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Lactate metabolism in the perfused rat hindlimb

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1. A preparation of isolated rat hindleg was perfused with a medium consisting of bicarbonate buffer containing Ficoll and fluorocarbon, containing glucose and/or lactate. The leg was electrically prestimulated to deplete partially muscle glycogen. The glucose was labelled uniformly with ^{14}C and with ^{3}H in positions 2, 5 or 6, and lactate uniformly with ^{14}C and with ^{3}H in positions 2 or 3. 2. Glucose carbon was predominantly recovered in glycogen, and to a lesser extent in lactate. The $^{3}H/^{14}C$ ration in glycogen from [5-^{3}H,U-^{14}C]- and [6-^{3}H,U-^{14}C]-glucose was the same as in glucose. Nearly all the utilized ^{3}H from [2-^{3}H]glucose was recovered as water. Insulin increased glucose uptake and glycogen synthesis 3-fold. 3. When the muscle was perfused with a medium containing 10mM-glucose and 2mM-lactate, there was little change in lactate concentration. ^{14}C from lactate was incorporated into glycogen. There was a marked exponential decrease in lactate specific radioactivity, much greater with [^{3}H]- than with [^{14}C]-lactate. The 'apparent turnover' of [U-^{14}C]lactate was 0.28μmol/min per g of muscle, and those of [2-^{3}H]- and [3-^{3}H]-lactate were both about 0.7μmol/min per g. With 10mM-lactate as sole substrate, there was a net uptake of lactate, at a rate of about 0.15μmol/min per g, and the apparent turnover of [U-^{14}C]lactate was 0.3μmol/min per g. The apparent turnover of [^{3}H]lactate was 3–5 times greater. 4. When glycogen synthesis was low (no prestimulation, no insulin), the incorporation of lactate carbon into glycogen exceeded that from glucose, but at high rates of glycogen deposition the incorporation of lactate carbon was much less than that of glucose. 5. Lactate incorporation into glycogen was similar in fast-twitch white and fast-twitch red muscle, but was very low in slow-twitch red fibres. 6. We find that (*a*) pyruvate in muscle is incorporated into glycogen without randomization of carbon, and synthesis is not inhibited by mercaptopicolinate or cycloserine; (*b*) there is extensive lactate turnover in the absence of net lactate uptake, and there is a large dilution of ^{14}C-labelled lactate from endogenous supply; (*c*) there is extensive detritiation of [2-^{3}H]- and [3-^{3}H]-lactate in excess of ^{14}C utilization.

The conversion of lactate into muscle glycogen was first demonstrated by Meyerhof *et al.* (1925) in frog muscle, and subsequently shown to occur in skeletal muscle of rabbits (Bendall & Taylor, 1970), rat (McLane & Holloszy, 1979) and man (Hermansen & Vaage, 1977). However, the relative roles of glucose and lactate in this synthesis are not clear. The pathways of lactate metabolism in muscle are not well defined. It is yet obscure how pyruvate is converted into phosphoenolpyruvate, and it is not known to what extent lactate is a fuel for muscle metabolism. In an attempt to clarify these issues we perfused the isolated hindleg of rats with ^{14}C- and ^{3}H-labelled glucose and lactate, and measured net uptake of substrates and net glycogen deposition and the incorporation of tracer into products.

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Experimental

Perfusion

The pump and apparatus were similar to those described by Sugano *et al.* (1978). The surgical preparation of the hindquarter was modified from the method of Ruderman *et al.* (1971). The rats

(190–210g body wt.) were anaesthetized with pentobarbital (6.5mg/100g, intraperitoneally). After a midline abdominal incision of the skin, the superficial epigastric vessels were ligated and the abdomen was incised from the pubic symphysis to the xiphoid process. The inferior mesenteric and caudal arteries and the lumbar, inferior epigastric, internal iliac, internal spermatic, deferential and superior vesicle vessels were ligated. The epididymis, testes and part of the descending colon were excised. The neck of the bladder, prostate, seminal vesicle and residual portion of the descending colon were ligated together. A ligature was placed at the base of the tail. The vessels supplying the subcutaneous region were ligated while the skin was carefully removed over the lower part of the animal.

After surgery the femoral and sciatic nerves were stimulated for 3×2.5min at 4 cycles/s, 40V and 5ms duration, with a 5min rest period between stimulations. This treatment decreased the glycogen content by about half. The aorta was then cannulated and the vena cava cut, and the right and left common iliac veins were cannulated. The hindquarter was perfused for 10min at a rate of about 35ml/min with Krebs–Henseleit (1932) buffer at 37°C, gassed with O_2/CO_2 (19:1). The left leg was then rapidly frozen with aluminium tongs cooled with liquid N_2, and the leg was cut off for determination of the initial glycogen content. The left common iliac artery was ligated and the carcass transferred into the perfusion chamber. Recirculating perfusion was performed for 2h with 150ml of Krebs–Henseleit bicarbonate medium containing 6g of Ficoll (Sigma), 30ml of fluorocarbon emulsion (FC-43; Green Cross Co., purchased from Alpha Therapeutics, Los Angeles, CA, U.S.A.) and glucose and/or lactate, with or without insulin. The medium was gassed with humidified O_2/CO_2 (19:1). O_2 uptake was determined with Clark oxygen electrodes in the inflow and the effluent. The medium was sampled at 15-min intervals. At the end of the perfusion, the leg was frozen as described above. In some experiments samples of the soleus and plantaris muscle were separated by inspection from the frozen leg, and white-muscle fibres were obtained from the surface area of the gastrocnemius and vastus lateralis muscle. Freezing had no apparent effect on the metabolism of the second leg (results not shown).

