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EFFECT OF ANOXIA, 2,4-DINITROPHENOL AND SALICYLATE ON XYLOSE TRANSPORT BY ISOLATED RAT SOLEUS MUSCLE

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Summary

1. These studies examined the theory that ATP served to regulate muscle sugar transport by a feedback mechanism. Xylose uptake by isolated rat soleus muscle was determined over a 5-min period following preincubation at 37°C for various times in the presence of insulin (0.1 unit/ml), 2,4-dinitrophenol (0.5 or 0.05 mM) or salicylate (5 mM) or under anaerobic conditions.

2. Xylose uptake, measured in freshly isolated soleus muscles, was approximately 3.5—4.0 μmol/g per h. When the muscles were preincubated at 37°C, this rate fell by 50% during the first 30 min and then slowly increased.

3. The stimulatory effect of insulin was evident within 2 min in freshly isolated soleus muscle and increased on preincubation, reaching a maximum value (approx. 14 μmol/g per h) after 20 min.

4. There was a 10-min lag period before xylose uptake was stimulated by anoxia. This lag period was approximately doubled when the incubation temperature was lowered from 37 to 27°C. The stimulatory effect of anoxia was promptly reversed when muscles were transferred from anaerobic to aerobic conditions.

5. There was a 5-min lag period before xylose uptake was stimulated by 2,4-dinitrophenol (0.05 mM) or by sodium salicylate (5 mM). At a concentration of 0.5 mM, 2,4-dinitrophenol stimulated xylose uptake in freshly isolated muscle. Whereas the stimulatory effects of insulin, anoxia and salicylate all tended to plateau with time, the effect of 2,4-dinitrophenol tended to peak and then decline.

6. There was no obvious relationship between total muscle ATP levels and xylose uptake. The stimulatory effect of anoxia, 2,4-dinitrophenol or salicylate on xylose uptake was not preceded by the fall in muscle ATP. Similarly, ATP levels did not change when xylose uptake was stimulated by anoxia at 27°C, or when xylose uptake was restored to basal values by transferring muscles from anaerobic to aerobic conditions.

7. It was argued that the presence of the myofibrils could act as a permeability barrier, which would limit the access of ATP produced within the interior

of the cell to a regulatory site on, or close to, the sarcolemma. On the other hand, it is conceivable that the ATP produced on the periphery of the fibre by the subsarcolemmal mitochondria could play a more specific role in the feedback regulation of sugar transport.

8. Insulin stimulated xylose uptake in the presence of 2,4-dinitrophenol (0.5 mM) when this was measured in freshly isolated muscle, but not after a period of preincubation. This suggested that there may be some ATP-dependent process involved in the stimulatory effect of insulin.

Introduction

As membrane transport of glucose constitutes the initial rate limiting step in its utilization by muscle, it might be expected that the regulation of glucose transport would be related to the intracellular metabolism of the sugar [1]. A number of systems have been proposed to explain how intracellular events may control sugar transport [1,2]. Randle and Smith [3] observed that sugar transport was stimulated by anoxia and uncouplers of oxidative phosphorylation. From this they concluded that intracellular ATP might serve to control, by a feedback mechanism, the entrance of glucose into the muscle cell [3,4]. This possibility has recently been given further impetus by Chang and Cuatrecasas [5], who found that the addition of ATP to the incubation medium inhibited glucose utilization by adipocytes. It has been suggested that the action of ATP may involve the phosphorylation of a membrane protein [4,6]. Alternatively, the depletion of ATP could stimulate sugar transport through effects on intracellular calcium levels, either as a consequence of the inhibition of the sodium pump [1,7] or perhaps more directly [8].

However, it has yet to be established that the stimulation of sugar transport by anoxia and the uncouplers was a direct consequence of the lowering of intracellular ATP levels. There does not appear to be any relationship between the increase in sugar transport and the magnitude of the fall in ATP concentration [9,10]. Other experiments have shown that anoxia stimulated glucose uptake under conditions where there was no apparent change in the level of ATP [10,11].

Previous studies in this laboratory examined the effects of anoxia and uncouplers on glucose uptake by rat soleus muscle [10]. These studies have now been extended, by measuring sugar transport itself, using D-xylose. The experiments reported in this paper examined the temporal relationship between the stimulation of sugar transport, by anoxia, 2,4-dinitrophenol and salicylate, and their effect on muscle ATP. As shown below, there was no obvious relationship between muscle ATP levels and xylose uptake. Nevertheless, if one considers the overall availability of intracellular ATP, it is still feasible that a small localized source of ATP could serve to regulate sugar transport.

Methods

Soleus muscles, each weighing approx. 30 mg, were obtained from Wistar rats (body weight 70—90 g) fed ad lib. The animals were stunned with a blow

on the head and decapitated. Approx. 1 min was required for the dissection of each muscle. As each muscle was removed it was weighed and incubated in a 25 ml beaker containing the appropriate incubation medium; the incubation of each muscle was timed individually. Xylose uptake was determined by incubating the muscles for 5 min at 37° C in 1.0 ml of Krebs bicarbonate buffer, pH 7.4, containing 10 mM D-[U-^{14}C]xylose (specific activity 0.03 Ci/mol) and 30 mM D-[1-^{3}H]sorbitol (specific activity 0.017 Ci/mol), according to the procedure described by Sloan et al. [12]. In most experiments the muscles were preincubated in 0.95 ml of bicarbonate medium prior to the measurement of xylose uptake; the test sugars were then added in a volume of 50 μl. Details of the various preincubation conditions will be specified at the appropriate place in the text. Aerobic incubations were performed under an atmosphere of 95% O_2/5% CO_2; 95% N_2/5% CO_2 was used for anaerobic incubations. The distribution of each sugar was expressed as a space (calculated as μmol/g muscle ÷ μmol/ml medium); xylose uptake was calculated from the difference between the xylose and sorbitol spaces.

