BBA 75582

THE RELATIONSHIP BETWEEN THE TRANSPORT OF GLUCOSE AND CATIONS ACROSS CELL MEMBRANES IN ISOLATED TISSUES

VI. THE EFFECT OF INSULIN, OUABAIN, AND METABOLIC INHIBITORS ON THE TRANSPORT OF 3-*O*-METHYLGLUCOSE AND GLUCOSE IN RAT SOLEUS MUSCLES

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(Received September 28th, 1970)

SUMMARY

1. The permeability of muscle cells to 3-*O*-methylglucose has been assessed by measurements of influx and efflux in isolated rat soleus muscle.

2. The transport of 3-*O*-methylglucose seems in part to be mediated by the same saturable and phlorizin-sensitive system which is operative in the transfer of glucose across the plasma membrane.

3. For the evaluation of the time-course of changes in 3-*O*-methylglucose transport, the rate coefficient of efflux was found to constitute a very sensitive parameter, which is increased by a variety of conditions known to stimulate the uptake of glucose in muscle cells (insulin, contractions, metabolic inhibitors, hyperosmolarity, trypsin). Since phlorizin markedly suppresses the effect of these stimuli, a major part of the increase in the rate coefficient of efflux seems to represent an acceleration of carrier-mediated 3-*O*-methylglucose transport.

4. The time-course of 3-*O*-methylglucose efflux suggests that the individual fibers in the soleus muscle may differ considerably with respect to sugar permeability.

5. Whereas the decrease in K^+ content produced by 2,4-dinitrophenol (0.05–0.5 mM) was associated with a prompt rise in 3-*O*-methylglucose efflux, similar losses of K^+ induced by ouabain (1 mM) did not lead to any immediate change in the basal or insulin-stimulated rate of 3-*O*-methylglucose efflux. A significant stimulation (30 %) could only be detected after an exposure to ouabain of 90 min.

INTRODUCTION

The isolated soleus muscle of young rats has been introduced by CHAUDRY AND GOULD[1] as a tool for studying sugar transport. It was demonstrated that glucose uptake in this muscle occurs by a carrier-mediated and insulin-sensitive process. Moreover, since the preparation has small dimensions, intact fibers and convenient tendons by which it may be handled, it seemed very suitable for the measurement and characterization of sugar and ion fluxes.

There are conflicting reports on the significance of Na^+–K^+ transport for sugar permeability in insulin-sensitive tissues. Inhibition of active Na^+–K^+ transport with cardiac glycosides was found to produce little or no change in the basal or insulin-stimulated uptake of glucose and other sugars in diaphragm muscle[2–4]. More recently, however, these compounds have been reported to stimulate glucose uptake in rat hemidiaphragm[5] and the uptake of 3-*O*-methylglucose in an intact diaphragm preparation[6]. Moreover, ouabain augments glucose metabolism in fat cells[7,8] and accelerates 3-*O*-methylglucose efflux from epididymal fat pads[9]. The fact that K^+ loss induced by a variety of conditions is associated with a rise in the transport of glucose and other sugars in adipocytes[9–11], muscle cells[12] and the heart[13] suggests that a decrease in intracellular K^+ concentration (or inhibition of active Na^+–K^+ transport) might constitute a significant stimulus for the sugar transport system in insulin-sensitive tissues. The aim of the present study has been to examine these possibilities using a peripheral skeletal muscle.

It has previously been shown that sugar permeability can conveniently be studied by following the efflux of nonmetabolized sugars from preloaded tissues[9,13–15]. The present results show that in soleus muscle, 3-*O*-methylglucose efflux is very sensitive to insulin and to a variety of other factors which are known to affect glucose uptake in skeletal muscle.

Inhibition of energy production by 2,4-dinitrophenol causes both K^+ loss and stimulation of the phlorizin-sensitive component of 3-*O*-methylglucose efflux. However, a similar decrease in K^+ content induced by ouabain had little or no effect on basal and insulin-stimulated 3-*O*-methylglucose efflux. The results indicate that in the soleus muscle there is no direct link between active Na^+–K^+ transport and sugar permeability.

METHODS

Small muscles were chosen to minimize the contribution of extracellular diffusion to the overall efflux process. Fed male and female Wistar rats, which had been weaned for 1 week and which were judged to be fully established on a diet of Altromin pellets, were used throughout. The animals were all in the weight range 60–70 g. They were killed by decapitation and the skin removed from the hind limbs. The calcaneous bone was gripped with a pair of forceps and its distal attachments severed. By gently pulling in a proximal direction the entire soleus muscle could be exposed. A small part of the tibia with the origin of the soleus muscle was dissected out using a pair of iridectomy scissors. With another pair of forceps the bony part of the upper insertion was lifted and the muscle loosened from its surroundings by severing the thin adhering fasciae. The distal tendon was dissected out together with a few mm of the achilles tendon which was used for the subsequent handling. After removal of the bony attachments all muscles were washed for at least 5 min in Krebs–Ringer bicarbonate buffer, blotted on filter paper moistened with the same medium and then transferred to the incubation mixture.

The basic incubation medium was Krebs–Ringer bicarbonate buffer[16] containing half the recommended concentration of Ca^{2+} and 1 mM pyruvate. Unless otherwise stated all experiments were carried out at 30° using centrifuge tubes containing 1–3 ml of buffer which were continuously gassed with a humidified mixture of O_2 and CO_2 (95:5, by vol.).

