A paired-tracer dilution method for characterizing membrane transport in the perfused rat hindlimb

Effects of insulin, feeding and fasting on the kinetics of sugar transport

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(Received 15 March 1983/Accepted 14 June 1983)

1. We have applied the paired-tracer dilution method to the study of transport processes in a mixed mammalian muscle preparation, the perfused rat hindlimb. 2. The method is suitable for the characterization of the kinetic parameters of sugar and amino acid transport and its regulation by hormones, contractile activity, hypoxia, etc. 3. Insulin stimulates sugar transport by increasing the $V_{\max}$ of the process 2–3-fold, but its affinity is unchanged. 4. Starvation increases the affinity of sugar transport in perfused skeletal muscle.

Sugars and other metabolites are taken up into skeletal muscle by the operation of three processes. These are: diffusion across the extracellular space; membrane transport (i.e. the crossing of plasma membranes by metabolites through a carrier-mediated process); and, in the case of sugars at least, post-membrane metabolism, such as glucose phosphorylation. The major aim of the present work is to characterize membrane transport in rat skeletal muscle of a glucose analogue, 2-deoxyglucose, and to investigate the effects of insulin.

Many previous workers have studied sugar transport in muscle but there is no general agreement concerning the kinetic parameters of transport, or the effects on them of insulin. This may reflect the wide variety of preparations and techniques used (Norman *et al.*, 1959; Chaudry & Gould, 1969; Daniel *et al.*, 1975; Narahara & Özand, 1963; Schudt *et al.*, 1976; Baker & Carruthers, 1983).

The perfused rat hindquarter preparation offers many advantages for the study of sugar uptake (Ruderman *et al.*, 1971; Berger *et al.*, 1975), but the enzymic measurement of glucose disappearance from recirculated perfusate or of arteriovenous differences provides estimates of net glucose uptake only and not of membrane transport.

In the present study we have used a paired-tracer dilution method (Yudilevich & Mann, 1983) to measure the transport of 2-deoxyglucose (and also of alanine and leucine) into skeletal muscle. We were particularly interested in using the technique to investigate the effects of insulin on the kinetics of transport of 2-deoxyglucose (as an analogue of glucose) in mixed muscle of the perfused rat hindlimb, since this preparation may be a model more appropriate to the clinical situation than those using isolated incubated muscles predominantly of one fibre type. A preliminary communication of this work has been presented (Bylund-Fellenius *et al.*, 1982).

Methods

Hindlimb preparation

We used a rat hindlimb preparation (Walker *et al.*, 1982) in which entry and exit from the rat hindlimb circulation are gained at the level of the thigh using the femoral artery and vein. In this preparation there is total recovery of perfusate in the venous outflow. Female Sprague–Dawley rats (250 g) were anaesthetized with nembutal (30 mg/kg body wt.), one hindlimb (about 6 g of muscle in these rats) was skinned and the femoral artery and vein were cannulated directly. A ligature was placed around the leg above the cannulation and perfusion

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was immediately started with warmed (38°C) oxygenated (O₂/CO₂, 19:1) Krebs–Henseleit bicarbonate buffer, pH 7.4, containing 6% (w/v) bovine serum albumin, and glucose (1–50 mM). Bovine serum albumin concentration was raised to 6% from the 4% normally used in order to counteract oedema. It had been originally intended to use erythrocytes in the medium, but it was difficult to overcome problems of quenching of radioactivity in the samples. Comparison of phosphocreatine and ATP concentrations in the perfused hindlimb muscle with those in rat muscle suggested that there was no oxygen deficit in resting muscle down to an oxygen delivery of 0.3 μ mol/min per hindlimb, i.e. a flow of 0.5 ml of perfusate/min per hindlimb. The perfusate was pumped without recirculation at 2 ml/min per hindlimb.