For the measurement of ATP, the muscles were blotted briefly on filter paper and immediately frozen between aluminium plates chilled in solid CO_2. Each muscle was then homogenized in 1.0 ml of ice-cold 10% trichloroacetic acid, 1.0 ml of 0.1 M HCl was added and mixed, and the mixture centrifuged for 10 min in a bench centrifuge. The supernatant solution was extracted four times with an equal volume of diethyl ether (water-saturated), and neutralized with 1 M Tris base. ATP was determined spectrophotometrically, using hexokinase and glucose-6-phosphate dehydrogenase. Lactate was determined in samples of the incubation medium using the method of Barker and Summerson [13].

To minimise the effect of biological variation between individual animals, wherever possible the experiments were designed using the paired-control technique. One muscle from each pair was incubated under test conditions, while the other served as the control. The results of these experiments were analyzed for statistical significance using Student's *t*-test as applied to paired samples. Where it was not possible to use the paired-control technique, muscles taken from litter mates were randomly distributed among the experimental groups and the results subjected to statistical analysis using the standard Student's *t*-test.

Materials. D-[U-^{14}C]Xylose and D-[1-^{3}H]sorbitol were obtained from the Radiochemical Centre, Amersham. Beef insulin, twice recrystallized, was a gift from the Commonwealth Serum Laboratories, Melbourne. All enzymes were obtained from C.F. Boehringer, Mannheim.

Results

Effect of anoxia on xylose uptake: lag period

Xylose uptake was determined in the first series of experiments using the standard two-stage procedure described by Sloan et al. [12]. This involved a 45-min period of preincubation at 0° C to allow the test sugars to diffuse into the extracellular space, followed by a 5-min incubation at 37° C during which xylose was transported into the cell. This technique enabled the measurement of xylose uptake under essentially linear conditions.

Using this procedure, it was not possible to demonstrate the stimulatory effect of anoxia on xylose transport (Table I). Several attempts were made to achieve a higher degree of anoxia by using sealed vessels, or by bubbling the gas directly into the incubation medium, but none of these proved successful. If the final period of incubation at 37°C were extended to 30 min, then the effect of anoxia was now apparent. Presumably, the brief period at 37°C was not sufficient to achieve an adequate state of hypoxia in the muscle, despite the fact that the muscles had been subjected to anaerobic conditions throughouth the 45-min preincubation period at 0°C. When soleus muscles were preincubated for 60 min at 17°C before proceeding with the standard two-stage incubation, the stimulatory effect of anoxia was clearly evident.

From these experiments it was apparent that the effect of anoxia required a period of metabolism under anaerobic conditions before becoming manifest. According to the ATP-feedback theory, this lag period would represent the time required to deplete muscle ATP levels. Further experiments were undertaken to examine the lag period and its relationship to the ATP content of the muscle. For these experiments the original incubation procedure was modified by the omission of the 45-min period at 0°C, so that xylose uptake was measured without preequilibration with the test sugars. In view of this modification, the time curve of xylose uptake measured without pre-equilibration was determined and this is shown in Fig. 1. Under basal conditions, xylose uptake was essentially linear over the period 0—5 min. After incubation for 5 min the sugar had equilibrated in an intracellular space of 29 μl/g muscle; this was equivalent to an uptake rate of 3.5 μmol xylose/g per h.

Xylose uptake was stimulated by insulin. This effect was evident within 2 min ($P < 0.05$) and increased with further exposure to the hormone. Whereas the average rate of xylose uptake measured over the entire 5-min period was 6.6 μmol/g per h, this could be resolved into an initial rate of 5.3 μmol/g per h over the first 3 min, followed by a rate of 8.9 μmol/g per h during the final

TABLE I

EFFECT OF ANOXIA ON XYLOSE UPTAKE

Xylose uptake was determined in soleus muscle pairs, incubated under the conditions shown. In each of the three experiments, [U-^{14}C]xylose (10 mM final concentration, specific activity 0.03 Ci/mol) and [1-^{3}H]sorbitol (30 mM final concentration, specific activity 0.017 Ci/mol) were added to the incubation medium at the commencement of incubation II. Values are mean ± S.E. of four determinations.

Incubation details				Xylose uptake (μmol/g)	
I 17° (min)	II 0° (min)	III 37° (min)	Atmos		
—	45	5	O_2/CO_2	0.46 ± 0.04	
			N_2/CO_2	0.52 ± 0.05	n.s.
—	45	30	O_2/CO_2	1.30 ± 0.02	
			N_2/CO_2	2.20 ± 0.10	$P < 0.005$
60	45	5	O_2/CO_2	0.30 ± 0.02	
			N_2/CO_2	0.80 ± 0.06	$P < 0.005$

n.s., not significant.

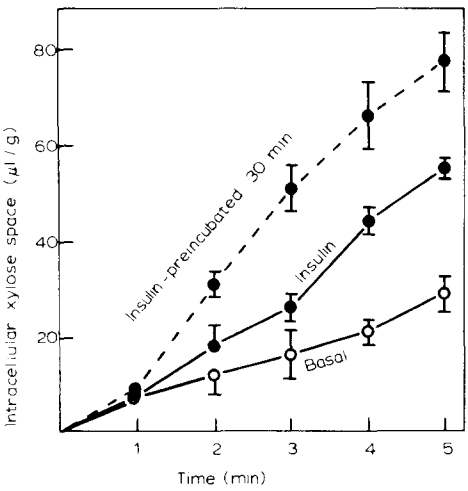

Fig. 1. Xylose uptake by isolated soleus muscle: time course. Soleus muscles were incubated for up to 5 min at 37°C under aerobic conditions in 1 ml of Krebs bicarbonate buffer containing 10 mM [U-^{14}C]-xylose (specific activity 0.03 Ci/mol) and 30 mM [1-^{3}H]sorbitol (specific activity 0.017 Ci/mol) in the presence and absence of insulin (0.1 unit/ml). Intracellular xylose was calculated as the difference between the xylose and sorbitol spaces. ○———○, freshly isolated muscles without insulin; ●———●, freshly isolated muscles with insulin; ●- - - - -●, muscles preincubated with insulin for 30 min at 37°C. Each point represents the mean of 4—6 determinations ± S.E.