For the measurement of 3-*O*-methylglucose uptake and extracellular space with inulin and mannitol, the muscles were removed from the incubation medium after the selected period of time, lightly blotted on filter paper according to a standardized procedure and weighed after removal of the achilles tendon. They were then homogenized in 5 % trichloroacetic acid using a Potter–Elvehjem tube, and, after centrifugation, aliquots of the clear supernatant were withdrawn for counting or for the determination of Na^+ and K^+ by flame photometry[9].

The methods for the measurement of glucose uptake and for the isolation and determination of ^{14}C activity in glycogen have been described in previous reports[3,4].

For efflux experiments, muscles were loaded for 60 min in buffer containing the labelled substrate, and then after light blotting, attached by the achilles tendon to a rigid polyethylene tube. This served both to transfer the muscles between the wash tubes and as a gas distribution tube. Each wash tube contained 3 ml of buffer, a volume more than 100 times greater than the water content of the muscles.

For experiments in which muscles were electrically stimulated, platinum electrodes were attached to the cannulae, one immediately above the attachment of the muscle and one at the tip of the cannula. The impulses were supplied from a Grass (Model S4B) stimulator. Fuller details of this arrangement will be given in a subsequent paper[17].

After the efflux period, the muscles were treated as in the uptake experiments and homogenized in 4 ml of 5 % trichloroacetic acid containing carrier 3-*O*-methylglucose (0.1 mM). Trichloroacetic acid and 3-*O*-methylglucose were added to the wash tubes to give the same final concentrations. 1.5-ml aliquots of homogenate and buffer were counted, and the rate coefficient of efflux (fraction of ^{14}C activity lost per min) was calculated as previously described[9,15].

The validity of results obtained in efflux experiments is very dependent on the purity of the radioactive compound used, and it is essential that this is not metabolized. Preliminary experiments showed that when soleus muscles were incubated for 60 min in the standard loading medium containing 3-*O*-[^{14}C]methylglucose, there was no detectable conversion of ^{14}C activity into CO_2 or glycogen, even when insulin (100 mU/ml) was added. Under these conditions a contamination with less than 0.01 % of [^{14}C]glucose would have been detected. Descending chromatography[14] of the trichloroacetic acid extracts of the tissue after an efflux experiment of 150 min duration showed that more than 90 % of the ^{14}C activity could be recovered from a single spot moving with the same speed as the 3-*O*-[^{14}C]methylglucose used for loading.

Chemicals, isotopes and hormones

All chemicals were of analytical grade. 3-*O*-Methylglucose was obtained from Calbiochem (Los Angeles), pancreatic trypsin (EC 3.4.4.4) from Nutritional Biochemicals Corp. (Cleveland), soybean trypsin inhibitor (type II s) from Sigma (St. Louis) and ouabain from Merck (Darmstadt). The ethacrynic acid was a gift from Merck, Sharp and Dohme (Holland). The reagents for the enzymatic determination of glucose were products of Kabi AB (Sweden). 3-*O*-[^{14}C]Methylglucose (specific activity 50 mC/mmole), [*carboxy*-^{14}C]inulin (specific activity 10 mC/mmole) and D-[1-^{14}C]mannitol (specific activity 25 mC/mmole) were purchased from New England Nuclear (Boston). The D-[$^{14}C_6$]glucose (specific activity 309 mC/mmole), [$^{14}C_3$]pyru-

vate (specific activity 17.3 mC/mmole) and part of the 3-*O*-[^{14}C]methylglucose were obtained from the Radiochemical Centre, Amersham. Mono-component pork insulin lot No. SL-11669 MC, (25 I.U./mg, purified by chromatography) was a gift from The Novo Research Laboratories (Copenhagen).

RESULTS

Tissue viability

It should be emphasized that unless their isolation is performed carefully and with a minimum of direct handling, soleus muscles tend to develop contractures. Since these were most frequently seen in muscles obtained from fasted rats, fed animals were used throughout, and 1 mM pyruvate was added to the incubation medium as metabolizable substrate. Furthermore, except where otherwise stated, muscles which did not remain relaxed throughout the experiment were discarded. These precautions are important because spontaneous contractures as well as those induced by electrical stimulation, can be associated with an increase in the rate of influx and efflux of 3-*O*-methylglucose.

All experiments were performed at 30° in order to reduce the oxygen demand, and at that temperature, a certain decrease in K^+/Na^+ ratio might be anticipated.

TABLE I

K^+ CONTENT OF RAT SOLEUS MUSCLE BEFORE AND AFTER INCUBATION

All incubated tissues were loaded for 60 min in Krebs–Ringer bicarbonate buffer containing 1 mM pyruvate and, in efflux experiments, 1 mM 3-*O*-methylglucose. The K^+ content was determined before or after washing for a further 150 min. Additions to the wash solutions were introduced after 90 min of washout (for the K^+-free experiments, K^+ omitted after 80 min of washout). The results are given as μmoles of K^+ per g wet wt. ± S.E. with the number of muscles given in parentheses.

Conditions	*μmoles K^+/g wet wt.*	*Significance of difference between controls and experimental, P*
Freshly dissected	89.8 ± 1.1 (20)	
After loading	73.9 ± 0.9 (12)	
After loading and washing for 150 min		
Controls	74.3 ± 1.1 (17)	
K^+-free medium	60.7 ± 0.9 (18)	< 0.001
Ouabain (1 mM)	55.0 ± 1.9 (10)	< 0.001
2,4-Dinitrophenol (0.05 mM)	56.8 ± 1.3 (6)	< 0.001
2,4-Dinitrophenol (0.5 mM)	42.0 ± 0.8 (4)	< 0.001
2,4-Dinitrophenol (0.05 mM) + phlorizin (5 mM)	52.6 ± 1.5 (6)	< 0.001
Phlorizin (5 mM)	72.9 ± 1.2 (8)	N.S.*
Cyanide (2 mM)	72.3 ± 1.6 (7)	N.S.
Salicylate (5 mM)	73.8 ± 3.4 (3)	N.S.
Ethacrynic acid (1 mM)	74.6 ± 2.1 (3)	N.S.
Trypsin (1 mg/ml)	75.0 ± 1.7 (3)	N.S.
Insulin (100 mU/ml)	78.6 ± 1.2 (12)	< 0.02

* N.S., not significant.