Analytical

The perfusate was deproteinized with $HClO_4$, neutralized with KOH, and the supernatant analysed for glucose, lactate and pyruvate (Golden *et al.*, 1982). The frozen muscle was extracted with boiling 5M-KOH, the glycogen precipitated with 2vol. of 95% (v/v) ethanol, redissolved in 2ml of 0.1M-KOH and re-precipitated with ethanol, and the residue dissolved in water. Samples were used for assays of glycogen and radioactivity (Golden *et al.*, 1982). Enzymes and chemicals were purchased from Sigma (St. Louis, MO, U.S.A.).

Fractionation of labelled compounds

This was essentially as described by Golden *et al.* (1982). Briefly, the neutralized medium was passed through three columns on top of each other, of Dowex 50 (H^+ form), Dowex 1 (acetate form) and Dowex 1 (borate form). The eluate contained ^3H_2O. Amino acids were eluted from the top column with 2M-NH_3; lactate was eluted from the middle column with 2M-acetic acid, followed by 2M-ammonium formate, pH3, for the elution of pyruvate. Glycerol was eluted from the borate column with 0.04M-ammonium tetraborate, followed with elution with 0.5M-acetic acid for glucose. Glycogen glucose was degraded as described by Genovese *et al.* (1970) and Schmidt *et al.* (1970).

Tracers

[2-^{3}H]- and [3-^{3}H]-lactate were prepared as described elsewhere (Okajima *et al.*, 1981). [^{3}H]Lactates are unstable on prolonged storage, even at −20°C, and were tested for purity as follows: the radioactive lactate samples were incubated with 5μmol of lactate carrier for 1.5h in 0.1M-phosphate buffer, pH7.4, 10mM-acetaldehyde, 20mM-glutamate, 0.1mM-NAD^+, 100 units of lactate dehydrogenase (pig heart), 80 units of glutamate–pyruvate transaminase and 180 units of alcohol dehydrogenase. (A 20:1 molar ratio of lactate to NAD^+ is required to transfer quantitatively the ^3H from [2-^{3}H]lactate to ethanol.) The solution was passed through two columns, one ($4cm \times 0.5cm$) of Dowex 50 (H^+ form) over one ($5cm \times 0.5cm$) of Dowex 1 (acetate form), and eluted with 5ml of water. The Dowex 50 column was then eluted with 5ml of 2M-NH_3. A recovery of over 95% of the initial [2-^{3}H]lactate as ethanol in the water eluate and of [^{14}C]- and [^{3}H]-lactate in the NH_3 eluate indicates satisfactory tracer purity. If necessary, lactate was purified by passage through a Dowex 1 (acetate form) column. Contaminants were eluted with 0.25M-acetic acid, and lactate was eluted with 2M-acetic acid. A small amount of NH_3 was added and the solution evaporated to dryness. Labelled glucoses and [U-^{14}C]lactate were purchased from New England Nuclear, and purified as described by Golden *et al.* (1982). [2-^{14}C]Lactate was prepared by enzymic reduction of [2-^{14}C]-pyruvate (Okajima *et al.*, 1981).

Calculations

The muscle weight of one hindleg was determined in a separate set of experiments and ranged from 10.5 to 13g ($n = 11$). A weight of 12g was used for calculations. The $HClO_4$-insoluble defatted dry weight of muscle tissue was 21.5% of wet weight. Results are expressed as μmol per leg or per g of muscle.

The total volume of the system (medium and tissue water) was obtained by dividing the tracer dose by the specific radioactivity extrapolated to zero time from the 15–30min period. The calculated tissue space (total volume minus medium) was about 5ml for glucose, and 17ml for lactate, indicating a higher intracellular lactate concentration than that in the medium.

Apparent turnover of lactate was calculated by dividing the tracer dose by the area under the specific-radioactivity curve (Katz *et al.*, 1981). The specific-radioactivity curves for [^{14}C]lactate could be fitted for the 2h period by two exponentials, and that for [^{3}H]lactate by one exponential.

Results

Glucose utilization

The prestimulated leg was perfused with 10mM-glucose, 2mM-lactate and 0.2mM-pyruvate, with and without insulin. The glucose was labelled uniformly with ^{14}C and in positions 2, 5 and 6 with ^{3}H. Results are summarized in Fig. 1 and Table 1. Glucose uptake was stimulated markedly by insulin (Fig. 1). Without insulin, glucose uptake was initially about 0.3μmol/min per g of muscle, but declined markedly after 30min. With insulin, uptake was 0.6μmol/min per g for the first hour and declined somewhat during the second hour. Glycogen synthesis with and without insulin was