2 min. When the muscles were preincubated in the presence of insulin for 30 min at 37°C, the overall rate was increased to 9.2 μmol/g per h. The rapid response to insulin shown in this experiment compares favourably with that recently reported by Crofford [14] using adipocytes. The increased effect of insulin with further exposure of the tissue to the hormone has also been observed by many other workers [14—16]. It is generally believed that this progressive increase in the effect of insulin reflects the association between the hormone and its receptor; however, the possibility that this may also involve some metabolic step subsequent to the binding of insulin cannot be excluded [17].

Effect of insulin and anoxia: time course

The effects of insulin and anoxia on xylose uptake were studied sequentially in the following manner. Soleus muscles were incubated for up to 55 min at 37°C in 95 ml of bicarbonate medium, either aerobically in the presence and absence of insulin, or anaerobically. At the appropriate time, xylose and sorbitol were added in a volume of 50 μl and xylose uptake was determined over the next 5 min.

The rate of xylose uptake, measured under basal conditions, did not remain constant throughout the course of this experiment (Fig. 2). During the first 30 min the rate decreased by about 50%; thereafter it appeared to increase slowly, but did not regain the original value even after 55 min. This effect appeared very early. In a separate experiment it was observed that the mean xylose uptake of seven muscle pairs fell from 4.3 ± 0.2 to 3.1 ± 0.4 μmol/g per h ($P <$ 0.02) after incubation for 5 min at 37°C. It is very likely that the elevated

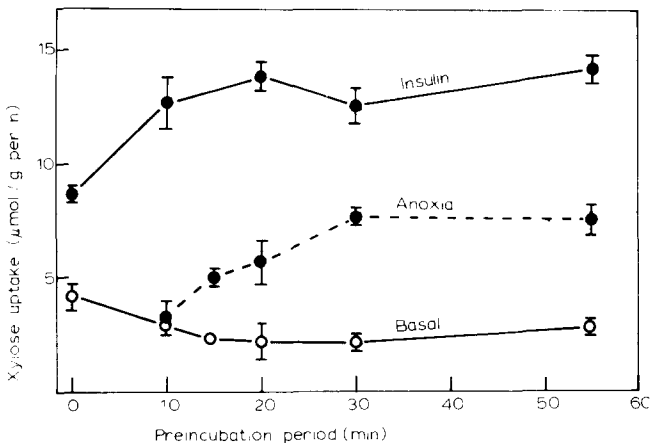

Fig. 2. Effect of preincubation in the presence of insulin, or under anaerobic conditions on xylose uptake. Soleus muscles were preincubated for up to 55 min at 37°C (a) aerobically in the absence (○———○) or presence (●———●) of insulin, or (b) under anaerobic conditions (●- - - - -●). At the times shown, xylose and sorbitol were added in a volume of 50 μl and xylose uptake determined over the next 5 min. The basal values shown are mean ± S.E. of 15—40 determinations; other values are mean ± S.E. of four determinations.

xylose uptake measured in freshly isolated soleus muscle was due to the stress associated with killing the animal and removing the muscles. The precise explanation for this effect, however, is not yet known.

As foreshadowed by the experiment presented in Fig. 1, insulin stimulated xylose uptake by soleus muscle without prior exposure to the hormone, and this effect increased when the muscles were preincubated in the presence of the hormone (Fig. 2). The maximum stimulatory effect of insulin was achieved 10—20 min after the tissue had been exposed to insulin. In marked contrast to the effect of insulin, there was an appreciable lag period before xylose uptake was stimulated in muscles incubated under anaerobic conditions (Fig. 2). Increased uptake was only evident in those muscles preincubated for at least 15 min. This lag period was temperature dependent. As shown in Fig. 3, the duration of the lag period was approximately doubled when the temperature of the incubation was reduced from 37 to 27°C. The effect of anoxia on xylose uptake could be reversed by transferring anaerobic muscles to an aerobic environment (Fig. 4). This reversal occurred more rapidly than the stimulatory effect of anaerobiosis; the lag period in this case was only 5 min *.

Effect of anoxia on muscle ATP levels

ATP levels in soleus muscles incubated under the same conditions as those shown in Figs. 2—4 were determined, and these results are shown in Fig. 5. The

* It is not immediately obvious that there was a lag period prior to the reversal of the effect of anoxia shown in Fig. 4. However, as the values for xylose uptake at each point shown represent the uptake of xylose during the subsequent 5 min, this would imply that xylose uptake was not depressed during the initial 5-min period after the muscles had been transferred to aerobic incubation conditions. Similar considerations apply to the lag period preceding the effects of 2,4-dinitrophenol and salicylate in Figs. 6 and 7.