The paired tracer dilution method

The method has been recently reviewed (Yudilevich & Mann, 1982). A mixture of two tracers, an extracellular marker and the transportable substance, is injected into the arterial circulation, close to the tissue, and the venous effluent is collected in aliquots. The unidirectional tracer uptake (U) into the tissue is the difference between the percentages of the doses of extracellular reference tracer and transportable tracer (i.e. 2-deoxyglucose) present in the venous effluent at any particular time (see Figs. 1a and 1b). The unidirectional transport rate v_t is equal to $\dot{Q} \times C \times -\ln(1 - U_{\max.})$, where $\dot{Q}$ is perfusate flow in

ml/min $\times$ (g of muscle)⁻¹, C is the perfusate concentration of unlabelled transportable substance (in the present case glucose) and $U_{\max.}$ is the maximum unidirectional uptake (Bustamante *et al.*, 1981). When $U_{\max.}$ is below 25% this reduces to a mathematical expression similar to the Fick equation, i.e.

$$v_t = \dot{Q} \times C \times (U_{\max.})$$

2-Deoxyglucose was chosen as an analogue of glucose since its transport characteristics in muscle tissues are similar to those of glucose (Kipnis & Cori, 1959; Morgan & Whitfield, 1974), but although it is phosphorylated it is not metabolized beyond 2-deoxyglucose 6-phosphate. This is an advantage since labelled metabolites, e.g. lactate, in the venous effluent could not be distinguished from labelled glucose without further processing of the samples. Mannitol (M_r 180) was used as the extracellular reference tracer. Perfusate [100 ml containing 5 μ Ci of ³H-labelled 2-deoxyglucose (37.3 Ci/mmol) and 1 μ Ci of mannitol (45 mCi/mmol) (New England Nuclear, Dusseldorf, Germany) in Krebs–Henseleit bicarbonate buffer] was injected through latex tubing connected to the arterial cannula, and 5 s later collection of the venous effluent into plastic scintillation minivials was begun. Four drops were counted into each tube.

Because of the presence in the perfusate of albumin, a tissue solubilizer, Soluene (Packard Inc.) (0.5 ml/tube) was used. To achieve stability of samples it was necessary immediately to mix the

Fig. 1. (a) Dilution for D-[¹⁴C]mannitol (1 μ Ci) as extracellular tracer and D-2-deoxy[³H]glucose (5 μ Ci) as transported tracer during perfusion of rat hindlimb and (b) unidirectional uptake curves for 2-deoxyglucose during perfusion at 1 ml/min per hindlimb in the presence of 2 mM- and 25 mM-D-glucose

solubilizer with the perfusate in the tubes using a vortex mixer, add scintillant (Dimilume-30; Packard Inc.) to the resulting emulsion, then immediately mix again. The capped tubes were counted for ^3H and ^{14}C using channel settings that allowed no spill-over of counts from the ^3H to the ^{14}C channel and only approx. 10% of ^{14}C to the ^3H channel. Counts were corrected for quenching and spill-over between channels. A computer programmed to make the simple arithmetical manipulations of the data was used to produce dilution and unidirectional uptake curves for each experiment (Figs. 1a and 1b).

Each preparation could be used for repeated transport measurements with successive injections and collections being made at 10 min intervals. Since the amounts of 2-deoxyglucose injected were extremely small (about 142 pmol/injection) accumulation of 2-deoxyglucose 6-phosphate with repeated injections was negligible. The background radioactivity of the venous effluent could be corrected for by taking baseline samples immediately before injection.

Studies on the effects of insulin, feeding and starvation on glucose transport

Rats, either fed or 48 h starved, were perfused at glucose concentrations of 1, 2, 5, 10, 15 and 50 mM. The effects of insulin were studied at a concen-

tration of 0.1 unit/litre of perfusate. The insulin concentration of the perfusate was checked by radioimmunoassay. All perfusions began with 10 min equilibration using 5 mM-glucose, but thereafter glucose concentrations were chosen at random from a collection of perfusate containers. Two injections and collections were made at each concentration.