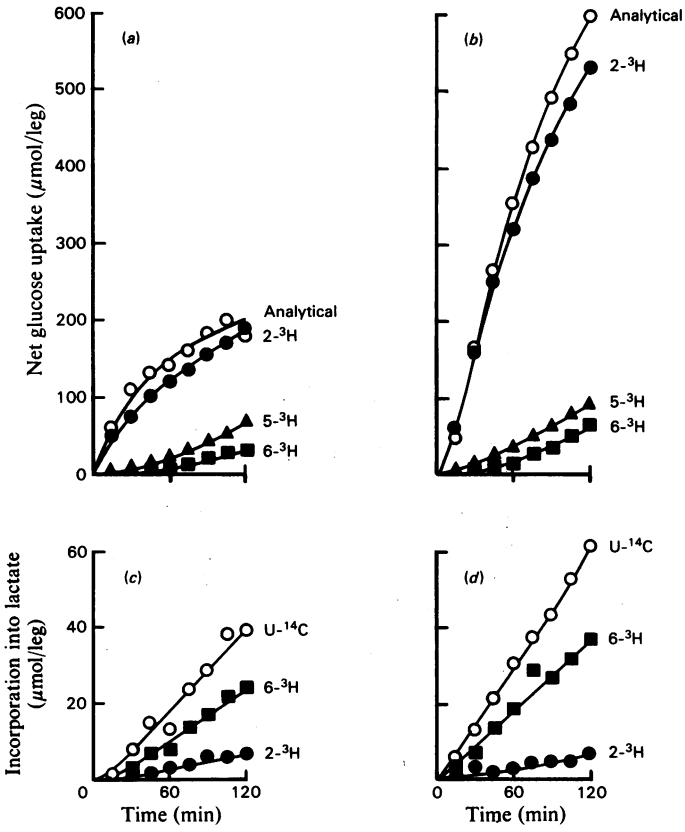

Fig. 1. *Effect of insulin of glucose utilization by perfused rat hindleg*
The prestimulated hindleg was perfused with 10mM-glucose, 2mM-lactate and 0.2mM-pyruvate. The glucose was labelled uniformly with ^{14}C or with ^{3}H in position 2, 5 or 6. Insulin was 10munits/ml. Results are expressed as μmol of C_6 units or as μmol of ^{3}HOH. (*a*) Net glucose uptake as determined by chemical analysis (○) and by apparent yield of ^{3}HOH from (●) [2-^{3}H]-, (▲) [5-^{3}H]- and (■) [6-^{3}H]-glucose. (*b*) Same as (*a*), but in the presence of insulin. (*c*) Apparent incorporation of ^{14}C and ^{3}H into lactate, from (○) [U-^{14}C]- or from (●) [2-^{3}H]- or (■) [6-^{3}H]-glucose. (*d*) Same as (*c*), but in the presence of insulin.

Table 1. *Distribution of ^3H and ^{14}C from glucose in prestimulated resting hindlimb*

The hindlimb was perfused for 2h with [2-^{3}H,U-^{14}C]-, [5-^{3}H,U-^{14}C]- or [6-^{3}H,U-^{14}C]-glucose with 10mM-glucose, 2mM-lactate and 0.2mM-pyruvate. Results are means ± S.E.M., averages of eight experiments with ^{14}C, or of two to three experiments with each ^{3}H tracer. Mean concentrations of substrates are those at 1 h of perfusion: (−) refers to uptake and (+) to production.

Insulin (10munits/ml) ...	Absent	Present
Mean [glucose] (mM)	9.4 ±0.2	7.2 ±0.01
Mean [lactate] (mM)	2.3 ±0.2	1.9 ±0.1
Mean [pyruvate] (mM)	0.13±0.01	0.14±0.01
Initial glycogen (μmol/leg)	186±23	153±15
Δ Glucose (μmol/2h per leg)	−188±27	−595±58
Δ Lactate (μmol/2h per leg)	67±30	−4±31
Δ Pyruvate (μmol/2h per leg)	−12± 4	−17± 1
Δ Glycogen (μmol/2h per leg)	161±19	561±39
O_2 consumption (μmol/2h per leg)	730±50	1085±10
Recovery (%) of tracer dose of ^{14}C in:		
Glucose	89 ±2.9	60 ±10
Glycogen	7.4±0.9	23.6± 1.3
Lactate	2.3±0.6	3.6± 0.5
Total	99 ±2.1	87 ± 4.0
Recovery (%) of tracer dose of ^{3}H in ^{3}HOH		
2-^{3}H	12	35
5-^{3}H	4.2	7.7
6-^{3}H	2.5	4.3
^{3}H/^{14}C ratio in lactate*		
2-^{3}H	0.18	0.11
5-^{3}H	0	0
6-^{3}H	0.58	0.60
^{3}H/^{14}C ratio in glycogen*		
2-^{3}H	0.05	0.03
5-^{3}H	0.90	0.99
6-^{3}H	1.00	1.11

* The ^{3}H/^{14}C ratios are those at the end of the experiment, relative to those in substrate glucose (^{3}H/^{14}C ratio in glucose set to 1.00).

0.1 and 0.4μmol/min per g respectively. Glucose uptake as measured by chemical analysis and ^{3}HOH production from [2-^{3}H]glucose was much the same, and thus ^{3}HOH production from this tracer may be used conveniently to measure glucose utilization by skeletal muscle. The specific radioactivities of labelled glucoses remained virtually unchanged, and thus there is no production or recycling of glucose.

Insulin over the 2h period increased glucose uptake 3-fold. There was a small formation of lactate without insulin, but virtually no change in lactate concentration in its presence. Glycogen was the major product, and with insulin it accounted for 94% of the glucose uptake, as measured by chemical analysis. $^{14}CO_2$ production was not measured. The most reliable estimate of the upper limit of glucose oxidation is provided by ^{3}HOH yield from [6-^{3}H]glucose. After correction for the ^{3}H retention in lactate, less than 10% of the utilized glucose was oxidized to CO_2, and thus it appears that most of the metabolic fuel of the resting leg was provided by tissue lipids. Berger *et al.* (1976) recovered in the presence of insulin 7%, and Karlsson *et al.* (1976) 5%, of the utilized glucose in CO_2. The glucose-uptake and glycogen-synthesis rates in our preparation are somewhat higher than those reported for perfused hindleg preparations (Karlsson *et al.*, 1976; Berger *et al.*, 1976) and in the range of rates reported *in vivo* (Conlee *et al.*, 1978; Terjung *et al.*, 1974). There was very little ^{3}H from position 2 retained in glycogen, and it appears thus that the activity of hexose-6-phosphate isomerase is very high relative to glucose flux (Katz & Rognstad, 1976). ^{3}H from positions 5 and 6 was completely retained in glycogen. This excludes recycling between fructose 6-phosphate and fructose 1,6-bisphosphate.

Although in the presence of insulin there was no net production of lactate, some 10% of the utilized ^{14}C was recovered in this compound. As expected, there was virtually no ^{3}H from position 5, but there was a substantial retention of ^{3}H from position 6 of glucose in lactate.

Lactate utilization

Perfusions with a medium containing 10mM-glucose and 2mM-[2-^{3}H,U-^{14}C]- or [3-^{3}H,U-^{14}C]-lactate are illustrated in the experiments of Fig. 2. There was a small net production of lactate (0.06μmol/min per g) during the first hour and no change in lactate content during the second hour. However, there was a marked exponential decrease in the [^{14}C]lactate specific radioactivity and a much more rapid decline in that of ^{3}H. Assuming a steady state, the apparent replacement rates were calculated to be 3.4 and 9.1μmol/min per leg for [U-^{14}C]- and [2-^{3}H]-lactate respectively. The apparent replacement of [3-^{3}H]lactate was somewhat less than that of the [2-^{3}H]lactate.