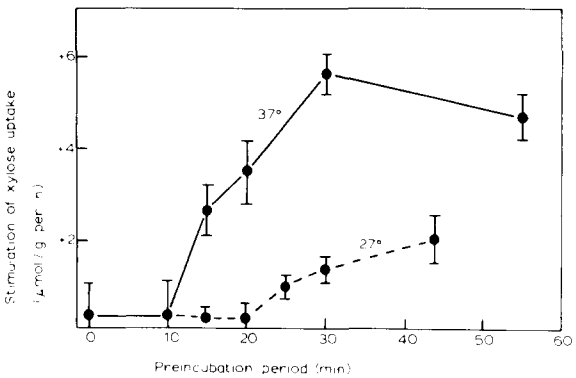

Fig. 3. Effect of anoxia on xylose uptake. Soleus muscles were preincubated for up to 55 min under anaerobic conditions at either 37°C (●———●) or 27°C (●- - - - -●). At the times shown, xylose and sorbitol were added in a volume of 50 μl and the incubation continued for a further 5 min at the same temperature. To compensate for variations in basal uptake rates due to muscle variability, the different incubation temperatures and changes in the rate during the course of the incubation period, the results shown have been corrected by subtracting the xylose uptake measured in paired muscles incubated under aerobic conditions. Values are mean ± S.E. of five determinations.

ATP content of freshly isolated soleus muscle was 3.4 ± 0.2 μmol/g (n = 6). When muscles were incubated under aerobic conditions the ATP level remained constant for 30 min and then slowly declined. Similar values were obtained from paired muscles incubated in the presence and absence of insulin.

There was a 15-min period before the effect of anoxia on muscle ATP became evident. After 20 min the level of ATP in anaerobic muscle was significantly lower than in corresponding aerobic control muscles ($P < 0.005$). This decrease in ATP level thus occurred during the same time period that xylose

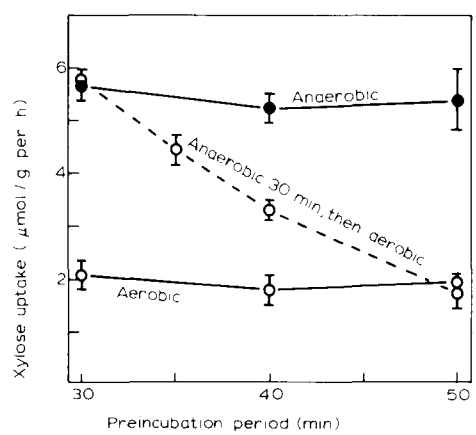

Fig. 4. Reversibility of the effect of anoxia on xylose uptake. Soleus muscles were preincubated for 30 min at 37°C under anaerobic conditions, the incubation medium was then briefly flushed with O_2/CO_2 and the incubation continued at 37°C under anaerobic conditions. Control muscles were incubated under either aerobic or anaerobic conditions throughout. At the times shown, xylose and sorbitol were added in a volume of 50 μl and the incubation continued for a further 5 min. Values are mean ± S.E. of 5—7 determinations.

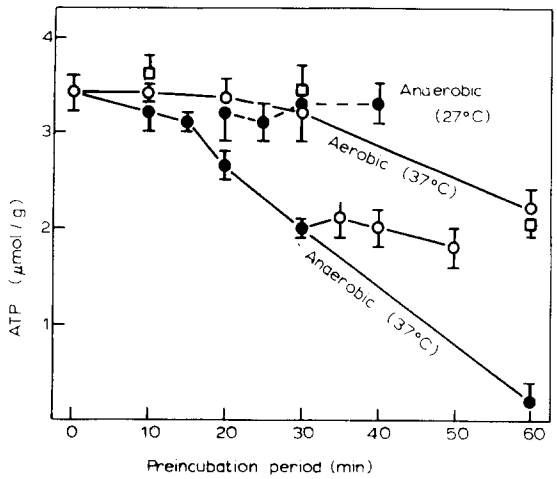

Fig. 5. Effect of anoxia on muscle ATP levels. ATP was determined in soleus muscles incubated under similar conditions to those shown in Figs. 2—4. These were (a) aerobic, 37°C (○———○); (b) aerobic, 37°C + 0.1 unit insulin/ml (□); (c) anaerobic, 37°C (●———●); (d) anaerobic, 27°C (●- - - - -●); (e) anaerobic, 37°C for 30 min, then aerobic, 37°C (●——●——○——○). Values are mean ± S.E. of 4—6 determinations.

uptake was first stimulated. In marked contrast, there was no effect of anoxia on muscle ATP levels when the temperature was reduced to 27°C. Similarly, whereas the stimulatory effect of anoxia on xylose uptake was reversed when anaerobic muscles were further incubated under aerobic conditions (Fig. 4), there was no corresponding increase in the ATP content (Fig. 5). Instead, ATP tended to be maintained at a depressed level following the restoration of aerobic conditions.

Effect of 2,4-dinitrophenol and salicylate

The next experiments examined the stimulatory effects of the two uncouplers, 2,4-dinitrophenol and sodium salicylate, on sugar transport in relationship to their effects on muscle ATP levels (Figs. 6 and 7). At a concentration of 0.5 mM the effect of 2,4-dinitrophenol was already evident within the initial 5-min assay period (Fig. 6). When the muscles were pre-exposed to 2,4-dinitrophenol (0.5 mM), the magnitude of this effect first increased and then decreased. This biphasic response to 2,4-dinitrophenol appeared to be related to the associated changes in muscle ATP. Thus, the level of ATP fell by 65% during the first 5 min of the incubation and continued to fall during the period when the rate of xylose uptake was increasing. The subsequent decrease in xylose uptake occurred after the level of ATP had declined to an unmeasurable value.

