

Further Studies on the Accumulation of Energy Substrates by Two-cell Mouse Embryos

R. G. Wales^A and D. G. Whittingham^{AB}

^A Department of Veterinary Physiology, University of Sydney, Sydney, N.S.W. 2006.

^B Present address: Physiological Laboratory, Cambridge, CB2 3EG, England.

Abstract

The accumulation of substrate carbon by two-cell mouse embryos after incubation for 30 min in isotopically labelled substrates was compared by two different methods. The embryos were centrifuged either through an isotonic sucrose solution to free them from radioactive medium before counting or in radioactive medium in a capillary tube when substrate uptake was calculated from the relative counts in packed embryos and incubation medium.

Measurements of the accumulation of substrate carbon from [$1\text{-}^{14}\text{C}$]pyruvate gave the same values by both methods, but the accumulation of C-1 of lactate was much greater after centrifuging the embryos in a capillary tube. Fractionation of the intracellular isotope accumulated from lactate showed that the increased radioactivity accumulated was associated with the parent substrate. A similar difference was found in the accumulation of C-1 of acetate with the two methods of recovery, indicating that both substrates diffuse freely between the medium and embryo. Incorporation of acetate into two- and eight-cell mouse embryos was similar. No intracellular substrate carbon was accumulated from [$1,5\text{-}^{14}\text{C}$]citrate by two-cell mouse embryos during 30 min incubation.

Introduction

Measurements of the accumulation of substrate carbon by preimplantation mouse embryos after incubation in isotopically labelled substrates have provided important information on the uptake and metabolism of energy substrates by the developing mouse embryo. Wales and Whittingham (1967) found no significant difference in the ability of one- and two-cell mouse embryos to accumulate either lactate or pyruvate and concluded that the greater utilization of pyruvate compared with lactate enables the former substrate to support development through the first cleavage division *in vitro*. Furthermore, the finding that uptake of C-1 of these substrates was concentration-dependent and was not significantly affected by cooling to 5°C suggested that these substrates enter the cell by diffusion, rather than by an active process as has been reported for the uptake of malate (Wales and Biggers 1968) and glucose (Wales and Brinster 1968). More recent studies in which fractionation of the intracellular compounds accumulating from lactate and pyruvate was performed (Wales and Whittingham 1973*a*) have indicated that the accumulation of C-1 of pyruvate appeared to be due to the activity of the enzyme alanine aminotransferase (EC 2.6.1.2) rather than substrate diffusion. In the case of lactate, most of the intracellular C-1 accumulated was associated with the parent substrate. However, the intracellular concentration of the substrate calculated from the uptake of [$1\text{-}^{14}\text{C}$]lactate was always much lower than that present in the incubation medium, suggesting either a loss of substrate during recovery from the incubation medium or limited transfer across the cell membrane.

In the present experiments the accumulation of carbon from energy substrates during short periods of incubation has been examined and the loss of isotope from embryos during recovery was assessed by comparing the method used in previous studies with a technique that does not require the removal of embryos from the incubation medium prior to assay.

Materials and Methods

General

Two-cell mouse embryos were obtained from superovulated random-bred Swiss mice, 46–68 h after the injection of human chorionic gonadotrophin (Brinster 1963). The embryos were collected in a modified Krebs–Ringer bicarbonate solution containing 25 mM sodium DL-lactate, 0.25 mM sodium pyruvate, 1 mg/ml bovine serum albumin, 60 µg/ml penicillin and 50 µg/ml streptomycin (Brinster 1965a). Except where stated otherwise, the embryos were washed in two changes of a similar medium devoid of energy substrates (2 ml/wash) before incubation in a medium containing the isotopically labelled substrates. Preliminary experiments using lactate and pyruvate showed that uptake of substrate by embryos was not affected by washing through substrate-free medium unless the embryos were left in medium devoid of energy substrates for extended periods. In the present experiment embryos were transferred to media containing labelled substrates within 2 min of washing.

Media

Isotopically labelled pyruvate, lactate and citrate were obtained from Amersham Laboratories and labelled acetate was obtained from Volk Radiochemical Company. Prior to use the radioactive substrates were stored as small dried aliquots at -40°C to minimize decomposition (Wales and Whittingham 1971). For the experiments the labelled substrates were added to modified Krebs–Ringer bicarbonate solution containing 1 mg/ml bovine serum albumin, 60 µg/ml penicillin and 50 µg/ml streptomycin, the NaCl content being adjusted to maintain isotonicity. The specific activities and concentrations of the substrates in the different media were

1. Sodium $[1-^{14}\text{C}]$ pyruvate: 11.8 mCi/mmol at a concentration of 0.5 mM and 1.18 mCi/mmol at a concentration of 5.0 mM.
2. Sodium $[1-^{14}\text{C}]$ -DL-lactate: 1.3 mCi/mmol at concentrations of 25.0 and 5.0 mM.
3. Sodium $[1,5-^{14}\text{C}]$ citrate: 19.2 mCi/mmol at a concentration of 0.5 mM.
4. Sodium $[1-^{14}\text{C}]$ acetate: 5.2 mCi/mmol at concentrations of 4.0 and 25.0 mM.