From Table I it can be seen that within the first 60 min of incubation, the muscles reach a steady state with respect to K^+ content which is maintained for up to 240 min. As the muscles showed an initial gain in wet weight of 10.9 ± 0.8 %, the real decrease in total K^+ content was only 9 %. After 60 min of incubation, the intracellular K^+ concentration (calculated on the basis of inulin space) was 142.2 ± 2.8 mM which is 92 % of that reported for fresh muscles[18]. This, together with the relatively large inulin space (28.3 ± 0.7 %), suggests that the muscles show an initial increase in extracellular water associated with a minor decrease in the capacity to maintain a concentration gradient for K^+ across the plasma membrane.

This contention is further supported by the observation that the muscles retained excitability for up to 240 min and that the K^+ loss induced during incubation at 0° for 90 min was almost completely restored after a further 90 min at 30°.

Under the conditions used for efflux measurements, the muscles were found to convert [$^{14}C_3$]pyruvate into $^{14}CO_2$ at a constant rate for up to 4 h (0.60 ± 0.03 μmole/g wet wt. per h). Within the same interval of time, the stimulating effect of insulin (1 mU/ml) on the incorporation of [$^{14}C_6$]glucose into glycogen was not significantly altered.

On the basis of these tests it was assumed that the experimental conditions caused no major derangement of functional integrity.

The uptake of glucose and 3-O-methylglucose

When incubated under the conditions used for loading with 3-*O*-methylglucose, soleus muscles were found to take up glucose at a rate of 0.8 μmole/g wet wt. per h (Table II). Allowing for differences in concentration, this is in good agreement with the value estimated on the basis of the data reported by CHAUDRY AND GOULD[1], who used the same incubation medium and muscle size.

Insulin (1 mU/ml) stimulated both the uptake of glucose and its incorporation into glycogen, whereas in the presence of 3-*O*-methylglucose (20 mM) these parameters were significantly decreased. Conversely, as is apparent from Fig. 1, the uptake of

TABLE II

EFFECT OF INSULIN AND 3-*O*-METHYLGLUCOSE ON THE UPTAKE AND INCORPORATION OF GLUCOSE INTO GLYCOGEN

Soleus muscles were incubated for 60 min at 30° in 1 ml of Krebs–Ringer bicarbonate buffer containing 1 mM pyruvate, 1 mM D-[$^{14}C_6$]glucose (0.2 μC/ml) with and without the additions indicated. The results are given as mean ± S.E. with the number of observations given in parentheses.

Addition	*Glucose disappearance (μmoles/g wet wt. per h)*	*P*	*μmoles of glucose incorporated into glycogen/g wet wt. per h*	*P*
Control	0.85 ± 0.08 (6)	<0.001	0.141 ± 0.027 (6)	<0.001
Insulin (1 mU/ml)	1.98 ± 0.11 (6)		0.671 ± 0.049 (6)	
Control	0.81 ± 0.02 (5)	<0.001	0.185 ± 0.014 (5)	<0.001
3-*O*-Methylglucose (20 mM)	0.58 ± 0.03 (5)		0.098 ± 0.010 (5)	
Control	0.68 ± 0.03 (5)	N.S.		
Ouabain (1 mM)	0.67 ± 0.03 (5)			

3-*O*-methylglucose is diminished by the addition of glucose. In the presence of phlorizin (5 mM) the basal uptake of 3-*O*-methylglucose was reduced to approximately the same level. The stimulating effect of insulin is considerably reduced by either glucose (20 mM) or phlorizin (5 mM). At 0°, the uptake of 3-*O*-methylglucose in the water space not available to inulin was significantly lowered (to around 1/8 of the control level).

In the presence of ouabain (1 mM), the uptake of 3-*O*-methylglucose within 60 min was 0.451 ± 0.009 μmole/g wet wt., which is almost identical with the control value (0.439 ± 0.008). Furthermore, in the same time interval, the glycoside produced no change whatsoever in the uptake of glucose (Table II).

As already reported for glucose[1], the uptake of 3-*O*-methylglucose was found to show saturation phenomena. However, since the apparent K_m for this process seemed to be greater than 40 mM, the validity of a kinetic characterization is limited by the necessity of measuring sugar transport at concentrations which would increase the osmolarity by an amount sufficient to cause stimulation of both glucose and 3-*O*-methylglucose transport[15].

