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Studies on the sugar carrier in skeletal muscle: Modification of carrier mobility by nitrogen mustard *in vitro*

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SUMMARY

Application of the anti-neoplastic agent nitrogen mustard *in vivo* or *in vitro*, enhances the uptake of the non-metabolizable sugar 3-*O*-methylglucose by rat hemidiaphragm *in vitro*. This effect has also been observed with other alkylating agents such as phenoxybenzamine and 2-hydroxy-5-nitrobenzyl bromide and is ascribed to a structural modification of that region of the cell membrane which regulates the mobility of the carrier–sugar complex.

The carrier-mediated uptake of glucose by skeletal muscle is believed to occur by a process of facilitated diffusion. The efficiency of the sugar carrier depends to a large extent on its stereoselectivity and on its affinity for various monosaccharides[1,2], but it is conceivable that regulation of its action could also be effected through modulation of the stereochemical and electrostatic factors which determine the mobility of the carrier–sugar complex. Thus insulin increases the apparent maximum velocity of the transport process without altering the carrier affinity[3]. Similarly, we have shown that carrier mobility may be altered by manipulation of the ionic composition of the extracellular fluid[4]. Recently, in the course of pharmacological studies, we observed that in isolated rat diaphragm, the transport of the non-metabolizable sugar derivative 3-*O*-methyl-D-glucose is appreciably enhanced by pretreatment of the tissue with the anti-neoplastic agent nitrogen mustard (mechlorethamine), or with either of two other alkylating agents of widely differing structure such as 2-methoxy-5-nitrobenzyl bromide or the α-adrenergic blocking agent phenoxybenzamine.

Intact hemidiaphragms were obtained[5] from young rats (35–45 g) of a Long-Evans strain. Hemidiaphragms were individually preincubated for 20 min in Krebs–Henseleit[6] solution at 37°, with alkylating agent added to one half only, which then served as test, while the other untreated tissue served as control. Subsequently, tissues were further incubated for 30 min without alkylating agent in fresh Krebs–Henseleit solution

TABLE 1

THE EFFECT OF ALKYLATING AGENTS ON THE DISTRIBUTION OF 3-*O*-METHYL-D-GLUCOSE IN RAT HEMIDIAPHRAGM

3-*O*-Methylglucose space (%) = virtual space − extracellular (mannitol) space.
Mannitol space: 24.8 ± 1.6. Values are means ± S.E. for at least five animals per group and $P < 0.01$ for differences between 3-*O*-methylglucose space for test and control.

Alkylating agent	*3-O-Methylglucose space for rat hemidiaphragm*						
	In vitro				*In vivo*		
	Control	*MCEA*	*POB*	*MNBB*	*Control*	*MCEA*	*POB*
No insulin	7.0 ± 1.1	28.0 ± 3.0	14.1 ± 1.52	26.2 ± 2.62			
+ insulin	17.0 ± 1.5	39.0 ± 4.0	32.03 ± 2.95	32.2 ± 3.37	19.13 ± 1.88	48.47 ± 1.85	42.46 ± 1.67

Abbreviations: see legend of Fig. 1.

to which had been added the appropriate quantity of 3-*O*-methylglucose and insulin, as well as tracer quantities of 3-*O*-[^{14}C]methylglucose and [^{3}H]mannitol, the latter for the determination of effective extracellular spaces[7]. Uptake of isotopes by the tissue was measured by double-label liquid-scintillation techniques[7] and sugar uptake was evaluated as "percentage penetration" P (Fig. 1), *i.e.* the concentration of 3-*O*-methylglucose in the intracellular water, expressed as a percentage of the final concentration in the incubation medium[7]. The response of the transport system to alkylating agent was calculated as $(P_{test} - P_{control})/P_{control}$ (Fig. 2). Statistical evaluation was by a two-tailed "t"-test[8], and in the kinetic studies, results were plotted by means of linear regression techniques[9].

Preincubation with mechlorethamine (0.32 mM) stimulates the uptake of 3-*O*-methylglucose by rat hemidiaphragm *in vitro*, whether insulin (100 μunits/ml) is present or not (Fig. 1 and Table I). Phenoxybenzamine and 2-methoxy-5-nitrobenzyl bromide cause a similar response when applied to the tissue at a concentration of 0.1 mM. Treatment with the prehydrolyzed alkylating agents has no stimulatory effect. Intraperitoneal administration of mechlorethamine (10 μg/g) or phenoxybenzamine (7.5 μg/g) at 12–16 h prior to sacrifice of the animals, eliminates the necessity for preincubation with the drug (Fig. 1). The relationship between response and the quantity of mechlorethamine applied *in vitro*, is demonstrated in Fig. 2. This treatment does not alter the passive permeability of the cell membrane to mannitol, nor the preference of the carrier for 3-*O*-methylglucose over arabinose, and the stereoselectivity which favours L-arabinose rather than the D-form, is maintained. The sugar transport process still follows saturation kinetics and the response to mechlorethamine is characterized by an increase in the apparent maximum velocity of the transport process (Fig. 3), with little, if any effect on the affinity of the carrier. *In vivo* mechlorethamine causes cross-linking of DNA[10] and, like phenoxybenzamine[11], interacts with the functional groups which occur in proteins and phospholipids. 2-Methoxy-5-nitrobenzyl bromide has a high, but not absolute specificity