Calculation of kinetic constants for 2-deoxyglucose and glucose transport

We chose to use three perfusate glucose concentrations below the expected glucose concentration (5–10 mM) for half maximal inhibition of transport of 2-deoxyglucose and three above (i.e. 1, 2, 5, 10, 25 and 50 mM). The kinetic constants were calculated by means of a computer program designed to solve a single Michaelis–Menten equation using non-linear regressions for calculated 2-deoxyglucose transport rates at varying perfusate glucose concentrations (Atkins & Gardner, 1977; Marquardt, 1963). Strictly speaking, the concentration of glucose at which 2-deoxyglucose transport is half-maximal is the K_i of the process rather than the K_m , but for the present purposes we have assumed them to be identical. If two substrates such as glucose and 2-deoxyglucose share a common carrier site, the inhibition constant K_i for glucose acting as an

Fig. 2. (a) Unidirectional uptake curves of 2-deoxyglucose in the presence of 5 mM-glucose and 0.1 unit of insulin/litre during perfusion of starved rat hindlimb at 1, 2, 4 and 8 ml/min and (b) transport of sugar calculated from influx, flow rate and perfusate glucose concentration

Data are derived from experiments performed with an injection of 5 μCi of 2-deoxy[^3H]glucose and 1 μCi of [^{14}C]mannitol.

inhibitor of transport of 2-deoxyglucose should be the same as the K_m for its own transport (Neame & Richards, 1972; Christensen, 1975). Our analysis showed the best fit of our data when calculations were made using a single Michaelis–Menten equation, and we take this as evidence of a single carrier site.

Results

Effects of perfusate flow on glucose uptake in perfused hindlimb (Fig. 2)

It has been suggested that glucose uptake by skeletal muscle depends upon total glucose delivery (Grubb & Snarr, 1977; Schultz *et al.*, 1977). In order to check on effects of perfusate flow, experiments were carried out using two injections-and-collections each, at flow rates of 1, 2, 4 and 8 ml/min with 5 mM-glucose in the perfusate. The protocol was repeated in two further preparations with similar results. The results shown in Figs. 2(a) and 2(b) demonstrate that perfused rat hindlimb preparation autoregulates 2-deoxyglucose transport independently of flow. In subsequent experiments flow was set at 2 ml/min per hindlimb for rats of 250 g body wt., i.e. approx. 0.33 ml/min per g of perfused muscle.

Effects of insulin and feeding and fasting on 2-deoxyglucose uptake (Table 1)

The results in Fig. 1 clearly show that 2-deoxyglucose transport is depressed by competition with unlabelled D-glucose in the perfusate. Calculation of the extent of inhibition of the transport of 2-deoxyglucose at different glucose concentrations showed that the transport followed simple Michaelis–Menten kinetics, suggesting the existence of a single common carrier for the sugars. The presence of 0.1 unit of insulin/litre increased the

transport rate by approx. 2.5-fold and kinetic analysis of the data indicates that this is an effect on the maximal velocity of 2-deoxyglucose transport, whereas the relative affinity of the transport process for 2-deoxyglucose is unaltered. The effects of insulin seem to be independent of the state of feeding or fasting in the animal, but fasting itself alters both the maximal transport rate and the relative affinity of transport of 2-deoxyglucose (Table 1).

Other applications for the tracer dilution technique in perfused rat hindlimb

We have made preliminary experiments using the technique to study the effects of hypoxia and muscular contraction on sugar transport (Figs. 3a and 3b) and have also demonstrated that the technique may be used to study amino acid transport into muscle (Figs. 4a and 4b). The technique is sensitive enough to detect physiologically important modulations of sugar transport and could be used to dissect out the control

Table 1. *Summary of rat hindlimb glucose kinetics in fed and fasted animals*

Values (means $\pm$ S.E.M.) are derived from linear regression analysis of transport data assuming single-site Michaelis–Menten kinetics and that 2-deoxyglucose is an appropriate tracer for glucose (see the text for discussion).

Condition of rat preparation	K_m (mM)	V_{max} (μ mol/min per g)
Fed, no insulin, rest ($n=6$)	10.50 ± 0.87	0.251 ± 0.01
Fed, 0.1 unit of insulin/litre, rest ($n=4$)	9.92 ± 0.90	0.67 ± 0.07
Starved, no insulin, rest ($n=6$)	4.14 ± 0.93	0.37 ± 0.04
Starved, 0.1 unit of insulin/litre, rest, ($n=5$)	5.96 ± 1.00	1.03 ± 0.20

Fig. 3. *Unidirectional uptake curves for 2-deoxyglucose in the presence of 10 mM-glucose*

(a) At rest under normoxic (pO_2 , 65 kPa) and hypoxic (pO_2 , 10 kPa) conditions and (b) at rest and during muscular contraction induced by direct muscle stimulation 10V, 60Hz, 1 train/s, 0.5 ms stimuli in perfused rat hindlimb.