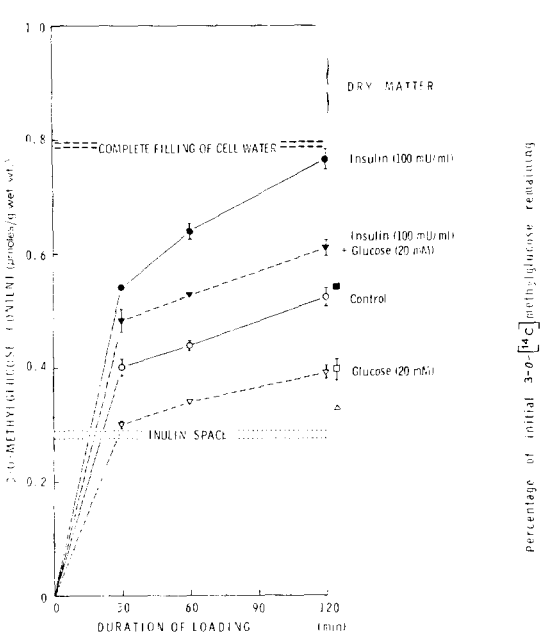
More significantly, measurements of the uptake of nonmetabolized sugars detect only the net result of influx and efflux, and as pointed out by NARAHARA AND ÖZAND[14], already at 10% filling of the cytoplasm, the efflux introduces a significant error. Under basal conditions, 22% intracellular filling is already achieved within 30 min (Fig. 1), which is the minimum interval of time required for equilibration in the extracellular space. Therefore, the effects of conditions which stimulate sugar transport can be considerably underestimated, especially when their action has a time-lag. The fact that the wet weight of soleus muscles shows an initial increase indicates that further inaccuracies can be introduced by the difficulty in defining the size of the extracellular and intracellular spaces.

For these reasons, an attempt was made to measure the transport of 3-*O*-methylglucose in mainly one direction by following the efflux of the labelled sugar from preloaded muscles. The concentration selected for the loading of the tissue (1 mM) is well below the estimated apparent K_m, and the intracellular filling obtained at 1 mM did not differ significantly from that measured at 10 mM. It should be noted that 3-*O*-methylglucose (1 mM) did not produce any change in the Na^+–K^+ content of the muscles.

3-O-Methylglucose efflux—effect of insulin and phlorizin

In order to establish conditions where the rate of 3-*O*-methylglucose release would mainly reflect the transport across the plasma membrane, the time required for the clearing of extracellular space was determined using an extracellular marker, mannitol, which has a molecular weight similar to that of 3-*O*-methylglucose. The washout of mannitol was found to proceed exponentially with a half-time of approx. 6–7 min. Assuming that the diffusion of mannitol and 3-*O*-methylglucose within the extracellular space proceeds at the same rate, more than 99% of the 3-*O*-methylglucose present in the extracellular space at the end of the loading period should be cleared within the first 50 min of washout.

Fig. 2 shows the change in 3-*O*-[^{14}C]methylglucose content during a washout in unlabelled buffer under basal conditions or in the presence of a supramaximal concentration of insulin (100 mU/ml). The marked decrease in 3-*O*-[^{14}C]methylglucose con-



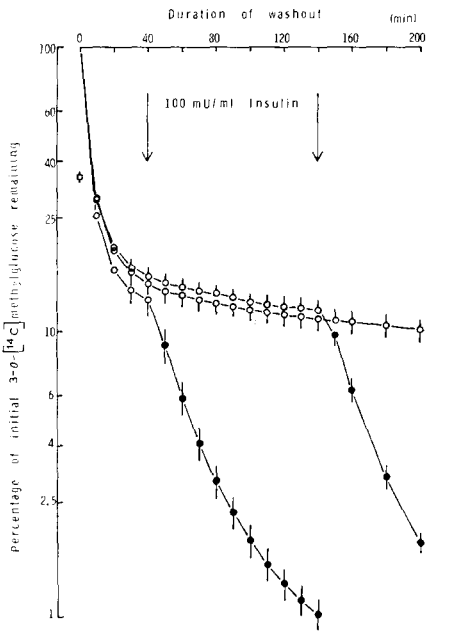


Fig. 1. Time-course of 3-*O*-methylglucose uptake in soleus muscle. Muscles were loaded in 2 ml Krebs–Ringer bicarbonate buffer containing 1 mM 3-*O*-methylglucose, 0.2 μC/ml 3-*O*-[^{14}C]methylglucose and 1 mM pyruvate with the following additions: ○—○, controls; ▽---▽, 20 mM glucose; □, 5 mM phlorizin; ●—●, 100 mU/ml insulin; ▼---▼, 100 mU/ml insulin + 20 mM glucose; ■, 100 mU/ml insulin + 5 mM phlorizin. All tissues were incubated at 30° with the exception of a single group which was loaded for 120 min at 0° (△). Each point represents the mean of 3–6 observations, and vertical bars are used to indicate the S.E. where this exceeds the size of the symbol. Total cell water and inulin space after 60 min loading are represented by pairs of horizontal lines indicating the mean ± S.E. for 28 muscles.

Fig. 2. 3-*O*-[^{14}C]glucose content of soleus muscles during washout. Muscles were loaded for 60 min in Krebs–Ringer bicarbonate buffer containing 1 mM 3-*O*-methylglucose, 2 μC/ml 3-*O*-[^{14}C]methylglucose, and 1 mM pyruvate. After loading the muscles were mounted on rigid polythylene tubes, and the washout was made by transferring the tissues through a series of centrifuge tubes containing 3 ml of the same buffer with 1 mM pyruvate but no 3-*O*-methylglucose. The ^{14}C activity remaining in the tissues is given as a percentage of the total content at the onset of the washout. The initial level of 3-*O*-methylglucose present in the space not available to inulin is indicated by an open square. For two groups of muscles, 100 mU/ml insulin was included in the efflux medium as indicated by arrows and closed symbols. Each point represents the mean of 4 observations with bars indicating S.E.

tent following the addition of the hormone indicates that the slow component of the washout curve represents transport out of an intracellular compartment, and not diffusion from deeper parts of the interstitial space. This contention is further supported by the fact that phlorizin or cooling promptly suppresses 3-*O*-methylglucose release (Fig. 3).

