Effect of Inhibitors on D-Xylose Permeability in Rat Diaphragm Muscle

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ABSTRACT The influence of metabolic inhibitors and low temperatures upon D-xylose transfer has been studied in rat diaphragm muscle preparations *in vitro*. Using intact fiber preparations, it has been confirmed that at body temperature metabolic inhibitors like DNP have an insulin-like action in that they permit D-xylose to distribute into previously unavailable intracellular aqueous regions; inhibitors, unlike insulin, disturb cation distribution in association with increased sugar penetration. Although the studies with inhibitors suggest an energy requirement for maintenance of D-xylose exclusion, the D-xylose exclusion mechanism is effectively maintained at 0° for many hours, and under these conditions, inhibitors have little or no effect on D-xylose distribution, though they do produce potassium loss. In cut muscle fiber preparations, in which insulin significantly increases the rate at which D-xylose equilibrates between cell water and external medium, DNP does not increase the rate of D-xylose entry, but does abolish the effect of insulin in this preparation. The results suggest that insulin action upon sugar permeability in muscle involves two barrier systems; some of the characteristics of these systems have been defined.

In previous work we have studied the *in vitro* sugar permeability characteristics of rat diaphragm muscle preparations, wherein the fibers were either intact or cut at both ends (1). As first shown by Kipnis and Cori (2), intact fiber preparations retain permeability characteristics similar to those which obtain *in vivo*, whereas cut fiber preparations exhibit altered permeability characteristics. Thus, in intact fibers, sucrose and mannitol do not penetrate significantly into the cell water, while "non-utilizable" sugars like D-xylose and D-galactose are excluded from about 75 per cent of the total cell water of the preparation; insulin markedly increases the distribution of D-xylose and D-galactose in cell water, without influence on the exclusion of sucrose or mannitol. In cut fibers, on the other hand, the evidence indicates that (*a*)

mannitol and sucrose penetrate into the fibers *via* the cut ends and distribute in sizable fractions of the cell water, (*b*) D-xylose and D-galactose enter and distribute in the total cell water, and (*c*) insulin increases the rate of D-xylose and D-galactose equilibration between extra- and intracellular water.

In an attempt to elucidate the action of insulin upon sugar transfer, we have studied the influence of dinitrophenol (DNP) and other metabolic inhibitors as well as other factors upon D-xylose entry into intact muscle fibers; in addition we have compared the effect of DNP upon D-xylose entry in the cut as well as in intact muscle fibers, in the presence and absence of insulin. While these studies were in progress, Randle and Smith (3) reported that anaerobiosis and inhibitors like dinitrophenol, arsenite, and cyanide have an "insulin-like" effect in that they facilitate D-xylose penetration into the cell water of intact preparations.

METHODS

The methods of preparing intact and cut fiber rat diaphragm muscle preparations and estimating pentose, sucrose, and inulin, as well as evaluating D-xylose penetration into the cell water of the preparation have been previously described (1). We have again assumed that sucrose is a measure of extracellular space in the intact diaphragm preparation and that inulin measures the extracellular space in cut hemidiaphragms.

The cut hemidiaphragm preparations were incubated in 5 ml. of Krebs-Ringer phosphate buffer containing 5 mg. per ml. D-xylose plus 7 mg. per ml. inulin. The intact preparations were incubated in 50 ml. of a modified Krebs-Ringer phosphate, containing the following (in milliequivalents per liter); NaCl (128), KCl (5.1), $CaCl_2$ (2.7), $MgSO_4$ (1.0), Na phosphate buffer (20) pH 7.2, and D-xylose at either 4 or 5 mg. per ml. plus sucrose at 8 or 10 mg. per ml. Unless otherwise stated all incubations were at 37.5° with shaking, with 100 per cent oxygen as gas phase. In the experiments involving insulin, a crystalline Zn insulin Lilly preparation was used at a maximal concentration of 0.3 unit per ml.

In certain of these experiments, the distribution of monovalent cations in muscle fibers was studied using methods previously described (4). Sodium and potassium were measured with a flame photometer using lithium ion as internal standard; lithium was estimated by flame photometry using the direct method. The intracellular concentration of ions was calculated on the assumptions that sucrose and inulin are a measure of the extracellular compartment of the tissue in intact and cut preparations respectively, and that the cation concentrations in the medium and in the extracellular space are equivalent.