Determination of Substrate Carbon Accumulated by Mouse Embryos

(i) Method 1

This method, originally used by Wales and O'Shea (1966) and modified for collection of mouse embryos free from radioactive substrate present in the incubation medium, has been adequately described elsewhere (Wales and Biggers 1968). Depending upon the experiment and the number of embryos available, between 60 and 250 embryos were placed in 20-µl drops of radioactive medium under 10 ml of paraffin oil contained in a plastic Petri dish. The embryos were incubated for 30 min at 37°C before recovery by centrifugation through isotonic sucrose. After centrifugation the embryos were dispersed from the broken tip of the centrifuge tube by adding 0.4 ml of water to the scintillation vial and shaking vigorously. Each sample was assayed for radioactivity by liquid scintillation techniques (Wales and Whittingham 1970). The accumulation of substrate carbon from the labelled atoms was calculated from the counts in the embryos and the specific activity of the substrate in the incubation medium.

(ii) Method 2

After being incubated at 37°C in the specifically labelled substrates for 30 min (see method 1), the embryos plus a small quantity of radioactive medium were taken up into a finely drawn Pasteur pipette of uniform diameter (approx. 110–140 µm internal diameter). The end of the pipette was sealed after drawing air into it for a distance of approximately 5 mm (see Fig. 1a). The finely drawn part of

the pipette was carefully broken away from the upper wider region to make a small centrifuge tube and centrifuged in a protective support at 500 *g* for approximately 5 min. After centrifugation the embryos were packed above the air space and a small volume of medium usually occupied the bottom of the tube next to the sealed end (Fig. 1*b*). Using a micrometer fitted into the eyepiece of the dissecting microscope, the length of the tube containing the embryos was measured, cut out with a diamond pencil and placed in a scintillation vial. Measured lengths of tube containing medium only were taken from above the embryos and also from below the eggs and air space (Fig. 1*b*). All samples were placed in scintillation vials and assayed for radioactivity by liquid scintillation techniques.

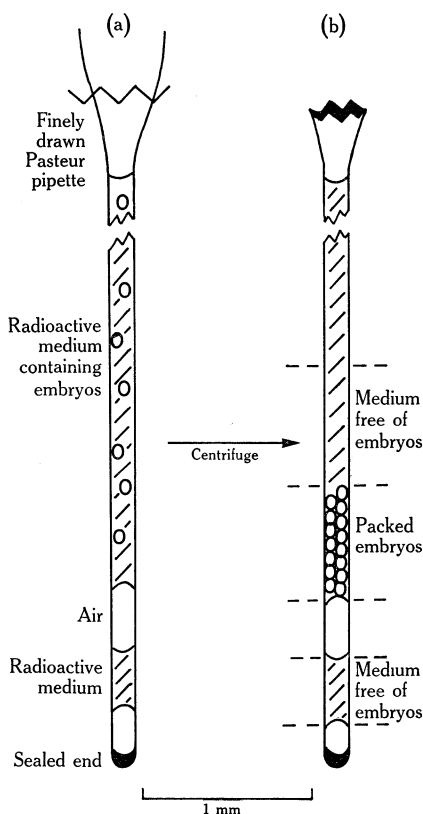


Fig. 1. Diagram of recovery procedure for method 2 (for detailed description of the method see Materials and Methods section). (a) Micropipette before centrifugation. (b) Micropipette after centrifugation.

If the diameter of the drawn Pasteur pipette was not uniform, the counts per minute per unit length of tube containing medium alone differed greatly between the samples from above and below the embryos, and the replicate was discarded. The volume of a unit length of tube containing medium alone was calculated knowing the counts in a standard volume of incubation medium. From this the volume of the sample containing the embryos was determined and assuming that the mean volume of the two-cell mouse embryo is 382 000 μm^3 (Lewis and Wright 1935) the volume of medium surrounding the packed embryos was determined by difference. The radioactivity contained in the embryos was obtained by subtracting the counts per minute for the medium surrounding the embryos from total counts per minute of the sample. Finally, the intracellular accumulation of substrate carbon from the labelled site was calculated from the counts in the embryos and the specific activity of the substrate in the incubation medium.