The very rapid fall in ATP level induced by 2,4-dinitrophenol (0.5 mM) precluded any evaluation of its relationship to the onset of increased sugar transport. For this it was necessary to lower the concentration of 2,4-dinitrophenol to 0.05 mM. At this lower concentration there was no effect of 2,4-dinitrophenol on xylose uptake when this was measured in freshly isolated muscles. There was a small but significant ($P < 0.05$) increase in xylose uptake after the muscles had been exposed to 2,4-dinitrophenol (0.05 mM) for 5 min. Once

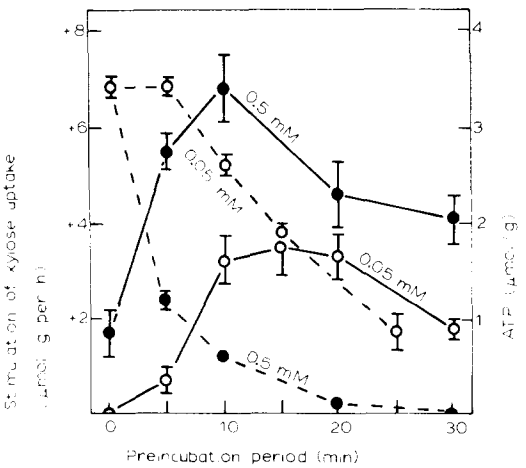

Fig. 6. Effect of 2,4-dinitrophenol on xylose uptake and muscle ATP levels. Xylose uptake (solid lines) was determined in soleus muscles after preincubation for up to 30 min at 37°C in the presence of 2,4-dinitrophenol, 0.05 mM (○———○) or 0.5 mM (●———●). The results shown have been corrected by subtracting the xylose uptake measured in paired control muscles incubated in the absence of 2,4-dinitrophenol. ATP (dashed lines) was determined in a parallel series incubated in the presence of 2,4-dinitrophenol, 0.05 mM (○- - - - -○) or 0.5 mM (●- - - - -●). Values are mean ± of 4—5 determinations.

again the magnitude of this effect first increased and then declined as the period of pre-exposure to the uncoupler was extended. There was no effect of 2,4-dinitrophenol (0.05 mM) on muscle ATP levels during the first 5 min of the incubation. Muscle ATP fell by 24% ($P < 0.01$) over the next 5 min and

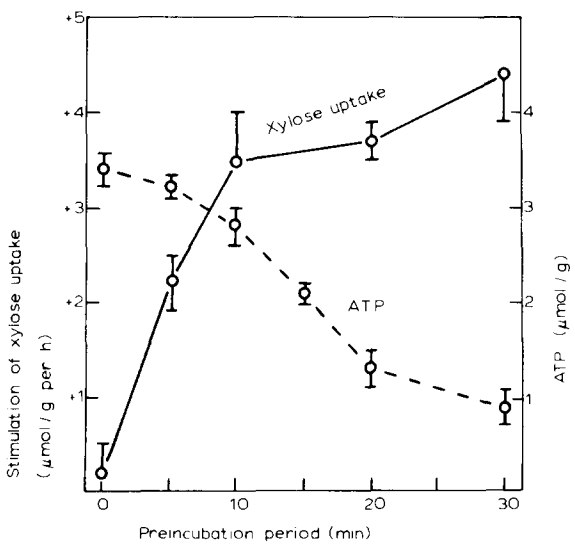

Fig. 7. Effect of sodium salicylate on xylose uptake and muscle ATP levels. Xylose uptake (○———○) was determined in soleus muscles after preincubation for up to 30 min at 37°C in the presence of 5 mM sodium salicylate. The results shown have been corrected by subtracting the xylose uptake measured in paired control muscles. ATP (○- - - - -○) was determined in a parallel series following incubation in the presence of salicylate for the times shown. Values are mean ± S.E. of 4—5 determinations.

continued to decline thereafter. The stimulatory effect of 2,4-dinitrophenol (0.05 mM) on xylose uptake decreased during the period when the level of ATP had fallen to less than 25% of its original value.

There was also a 5-min lag period before xylose uptake was stimulated by sodium salicylate (5 mM) (Fig. 7). Xylose uptake was increased further as the period of pre-exposure was extended up to 10 min and thereafter remained essentially constant. The effect of salicylate on muscle ATP levels was similar to that of 2,4-dinitrophenol (0.05 mM). Thus, the ATP content of those muscles incubated in the presence of salicylate remained constant for the first 5 min and then declined. The observed decrease in ATP level during the period 5—10 min was not statistically significant ($0.10 > P > 0.05$).

Effect of iodoacetate

The involvement of ATP in the control of sugar transport has thus far been considered in terms of the feedback system proposed by Randle and Smith [3]. However, as discussed more fully below, the stimulation of xylose uptake by anoxia and the uncouplers was also consistent with an alternative possibility, namely, that sugar transport may be an active process with a specific requirement for ATP produced by glycolysis. If the stimulatory effect of anoxia on xylose uptake were due to the increased production of glycolytic ATP, then it was argued that this should be inhibited by iodoacetate. However, iodoacetate (2 mM) actually increased xylose uptake measured under anaerobic conditions (Table II). Concurrent experiments showed that under these conditions iodoacetate blocked glycolysis and depleted the level of muscle ATP.

Combined effects of insulin, anoxia and 2,4-dinitrophenol

Whereas it has been suggested that the stimulatory effects of anoxia and the uncouplers on sugar transport were mediated through the lowering of ATP levels [3,4], this does not appear to apply to the action of insulin [4,10]. A number of experiments were performed to determine whether the effect of insulin was still demonstrable under anaerobic conditions, or in the presence of 2,4-dinitrophenol (0.5 mM) and, conversely, whether anoxia or 2,4-dinitrophenol had any effect on xylose uptake in insulin-treated muscles. In view of the observed differences between the effects of these three agents on xylose uptake and on muscle ATP content, the conditions used for these experiments were varied, with reference to the data shown in Figs. 2—6, to ensure that the most appropriate procedures were used in each case.

