THE UPTAKE OF HEXOSES BY PRE-IMPLANTATION MOUSE EMBRYOS *IN VITRO*

R. G. WALES* AND R. L. BRINSTER

*King Ranch Laboratory of Reproductive Physiology, School of Veterinary Medicine,
University of Pennsylvania, Philadelphia, Pa, U.S.A.*

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Summary. The accumulation of substrate carbon by mouse embryos was measured following incubation in U- ^{14}C -glucose. Following a 30-min incubation period 273×10^{-14} and 301×10^{-14} g atoms of substrate carbon per embryo were found in 2- and 8-cell embryos respectively. By comparison, the figures for unfertilized and fertilized ova were 14×10^{-14} and 45×10^{-14} g atoms of substrate carbon.

The intracellular concentration of substrate carbon was time-dependent in both 2- and 8-cell embryos. After an 80-min incubation, substrate carbon in the 8-cell embryo was almost double that in the 2-cell embryo. Accumulation did not occur during incubations at 5°C and there was competition between glucose and galactose for uptake. The results are discussed in relation to the energy requirements of the developing zygote.

INTRODUCTION

During development of in-vitro techniques for the cultivation of mouse embryos at the pre-implantation stages of development, it was found that the late 2-cell embryo would not develop to the blastocyst when glucose was the sole energy source (Brinster, 1965a). On the other hand, 8-cell embryos would continue development when this substrate was provided (Brinster & Thomson, 1966). Recent studies using L-malate (Wales & Biggers, 1968) have indicated that the cell membrane undergoes a change in permeability to this substrate between the first and third cleavage divisions. This raises the question as to whether a similar change in permeability to glucose occurs at this time and is responsible for the difference in the ability of glucose to support development at these two stages.

In an attempt to throw light on this problem, the intracellular accumulation of glucose carbon has been studied at various stages in development. Some studies on the accumulation of galactose are also included.

* Present address: Department of Veterinary Physiology, University of Sydney, Sydney, N.S.W., Australia.

MATERIALS AND METHODS

Mouse embryos were obtained from random-bred Swiss mice which had been superovulated by an intraperitoneal injection of 10 i.u. of serum gonadotrophin (Gestyl, Organon), followed 48 hr later by an intraperitoneal injection of 10 i.u. of chorionic gonadotrophin (Pregnyl, Organon) (Brinster, 1963). Successive developmental stages of the embryos were removed from the Fallopian tubes of mice at specific times after ovulation. The cumulus cells which surrounded unfertilized ova and single-cell embryos were removed by incubation in hyaluronidase solution (Brinster, 1965b). The basic medium used in the study and the method of washing the embryos free of substrate before incubation have been described previously (Wales & Biggers, 1968). For the studies of the uptake of substrates, incubations were carried out in tissue culture dishes containing a small drop of radio-active medium in 10 ml of light paraffin oil. In the first experiments, 200 to 300 2-cell embryos and eighty to 150 8-cell embryos were used for each treatment, but in later experiments fifty embryos/treatment were used for these stages and 150/treatment for 1-cell embryos. Incubations to study the production of carbon dioxide were set up in small test tubes as previously described (Brinster, 1967). For these experiments 300 to 400 2-cell embryos and 100 to 150 8-cell embryos were used.

The U- ^{14}C -glucose (Nuclear Chicago Corp.) was diluted with basic diluent to give a concentration of $5.6\text{ }\mu\text{mole/ml}$ and a specific activity of $2.9\text{ }\mu\text{C}/\mu\text{mole}$. For the experiment where a low concentration of glucose was used, the above solution was further diluted with basic, substrate-free medium to the desired concentration. The 1- ^{14}C -galactose (Nuclear Chicago Corp.) was used at a concentration of $4.5\text{ }\mu\text{mole/ml}$ and a specific activity of $3.4\text{ }\mu\text{C}/\mu\text{mole}$.

To measure the intracellular accumulation of substrate carbon, embryos, at the completion of incubation in the radio-active medium, were collected free of incubation medium and prepared for radio-active assay by liquid scintillation techniques as previously described (Wales & Biggers, 1968). Incubations at 5°C were carried out in a refrigerated room. After collection, the embryos were cooled to 5°C over 30 min in a diluent containing lactate (25 mM) and pyruvate (0.25 mM). They were then washed free of substrate at 5°C before incubation in the radio-active medium.