RESULTS

Table I illustrates results obtained when intact diaphragm preparations are incubated at 37.5° for one hour with DNP, azide, or iodoacetate. It can

be seen, in confirmation of Randle and Smith (3), that these metabolic inhibitors increase the distribution of D-xylose in the cell water of intact fiber preparations without marked influence on extracellular space as evaluated by sucrose. It should be noted, however, that there are striking differences between inhibitors and insulin with regard to the cellular distribution of potassium and sodium. Both DNP and azide decrease the intracellular potassium; the potassium loss with DNP, but not with azide, is associated with a gain of sodium, but the sodium entry does not balance the potassium loss.

TABLE I

THE EFFECT OF METABOLIC INHIBITORS ON THE DISTRIBUTION OF D-XYLOSE AND SUCROSE IN INTACT DIAPHRAGM PREPARATIONS INCUBATED FOR 1 HOUR AT 37.5°

	D-Xylose		Intracellular cations	
	Extracellular sucrose space*	Distribution in cell water‡	K	Na
	ml. per 100 gm.	*Per cent*	*mM per liter*	
Normal	23.5±4(25)§	24.0±1(7)	133±4(7)	37.1±2(7)
Insulin	23.5±4(24)	65.5±1(7)	153±6(7)	32.3±3(7)
DNP (0.2 mM)	20.7±2(8)	81.6±6(6)	46.7±7(6)	82.0±6(6)
DNP (0.02 mM)	24.0±3(5)	73.8±4(5)	65.4±2(5)	57.6±4(5)
Na azide (1.0 mM)	23.6±1(7)	85.0±6(11)	72.7±3(11)	39.2±4(9)
" plus insulin	24.0±1(6)	84.9±6(6)	—	—
Iodoacetate (1.0 mM)	19.5±1(4)	81.0±3(4)	—	—
" plus insulin	19.3±1(4)	81.0±3(4)	—	—

* Volume of sucrose distribution in muscle (wet weight).

‡ Calculated on the assumption that sucrose is a measure of the extracellular water.

§ Mean, standard error, and number of determinations.

Thus, both of these inhibitors reduce the total intracellular concentration of monovalent cations. It may be noted in Table I, however, that in the presence of inhibitors (*a*) intracellular potassium while lowered, nevertheless is still accumulated against an apparent gradient, and that external sodium is still excluded, in large part, from the fiber water at a time when the D-xylose exclusion mechanism does not appear to be operative, and (*b*) sucrose still is excluded from the cell. Insulin, in marked contrast to the inhibitors, increases intracellular potassium and tends to decrease intracellular sodium. The metabolic poisons studied thus appear to exert a generalized deleterious effect upon the permeability properties of the intact muscle fibers, whereas insulin acts to facilitate sugar entry without disturbing cation distribution.

Following treatment with DNP, the intact diaphragm preparation is similar to the cut hemidiaphragm in that D-xylose enters and equilibrates in most or all the cell water in both preparations. Table II shows the rate of D-xylose entry into the cell water of intact preparations pretreated with inhibitor and

of hemidiaphragms, with or without DNP, in the presence and absence of added insulin. The intact diaphragm preparations were preincubated with DNP (0.2 mM) for 1 hour at 37.5° in the absence of D-xylose, then transferred to fresh medium containing D-xylose plus inhibitor; the hemidiaphragm preparations were not pretreated with DNP. These results may be summarized as follows: (*a*) In hemidiaphragms, DNP has no detectable influence on the rate of sugar entry in the absence of added insulin, but does abolish the effect of insulin upon D-xylose entry. (*b*) The rate of entry of D-xylose

TABLE II

THE RATE OF D-XYLOSE ENTRY INTO THE CELL WATER OF HEMIDIAPHRAGMS AND INTACT DIAPHRAGM PREPARATIONS PRETREATED WITH DNP

	Per cent distribution in cell water at varying intervals of incubation (min.)*			
	7.5	15	30	60
Cut fibers				
No additions	43±2(6)‡	64±2(6)	78±2(4)	94±6(4)
Insulin	52±3(7)	79±2(6)	92±3(5)	100±3(4)
DNP	38±2(10)	64±3(6)	79±3(4)	96±4(6)
DNP + insulin	41±2(10)	65±4(6)	77±4(6)	96±2(6)
Pretreated intact fibers§				
DNP	27±1(4)	52±2(4)	82±2(4)	92±3(4)
DNP + insulin	29±1(4)	54±2(4)	83±4(4)	93±3(4)

* Based upon the assumption that inulin is a measure of extracellular space in the cut fibers of hemidiaphragms; in the intact fiber preparations, the sucrose space after 1 hour of incubation in DNP (*cf*. Table I) was assumed to represent the extracellular space during the 2nd hour of incubation.

‡ Mean, standard error, and number of determinations.