The incorporation of tracer is summarized in Table 2. In 2h over half of the ^{14}C was utilized. About one-third of the utilized ^{14}C was in glycogen, and a small fraction each in pyruvate, amino acids (presumably mainly alanine and glycerol. Most of the unaccounted-for radioactivity, about half of the utilized [^{14}C]lactate, is most likely in CO_2. From the apparent replacement rates and isotope yields, the apparent rate of lactate incorporation into glycogen was calculated to be approx. 0.09, and that into CO_2 estimated as 0.15μmol/min per g. Nearly 90% of the [2-^{3}H]- and 80% of the [3-^{3}H]-lactate were utilized, with nearly all the utilized ^{3}H appearing as water.

In Fig. 3 and Table 3, experiments are shown with perfusion of the hindleg with 10mM-lactate. Such concentrations of lactate are attained after vigorous exercise, but the condition is unphysiological inasmuch as there was no glucose. There was a net uptake of lactate at a rate of 0.17μmol/min per g. There was net glycogen synthesis, 0.06μmol/min per g of glucose equivalents. The

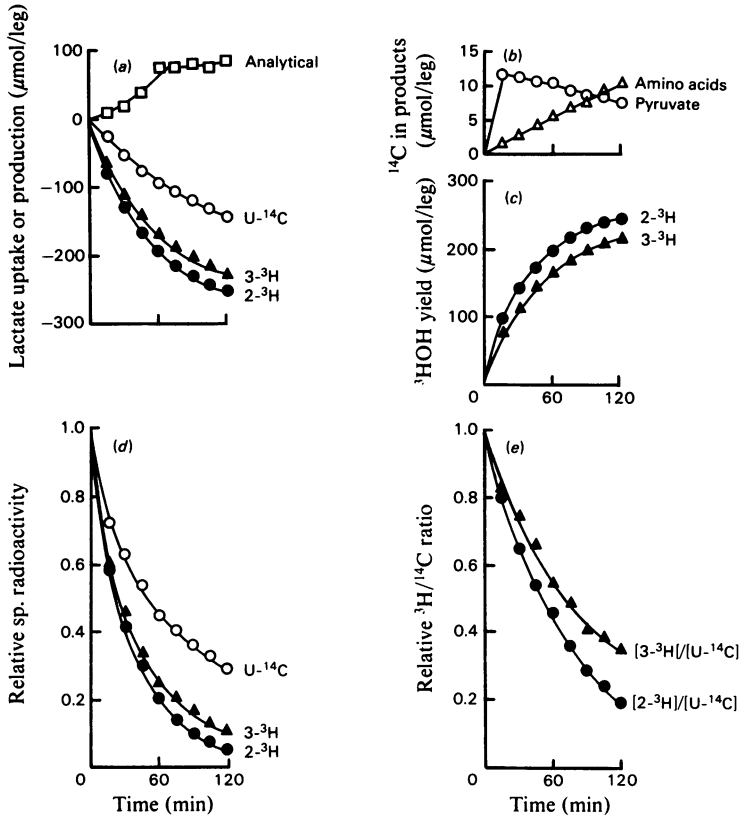

Fig. 2. *Lactate metabolism by perfused hindlimb in the presence of glucose*

The prestimulated hindleg was perfused with 10mM-glucose, 0.2mM-pyruvate and 2mM-lactate, labelled uniformly with ^{14}C and with ^{3}H in either position 2 or 3. No insulin was present. Results are expressed as μmol of C_3 units or μmol of H_2O. The initial specific radioactivity and ^{3}H/^{14}C ratio in lactate are set as 1.00. (*a*) Net lactate uptake or production as determined by chemical analysis (□) and by apparent utilization of (○) [U-^{14}C]-, (●) [2-^{3}H]- or (▲) [3-^{3}H]-lactate. (*b*) Apparent incorporation of ^{14}C into pyruvate (○) and amino acids (△). (*c*) Apparent ^{3}HOH yield from (●) [2-^{3}H]- and (▲) [3-^{3}H]-lactate. (*d*) Specific radioactivity of labelled lactate in perfusate. (*e*) ^{3}H/^{14}C ratios in perfusate of (●) [2-^{3}H,U-^{14}C]- and (▲) [3-^{3}H,U-^{14}C]-lactate.

Table 2. *Metabolism of labelled lactate in the presence of glucose*

Experiments were as in Table 1 (without insulin) but with the label in lactate. Prestimulated hindleg was perfused for 2h with glucose, lactate and pyruvate. Results are means for two experiments each with [2-^{3}H,U-^{14}C]- and [3-^{3}H,U-^{14}C]-lactate: (−) indicates uptake and (+) production.

	Concn. (mM)	
	Initial	Final
Glucose	8.7	7.3
Lactate	2.0	2.5
Pyruvate	0.14	0.15
Δ Glucose (μmol/2h per leg)	−181	
Δ Lactate (μmol/2h per leg)	+74	
Δ Pyruvate (μmol/2h per leg)	+4	
Δ Glycogen (μmol/2h per leg)	+168	
O_2 uptake (μmol/2h per leg)	698	

	Tracer recovery (% of dose)		
Label in lactate ...	U-^{14}C	2-^{3}H	3-^{3}H
Lactate	45	10	17
Pyruvate	2.7	0	0.8
Amino acids	3.6	0	0.9
Glycerol	0.9	0.1	0.4
Glycogen	16	0.2	2.2
Water	–	86	79
Total	68	96	100
Final relative sp. radioactivity of lactate*	0.29	0.05	0.11
Final ^{3}H/^{14}C ratio*		0.19	0.35

* The specific radioactivity and the ^{3}H/^{14}C ratio in lactate at zero time were set to 1.00.

specific radioactivity of [U-^{14}C]lactate at the end of the 2h period declined by 20% (Fig. 3). The apparent replacement was about 0.3 μmol/min per g, as compared with 0.17 measured by analysis. The apparent turnover of [^{3}H]lactate was 1.5 μmol/min per g for [2-^{3}H]lactate and 0.9 μmol/min per g for [3-^{3}H]-lactate. About 30% of the utilized U-^{14}C radioactivity appeared in glycogen.