The slow component of the unstimulated efflux has an intercept which is significantly lower than the estimated intracellular concentration at the start of washout (Fig. 2). Moreover, unlike the situation in epididymal fat pads[9], the rate coefficient of 3-*O*-methylglucose release continues to fall for at least 180 min after the onset of the efflux period (Fig. 3). This suggests that it is not valid to treat the intracellular volume of all the cells in a given muscle as a single homogeneous compartment.

In the interval 60–80 min after the start of washout, the rate coefficients measured in 128 experiments showed a distribution closely corresponding to the calculated Gaussian, and a probit test confirmed that the observations were normally distributed. In the range 25–40 mg there was no significant correlation between the rate coefficient and muscle wet weight.

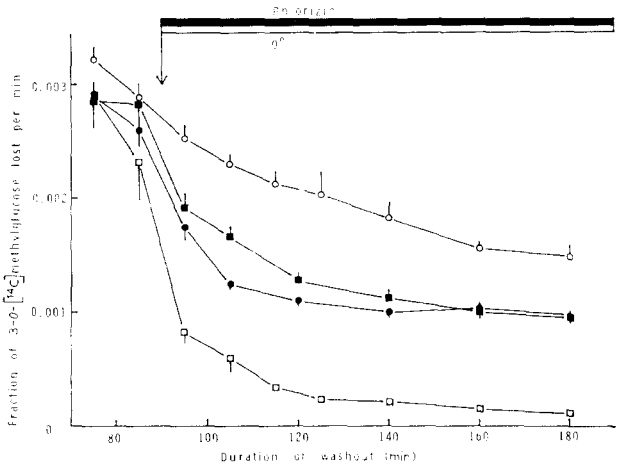


Fig. 3. Effect of phlorizin and cooling on the rate coefficient of 3-*O*-methylglucose release. Muscles were loaded and washed as described under Fig. 2. The fraction of ^{14}C activity lost per min from the tissue during each interval of the washout period is shown as a function of time. ○—○, controls; ■—■, 1 mM phlorizin; ●—●, 5 mM phlorizin; □—□, 0°. Each point represents the mean of 4–9 observations with bars indicating S.E. when this value exceeds the size of the symbols.

The addition of phlorizin (5 mM) to the efflux medium promptly reduced the rate coefficient to about half the control level (Fig. 3). A similar decrease with a slightly slower onset was observed with 1 mM. Lowering the temperature to 0° decreased the rate coefficient to a level considerably below that measured in the presence of the glycoside. However, this difference is not necessarily an indication that there is a large proportion of carrier-mediated transport unaffected by phlorizin. In spite of contrary assumptions in the literature, a Q_{10} of 2 would be quite consistent with nonmediated membrane diffusion[19]. From the results shown in Fig. 3 the total 3-*O*-methylglucose efflux seems to have a Q_{10} of around 2.2, which is in the same range as the Q_{10} of 2.0 for the uptake of the sugar (Fig. 1).

Fig. 4 shows the effect of various concentrations of insulin in the efflux medium. A definite dose–response relationship was found in the range, 0.1–5 mU/ml, which is closely similar to that reported for the uptake of 3-*O*-methylglucose in the intact rat diaphragm[6]. The relatively small stimulation obtained with 0.1 mU/ml may be due to adsorption of insulin to glassware. In the presence of bovine serum albumin (0.1 %), the peak response to 0.1 mU/ml was increased several fold to 0.013 min^{-1}, but no significant change in the effect of 1 mU/ml was observed. With albumin, insulin at concentrations down to 0.01 mU/ml produced a detectable stimulation of 3-*O*-methylglucose efflux.

The effect of insulin could be detected within 5 min, the peak response being reached around 15 min after the start of exposure. The increase in rate coefficient

was not sustained but was followed by a drop towards the control level. Efflux experiments performed with the extensor digitorum longus muscle from the same animals yielded an almost identical time-course, indicating that this phenomenon is not specific to muscles containing a large proportion of red fibers (P. G. KOHN AND T. CLAUSEN, unpublished observations). The same pattern, but even more accentuated, was obtained in efflux experiments with epididymal fat pads, and it was suggested that this could be the outcome of extracellular diffusion being the rate-limiting factor for efflux[9]. However, experiments performed with soleus muscles showed that when exposed to insulin, small muscles (15 mg) showed an even more rapid reversal of the rate coefficient than large ones (45 mg).

As can be seen from Fig. 5, phlorizin in the concentration range, 0.2–5 mM, produces a graded suppression of the insulin-induced rise in rate coefficient. When tested with insulin at a supramaximal concentration (100 mU/ml), phlorizin (5 mM) was found to reduce the peak response to insulin by more than 90 %.

When added after the onset of an insulin-induced rise in rate coefficient, phlorizin promptly suppressed the release of 3-*O*-methylglucose. This indicates that the glycoside does not exert its action by preventing the access or binding of insulin to the plasma membrane. Furthermore the effect of insulin on $^{22}Na^+$ efflux was found to be completely unaffected by phlorizin[20].