Fig. 4. Unidirectional uptake curves of (a) leucine and (b) alanine during perfusion of rat hindlimb at 2 ml/min. Tracer injectate contained about 5 μ Ci of L-[4,5- 3 H(n)]leucine (5.0 Ci/mmol) or L-[3- 3 H]alanine (70–85 Ci/mmol) as well as 1 μ Ci of [14 C]mannitol (145 mCi/mmol) in the presence of 5 mM-glucose, which was also present in the perfusate.

mechanisms for both sugar and amino acid transport. An important facet of the use of tracers which are not trapped intracellularly is that if collections are made for sufficiently long periods following injection, efflux of the labelled substrate (or its metabolites) out of the tissue may be quantified (see Mann *et al.*, 1982). Such information about both inward and outward transport rates (e.g. see Fig. 4b) could provide information not only on trans-stimulation or trans-inhibition of uptake but also on muscle metabolism over the very short term. For example, in a study by similar methods of the perfused salivary gland, efflux of 3 H-labelled metabolites of alanine could be abolished with amino-oxyacetate, an inhibitor of alanine aminotransferase (Mann *et al.*, 1982).

Discussion

The present results demonstrate that the tracer dilution technique can be used with advantage to obtain information about the kinetics of transport of sugars (and of other low molecular weight substances) into perfused rat skeletal muscle. The relevance of the results obtained for kinetic characteristics of sugar transport in muscle depend to a large extent upon the inherent limitations of the preparation used and upon the similarity of the

transport parameters of 2-deoxyglucose and glucose.

The perfused rat hindlimb preparation is more likely to reflect the characteristics of skeletal muscle in adult mammals, including man, than are preparations of, for example, frog sartorius, rat heart, diaphragm, and soleus muscle, barnacle muscle or cultured chick myoblasts used by previous investigators (Table 2). The preparation is composed of a mixture of muscle fibres with metabolic characteristics broadly similar to those found in man and in similar proportions (Baldwin *et al.*, 1972; Saltin, 1977). In addition, glucose transport in the perfused preparation is less likely to suffer from diffusion limitations than are incubated muscle preparations.

Whether or not 2-deoxyglucose is a perfect analogue of glucose for transport studies in perfused muscle is not easily resolvable with the present technique because of the problems of labelled lactate efflux following transport of labelled glucose. However, numerous investigators have demonstrated that in adipose tissue, heart and rat diaphragm there is a striking similarity between the characteristics of the transport of the two sugars (Park *et al.*, 1968; Morgan & Whitfield, 1974; Kipnis & Cori, 1959).

The present results suggest that in the physiological circumstances pertaining in the rat the main modulator of glucose uptake would be the avail-

Table 2. *Published values for glucose transport parameters in muscle*

Units of K_m are mM and of V_{max} are $\mu\text{mol/min}$ per g of muscle. Values are calculated from authors' values in other units.

Muscle and conditions	No insulin		Supramaximal insulin	
	K_m	V_{max}	K_m	V_{max}
Incubated rat diaphragm (Norman <i>et al.</i> , 1959)	22	0.77	9	0.77
Incubated rat soleus (Chaudry & Gould, 1969)	35	1	12	1
Rat pectoralis major <i>in vivo</i> (Daniel <i>et al.</i> , 1975)	34	1.2	300*	20*
Perfused rat heart (Cheung <i>et al.</i> , 1978)	4	1.2	7*	60*
Incubated frog sartorius (19°C), 3-O-methylglucose (Narahara & Özand, 1963)	4	0.05	4	0.15
Chick myotubes (Schudt <i>et al.</i> , 1976)	13†	10†	32†	32†
Barnacle giant muscle fibres, 3-O-methylglucose (Baker & Carruthers, 1983)	4.2	17‡	4.5	42‡

* Values are approximate due to difficulty of estimating maximum transport rates in presence of insulin.