TABLE II

Xylose uptake, muscle ATP and medium lactate were all determined after preincubation for 30 min under anaerobic conditions in the presence and absence of 2 mM iodoacetate. Values are mean ± S.E. of four determinations.

	Xylose uptake (μmol/g per h)	ATP (μmol/g)	Lactate (μmol/g)
Control	3.6 ± 0.6	1.3 ± 0.3	11.8 ± 0.7
Iodoacetate	8.1 ± 0.5	0.03 ± 0.03	1.6 ± 0.4
	$P < 0.01$	$P < 0.05$	$P < 0.001$

TABLE III

EFFECT OF INSULIN AND ANOXIA ON XYLOSE UPTAKE

Xylose uptake was determined in soleus muscles following preincubation at 37°C for 30 min under aerobic or anaerobic conditions, in the presence and absence of insulin (0.1 unit/ml). Values are mean ± S.E. of five determinations.

Incubation conditions		Xylose uptake (μmol/g per h)	
Atmos	Insulin		
N_2/CO_2	—	4.0 ± 0.6	
N_2/CO_2	+	15.3 ± 1.0	$P < 0.001$
O_2/CO_2	+	17.9 ± 1.3	
N_2/CO_2	+	15.6 ± 1.1	n.s.

n.s., not significant.

When soleus muscles were preincubated for 30 min under N_2/CO_2 to maximize the effect of anoxia, xylose uptake was further stimulated by insulin (Table III). Conversely, when xylose uptake was stimulated by insulin, anoxia had no further effect. This result contrasted with the earlier observation of Chaudry and Gould [10] that anoxia stimulated glucose uptake by insulin-treated soleus muscle. According to Morgan et al. [18], these differing results may be explained in terms of the stimulatory effect of anoxia on glucose phosphorylation.

In view of the biphasic effect of 2,4-dinitrophenol on xylose uptake (Fig. 6), the effect of insulin on 2,4-dinitrophenol-treated muscles was studied at three time points. As shown in Fig. 6, 2,4-dinitrophenol (0.5 mM) stimulated xylose uptake, measured without prior exposure to the uncoupler. Under these conditions, insulin effected a further increase in xylose uptake (Table IV). When the effect of 2,4-dinitrophenol was maximized by preincubating the muscles for 10 min, there was no further effect of insulin. Similarly, when the period of preincubation was increased to 55 min (by which time the uncoupler had com-

TABLE IV

EFFECT OF INSULIN ON XYLOSE UPTAKE IN THE PRESENCE OF 2,4-DINITROPHENOL

Xylose uptake was determined in soleus muscle pairs following preincubation at 37°C under aerobic conditions in the presence of 2,4-dinitrophenol (0.5 mM) ± insulin (0.1 unit/ml). Values are mean ± S.E. of five determinations.

Incubation conditions		Xylose uptake (μmol g per h)	
Preincubation period (min)	Insulin		
0	—	5.5 ± 0.5	
0	+	7.7 ± 0.7	$P < 0.02$
10	—	8.6 ± 0.7	
10	+	9.6 ± 0.2	n.s.
55	—	6.7 ± 0.3	
55	+	8.2 ± 0.5	n.s.

n.s., not significant.

TABLE V

EFFECT OF 2,4-DINITROPHENOL ON XYLOSE UPTAKE IN INSULIN-TREATED OR ANAEROBIC MUSCLES

Xylose uptake was determined in soleus muscle following preincubation for 30 min at 37°C either aerobically in the presence of insulin (0.1 unit/ml) or anaerobically. Where indicated, 2,4-dinitrophenol (0.5 mM) was added 5 min before the end of the preincubation period. Values are mean ± S.E. of five determinations.

Incubation conditions	Xylose uptake (μmol/g per h)	
Insulin	13.6 ± 1.0	
Insulin + 2,4-dinitrophenol	9.7 ± 0.4	$P < 0.02$
Anaerobic	5.4 ± 0.8	
Anaerobic + 2,4-dinitrophenol	6.6 ± 0.8	$P < 0.05$

pletely depleted muscle ATP levels), there was no further effect of insulin on xylose uptake. The experimental conditions were varied somewhat to examine the effect of 2,4-dinitrophenol on xylose uptake in insulin-treated or anaerobic muscles (Table V). In this case the muscles were preincubated with insulin or under anaerobic conditions for 30 min to ensure a maximal effect. To avoid the complete depletion of ATP levels, 2,4-dinitrophenol (0.5 mM) was added only 5 min before the end of the preincubation period; xylose uptake was measured 5 min after the addition of the uncoupler. Under these conditions, 2,4-dinitrophenol inhibited xylose uptake in insulin-treated muscles and stimulated xylose uptake in anaerobic muscles.

Discussion

The experiments described in this paper were undertaken to reexamine the theory that ATP served to control muscle sugar transport by a feedback mechanism. An important feature of these studies was the measurement of xylose uptake over a very short period of time. In this way, changes in the rate of uptake, induced by incubating the muscles under a variety of conditions, could be determined sequentially during the course of the incubation.

Because the measurement of xylose uptake required a finite period of time, it was to be expected that under certain conditions the rate of transport would not remain constant during the assay period itself. This was clear from the experiment shown in Fig. 1. A period of 5 min was adopted for the measurement of xylose uptake. This was a compromise between a period short enough to minimize the effect of rate changes during the course of the assay, and yet long enough to enable xylose uptake to be measured with adequate precision. As xylose uptake will not always be linear during the course of the assay, the present procedure measures the average rate over the uptake period rather than the initial rate of xylose uptake. Furthermore, the method assumes that there is no discrepancy between the distribution of xylose and sorbitol in the extracellular space at the end of the 5 min assay period. As previously reported [12], this is not so; when uptake rates are high this will tend to underestimate the true value. Nevertheless, as shown by the results presented in this paper, the sequen-

tial measurement of short-term xylose uptake offers a new approach to the study of muscle sugar transport and its regulation.