The considerable suppression of the insulin-stimulated 3-*O*-methylglucose efflux

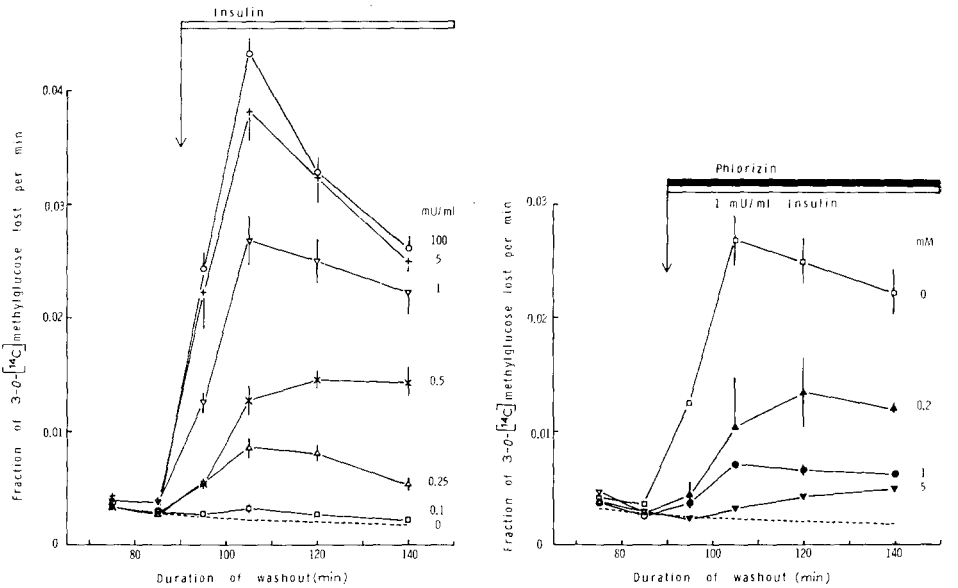


Fig. 4. Effect of insulin on 3-*O*-methylglucose release. Experimental conditions as in Fig. 2. Insulin (0.1–100 mU/ml) was present in the efflux medium during the period indicated by the horizontal bar. The rate coefficient of washout in basal buffer is given by the dashed line. Each point represents the mean of 4–10 observations with bars indicating S.E.

Fig. 5. Effect of phlorizin and insulin on 3-*O*-methylglucose release. Experimental conditions as in Fig. 2. Insulin (1 mU/ml) and phlorizin at the indicated concentrations were present in the efflux medium during the period indicated by the horizontal bars. The rate coefficient of washout in basal buffer is given by the dashed line. Each point represents the mean of 3 observations with bars indicating S.E.

by phlorizin, together with the observation that at 0° insulin produced no increase in rate coefficient whatsoever, indicates that the hormone augments 3-*O*-methylglucose efflux by accelerating the carrier-mediated component of this process.

3-O-Methylglucose efflux—effects of other stimuli and K^+-depletion

It has earlier been shown that phlorizin can abolish the stimulation of 3-*O*-methylglucose efflux induced by hyperosmolarity[15]. From Figs. 6 and 7 it can be seen that the glycoside can also suppress the increase in 3-*O*-methylglucose efflux caused by electrical stimulation or exposure to trypsin. (The increase in 3-*O*-methylglucose release produced by trypsin is probably not the result of contamination with insulin, since soybean trypsin inhibitor abolished the stimulating effect of the enzyme, but not that of the hormone.) The collective evidence indicates that phlorizin inactivates the system mediating the transport of 3-*O*-methylglucose across the plasma membrane, and consequently, this compound has been used as a tool for assessing whether a rise in the rate coefficient of 3-*O*-methylglucose release is due to a nonspecific leak from the cytoplasm or represents an activation of the sugar transport system in the plasma membrane.

In soleus muscle, 2,4-dinitrophenol produces a decrease in K^+ content (Table I), which is in agreement with that reported for rat diaphragm[11] and epididymal adipose tissue[9]. This is associated with an increase in the rate of 3-*O*-methylglucose release (Fig. 8). The fact that phlorizin suppresses the rise (without preventing the K^+ loss) indicates that it is not the outcome of nonspecific cell damage.

Cyanide (2 mM) and salicylate (5 mM) increased the rate coefficient of 3-*O*-

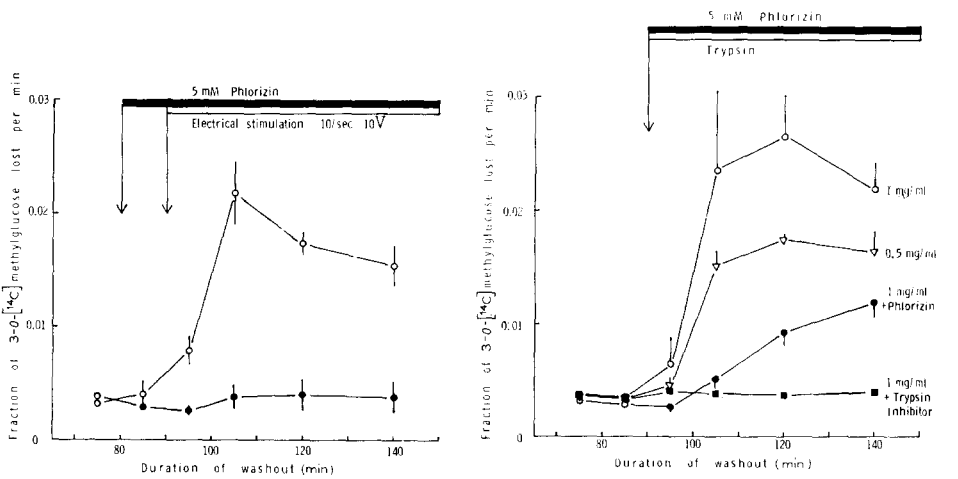


Fig. 6. Effect of electrical stimulation on 3-*O*-methylglucose release. Experimental conditions as in Fig. 2. In the interval indicated by the open horizontal bar all muscles were stimulated by rectangular pulses of 1 msec duration *via* platinum electrodes situated at either end of the muscles. In one group of muscles, phlorizin was present in the efflux medium (●—●). Each point represents the mean of 3 observations with bars indicating 2 × S.E.