† Values calculated assuming protein content of cultured myotubes as 180 mg/g wet wt.

‡ Units are pmol of 3-O-methylglucose/s per cm^2 of muscle membrane.

ability of carrier sites. If the relative affinity of the transport process for 2-deoxyglucose (strictly a K_t and not K_m) can be assumed to be similar to the K_m for glucose transport, then this appears to be in the physiological range of rat blood glucose concentration and does not appear to be altered by the presence or absence of insulin. These results are unlike those obtained for incubated rat diaphragm, soleus or pectoralis major muscle *in vivo* (Norman *et al.*, 1959; Chaudry & Gould, 1969; Daniel *et al.*, 1975) or cultured chick myotubes (Schudt *et al.*, 1976) in which the K_m in the absence of insulin was found to be well above the physiological range (Table 2). We have no definitive explanation of this major difference. However, part of the difference may be due to the occurrence of poorly stirred layers of extracellular fluid in incubated preparations, which would lead to an overestimate of the sugar concentration at the transport surface, and thus to falsely high K_m estimates. If the K_m for muscle sugar transport were above the normal circulating glucose concentration (as it is likely to be for the liver)

control of net glucose uptake would reside in control of glucose delivery, i.e. net glucose entry to the hepatic venous blood. This would be efficient when blood glucose is elevated, but may not be so when blood glucose is low, e.g. in fasting or after exhaustive exercise, when there is evidence that the rate of muscle glycogen storage is maximized (Ivy & Holloszy, 1981; Fell *et al.*, 1980; Maehlum *et al.*, 1978).

In the diaphragm (Norman *et al.*, 1959) and soleus (Chaudry & Gould, 1969) preparations the presence of supramaximal insulin caused no effect on the maximal transport rate but doubled and tripled respectively the affinity of the process (Table 2). The study by Daniel *et al.* (1975) in rat pectoralis major muscle *in vivo* suggested that insulin not only massively increased the maximal activity of transport (by 15-fold) but also decreased the affinity by 10-fold. Similar results were obtained by Schudt *et al.* (1976) for chick myotube cultures. Our results, showing no change in transport affinity, but a rise in maximal transport rate, in the presence of insulin are similar to those obtained in one study of perfused rat heart (Cheung *et al.*, 1978) and in giant muscle fibres of the barnacle (Baker & Carruthers, 1983). We therefore believe that the present results are more likely to represent the true physiological circumstances, partly because of the preparation in which they were obtained, but also because the results fit in more easily with what is known about the physiology of glucose homeostasis and the effects of insulin in increasing the mobility of sugar carriers within the cell membrane (Wardzala & Jeanrenaud, 1981).

The effects of starvation in increasing the affinity of the transport process for 2-deoxyglucose were initially surprising to us. This may be partly due to the increased insulin sensitivity demonstrated by different methods in the perfused rat hindquarter (Brady *et al.*, 1981).

In conclusion, we have developed a method for examination of rates of transport of sugars and other low molecular weight substances such as amino acids into perfused skeletal muscle. The method appears to be capable of giving useful information about the physiological control of transport processes.

This work was largely carried out during a sabbatical spent by M.J.R. in the Department of Surgery I, Sahlgrens Hospital, Göteborg, Sweden. The work was supported generously by the Stanley Thomas Johnson Foundation and the Swedish Medical Research Council (no. 536). Work carried out in the U.K. in collaboration with G. E. M. was supported by the Medical Research Council of Great Britain and the Wellcome Trust. The advice and generous assistance of Dr. Michael Gardner in conducting the computer analysis of the transport data is gratefully acknowledged. We thank Professors D. L.

Yudilevich and R. H. T. Edwards for help and encouragement. The technical expertise of M. Andersson, A. Arvidsson and L. Hellenäs was invaluable. M. J. R. is a Wellcome Senior Lecturer.