The much higher apparent replacement of [^{3}H]- than of [^{14}C]-lactate suggested that the ^{3}H loss occurred by some exchange reactions rather than by metabolism. The exchange of hydrogen on C-3 of pyruvate with protons, catalysed via pyridoxamine by alanine–glutamate transaminase, has been shown (Oshima & Tamiya, 1961). The detritiation of [3-^{3}H]pyruvate by this reaction occurs in hepatocytes (Rognstad & Wals, 1976), and it was inhibited by the transaminase inhibitors aminooxyacetate and cycloserine. The effect of cycloserine is shown in Table 3: 1 mM-cycloserine did not affect lactate uptake, but inhibited glycogen synthesis by 50%. It drastically depressed ^{3}H loss from position 3 of lactate. The final ^{3}H/^{14}C ratio of [3-^{3}H,U-^{14}C]-lactate increased in the presence of 1 mM-cycloserine from 0.45 to 0.92, and thus the apparent turnover of the two tracers became similar.

Role of lactate in glycogen synthesis

To determine the relative roles of lactate and glucose as precursors for muscle glycogen, the hindleg was perfused with [U-^{14}C]lactate and [6-^{3}H]glucose. We have shown (Table 1) that the yields of ^{14}C and ^{3}H in glycogen from [6-^{3}H,U-^{14}C]glucose were equal. Table 4 shows the net (analytical) uptake of the substrates and synthesis of glycogen, and the recovery of tracers in the products. When the glycogen content of the leg was not depleted by prestimulation and in the absence of insulin, uptake of glucose was limited (10–15 μmol/h per g) and glycogen synthesis from glucose was very low. Under these conditions the relative contribution of lactate to glycogen carbon was substantial, and at high lactate concentration exceeded that of glucose. Significant synthesis occurred in muscle depleted of glycogen by prior muscle contraction, and it was increased several-fold in the presence of insulin. In prestimulated muscle, in the absence of insulin, the apparent relative contribution of lactate carbon was sub-

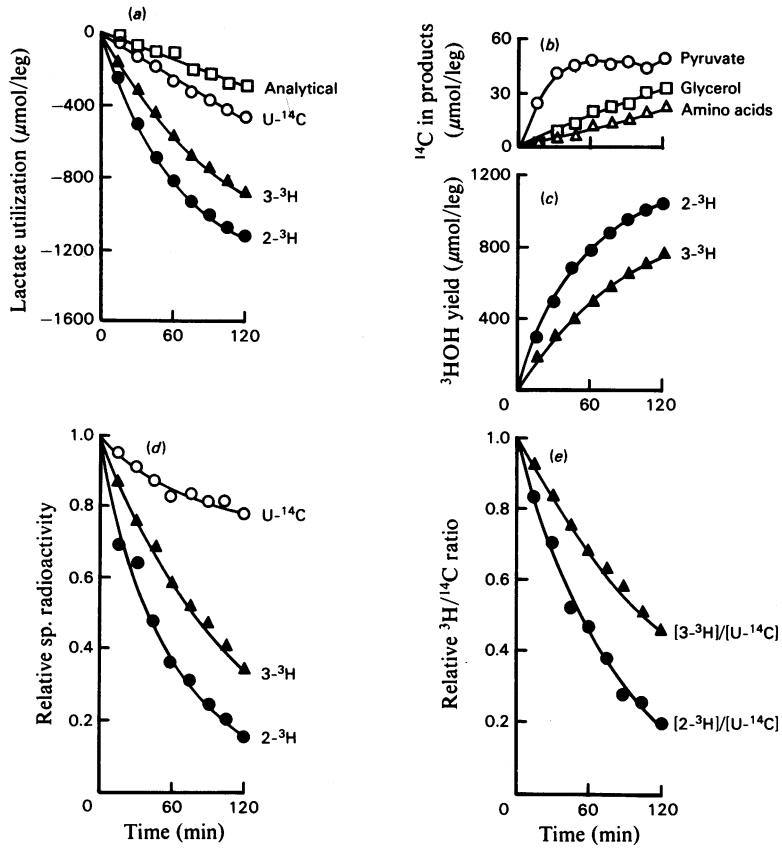

Fig. 3. *Lactate metabolism as sole added substrate by perfused hindleg.*
The hindleg was perfused with 9.0–9.4mM-lactate without insulin. The lactate was labelled uniformly with ^{14}C and with ^{3}H in either position 2 or 3. Results are expressed as μmol of C_3 units or μmol of H_2O. Initial specific radioactivity and $^{3}H/^{14}C$ ratio in lactate are set to 1.00. (*a*) Net lactate utilization as determined by chemical analysis (□) and apparent utilization of (○) [U-^{14}C]-, (●) [2-^{3}H]- or (▲) [3-^{3}H]-lactate. (*b*) Apparent incorporation of ^{14}C into pyruvate (○), glycerol (□) and amino acids (△). (*c*) ^{3}HOH yield from (●) [2-^{3}H]- and (▲) [3-^{3}H]-lactate. (*d*) Specific radioactivity of labelled lactate in perfusate. (*e*) $^{3}H/^{14}C$ ratios in perfusate of (●) [2-^{3}H,U-^{14}C]- and (▲) [3-^{3}H,U-^{14}C]lactate.

stantial, and at a high concentration (8.7mM-glucose and 17mM-lactate), about equal to that from glucose.