Fig. 7. Effect of trypsin on 3-*O*-methylglucose release. Experimental conditions as in Fig. 2. Trypsin with or without phlorizin or trypsin inhibitor was present in the efflux medium during the period indicated by the horizontal bars. ○—○, 1 mg/ml trypsin; ▽—▽, 0.5 mg/ml trypsin; ●—●, 1 mg/ml trypsin + 5 mM phlorizin; ■—■, 1 mg/ml trypsin + 3 mg/ml soybean trypsin inhibitor. Each point represents the mean of 3 observations with bars indicating S.E.

methylglucose efflux by, respectively, 160 and 830 % within 40 min. Again phlorizin (5 mM) produced a significant suppression of these stimuli.

Ethacrynic acid, which produces a decrease in respiration and glycolysis in other cells[21], was found to suppress glycogen synthesis and the conversion of [^{14}C]-pyruvate into CO_2 in soleus muscle (data not presented). This metabolic inhibitor also accelerated 3-*O*-methylglucose efflux (Fig. 9), but like cyanide and salicylate, without producing any significant change in K^+ content (Table II).

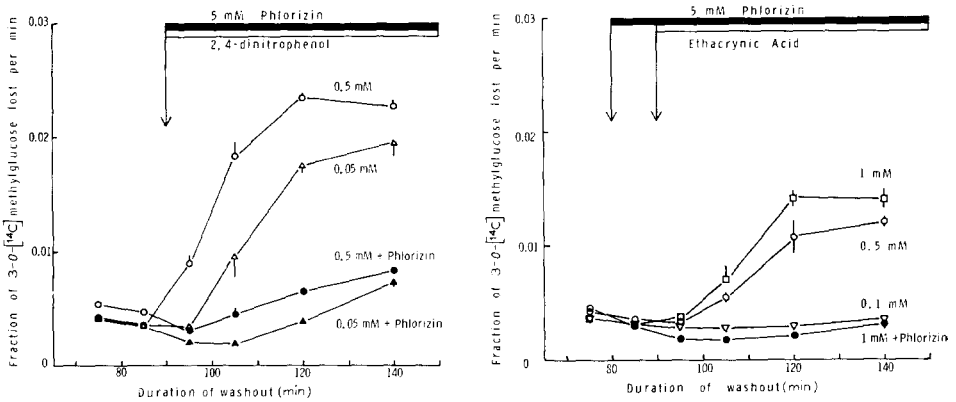


Fig. 8. Effect of 2,4-dinitrophenol on 3-*O*-methylglucose release. Experimental conditions as in Fig. 2. 2,4-Dinitrophenol with and without phlorizin was present in the efflux medium during the period indicated by the horizontal bars. ○—○, 0.5 mM 2,4-dinitrophenol; △—△, 0.05 mM dinitrophenol; ●—●, 0.5 mM dinitrophenol + 5 mM phlorizin; ▲—▲, 0.05 mM dinitrophenol + 5 mM phlorizin. Each point represents the mean of 4–6 observations with bars indicating S.E.

Fig. 9. Effect of ethacrynic acid on 3-*O*-methylglucose release. Experimental conditions as in Fig. 2. Ethacrynic acid with and without phlorizin was present in the efflux medium in the intervals indicated by the horizontal bars. □—□, 1 mM ethacrynic acid; ○—○, 0.5 mM ethacrynic acid; ▽—▽, 0.1 mM ethacrynic acid; ●—●, 1 mM ethacrynic acid + 5 mM phlorizin. Each point represents the mean of 3 observations with bars indicating S.E.

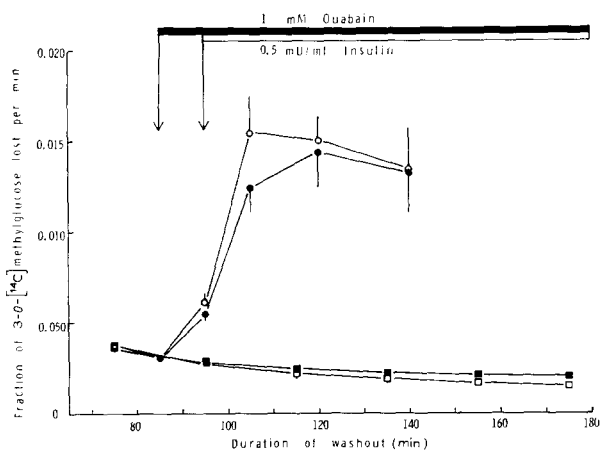


Fig. 10. Effect of ouabain and insulin on 3-*O*-methylglucose release. Experimental conditions as in Fig. 2. Ouabain with and without insulin was present in the efflux medium during the period indicated by the horizontal bars. □—□, controls; ■—■, 1 mM ouabain; ○—○, 0.5 mU/ml insulin; ●—●, 0.5 mU/ml insulin + 1 mM ouabain. Each point represents the mean of 3 observations with bars indicating S.E.

On the other hand, incubation in a K^+-free buffer or the addition of ouabain (1 mM) decreased the K^+ content by about the same amount as dinitrophenol (0.05 mM) (Table I). However, in the presence of ouabain the rate coefficient for 3-*O*-methylglucose efflux showed only a slow rise which after 90 min was still barely significant (Fig. 10). (The effects of K^+ omission are presented elsewhere[20].) In addition, the glycoside produced no change in the stimulation of 3-*O*-methylglucose by insulin in the range, 0.5–100 mU/ml (data for 0.5 mU/ml are shown in Fig. 10). Since ouabain (1 mM) under identical conditions was found to suppress $^{22}Na^+$ efflux to 1/3 of the control level within 30 min after its addition[20], it seems unlikely that inhibition of active Na^+–K^+ transport has any direct effect on basal or insulin-stimulated 3-*O*-methylglucose transport in soleus muscle.