Fractionation Procedures

In the final experiment the intracellular substrate carbon accumulating from $[1-^{14}\text{C}]\text{lactate}$ was fractionated into a variety of biochemical components as described previously (Wales and Whittingham 1970, 1973a). After incubation and recovery of the embryos, the metabolites in the acid-soluble

fraction were fractionated into basic, acidic and neutral compounds by applying aliquots of this fraction to ion-exchange resins. The acidic fraction eluted from the ion-exchange resin was reduced in volume and an aliquot applied to a silicic acid column to separate and identify carboxylic acids (O'Shea and Wales 1968). The identity of the carboxylic acids separated by this method was further checked by applying another aliquot of the acid-soluble fraction to Whatman No. 1 paper and chromatographing in a descending system using n-butanol-acetic acid-water (4:1:5) as solvent. Estimates of intracellular incorporation of compounds determined by method 2 were then corrected for counts in the medium collected with the embryos.

Table 1. Effect of the presence of unlabelled substrate in the sucrose rinse upon the accumulation of the specifically labelled substrate by two-cell mouse embryos incubated for 30 min

Mean values \pm standard errors of the means are given

Substrate in incubation medium (mM)	Substrate in sucrose rinse (mM)	Number of replicates	Substrate carbon accumulated from position labelled (pg-atoms/embryo)
[1- ¹⁴ C]Pyruvate (0.5)	Pyruvate (0.5)	6	0.29 \pm 0.02
[1- ¹⁴ C]Pyruvate (0.5)	None	6	0.28 \pm 0.02
[1- ¹⁴ C]Lactate (25.0)	Lactate (25.0)	4	0.65 \pm 0.05
[1- ¹⁴ C]Lactate (25.0)	None	4	0.51 \pm 0.04
[1,5- ¹⁴ C]Citrate (0.5)	Citrate (0.5)	3	0.0023 \pm 0.0004
[1,5- ¹⁴ C]Citrate (0.5)	None	3	0.0007 \pm 0.0003
[1- ¹⁴ C]Acetate (4.0)	Acetate (4.0)	3	0.13 \pm 0.01
[1- ¹⁴ C]Acetate (4.0)	None	3	0.13 \pm 0.01

Results

Measurement of Uptake by the Use of a Sucrose Rinse

The accumulation of substrate carbon by two-cell mouse embryos incubated for 30 min in sodium [1-¹⁴C]pyruvate (0.5 mM), sodium [1-¹⁴C]-DL-lactate (25.0 mM), sodium [1,5-¹⁴C]citrate (0.5 mM) or sodium [1-¹⁴C]acetate (4 mM) was measured by method 1 (see Materials and Methods section). In addition, the effects of the presence or absence of unlabelled substrate in the isotonic sucrose rinse was tested and the results are summarized in Table 1.

There was a substantial uptake of C-1 of pyruvate and lactate during the 30-min period of incubation. No increase in the accumulation of C-1 of pyruvate was found when unlabelled pyruvate was added to the sucrose rinse. On the other hand, the accumulation of C-1 of lactate was consistently higher in all replicates when unlabelled lactate was present in the sucrose rinse. Although there was some variation between replicates in the response to added lactate, there was an average increase of 25% in the accumulation of substrate under these conditions. There was virtually no uptake of citrate by the embryos either with or without the addition of this substrate to the sucrose rinse. The values given for citrate uptake in Table 1 represent an average uptake of 2.0 net counts/min per 100 embryos (range 0-4 counts/min) above background.

Embryos accumulated 0.13 pg-atoms of substrate carbon from C-1 of acetate during the 30-min incubation but there was no effect of the addition of 4 mM unlabelled

acetate to the sucrose rinse. It was also found that the uptake of acetate by eight-cell embryos was not significantly different from that by two-cell embryos. In a further test, two-cell embryos were incubated for 30 min in medium containing 25 mM acetate. In this case accumulation of substrate carbon was increased five- to sixfold over that occurring when embryos were incubated in 4 mM acetate. The embryos accumulated 0.72 ± 0.04 pg-atoms of substrate carbon from C-1 of acetate (mean \pm S.E. for five replicates) when collected without the addition of acetate to the rinse. With the addition of acetate to the rinse, 0.77 ± 0.03 pg-atoms of substrate carbon were accumulated from C-1 of acetate. This response to added acetate in the rinse varied between replicates and was not statistically significant.