It should be noted that the apparent glycogen synthesis calculated from the ^{14}C and ^{3}H yields was less than the analytically measured glycogen deposition, and the discrepancy was most pronounced at low glycogen yields, when the apparent contribution of lactate carbon was equal to or higher than that of glucose. This suggests a large dilution of lactate carbon by unlabelled carbon (see the Discussion section. Thus apparent incorporation of lactate carbon, as based on tracer yields, may substantially underestimate the real contribution of lactate to glycogen synthesis.

We infer from these findings that in rat *in vivo*, at low lactate concentrations (up to 2mM) and normal insulin concentrations, there is a small contribution of lactate to glycogen synthesis in skeletal muscle. This may be more substantial in diabetes with low insulin concentrations or with insulin resistance. On the other hand, after exhaustive work (low glucose and insulin, very high lactate), lactate may become a major source for the replenishment of muscle glycogen. This is in accord with observations by Hermansen & Vaage (1977) in humans, where lactate concentration after exhaustion approached 20mM, and was the main source for the replenishment of glycogen.

Glycogen formation by different muscle types

Three types of muscle fibres are distinguished on the basis of contractility, mitochondrial content and enzyme profiles, i.e. fast-twitch red, slow-twitch red and fast-twitch white. To assess the role of these muscle types (Barnard *et al.*, 1971), after a

Table 3. *Metabolism of lactate as sole substrate by hindlimb*

The hindlimb was perfused (without insulin) with 10mM-[2-^{3}H,U-^{14}C]- or -[3-^{3}H,U-^{14}C]-lactate with or without cycloserine. Results are means of two experiments with each combination.

Label in lactate ...	[2-^{3}H,U-^{14}C]	[3-^{3}H,U-^{14}C]	[3-^{3}H,U-^{14}C]	[3-^{3}H,U-^{14}C]
Cycloserine (mM) ...	–	–	0.1	1.0
Lactate concn. (mM)				
Initial	9.4	9.0	9.4	8.9
Final	7.6	7.4	7.7	7.3
Δ Lactate (μmol/2h per leg)	−251	−226	−259	−230
Δ Pyruvate (μmol/2h per leg)	42	44	54	12
Δ Glycogen (as C_3 units) (μmol/2h per leg)	162	136	112	76
O_2 consumption (μmol/2h per leg)	860	881	893	873
^{14}C recovery (%) in:				
Lactate	65	66	66	73
Pyruvate	3.8	3.9	3.0	0.8
Amino acids	1.4	2.3	1.0	1.2
Glycerol	2.5	1.0	1.6	1.7
Glycogen	8.3	8.1	4.7	4.0
Total	81	81	77	81
^{3}H recovery (%) in:				
Lactate	14	32	55	68
Pyruvate	0	1.5	2.2	0.3
Amino acids	0	0.7	0.6	0.8
Glycerol	0.3	0.3	0.7	0.9
Glycogen	0.1	2.2	1.6	1.1
Water	79	59	36	24
Total	94	96	96	95
Final relative sp. radioactivity of lactate:*				
^{3}H	0.16	0.35	0.64	0.78
^{14}C	0.79	0.76	0.79	0.85
Final ^{3}H/^{14}C relative ratio of lactate*	0.20	0.45	0.83	0.92

* Initial specific radioactivity of lactate and the ^{3}H/^{14}C ratio of lactate in perfusion medium were both set to 1.00.

2h perfusion with [6-^{3}H]glucose and/or [U-^{14}C]-lactate the leg was rapidly frozen, and samples of the soleus (slow-twitch red), plantaris (fast-twitch red) and fast-twitch white fibres were separated by inspection. The glycogen content of the muscles frozen at zero time and after 2h of perfusion was determined, and the apparent incorporation of glucose and lactate carbon calculated from the yields of tracers and the mean specific radioactivities of glucose and lactate. The deposition of glycogen was lowest in the soleus (slow-twitch red) muscle, and it was negligible in the absence of insulin. The formation of glycogen by the plantaris and white muscle was similar in most experiments. The white fibres appear to lose most glycogen on stimulation, declining from 35 to about 10mg/g, compared with a decrease of 10–15mg/g in plantaris, and even less than that in the soleus muscle. The apparent relative contributions of glucose and lactate to glycogen carbon in the plantaris and white muscles were much the same as described in Table 4 for the whole leg. Lactate incorporation was high in the absence of insulin, and equalled or exceeded that of glucose at high lactate concentrations. In the presence of insulin the apparent contribution of lactate was 10–15% that of glucose when glucose concentration was 6.6mM and that of lactate 16mM, and was considerably less at normal lactate concentrations.

The pathway of phosphoenolpyruvate synthesis

Phosphoenolpyruvate is the first obligatory phosphorylated intermediate on the path of lactate to glycogen. In rat liver the key steps in this path are the mitochondrial phosphorylation of pyruvate to oxaloacetate catalysed by pyruvate carboxylase, the transfer of oxaloacetate from mitochondria to the cytosol, catalysed by a reversible transamination to aspartate, and conversion of cytosolic oxaloacetate into phosphoenolpyruvate, catalysed by phosphoenolpyruvate carboxykinase. It appears that none of these steps occurs in rat skeletal muscle, and the mechanism of phosphoenolpyruvate synthesis is still obscure.

Pyruvate carboxylase is absent from skeletal muscle (Opie & Newsholme, 1967). If there were a

Table 4. *Glycogen synthesis from glucose and lactate in resting hindlimb*

The hindleg was perfused for 2h with [6-^{3}H]glucose and [U-^{14}C]lactate. Net glycogen synthesis was determined from the glycogen content of the contralateral leg frozen at zero time and that at 2h. The amount (μmol) of [^{14}C]lactate incorporated into glycogen was determined by measuring the radioactivity of glycogen and specific radioactivity of lactate at 60min of perfusion. Results are averages from two to four experiments.