The value of this technique is evident when one considers that many of the agents which influence sugar transport may require an initial period to interact with the tissue before changes in transport rates become apparent. Such changes, which would tend to be diminished if uptake were measured over the entire incubation period, are accentuated by measuring short-term uptake after a period of preincubation. As the rate of basal uptake itself tends to fall during the course of the incubation period, this further serves to accentuate any stimulatory effect. This is well demonstrated by the experiment shown in Fig. 2. Xylose uptake, measured without preincubation, was stimulated 108% by insulin; after preincubation for 20 min the effect of insulin had increased to 515%.

Xylose uptake and muscle ATP levels

Using this technique, we have confirmed the earlier observation of Holloszy and Narahara [19] that there was a lag period before sugar transport was stimulated by anoxia. The duration of the lag period was increased when the temperature of the anaerobic incubation was lowered from 37 to 27°C. This suggested that the effect of anoxia on sugar transport was due to some metabolic change within the muscle cell. Similarly, the effects of the two uncouplers, 2,4-dinitrophenol (0.05 mM) and salicylate (5 mM), did not become evident for at least 5 min. The lag periods associated with the action of 2,4-dinitrophenol (0.5 and 0.05 mM) reported in this paper were identical to those found by Kohn and Clausen [20], measuring the efflux of 3-*O*-methylglucose from soleus muscle. However, they did not observe a biphasic response to this uncoupler.

If sugar transport were regulated by ATP, then one might have expected the ATP level to fall prior to the stimulation of xylose uptake by anoxia, 2,4-dinitrophenol or salicylate. In no experiment was this observed. Instead, ATP levels were seen to fall concurrently with the increase in xylose uptake. The extent of this fall was not particularly great, and ranged from 17 to 25% of the ATP originally present in the muscle. Significantly, the ATP level in muscles incubated for 60 min under aerobic conditions fell by 35% (Fig. 5), but xylose uptake was not stimulated in these muscles. Furthermore, under certain conditions it was found that the rate of sugar transport could be altered without any appreciable change in the level of ATP. Thus, when the muscles were incubated at 27°C, anoxia stimulated xylose uptake but had no effect on muscle ATP. Similarly, when muscles were incubated for 30 min under anaerobic conditions and then transferred to an aerobic atmosphere, the stimulatory effect of anoxia on xylose uptake was reversed but there was no change in the muscle ATP level. These observations inducated that there was no simple relationship between sugar transport and the total level of ATP in the muscle.

Specific role of mitochondrial versus glycolytic ATP

According to Wu and Racker [21], intracellular compartmentation may so limit the availability of ATP produced by glycolysis or by oxidative phosphorylation that a system may have a specific requirement for the ATP produced by one or other of these two sources. One may then ask whether this applies in the present case: whether the regulation of sugar transport in muscle is a func-

tion of a specific pool of ATP, either mitochondrial or glycolytic? It is not possible to measure separately the pools of "mitochondrial" or "glycolytic" ATP in the muscle. Nevertheless, if one considers the likely source rather than the total amount of the ATP in the muscle under the various conditions decribed above, it is possible to relate changes in xylose uptake to changes in the availability of either mitochondrial or glycolytic ATP.

Under anaerobic conditions, or in the presence of the uncouplers, the production of mitochondrial ATP would be curtailed in favour of glycolytic ATP. Under these conditions xylose uptake was stimulated. Conversely, upon the restoration of aerobic conditions the production of ATP would revert back to the mitochondria. Under these conditions the rate of xylose uptake declined. This suggests that if sugar transport was regulated by a feedback system as proposed by Randle and Smith [3], this must be a function specific to mitochondrial ATP. However, if one considers the reciprocal changes in glycolytic ATP, these results could equally suggest that sugar transport was an active process requiring specifically the ATP produced by glycolysis. This possibility was excluded by the demonstration that xylose uptake was not inhibited when anaerobic glycolysis was inhibited by iodoacetate.

These experiments appear to suggest that if ATP is involved with the regulation of sugar transport, this must be a function specific to mitochondrial, as distinct from glycolytic, ATP. However, there are other observations reported in this paper which suggest that, under certain conditions, glycolytic ATP may also function as a feedback inhibitor of sugar transport. Thus, both iodoacetate and 2,4-dinitrophenol (0.5 mM) increased xylose uptake under conditions where it was otherwise maximally stimulated by anoxia (Tables II and V). Similarly, 2,4-dinitrophenol (0.5 mM) alone, which tended to deplete the overall ATP level more rapidly than anoxia, also tended to stimulate xylose uptake to higher values than those measured in anaerobic muscles (Fig. 6).

There are a number of possible reasons why this should be so. The stimulatory effect of 2,4-dinitrophenol on the activity of mitochondrial ATPase [22] would not only inhibit the production of mitochondrial ATP, but would hasten the depletion of residual glycolytic ATP levels. Furthermore, as discussed further below, the uncoupler could have a more direct effect on sugar transport through changes in intracellular Ca^{2+} levels [8].

As far as iodoacetate is concerned, it is possible that this effect, like that of a number of other sulfhydryl reagents, may be due to its interaction with sulfhydryl groups on the surface of the cell [9,23,24]. However, one must also consider the possibility that these effects of iodoacetate and 2,4-dinitrophenol may have been due to their depletion of the residual intracellular (i.e. glycolytic) ATP.