Table 2. Accumulation of specifically labelled substrates by two-cell mouse embryos incubated for 30 min and recovered from the incubation medium by centrifugation in a capillary tube (method 2)

Mean values \pm standard errors of the means are given

Substrate in incubation medium	Substrate concentration (mM)	Number of replicates	Substrate carbon accumulated from position labelled (pg-atoms/embryo)
[1- ¹⁴ C]Pyruvate	0.5	4	0.32 ± 0.02
	5.0	2	2.36 ± 0.52
[1- ¹⁴ C]Lactate	5.0	2	2.01 ± 0.24
	25.0	7	6.27 ± 0.74
[1,5- ¹⁴ C]Citrate	0.5	2	0.00 ± 0.00
[1- ¹⁴ C]Acetate	4.0	4	0.80 ± 0.06

Measurement of Uptake without Removal from the Incubation Medium

The accumulation of substrate carbon by two-cell mouse embryos incubated for 30 min in sodium [1-¹⁴C]pyruvate, sodium [1-¹⁴C]-DL-lactate, sodium [1,5-¹⁴C]-citrate and sodium [1-¹⁴C]acetate was measured by the procedure described in method 2 (see Materials and Methods section), and the results are presented in Table 2. The accumulation of C-1 of pyruvate (0.5 mM) was similar to the values obtained using method 1. When the pyruvate concentration in the incubation medium was increased 10-fold, the accumulation of substrate carbon was increased 6- to 11-fold.

The accumulation of C-1 of substrate from medium containing 25 mM lactate was 6.27 pg-atoms/embryo, approximately 10 times greater than the values obtained by method 1 (see Table 1). When the lactate concentration was decreased to 5.0 mM, the accumulation of substrate carbon decreased to 2.0 pg-atoms/embryo and was still approximately four times greater than the value obtained by method 1 at the higher concentration. In the present experiment the estimate of intracellular concentration of substrate carbon was less than that of the extracellular concentration when the incubation medium contained 25.0 mM lactate, but when the medium contained 5.0 mM lactate the intra- and extracellular concentrations of substrate carbon were similar. No accumulation of substrate carbon from [1,5-¹⁴C]citrate (0.5 mM) by two-cell mouse embryos after 30 min incubation was found using this method.

The estimate of the uptake of acetate by this method was approximately five times that found when the embryos were separated from the incubation medium by method 1. With eight-cell embryos incubated in $[1-^{14}\text{C}]$ acetate, the uptake of acetate estimated by method 2 was also five to six times that measured by method 1.

Identification of Intracellular Metabolites Accumulated from Lactate

In view of the large differences in the intracellular accumulation of isotope from $[1-^{14}\text{C}]$ lactate when estimated by the two methods, two-cell embryos (150–200 per treatment) were incubated for 30 min in 25 mM $[1-^{14}\text{C}]$ lactate, recovered by the two methods and fractionated by the procedures described in the Methods section. The results for two replicates are given in Table 3.

Table 3. Comparison of the intracellular accumulation of C-1 of lactate in various fractions of embryos incubated for 30 min in $[1-^{14}\text{C}]$ lactate and recovered from the medium by two methods

Data are given for two replicates for each method. See Methods section for experimental details

Fraction assayed	Substrate carbon accumulated (pg-atoms/ embryo) following recovery of embryos by:	
	Method 1	Method 2
Total uptake	0.80, 0.72	6.50, 9.51
Acid-insoluble fraction	0.010, 0.004	0.005, 0.006
Acid-soluble fraction		
Basic compounds	0.14, 0.11	0.18, 0.15
Neutral compounds	0.01, 0.03	0.02, 0.04
Acidic compounds	0.66, 0.61	6.31, 9.32

As in the previous experiments, the estimate of incorporation using method 2 was 10 times that determined by method 1. This difference was due to a greater amount of incorporation into the acid-soluble fraction in embryos recovered by method 2. Labelled lactate was the only carboxylic acid identified in this fraction and accounted for the major portion of the radioactivity in the embryos recovered by method 2. Estimates of incorporation into the acid-insoluble fraction and into the basic and neutral compounds of the acid-soluble fraction were similar using the two methods of recovery.

Discussion

In the present experiments, recovery of embryos from the incubation medium by centrifugation through isotonic sucrose resulted in similar estimates of the accumulation of radioactivity from $[1-^{14}\text{C}]$ pyruvate to those found in previous studies (Wales and Whittingham 1967, 1973a). The adoption of an alternative method for measuring substrate uptake which did not involve the removal of embryos from the radioactive incubation medium did not affect the estimate of the accumulation of C-1 of pyruvate in the embryo. This, plus the finding that the addition of unlabelled pyruvate to the sucrose rinse did not affect estimates of uptake, suggests that little diffusible substrate is present in these embryos. Thus the inability to detect pyruvate in embryos following incubation in this substrate (Wales and Whittingham 1970, 1973a; Quinn and Wales 1973) is unlikely to have been due to its loss during recovery of the embryos.