Samples of medium were taken for radio-assay at the completion of all incubations in order to determine the actual concentration of substrate present during the incubation period. These concentrations are given in the tables. The accumulation of substrate carbon was then calculated from the counts in the embryos and the specific activity of the substrate in the incubation medium. Where necessary, the data of Lewis & Wright (1935) were used as an estimate of the volume of the vitellus, to calculate the concentration of isotope in the embryo.

The method used to measure the production of carbon dioxide has been described previously (Brinster, 1967). After a 4-hr incubation, samples were acidified with $0.2\text{ N-H}_2\text{SO}_4$ and the evolved carbon dioxide absorbed with 1 ml of $1\text{ M } p\text{-(diisobutyl-cresoxyethoxyethyl)dimethyl benzyl ammonium hydroxide}$ (hyamine hydroxide, Packard Instrument Company). The radio-activity was then assayed by liquid scintillation techniques.

RESULTS

The overall results for the accumulation of substrate carbon by 2- and 8-cell mouse embryos incubated in the presence of glucose (4.8 mM) are given in Table 1. After a 30-min incubation, 273×10^{-14} g atoms of substrate carbon

TABLE 1
ACCUMULATION OF SUBSTRATE CARBON BY MOUSE EMBRYOS DURING A 30-MIN INCUBATION IN A DILUENT CONTAINING 4.8 mM GLUCOSE AS SOLE ENERGY SOURCE

Stage of development	No. of observations	Substrate carbon accumulated (g atoms $\times 10^{-14}$ /embryo)
2-cell	9	273 ± 10
8-cell	12	301 ± 9

Mean values $\pm$ standard errors of the means are given.

accumulated in 2-cell embryos, while in 8-cell embryos, 301×10^{-14} g atoms were found. The difference between 2- and 8-cell embryos was just significant ($t_{19} = 2.1$, $P = 0.05$). By comparison the amount of substrate carbon found in 1-cell embryos under similar conditions (Table 2) was low although not as

TABLE 2
UPTAKE OF GLUCOSE (3.8 mM) BY UNFERTILIZED AND FERTILIZED MOUSE OVA DURING A 30-MIN INCUBATION IN THE PRESENCE OR ABSENCE OF LACTATE (5 mM) AND PYRUVATE (0.05 mM)

Stage of development	Lactate + pyruvate	Glucose carbon accumulated (g atoms $\times 10^{-14}$ /embryo)	
		Rep 1	Rep 2
Unfertilized	—	14	13
	+	13	15
Fertilized	—	46	51
	+	40	44

low as that found in unfertilized ova. The addition of low levels of lactate and pyruvate to the incubation medium as a readily available alternative energy source did not increase the accumulation of glucose carbon by either fertilized or unfertilized ova.

The effect of varying conditions on the intra-cellular levels of glucose carbon in 2- and 8-cell embryos is shown in Tables 3, 4 and 5. A change in the incubation temperature from 37° C to 5° C almost completely eliminated any uptake of substrate carbon at both stages of development (Table 3). In the second experiment (Table 4), a glucose concentration of 0.5 mM, instead of 5 mM, resulted in a fall in the intracellular level of glucose carbon to 40% of that found with the higher concentration. However, with 0.5 mM glucose, the concentration of substrate carbon in the cell was 2.5 times that in the medium.

TABLE 3
COMPARISON OF INCUBATION AT 37° C AND 5° C FOR
30 MIN ON THE ACCUMULATION OF SUBSTRATE CARBON
BY MOUSE EMBRYOS IN A DILUENT CONTAINING 4.9 mM
GLUCOSE

Stage of development	Temperature of incubation (°C)	Substrate carbon accumulated (g atoms $\times 10^{-14}$ /embryo)	
		Rep 1	Rep 2
2-cell	37	165	178
	5	4	2
8-cell	37	297	311
	5	6	9

By contrast, substrate carbon concentration was lower in the cell than in the medium when 5 mM glucose was added to the incubation medium.