Prestimulation ...	No	No	Yes	Yes	Yes	Yes
Insulin (10munits/ml) ...	−	−	−	+	−	+
Mean [glucose] (mM)	8.4	8.4	8.0	6.6	8.7	6.6
Mean [lactate] (mM)	1.9	16.0	2.2	2.2	17.0	16.0
Mean [pyruvate] (mM)	0.1	1.0	0.1	0.1	1.1	0.9
Δ Glucose (μmol/2h per leg)	−151	−119	−242	−727	−189	−613
Δ Lactate (μmol/2h per leg)	51	−242	42	24	−185	−238
Δ Pyruvate (μmol/2h per leg)	−6	−131	−5	−11	−140	−142
Δ Glycogen (μmol/2h per leg)	71	45	190	517	182	538
^{3}H recovered from [6-^{3}H]glucose (% of dose):						
Glucose	91	94	85	49	88	54
Glycogen	1	1	9	34	5	33
Lactate	3	3	2	4	3	6
Water	3	2	2	5	2	3
Total	98	100	98	92	98	96
^{14}C recovered from [U-^{14}C]lactate (% of dose):						
Lactate	42	76	43	35	77	76
Glycogen	8	2	14	6	4	3
Other	7	8	7	5	8	6
Total	57	86	64	46	89	85
Final relative sp. radioactivity of lactate*	0.37	0.78	0.26	0.25	0.80	0.79
Glycogen formed (μmol of glucose equivalents/2h per leg) from:						
Glucose	14	7	122	452	66	409
Lactate	21	29	44	18	59	42

* The specific radioactivity of lactate at zero time was set to 1.00.

mitochondrial synthesis of oxaloacetate or any other dicarboxylic acid, equilibration with the intermediates of the tricarboxylic acid cycle would lead to the randomization of ^{14}C from C-2 or C-3 of lactate in the intermediates and in glycogen. We thus isolated glycogen formed in perfused rat leg from [2-^{14}C]lactate and degraded the glycogen as described by Schmidt *et al.* (1970). The phosphoglyceric acid obtained from C-4, C-5 and C-6 of glycogen contained 97–98% of the ^{14}C on C-2 of this acid, indicating no randomization. The lack of randomization *in vivo* from [2-^{14}C]lactate in muscle glycogen of mice was also observed by Hiatt *et al.* (1958). The lack of inhibition of glycogen synthesis from lactate by the transaminase inhibitor cycloserine (Table 3) also contradicts a mitochondrial formation of oxaloacetate. Connett (1979) proposed the synthesis of cytosolic oxaloacetate, catalysed by malic enzyme. The activity of malic enzyme in skeletal muscle is fairly high (Newsholme & Williams, 1978), but the equilibrium of this reaction is toward decarboxylation, and there is no evidence for a significant formation of NADPH in muscle cells.

The conversion of oxaloacetate into phosphoenolpyruvate is catalysed by phosphoenolpyruvate carboxykinase. A low activity of this enzyme in skeletal muscle was described by various investigators (for references, see Duff & Snell, 1982). Duff & Snell (1982) have shown that the measurement of pyruvate carboxylation by spectrophotometric methods used by previous investigators was flawed, and the activity of the enzyme was an order of magnitude lower than previously reported. If phosphoenolpyruvate carboxykinase were essential for glycogen synthesis from lactate, the latter should be depressed by mercaptopicolinic acid, an effective inhibitor of this enzyme. We found no inhibition of glycogen synthesis from lactate by mercaptopicolinic acid in perfused hindleg (Table 6). Hiatt *et al.* (1958) suggested the formation of phosphoenolpyruvate by reversal of the pyruvate kinase reaction. In spite of the unfavourable equilibrium, Dyson *et al.* (1975) calculated that the rate of the reverse reaction would be adequate to support glycogen synthesis from lactate in muscle. Our negative findings are consistent with a direct synthesis of phosphoenolpyruvate from pyruvate and ATP, but there is no direct experimental evidence for such a pathway.

Table 5. *Contribution of glucose and lactate to glycogen in three types of skeletal muscle*
The hindleg was perfused for 2h with [U-^{14}C]lactate and [6-^{3}H]glucose. Glycogen deposition was obtained from the content of the legs frozen at zero time and at 2h. The incorporation of tracer was calculated from the tracer yield in glycogen and the specific radioactivity of labelled compounds at 60min of perfusion. Results are averages of two to four experiments for each condition.

Prestimulated	Insulin (10munits/ml)	Average substrate concn. (mM) Glucose	Lactate	Muscle	Initial glycogen (μmol/g)	Δ Glycogen (μmol of glucose/2h per g of muscle)	Apparent glycogen synthesis (μmol of glucose/2h per g of muscle) from Glucose	Lactate
No	–	8.4	1.9	Soleus	20.7	0.7	0.5	0.5
				Plantaris	27.0	8.7	0.5	1.5
				White	35.7	6.7	0.7	2.1
No	–	8.4	16.0	Soleus	27.1	1.8	0.4	0.8
				Plantaris	33.9	6.1	0.4	3.5
				White	33.3	5.0	0.5	3.1
Yes	–	8.0	2.2	Soleus	17.3	3.7	1.0	0.7
				Plantaris	17.3	6.2	4.0	3.6
				White	11.8	18.3	19.2	3.4
Yes	+	6.6	2.2	Soleus	14.8	31.5	29.9	0.3
				Plantaris	16.9	51.6	39.8	2.8
				White	9.9	37.2	29.2	2.2
Yes	–	8.7	17.1	Soleus	19.2	3.4	0.7	2.0
				Plantaris	14.9	13.5	2.5	4.9
				White	9.8	15.0	6.3	5.1
Yes	+	6.6	15.9	Soleus	18.0	35.1	24.0	3.3
				Plantaris	20.4	64.0	53.6	5.5
				White	8.1	42.4	33.7	4.4
Yes	–	None	8.3	Soleus	19.2	4.4	–	0.7
				Plantaris	22.3	6.8	–	4.3
				White	18.0	12.5	–	6.0

Table 6. *Effect of mercaptopicolinate on glycogen synthesis in prestimulated resting rat hindlimb*
The hindleg was perfused for 2h with 10mM-[6-^{3}H]glucose, 20mM-[U-^{14}C]lactate and 2mM-pyruvate without insulin. 3-Mercaptopicolinic acid when present was 0.5mM. Glycogen synthesis was determined chemically, and apparent synthesis from glucose and lactate was calculated respectively from the yields of ^{3}H and ^{14}C in glycogen. Means and individual values are shown.