Role of the subsarcolemmal mitochondria

Other workers, using avian erythrocytes [25] and rat thymocytes [26], have shown that there was a relationship between the depletion of ATP levels by anoxia and the stimulation of sugar transport consistent with the feedback regulation of transport by ATP. The situation in skeletal muscle does not appear to be so simple. From the evidence considered above it would appear that both mitochondrial and glycolytic ATP are capable of functioning as feedback

inhibitors, though not to the same extent. This difference would seem to imply that the putative regulatory site was somehow more accessible to mitochondrial ATP than to glycolytic ATP. It is not immediately obvious why this should be so.

Recent studies by Hoppeler et al. [27] have shown that the mitochondria in skeletal muscle are distributed in two morphologically distinct areas. Some (the "interfibrillar" mitochondria) are located within the interior of the fibre where they can be seen lying between the myofibrils in association with glycogen granules. Others (the "subsarcolemmal" mitochondria) appear to form aggregations on the periphery of the fibre in the area lying adjacent to the capillary space. We have also observed these two groups of mitochondria in rat soleus muscle. The obvious barrier to intracellular diffusion imposed by the presence of the myofibrils could explain why ATP produced in the interior of the cell should have limited access to a regulatory system which is presumably located on, or close to, the sarcolemma. However, this limitation should apply equally to the ATP produced by glycolysis and that produced, in the same general region, by the interfibrillar mitochondria. This suggests that regulation may be a function of some other source of ATP, more appropriately localized with respect to the sugar transport system. In this regard the subsarcolemmal mitochondria would appear to be ideally situated. The ATP produced by these mitochondria would be more readily available for the regulation of sugar transport than that produced, either aerobically or anaerobically, within the interior of the cell.

It should be stressed that at present there is still no direct evidence to link the stimulation of the sugar transport system with the depletion of muscle ATP, mitochondrial or otherwise. Nevertheless, if one considers the availability to the sugar transport system of the ATP produced by these various sources, it is possible to explain the various effects of anoxia, iodoacetate and the uncouplers reported above in terms of the feedback system proposed by Randle and Smith [3,4]. In this case, however, the major restraining influence on the rate of sugar transport would appear to be the ATP produced, in the immediate vicinity, by the subsarcolemmal mitochondria. When this is no longer available, as, for example, under anaerobic conditions, glycolytic ATP can exert a degree of restraint. However, diffusional limitations appear to prevent glycolytic ATP from functioning as efficiently as the ATP produced by the subsarcolemmal mitochondria. Xylose uptake was further increased when the restraining influence of glycolytic ATP was removed under the influence of iodoacetate or 2,4-dinitrophenol.

The present studies have not attempted to explore the mechanism whereby ATP may control sugar transport. Smith et al. [4] originally proposed that the action of ATP may involve the phosphorylation of the carrier or an associated membrane protein. In view of the current interest in the role of phosphorylation-dephosphorylation mechanisms in metabolic regulation [28,29], this remains an interesting possibility. Bihler and Sawh [30] have suggested that the effect of anoxia on sugar transport was due to the decreased availability of oxidative ATP for the Na^+ pump. According to Bihler [31], inhibition of the Na^+ pump would stimulate Na^+-Ca^{2+} exchange; stimulation of sugar transport would thus be a consequence of the altered distribution of intracellular Ca^{2+}.

Clausen et al. [8] suggest that many of the factors which stimulate sugar transport, including 2,4-dinitrophenol, do so by increasing the intracellular Ca^{2+} level. However, whether or not the effect of anoxia and the uncoupler on Ca^{2+} levels is direct, or is mediated via reduced levels of ATP, is not yet known.

ATP-dependent stimulation of xylose uptake

Reeves [26], studying the effects of anoxia and 2,4-dinitrophenol on the transport of 3-*O*-methylglucose in rat thymocytes, concluded that there were two ways in which ATP might be involved. Transport was stimulated when cellular ATP levels declined, but a minimal level of ATP was, nevertheless, required for the full expression of this stimulatory effect. The biphasic effect of 2,4-dinitrophenol on muscle xylose uptake shown in Fig. 6 would appear to be in accord with this proposal.

Significantly, insulin stimulated xylose uptake in 2,4-dinitrophenol-treated muscles during the early phase, when ATP was still present, but not later (Table IV). Similarly, 2,4-dinitrophenol inhibited uptake in the presence of insulin (Table V). These experiments suggest that ATP may also be required for the stimulation of sugar transport by insulin. This possibility is supported by the recent observation of Chaudry et al. [32], that glucose uptake by soleus muscle taken from rats during severe hemorrhagic shock was insensitive to insulin concencentrations less than 0.2 unit/ml. Noting that the ATP levels in muscles taken from shocked animals were severely depleted, they showed that sensitivity to insulin could be restored by the addition of Mg^{2+}-ATP to the incubation medium [33]. There was no effect of hemorrhagic stock on basal glucose uptake, nor was this affected by the addition of Mg^{2+}-ATP to the incubation medium [33]. This suggests that whatever this ATP-dependent process may be, it does not appear to be essential for the basic function of the sugar transport system itself.

Benjamin and Singer [34] found that insulin promoted the phosphorylation of a specific protein (mol. wt. 140 000) in adipocytes. The physiological significance of this observation has yet to be determined. If this phosphorylation were somehow involved in the mechanism whereby insulin stimulates sugar transport, this might offer an explanation for the ATP dependency suggested by the present results and those of Chaudry et al. [32,33]. Conversely, Chang et al. [6], who found that ATP inhibited insulin-stimulated sugar transport in adipocytes, associated this with the phosphorylation of two membrane proteins (mol. wts. 22 000 and 16 000). At present we do not know the extent to which these observations may be applied to skeletal muscle; however, the implication that ATP may play two separate roles in the regulation of sugar transport in the adipocyte is of obvious interest, in view of the suggestion offered here that there may also be two disparate effects of ATP in soleus muscle.

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