A third experiment was carried out to study the effects of varying the time of

TABLE 4
EFFECT OF A CHANGE IN GLUCOSE CONCENTRATION ON
THE ACCUMULATION OF SUBSTRATE CARBON BY MOUSE
EMBRYOS

Stage of development	Glucose concentration (mM)	Substrate carbon accumulated	
		(i) g atoms $\times 10^{-14}$ /embryo	(ii) μ g atoms/ml
2-cell	5.0	261	17
	0.5	111	7
8-cell	4.7	327	24
	0.5	126	9

Mean values for two replicates are given.

incubation (Table 5). For 2-cell embryos, aliquots of two collections of embryos were incubated 3, 9, 27 and 81 min. In the case of 8-cell embryos, insufficient numbers of embryos were collected for all times to be studied at once and a

TABLE 5
ACCUMULATION OF SUBSTRATE CARBON DURING VARY-
ING PERIODS OF INCUBATION IN 5.1 mM GLUCOSE

Incubation time (min)	Substrate carbon accumulated (g atoms $\times 10^{-14}$ /embryo)					
	2-cell		8-cell			
			Exp. 1		Exp. 2	
	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
3	53	60	68	60	—	—
9	137	122	172	158	—	—
27	248	248	319	281	245	336
81	286	393	—	—	519	615

complementary experiment comparing 27 and 81 min was carried out separately (see Table 5). Substrate carbon accumulated rapidly in 2-cell embryos during the first 9 min of incubation. Thereafter, the rate of its accumulation decreased. Its concentration doubled during the next 18 min and the levels at 81 min were only 40% above those at 27 min. At all times, there was more substrate carbon accumulation in 8-cell embryos than in 2-cell embryos, and the difference became greater as the time of incubation was increased. This was due to the fact that the rate of accumulation of substrate carbon in 8-cell embryos did not fall off as rapidly with time and the levels doubled between 27 and 81 min.

TABLE 6
METABOLISM OF HEXOSE CARBON BY MOUSE EMBRYOS INCUBATED
IN A DILUENT CONTAINING 3.6 mM $1\text{-}^{14}\text{C}$ -GALACTOSE

Stage of development	Carbon one of galactose accumulated in 30 min		CO_2 produced from carbon one during 4-hr incubation	
	No. of observations	$\text{g atoms} \times 10^{-14} / \text{embryo}$	No. of observations	$\text{moles} \times 10^{-14} / \text{embryo}$
2-cell	4	41.1 ± 1.9	3	21.3 ± 18.0
8-cell	4	45.5 ± 1.4	3	89.7 ± 2.2

Mean values $\pm$ standard errors of the means are given.

In the final experiments, the uptake and utilization of $1\text{-}^{14}\text{C}$ -galactose by mouse embryos was investigated. The accumulation of substrate carbon and the production of carbon dioxide from carbon position one of galactose are shown in Table 6. The accumulation of carbon from position one of galactose

TABLE 7
UPTAKE OF $1\text{-}^{14}\text{C}$ -GALACTOSE (3.7 mM) BY MOUSE EMBRYOS DURING 30-MIN INCUBATION IN THE PRESENCE OR ABSENCE OF LACTATE (5 mM) PLUS PYRUVATE (0.05 mM)

Stage of development	Lactate + pyruvate	Carbon one of galactose accumulated ($\text{g atoms} \times 10^{-14} / \text{embryo}$)	
		Rep 1	Rep 2
2-cell	—	41	43
	+	46	41
8-cell	—	48	48
	+	52	53

was one-sixth the total accumulation of carbon from $\text{U-}^{14}\text{C}$ -glucose. The levels in 8-cell embryos showed a tendency to be higher but the difference was not significant. On the other hand, the production of carbon dioxide by 8-cell embryos was higher than that from the 2-cell stage. In a further experiment (Table 7), the addition of low levels of lactate (5 mM) and pyruvate (0.05 mM) to the incubation medium did not significantly affect the accumulation of carbon one of galactose in either 2- or 8-cell embryos.

The effect of a combination of glucose and galactose on their respective accumulation was also studied. For each sample of embryos collected, equal numbers of embryos were transferred to four droplets of medium containing, respectively, radio-active glucose alone, radio-active glucose plus non-radio-active galactose, radio-active galactose alone and radio-active galactose plus non-radio-active glucose. By assay of the radio-activity incorporated into the

TABLE 8
ACCUMULATION OF HEXOSE CARBON BY MOUSE EMBRYOS
INCUBATED 30 MIN IN A DILUENT CONTAINING GLUCOSE
(4.8 mM) OR GALACTOSE (4.0 mM) ALONE AND IN
COMBINATION

Stage of development	Total glucose carbon accumulated		Carbon one of galactose accumulated	
	— galactose	+ galactose	— glucose	+ glucose
2-cell	250	230	39	19**
8-cell	285	208**	44	22**

Values are expressed as g atoms $\times 10^{-14}$ /embryo and are the means of two replicates.