§ Preincubated for 1 hour at 37.5° with DNP (0.2 mM).

into intact fibers pretreated with DNP is not increased by insulin. (*c*) The rate of D-xylose entry in intact fibers, pretreated with DNP, is essentially similar to that observed in cut fibers in the absence of added insulin.

Attempts to reverse the effect produced by pretreatment with DNP by "washing" the intact preparation in Ringer's several times prior to the second incubation period, were uniformly unsuccessful. In preparations incubated for 1 hour at 37.5° with DNP, washed twice with 50 ml. of fresh medium, and then incubated in medium without inhibitor, the entry rate of D-xylose was similar to that observed when inhibitor had been present in both incubation periods, and no effect of insulin on D-xylose entry could be detected. These results may be an expression of irreversible damage to the system, or be a consequence of the ineffectiveness of our washing procedure in removing DNP from the tissue.

To obtain information about the energy requirement for the maintenance of the D-xylose exclusion mechanism, intact diaphragm preparations were incubated in Ringer's medium with D-xylose at 0° for 1 to 8 hours. These results shown in Table III demonstrate that after 8 hours at 0°, D-xylose distributes in only 40 per cent of the cell water. If the D-xylose exclusion mechanism is damaged by preincubation with DNP (0.2 mM) at 37.5° for 1 hour and the preparation is subsequently incubated with fresh medium containing

TABLE III

THE DISTRIBUTION OF D-XYLOSE IN THE FIBER WATER OF PREPARATIONS INCUBATED AT 0°C, IN THE PRESENCE AND ABSENCE OF INHIBITORS

	Period of incubation	Extracelluar sucrose space	Per cent xylose distribution in cell water	Intracellular cations	
				K	Na
	hrs.			*μM per ml.*	
Normal medium	1	22.5±1(7)*	19±5(7)	143.3±5(7)	35.5±1(7)
	2	22.0±1(8)	24±6(8)	135.5±6(8)	45.0±5(8)
	4	22.1±1(4)	34±4(4)	142.0±3(4)	44.1±5(4)
	8	26.7±2(4)	40±3(4)	127.0±7(4)	50.2±8(4)
DNP (0.2 mM)	1	23.5±1(10)	14±1(10)	137.6±3(10)	41.0±2(6)
	2	22.0±1(4)	28±3(4)	97.2±2(4)	41.6±1(4)
NaN_3 (1.0 mM)	1	22.5±1(10)	23±1(10)	108.5±2(4)	29.1±1(4)
	2	21.5±1(4)	32±1(5)	85±5(4)	41.3±1(4)
Preincubated DNP at 37.5°‡	1	23.0±1(4)	53±2(4)	—	—
	2	23.5±2(4)	75±3(4)	—	—

* Mean, standard error, and number of determinations.

‡ Preincubated with 0.2 mM DNP at 37.5° for 1 hour and then transferred to fresh medium containing D-xylose at 0° and incubated for 2 hours as indicated.

D-xylose at 0° (*cf.* Table III), D-xylose penetration into the cell water can be readily demonstrated, the sugar distributing in 75 per cent of the cell water after 2 hours at 0°. It may be noted that D-xylose penetration at 0° into inhibitor-pretreated preparations is slow relative to the rate at 37.5° (half-time of complete equilibration being approximately five times greater at 0° than at 37.5°; compare Tables II and III). These results which demonstrate that at 0° the D-xylose exclusion mechanism retains its effectiveness for periods as long as 8 hours indicate that there is only a modest energy requirement for maintenance of the exclusion process at this temperature. Under these conditions, DNP and azide likewise have little effect on the D-xylose exclusion mechanism as can be seen in Table III. It should be noted, in Table III, that at 0° these inhibitors retain their ability to cause potassium

loss from muscle, azide (1 mM) producing a somewhat greater effect than DNP (0.2 mM). As was previously observed at 37.5° (*cf.* Table I) the potassium loss produced by these inhibitors is not accompanied by a corresponding gain of sodium; indeed, the sodium exclusion properties of the diaphragm preparations incubated at 0° are maintained almost as well, whether inhibitors are present or absent.

