convenient in that it measures only the accumulated 2-deoxyglucose 6-phosphate, unlike methods involving radioactivity, which require complex separations to differentiate between the accumulated 2-deoxyglucose 6-phosphate and 2-deoxyglucose (Graff *et al.*, 1978). The hexokinase reaction is modulated by changes in the intracellular concentrations of ATP, ADP, glucose 6-phosphate, free glucose and deoxyglucose (Colowick, 1973), but not by 2-deoxyglucose 6-phosphate (Crane & Sols, 1954). We observe a linear relationship between 2-deoxyglucose 6-phosphate concentration and time (Fig. 3). This means that changes in the concentrations of these effectors are probably not important in the regulation of hexokinase during the course of these experiments. This, in turn, suggests that here there is no change in the concentration of free deoxyglucose and that the measured rate of

hexokinase phosphorylation is equivalent to the rate of deoxyglucose transport.

Measurement of cytosolic pH

As the use of ^{31}P n.m.r. for the measurement of intracellular pH from the P_i resonances becomes widespread, it is important that the ascription of such values to particular intracellular compartments can be made with confidence. It is relatively easy to differentiate between the intracellular and buffer phosphate resonances and thereby measure two pH values (Garlick *et al.*, 1979). So far, it has been impossible to differentiate between the cytoplasmic and mitochondrial signals of an individual molecule in perfused organs, although in isolated liver cells such separation has been reported for the P_i resonances (Cohen *et al.*, 1978).

The present results show that the cytoplasmic pH measured from the 2-deoxyglucose 6-phosphate resonance is identical with that derived from the P_i signal under both normoxic and ischaemic conditions. Although the introduction of relatively large quantities of 2-deoxyglucose 6-phosphate into the heart alters the system to some extent, the validity of the pH measurements is further strengthened by the observation that both probes give identical values. Since P_i is known to be distributed between the cytoplasm and the mitochondria, whereas 2-deoxyglucose 6-phosphate is expected to be exclusively in the cytoplasm, we conclude that, both under normoxic and ischaemic conditions, the P_i resonance essentially reflects the cytoplasmic pH. There can be several reasons why we do not observe a separate mitochondrial P_i signal: (i) the proportion of intramitochondrial to cytoplasmic P_i is too low; (ii) in heart tissue the ΔpH across the mitochondrial membrane is too small; (iii) mitochondrial P_i gives too broad a signal to be observed. At present we cannot distinguish between these possibilities.

The present observations not only establish that, in heart tissue at least, cytoplasmic pH is being measured, but also that other possible effects, such as binding to proteins and membranes, can be excluded as being responsible for the measured chemical shifts of intracellular P_i.

Phosphorylase activity in ischaemia

We have observed differences in both the rate and the extent of ischaemic acidosis in hearts pre-perfused with 2-deoxyglucose. The question arises of how deoxyglucose exerts its effect.

Garlick *et al.* (1979) have demonstrated that H^+ production in ischaemia is related to glycogen content, as glycogen-depleted hearts exhibit a significantly smaller decrease in pH than do normal hearts. Consequently, if glycogenolysis is inhibited in the ischaemic heart, one would expect to see a smaller decrease in pH. Our observations of a smaller decline in pH in the presence of 2-deoxyglucose (Fig. 5) therefore suggest that the main effect of deoxyglucose is to impair the rate of glycogen breakdown in ischaemia.

In experiments on isolated enzymes, we have shown that 2-deoxyglucose 6-phosphate has no effect on phosphorylase kinase, and Lawrence & Larner (1978) have reported that it has no effect on phosphorylase *a* or on phosphorylase phosphatase. The accumulation of this sugar cannot therefore affect the proportion of phosphorylase in the *a* form, nor can it change the activity of this form of the enzyme. We have shown that it can inhibit the AMP-induced activity of phosphorylase *b*.

Assuming that the regulation of phosphorylase *b* activity in the perfused heart is the same as in the isolated-enzyme experiments, the accumulation of 2-deoxyglucose 6-phosphate should inhibit any AMP-induced phosphorylase *b* activity in the ischaemic heart. If a significant proportion of glycogen is normally degraded by phosphorylase *b*, then, in the presence of 2-deoxyglucose 6-phosphate, glycogenolysis and consequently H^+ production should be impaired during total global ischaemia. Impaired H^+ production is indeed observed (Fig. 5).

We therefore suggest that 2-deoxyglucose 6-phosphate does specifically inhibit the *b* form of phosphorylase in the perfused heart and that glycogenolysis catalysed by phosphorylase *b* is normally an important mechanism for ATP production in the ischaemic heart.

There are considerable difficulties in measuring reliably the proportions of phosphorylase *a* and phosphorylase *b* in tissue after conventional extraction methods. Cornblath *et al.* (1963) have discussed the problems arising from tight binding of the activator AMP to both forms of phosphorylase. Ideally one should also consider the possible effect of the tight binding of inhibitors such as glucose 6-phosphate, ADP and ATP. Even if these problems can be successfully overcome and an accurate phosphorylase *b*/phosphorylase *a* ratio obtained, it is still not possible to know the phosphorylase *b* activity in tissue, since the state of ligand binding to the enzyme cannot be known. Extraction procedures destroy intracellular compartments therefore the extractable amounts of various metabolites (particularly AMP and ADP) do not accurately reflect the free tissue concentrations of those metabolites (Veech *et al.*, 1979; Ackerman *et al.*, 1980), so an assessment of the activation state of phosphorylase *b* is impossible with conventional techniques. We believe that this n.m.r. measurement is currently the best indication of phosphorylase *b* activity in whole-tissue ischaemia.

In the normoxic myocardium, the activity of phosphorylase *a* is very low (Cornblath *et al.*, 1963) and phosphorylase *b* is assumed to be inhibited. The

observation that 2-deoxyglucose 6-phosphate considerably increases the amount of glycogen in the heart might suggest that there is always a certain amount of phosphorylase *b* activity present in the normoxic heart. This interpretation is not necessarily valid, as it has been shown that 2-deoxyglucose 6-phosphate increases the proportion of glycogen synthase I activity in adipocytes by a stimulation of glycogen synthase phosphatase (Lawrence & Larner, 1978). Our data do not indicate whether the increase in the glycogen content of hearts perfused with deoxyglucose is caused by a decrease in glycogen utilization or by an increase in glycogen synthesis.

Unlike glucose 6-phosphate, 2-deoxyglucose 6-phosphate does not exhibit co-operativity in its inhibition of phosphorylase *b* (Fig. 6). Battersby & Radda (1979) have reported that the co-operativity of the glucose 6-phosphate effect is abolished in hybrid dimers of phosphorylase *b* in which one monomer is affinity labelled with an analogue of AMP. They obtained a K_I of 600μM for glucose 6-phosphate inhibition of the AMP-induced enzyme activity. This is of a similar magnitude to the inhibition constant of 450μM that we observe for the effect of 2-deoxyglucose 6-phosphate on phosphorylase *b*. It appears that although the 2-hydroxy group is not essential for the inhibition of phosphorylase *b*, it is required for promoting intersubunit effects.

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