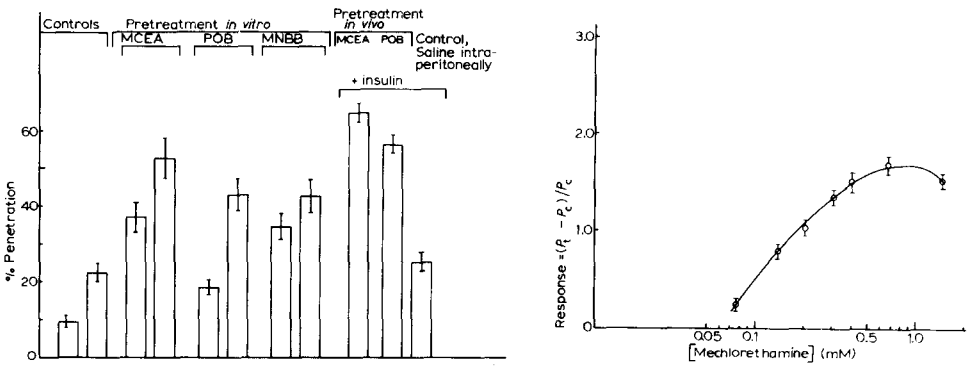

Fig. 1. Stimulatory action of alkylating agents on the uptake of 5 mM 3-*O*-methylglucose by rat diaphragm *in vitro*. Values given are means ± S.E., for at least five animals per group, and $P < 0.01$ for $(P_t - P_c)$. Abbreviations: MCEA, mechlorethamine; POB, phenoxybenzamine; MNBB, 2-methoxy-5-nitrobenzyl bromide.

Fig. 2. Concentration–response relationship for the stimulatory effect of nitrogen mustard on the uptake of 5 mM 3-*O*-methylglucose by rat diaphragm *in vitro*. Each point represents mean ± S.E. for at least three animals.

for tryptophan residues[12], as does the less stable parent compound 2-hydroxy-5-nitrobenzyl bromide. We find that, unlike the methylated compound 2-methoxy-5-nitrobenzyl bromide, which hydrolyzes relatively slowly[12], this compound decomposes rapidly on contact with aqueous solutions, and stimulates sugar uptake only very weakly in the concentration range 0.1 to 1.0 mM, where all the other alkylating agents which we have tested unequivocally stimulate sugar uptake. Acylating agents such as maleic anhydride do not affect the operation of the carrier *in vitro* at 1 mM, nor does L-1-tosylamide-2-phenylethyl-chloromethyl ketone★, an alkylating agent with reputed high specificity for histidyl residues[13]. *N*-Ethylmaleimide, which is believed to alkylate sulphydryl groups, inactivates the sugar carrier[7,14], as does the carboxy reagent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide[18,★]. One may assume that the appreciable stimulation of sugar transport caused by pretreatment of skeletal muscle *in vitro* with alkylating agents could be due to a perturbation of the membrane through modification of a reactive site in a regulatory region of the membrane associated with the sugar carrier, so as to alleviate the natural restraints which are imposed on the operation of the carrier. If each carrier site has a regulatory region susceptible to alkylating agents, the assumption of independent activation of individual carriers would considerably simplify kinetic interpretation of this phenomenon. More complex would be the obvious alternative, in which increased carrier mobility would be the consequence of a co-operative effect[15] of the alkylating agent in the membrane. Assuming the possibility of independent activation of individual carriers by mechlorethamine, we have tentatively calculated an apparent activation constant $K_a = 2.2 \cdot 10^{-4}$ M by a

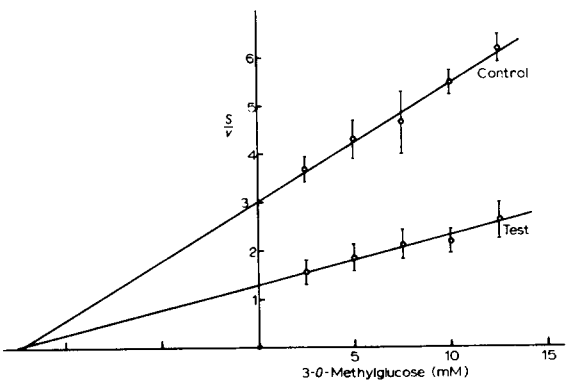

Fig. 3. Kinetic characteristics for the stimulatory effect of 0.32 mM mechlorethamine on the uptake of 3-*O*-methylglucose by rat diaphragm *in vitro*. Values given show means ± S.E. for at least four animals per point.

$$\frac{S}{V} = \frac{\text{external 3-}O\text{-methylglucose (mM)}}{\text{(external 3-}O\text{-methylglucose (mM))} \times \%\text{ penetration per 30 min}}$$

$$= \frac{1}{\%\text{ penetration per 30 min}}$$

★D. Ilse, unpublished observations.

modification of the approach of Dixon and Webb[16]. Although higher than may be expected for an alkylation, the magnitude of this constant is of the same order as the K_i for the interaction of the alkylating agent L-1-tosylamide-2-phenylethyl-chloromethyl ketone with chymotrypsin[17]. However, it is likely that the response of the sugar carrier to an alkylating agent would depend very much on the accessibility of the sensitive site. No doubt, the interpretation of the activating effect of mechlorethamine may indeed prove more complex than is suggested by the relatively good accommodation of the response within the limitations of Michaelis–Menten kinetics. Studies with isotopically labeled alkylating agents are in progress to determine more specifically the site of interaction of the alkylating agents with the membrane, and the kinetic characteristics associated with the increased mobility of the sugar carrier *in vitro*,

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