DISCUSSION

The present studies help to define certain aspects of insulin action upon sugar penetration in muscle, in that two insulin-responsive barrier systems appear to be involved. One, evident in the intact fiber preprations, which acts to exclude D-xylose from the cell water may be designated as inhibitor-sensitive since at 37.5° this restraining system can be removed by dinitrophenol and other metabolic inhibitors, as first shown by Randle and Smith (3). The other barrier system, evident in cut muscle fibers, does not exclude D-xylose, but is modified by insulin so that the rate of sugar equilibration is increased; this system may be designated as inhibitor-insensitive, since DNP had no significant effect on the rate of D-xylose entry in hemidiaphragms. If the inhibitor-sensitive mechanism in intact fibers is postulated to be localized at the cell membrane, it would appear to be necessary to postulate that in cut fibers, insulin influences an alternative barrier system, presumably cytoplasmic, which restricts intracellular permeation of sugars within the cell. While the available data do not permit a definitive localization of the site of insulin action upon sugar penetration, the suggestion arises as in our previous studies (1) that the effect of insulin on sugar permeability may involve modification of a cytoplasmic system. The substantial evidence in hemidiaphragm preparations for effects of insulin on glycogen synthesis and amino acid incorporation into protein which cannot be explained in terms of accelerated sugar transfer or amino acid transport (5, 6) supports the contention that insulin action involves modification of the cellular interior (7, 8) as well as the cell surface.

Although the locus of the D-xylose exclusion mechanism in intact fibers is uncertain, our studies on low temperature and inhibitors do provide a basis for defining certain characteristics of this system. The fact that at 37.5°, DNP and azide promote increased distribution of D-xylose in the cell water may be interpreted to indicate that at this temperature metabolic energy is required to maintain a system which excludes D-xylose. In essence, this is a restatement of the basic aspect of the theory proposed by Randle and Smith which postulates that the entry of sugars like D-glucose and D-xylose is restrained by a process dependent upon a substance generated during oxidative phosphorylation. Our studies reveal that at 0°, the system maintains its effectiveness for

long periods of time, and that under these conditions DNP and azide have little or no effect. It would therefore appear that at 0°, the D-xylose exclusion mechanism is relatively "stable" and has only a modest energy requirement for maintenance. As temperature is increased, however, the system appears to become "metastable" in the sense that maintenance at body temperature requires an adequate supply of metabolic energy. Such an explanation would serve to account for the lack of effect of DNP or azide upon D-xylose penetration at 0° in contrast to their marked effects at 37.5°.

The relationship between D-xylose exclusion and the permeability mechanisms involved in potassium accumulation and sodium exclusion is likewise difficult to assess. Although it is widely assumed that sodium efflux and potassium influx, controlled by an energy-requiring ion pump in the cell membrane, determine the distribution of cations in cells, certain results from the present experiments are not readily explicable in terms of this concept. Thus, while DNP and azide at 37.5° produce potassium loss, the expected equivalent gain in sodium is not observed, the discrepancy being particularly evident with azide. This is particularly evident at 0° where both of these inhibitors decrease the level of intracellular potassium without sodium gain. Moreover, preparations treated with these inhibitors do not exhibit marked changes in total water or in extracellular space as evaluated by sucrose, despite the fact that the level of intracellular sodium plus potassium appears to be significantly reduced; we have no explanation for this apparent discrepancy. In any case the mechanisms involved in sodium exclusion and potassium accumulation are similar to the D-xylose exclusion process to the extent that while they are "disturbed" by inhibitors at 37.5°, they are nevertheless maintained for many hours at 0°, in the absence of added inhibitors. The fact that azide and DNP nevertheless cause potassium loss at 0°, raises the question whether the effects of these compounds are exclusively due to their inhibition of cellular energetics.

Finally, there remains to be considered, the relationship of these findings to the theory of Randle and Smith, who have suggested that the entry of sugars like D-xylose into the cell is normally restrained by a process dependent upon a supply of a substance generated during oxidative phosphorylation, and that insulin acts in isolated diaphragm muscle by preventing access of this hypothetical substance to the process regulating sugar entry. While our limited data in intact diaphragm preparations might be explained in terms of this particular hypothesis, as well as alternative mechanistic formulations, it is important to note that our studies on hemidiaphragms are not consistent with the Randle-Smith thesis. Thus, if the established effect of insulin to increase the rate of D-xylose penetration into the cut fibers were due to the removal of a substance necessary for a process restraining D-xylose entry, one should expect DNP to produce an "insulin-like" effect in cut fibers

as well as in intact fibers. Our present evidence with D-xylose as well as previous evidence with D-galactose (9) clearly demonstrates, however, that DNP does not increase the rate of D-xylose (or D-galactose) penetration in hemidiaphragms as does insulin; indeed the only discernible effect of DNP upon penetration of these sugars in cut fibers is to abolish the effect of insulin upon this process. If insulin action upon penetration involves two barrier systems in the muscle fiber, as this study suggests, then it would follow that the Randle and Smith hypothesis is an incomplete description of insulin action upon sugar transfer in muscle.

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