** Significant effect of combination on carbon accumulation, $P < 0.01$.

embryos in each droplet, the accumulation of substrate carbon from both glucose and galactose, alone or in combination, could be calculated. The results for two replicates at each stage of development (Table 8) showed that the addition of galactose decreased the accumulation of glucose carbon slightly. On the other hand, the intracellular level of galactose carbon was halved by the addition of glucose to the incubation medium.

DISCUSSION

In these studies a great change in the accumulation of substrate carbon from glucose occurred about the time of fertilization and first cleavage, but after this time there was only a slight change in substrate accumulation. In other studies, it has been found that carbon dioxide production from glucose continues to increase through all stages of development (Brinster, 1967). Wales & Whittingham (1968) have found no difference between 1- and 2-cell embryos in their oxidative utilization of lactate or pyruvate; thus the available evidence points either to rate of transport or to rate of glycolysis as the mechanism governing the production of energy from glucose in the embryos.

In the case of malic acid, a difference in permeability between 2- and 8-cell mouse embryos (Wales & Biggers, 1968) explains why this compound will support development only from the 8-cell stage. The position appears to be quite different for glucose, where similar amounts of glucose carbon accumulate in the cell at the 2- and 8-cell stage during a 30-min incubation. At the same time, the increase in the accumulation of substrate carbon with time was far greater for 8- than 2-cell mouse embryos, indicating that differences exist between 2- and 8-cell mouse embryos in their metabolism of glucose. However, it is not clear if it is the greater accumulation of metabolic products in 8-cell

embryos or the greater oxidative utilization of glucose by 8- as compared to 2-cell embryos (Brinster, 1967) that is responsible for the ability of glucose to maintain development only after the third cell division.

Incubation of the embryos in glucose at the specific activity used resulted in the accumulation of approximately 3 d.p.m./embryo. At such a low level of radio-activity, it was impossible to make even a preliminary fractionation of the substrate carbon accumulated within the cell. Thus the nature of the accumulated products is a matter for debate. No doubt some of the carbon accumulates as the parent substrate. At the same time, some will have entered the carbon pools of the embryo and the net accumulation of isotope will depend on the size and number of these pools and on the rates of reaction replenishing and depleting them. Brinster (1967) has indicated that the pentose shunt is operative in mouse embryos at this stage of development, and thus some of the carbon accumulated may arise from synthetic reactions involving this pathway. In addition, Wales & Whittingham (1968) have found that there is intracellular accumulation of metabolic products following decarboxylation of pyruvate or lactate in the mouse embryo.

The mechanism of transfer of monosaccharides across membranes differs from tissue to tissue. Glucose uptake has been described as active transport in the isolated intestine of the guinea-pig (Riklis, Haber & Quastel, 1958) and in suspensions of isolated rat intestinal epithelial cells (Stern & Jensen 1966), but in erythrocytes and muscle has been explained on the basis of an exchange diffusion (Mawe & Hempling, 1965; Park, Reinwein, Henderson, Cadenas & Morgan, 1959) involving a membrane carrier but lacking an energy-consuming link. In embryos, elucidation of the mechanism of transport is made difficult because few cells are able to be harvested for study and as mentioned above it was impossible to identify what portion of the intracellular carbon accumulating is the parent substrate. However, a very substantial increase in the specific activity of the substrate may make it possible to recover sufficient isotope to allow identification of the materials accumulated in the embryo and thus to determine if an active transport system exists in embryos for the movement of glucose across the cell membrane. At the same time, the virtual absence of substrate in embryos incubated at 5° C indicates that little, if any, of the substrate enters the cell by diffusion.

Galactose, the naturally occurring isomer of glucose, behaves as a non-utilizable substance in most animal tissues (see Levine & Goldstein, 1955) and has been used as an indicator of hexose transport in spermatozoa (Flipse, 1962). The competition between glucose and galactose in embryos is similar to that reported in guinea-pig intestine (Riklis, Haber & Quastel, 1958), tissue culture cells (Rickenberg & Maio, 1960) and rabbit kidney cortex (Krane & Crane, 1959). However, the finding that mouse embryos utilize galactose to some extent makes it impossible at the present stage of our knowledge to differentiate between competition for uptake as is found in other tissues and competition for entry of metabolic products into the common carbon pools.

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