When exposed to ouabain for prolonged periods a number of muscles developed contractures, and in these tissues, the rate coefficient for efflux showed an increase of up to 3-fold above the control level after 240 min. It should be noted that dinitrophenol and ethacrynic acid induced contractures within considerably shorter periods of time (30 min).

DISCUSSION

The aim of the present study has primarily been to develop a sensitive method for the characterization of the processes of sugar transport in an intact peripheral muscle.

In adipocytes[22] and muscle cells[14], the uptake of 3-*O*-methylglucose seems to be mediated by a saturable mechanism, which is sensitive to phlorizin and insulin, and closely similar to, if not identical with the system mediating the transport of glucose across the plasma membrane. The present results show that in the isolated soleus muscle also, the uptake of 3-*O*-methylglucose is accelerated by insulin and suppressed by the addition of glucose or phlorizin. Conversely, the uptake of glucose is inhibited by 3-*O*-methylglucose, and it seems reasonable to assume that in the soleus muscle, these two sugars can share the same transport system. The suppression of both influx and efflux produced with phlorizin suggests that at least half of the basal 3-*O*-methylglucose transport occurs by a carrier-mediated process. Since phlorizin and cooling could abolish the stimulation produced by insulin, this hormone seems to accelerate mainly the carrier-mediated component of 3-*O*-methylglucose transport.

From the time-course of 3-*O*-methylglucose washout, it was estimated that within 200 min the half-time of efflux (after correction for the extracellular component) increases from around 25 to 530 min. This is not compatible with the assumption that the sugar is evenly distributed in a homogeneous intracellular pool. The uptake of 3-*O*-methylglucose proceeds with a rapid initial phase followed by a considerably slower filling, which suggests a similar heterogeneity with respect to influx. Unless each muscle cell contains compartments with widely different exchangeability for 3-*O*-methylglucose, this pattern may best be explained as an outcome of differences in sugar permeability between the individual fibers. When measured under identical conditions, the efflux of Na^+ and K^+ proceeded with a much more constant rate, which suggests that the heterogeneity of pools may be characteristic for sugar transport[20].

Following the addition of insulin, the rate coefficient of 3-*O*-methylglucose efflux showed a rapid increase which could be detected within 5 min. The fact that the rise in rate coefficient is not sustained could have several interpretations. Since this pattern was even more marked in small muscles, it is presumably not the result of diffusion being rate limiting for the access of insulin to or the egress of sugar from deeper cell layers. A decrease in insulin responsiveness with time seems unlikely because of the almost identical effects obtained both early and late in the efflux period (Fig. 2). Chromatography of extracts from insulin-treated muscles showed that retention of ^{14}C activity not representing 3-*O*-[^{14}C]methylglucose could not account for the reversal of the insulin-induced rise in rate coefficient. The phenomenon may, however, be another indication of heterogeneity with respect to sugar permeability. The possibility that individual cells within the same tissue may have very different rates of sugar transport is supported by the studies of GLIEMANN[23], who found large variations in the sensitivity of glucose uptake to insulin among single fat cells isolated from epididymal fat pads.

Since 3-*O*-methylglucose is not concentrated in the cytoplasm of muscle cells[14], it seemed logical to expect that the plasma membrane would display symmetry with respect to the transport of this glucose analogue. This contention is supported by the observation that conditions which stimulate the uptake of 3-*O*-methylglucose also accelerate its efflux[6,14,15]. Furthermore, the decrease in efflux produced by phlorizin is of the same order of magnitude as that obtained in uptake experiments, and at 0°, influx and efflux show similar degrees of suppression. The initial rates of uptake and release are difficult to determine with satisfactory precision but seemed to be roughly similar. (In the interval 0–30 min they were estimated to be 0.12 and 0.16 μmole/g wet wt./mM for influx and efflux, respectively.)

A major advantage of efflux measurements is the possibility of describing the time-course of changes in sugar permeability and their association with shifts in the rate of ion efflux or ionic content. The present results show that ouabain can induce a small increase in 3-*O*-methylglucose efflux, but this effect has a considerably longer time-lag than any of the other stimuli tested. Furthermore, there is a dissociation in time between the effect of ouabain on active Na^+–K^+ transport or K^+ content and that on 3-*O*-methylglucose efflux. Thus the rate coefficient of $^{22}Na^+$ efflux may be decreased by 70 %, and the K^+ content by at least 25 % before any significant change in 3-*O*-methylglucose efflux can be detected. From a comparison between the experiments with ouabain and metabolic inhibitors it is evident that there is no correlation between K^+ content (in the range 55–75 mmoles/g) and the rate of 3-*O*-methylglucose efflux.

When exposed to ouabain for more than 90 min, the muscles tended to contract, and at that stage a progressive stimulation of 3-*O*-methylglucose efflux was found. This is perhaps not so surprising in view of the fact that both spontaneous contractures and, to an even greater extent, those induced by electrical stimulation or metabolic inhibitors were associated with a marked increase in 3-*O*-methylglucose efflux. The late stimulating effect of ouabain on sugar transport is not necessarily the result of muscle contraction but may rather be related to one of the processes involved in the activation of the contractile process. HOLLOSZY AND NARAHARA[24] have suggested that the stimulation of 3-*O*-methylglucose transport produced by contractures is secondary to a rise in the concentration of Ca^{2+} in the cytoplasm.

ACKNOWLEDGEMENTS

The skilled technical assistance of Miss Kirsten Skårup and Miss Karin Skovgaard Sørensen is gratefully acknowledged.

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