References

- Atkins, G. L. & Gardner, M. L. G. (1977) *Biochim. Biophys. Acta* **468**, 127–145
- Baker, F. & Carruthers, H. (1983) *J. Physiol. (London)* **336**, 397–431
- Baldwin, A. M., Klinkerfuss, G. H., Terjung, R. L., Molé, P. A. & Holloszy, J. O. (1972) *Am. J. Physiol.* **222**, 373–378
- Berger, M., Hagg, S. & Ruderman, M. B. (1975) *Biochem. J.* **146**, 231–238
- Brady, L. J., Goodman, M. N., Kalish, F. N. & Ruderman, N. B. (1981) *Am. J. Physiol.* **240**, E184–E190
- Bustamante, J. C., Mann, G. E. & Yudilevich, D. L. (1981) *J. Physiol. (London)* **313**, 65–79
- Bylund-Fellenius, A. C., Idström, J. P., Mann, G. E., Rennie, M. J. & Scherstén, T. (1982) *J. Physiol. (London)* **324**, 18P–19P
- Chaudry, I. H. & Gould, M. K. (1969) *Biochim. Biophys. Acta* **177**, 527–534
- Cheung, J. T., Conover, C., Regan, D. M., Whitfield, C. F. & Morgan, A. G. (1978) *Am. J. Physiol.* **234**, E70–E78
- Christensen, H. N. (1975) *Biological Transport*, 2nd ed., Benjamin Cummins, Menlo Park, CA
- Daniel, P. M., Love, E. R. & Pratt, O. E. (1975) *J. Physiol. (London)* **247**, 273–288
- Fell, R. D., McLane, J. A., Winder, W. W. & Holloszy, J. O. (1980) *Am. J. Physiol.* **238**, R328–R332
- Grubb, B. & Snarr, J. B. (1977) *Proc. Soc. Exp. Biol. Med.* **154**, 33–36
- Ivy, J. & Holloszy, J. O. (1981) *Am. J. Physiol.* **241**, C200–C203
- Kipnis, D. M. & Cori, C. (1959) *J. Biol. Chem.* **234**, 171–176
- Maehlum, S., Felig, P. & Wahren, J. (1978) *Am. J. Physiol.* **235**, E255–E260
- Mann, G. E., Tradatti, C. E. & Yudilevich, D. L. (1982) *Biochim. Biophys. Acta* **692**, 157–160
- Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math.* **11**, 431–441
- Morgan, H. E. & Whitfield, C. F. (1974) *Curr. Top. Membr. Transport* **4**, 255–272
- Narahara, H. T. & Özand, P. (1963) *J. Biol. Chem.* **238**, 40–49
- Neame, K. D. & Richards, T. G. (1972) *Elementary Kinetics of Membrane Carrier Transport*, Blackwell Scientific, Oxford
- Norman, D., Menozzi, P., Reid, D., Lester, G. & Hechter, O. (1959) *J. Gen. Physiol.* **42**, 1277–1282
- Park, C. R., Crofford, O. B. & Kono, T. (1968) *J. Gen. Physiol.* **51**, 2965–2987
- Ruderman, N. B., Houghton, C. R. S. & Hems, R. (1971) *Biochem. J.* **124**, 639–651
- Saltin, B., Henriksson, J., Nygaard, E., Andersen, P. & Jansson, E. (1977) *Ann. N.Y. Acad. Sci.* **301**, 2–29
- Schudt, C., Gaertner, U. & Pette, D. (1976) *Eur. J. Biochem.* **68**, 103–111
- Schultz, T. A., Lewis, S. B., Westbie, D. K., Wallin, J. D. & Gerich, J. E. (1977) *Am. J. Physiol.* **233**, E514–E518
- Walker, P. M., Idström, J.-P., Scherstén, T. & Bylund-Fellenius, A. C. (1982) *Eur. J. Appl. Physiol.* **48**, 163–176
- Wardzala, L. J. & Jeanrenaud, B. (1981) *J. Biol. Chem.* **256**, 7090–7093
- Yudilevich, D. L. & Mann, G. E. (1982) *Fed. Prod. Fed. Am. Soc. Exp. Biol.* **41**, 3045–3053