3-Mercaptopicolinate	Δ Glycogen (chemical) (μmol of glucosyl units/leg)	Apparent glycogen formation (μmol of glucosyl units/leg) from [6-^{3}H]Glucose	[U-^{14}C]Lactate
Absent	166	80	48
	(145, 128, 225)	(108, 69, 63)	(27, 42, 75)
Present	139	66	51
	(148, 129)	(79, 52)	(41, 61)

Discussion

Use of tracers to measure glucose and lactate turnover in muscle

In perfused muscle, glucose uptake from the medium is the same whether measured chemically or by the yield of ^{3}HOH from [2-^{3}H]glucose, and the incorporations of ^{14}C and ^{3}H from position 6 of glucose into glycogen are nearly equal. Thus there is little recycling of glucose carbon in muscle, and the yields of ^{14}C and ^{3}H provide valid measurements of the turnover of glucose and incorporation into glycogen. On the other hand, with lactate there is a marked divergence between utilization of

lactate as measured chemically and with tracers, and a large difference between the fates of ^{14}C and ^{3}H. With glucose and lactate concentrations at near normal range, although there was a small production or no change in lactate content in the medium, there was a rapid exponential decrease of the specific radioactivity of [U-^{14}C]lactate, with an apparent turnover (in the absence of insulin) of 0.28 μmol/min per g. With 10 mM-lactate, in the absence of glucose, there was a net uptake of lactate at a rate of 0.17 μmol/min per g, but replacement as measured with ^{14}C was about 1.5 times that rate. Thus there must be an influx of unlabelled carbon into the lactate pool. Glycolysis may contribute up to 0.02 μmol/min per g. Alanine production by proteolysis was estimated by Odessey *et al.* (1974) and Ruderman & Berger (1974) to be 0.01–0.015 μmol/min per g. There could be an influx from the tricarboxylic acid-cycle intermediates. Lee & Davis (1979) showed in muscle the incorporation of ^{14}C from $^{14}CO_2$ and pyruvate into malate and lactate. This suggests an influx of carbon from intermediates of the tricarboxylic acid cycle into the lactate pool. Lee & Davis (1979) estimated such an influx to be about 0.01 μmol/min per g. The total influx from these sources appears to be insufficient to account for dilution of the specific radioactivity of lactate.

A possible mechanism for the dilution of the radioactivity could be an exchange of carbon atoms with those of intermediates of the tricarboxylic acid cycle without a net influx. A hypothetical mechanism could be a reversible formation of malate from pyruvate in the cytosol, and a rapid reversible equilibration with mitochondrial malate and other dicarboxylic acids common to the tricarboxylic acid cycle. This will be in effect of exchange of lactate carbon with that of CO_2 and acetyl-CoA. This mechanism is attractive, since it would account for the discrepancy between actual lactate uptake and apparent lactate turnover as measured with ^{14}C, and also would provide a mechanism for the detritiation of [2-^{3}H]lactate. However, there is no experimental support for this mechanism and lack of randomization of carbon from [2-^{14}C]lactate in glycogen by reactions of the tricarboxylic acid cycle is not consistent with an exchange with tricarboxylic acid-cycle intermediates.

Whatever the mechanism of dilution, we wish to stress that the incorporation of ^{14}C from radiolabelled lactate into glycogen may not be a measure of a true rate of synthesis, and great caution is required in the calculation of metabolic rates from such ^{14}C yields and in the quantitative estimates for the role of lactate in the synthesis of glycogen in muscle.

We (Katz *et al.*, 1981; Okajima *et al.*, 1981) have introduced [2-^{3}H]- and [3-^{3}H]-lactate as irreversible tracers for lactate turnover *in vivo*. The rates of turnover of these two tracers in rats were similar, about twice that for [U-^{14}C]lactate. In the leg perfused with 1–2 mM-lactate, the turnover of [3-^{3}H]lactate was nearly twice, and that of [2-^{3}H]-lactate 3 times that of [U-^{14}C]lactate, with 8 mM-lactate. Apparent turnover of [3-^{3}H]lactate was 3 times and that of [2-^{3}H]lactate was 5 times that of [U-^{14}C]lactate. Our results with transaminase inhibitors (Table 3 and the Results section) establish that extensive loss of ^{3}H from position 3 of lactate occurs by exchange reactions with the protons of the medium. It appears that the loss of ^{3}H from position 2 of lactate is an exchange reaction. In erythrocytes detritiation of [2-^{3}H]-lactate occurs during glycolysis (Rose & Warms, 1969). The detritiation in erythrocytes requires the presence of glucose; the mechanism involves the reversible oxidoreduction of glyceraldehyde phosphate to 1,3-bisphosphoglyceric acid with NADH. We found no detritiation of [2-^{3}H]lactate in excess of ^{14}C utilization in rat hepatocytes (S. Golden & J. Katz, unpublished work). The very high rate of detritiation of [2-^{3}H]lactate in muscle, especially in the presence of the high lactate concentration, indicates that the loss of ^{3}H is mainly by exchange. In muscle the detritiation occurs in the absence of glucose. The mechanism of the ^{3}H loss from position 2 of lactate remains to be established. The present findings throw serious doubt on the value of either [2-^{3}H]- or [3-^{3}H]-lactate for turnover studies *in vivo*.

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