Using the published data for the volume of the embryo (Lewis and Wright 1935), calculations show that the accumulated intracellular pyruvate that has not undergone decarboxylation exceeds the extracellular concentration of pyruvate by approximately 60%. Active transport of pyruvate into the embryo and its intracellular accumulation are unlikely to explain the elevated levels of C-1 in the embryos, as no accumulation of intracellular pyruvate could be detected in embryos cultured in the presence of radioactive pyruvate for 72 h (Wales and Whittingham 1970). More recent evidence (Wales and Whittingham 1973*a*) suggests that the accumulation of C-1 of pyruvate is due mainly to the activity of alanine aminotransferase, leading to the accumulation of alanine rather than pyruvate. Thus it would appear that the intracellular pool of pyruvate is small and rapid turnover through this pool accounts for the high rate of utilization of this substrate.

In estimating the accumulation of C-1 of lactate, addition of unlabelled substrate to the sucrose rinse led to an increase in the estimate of total substrate accumulated, suggesting that some loss by diffusion might have occurred. Much higher values for intracellular accumulation were obtained when embryos were not freed from the incubation medium by centrifugation through isotonic sucrose. All of this increase was due to an increase in the accumulation of the parent substrate. Apparently most of this intracellular lactate is lost during the passage of the embryo through the sucrose rinse. Interchange between the medium and the embryo must be rapid as the presence of unlabelled lactate in the sucrose rinse failed to stop an appreciable loss of labelled lactate from the cell.

There are inherent technical difficulties in the use of the second method described in this paper. However, as manipulative skill in performing the technique increased, more satisfactory recoveries of the embryos appeared to be achieved. In these circumstances estimates of the accumulation of lactate were higher, and in the last experiment the intracellular concentration of lactate approached the concentration of the substrate in the medium. In this experiment the mean estimate of the concentration of lactate in the embryos was 20.5 mM while the concentration of lactate measured in the medium was 23 mM. These estimates confirm the suggestion that lactate freely diffuses into the embryo. The other intracellular carbon pools into which lactate enters are more stable and the estimates of incorporation into these pools were similar with the two methods of recovery. Thus the more convenient method 1 gives an accurate assessment of the metabolic fate of lactate within the embryo but when an estimate of the intracellular concentration of the parent substrate is required the more tedious method 2 is warranted.

Uptake of citrate by the two-cell mouse embryo is negligible. The estimates of uptake found in the present experiments by method 1 are of the same order as those obtained by Kramen and Biggers (1971) and represent only a few counts above background for each batch of embryos incubated. Direct comparison between the two studies is difficult, however, since Kramen and Biggers (1971) inactivated their embryos with formalin prior to, rather than after, removal from radioactive medium. The two values for citrate incorporation are not due to loss of citrate during recovery, as determinations by method 2 also indicated no entry of citrate into the embryos, all radioactivity in the samples being accounted for by the medium surrounding the embryos. As the estimates of embryonic volume used in the study were based on the values of Lewis and Wright (1935) for the total volume within the zona pellucida,

the values determined by method 2 suggest that citrate does not accumulate in the perivitelline space. Such a result is unexpected and difficult to explain as the zona pellucida at this stage in development of the mouse embryo has been shown to be permeable to molecules as large as ferritin (Hastings *et al.* 1972).

Although acetate, like citrate, will not support development of the two-cell mouse embryo (Brinster 1965*b*), its uptake by the embryos at this stage of development differs considerably from that of citrate. Determinations by method 1 showed that significant amounts of substrate carbon accumulated in embryos from acetate and the extent of this accumulation depended on the concentration in the medium. Furthermore, the experiments using method 2 demonstrated that large losses of isotope occurred during recovery by centrifugation through isotonic sucrose, presumably due to the diffusion of acetate from the embryo. There was also little change in the incorporation of acetate between the two- and eight-cell stage. This is in contrast to the findings for certain intermediates of the tricarboxylic acid cycle (Wales and Biggers 1968; Kramen and Biggers 1971) and probably explains why these latter compounds rather than acetate can act as suitable energy sources at this stage of development (Brinster and Thomson 1966; Wales and Whittingham 1973*b*). The results of the present experiments suggest that acetate can enter the cell. Therefore, its inability to support development probably arises either from a limited conversion of acetate to acetyl-CoA or from an insufficient endogenous pool of oxaloacetate to act as acceptor for the acetyl-CoA formed.

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