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Turnover of Carbon Pools Labelled with [^{14}C]Glucose during *in vitro* Culture of Preimplantation Mouse Embryos

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Abstract

The pulse–chase technique was used to study the uptake and turnover of glucose carbon by mouse embryos *in vitro*. During a 1 h pulse the uptake of glucose into all embryonic fractions increased between the eight-celled and the morula–early blastocyst stages of development. Whilst most of the glucose carbon entered the non-glycogen, acid-soluble pool, significant amounts were isolated in acid-insoluble macromolecules and, at the later stage of development, in acid-soluble glycogen.

Most of the carbon which entered the non-glycogen, acid-soluble fraction of embryos during the pulse was lost during subsequent chase culture. Under most circumstances, chase culture also led to a loss of carbon from the acid-insoluble components of embryos. By contrast, the acid-soluble glycogen synthesized during the pulse was stable during subsequent chase culture providing a simple energy substrate was present in the medium.

The turnover of glucose carbon incorporated into the biochemical pools of morulae–early blastocysts was similar during chase in the presence of 0·28 mM glucose, 5 mM lactate plus 0·5 mM pyruvate to that found when the chase medium contained 5·56 mM glucose as sole exogenous substrate. When the chase was conducted in medium containing lactate and pyruvate but lacking glucose, more carbon was retained by embryos during the chase than under the conditions above. This effect was due mainly to an increased retention of label in the non-lipid, acid-insoluble fraction.

Introduction

The utilization of glucose as an energy source for the growth and development of preimplantation mouse embryos has been examined by measurement of CO_2 output and the production of organic acids, particularly lactate (Brinster 1967; Wales 1969). As well as a source of energy, glucose carbon is used by preimplantation embryos for the synthesis of macromolecules. During *in vitro* culture, for example, carbon from glucose is accumulated in embryonic energy stores such as glycogen (Brinster 1969; Pike and Wales 1982) and triglyceride (Flynn and Hillman 1978). Some glucose carbon is also incorporated into structural and informational macromolecules such as proteins, nucleic acids and phospholipids (Brinster 1969; Pike *et al.* 1977; Flynn and Hillman 1978; Pike and Wales 1982).

The studies of the uptake and incorporation of radioactive glucose during culture of preimplantation embryos has supplied valuable information relating to glucose anabolism during development. However, further analysis of the rates of degradation of the intracellular glucose carbon pools is required in order to determine the turnover of this substrate within the embryo. These data could help to explain the anomalous results which have been reported for the accumulation of glycogen in freshly collected and *in-vitro*-cultured mouse embryos. Glucose is incorporated into glycogen at a

high rate during culture of mouse morulae and blastocysts in medium containing both 0·28 and 5·56 mM glucose (Brinster 1969; Pike and Wales 1982). On the other hand, the glycogen content of uterine embryos does not rise during this period (Stern and Biggers 1968; Ozias and Stern 1973). The difference in glycogen metabolism *in vitro* compared with that *in vivo* could be explained by a limiting level of glucose in the luminal fluids of the mouse reproductive tract. Data for the concentration of glucose in oviducal and uterine fluid in other mammals (Holmdahl and Mastroianni 1965; Wales 1973) and the high rate of glycogen synthesis in the presence of glucose concentrations as low as 0·28 mM suggest that limitation of substrate *in vivo* is unlikely (Pike and Wales 1982). An alternative explanation is that the embryo utilizes glucose for rapid glycogen synthesis but links this with a concomitant high rate of glycogen degradation to maintain a constant level of embryonic glycogen over this period.

An estimate of the contribution of degradation to the net accumulation of glucose within the embryo is afforded by study of the turnover rate of glucose carbon pools during culture of embryos which have previously been incubated for a short period in radioactive substrate. The method was originally developed for investigations into bacterial metabolism and termed the pulse–chase technique. The results of preliminary experiments using this technique to define suitable culture periods for subsequent studies of glucose incorporation and turnover in mouse embryos have been reported (Pike *et al.* 1973). The aim of the present study was to employ the pulse–chase technique to examine further the metabolism of glucose by preimplantation mouse embryos during *in vitro* culture.

Materials and Methods

General

Preimplantation embryos were obtained from albino mice 8–12 weeks old and superovulated by intraperitoneal injection of 5–10 i.u. of pregnant mare's serum gonadotrophin (Folligon, Intervet International B.V., Boxmeer, Holland) followed after 48 h by intraperitoneal injection of 5–10 i.u. of human chorionic gonadotrophin (hCG) (Chorulon, Intervet International B.V., Boxmeer, Holland). Following hCG administration the mice were mated and the reproductive tracts of mated mice flushed approximately 60 and 84 h after ovulation to recover embryos at the eight-celled and morula–early blastocyst stage respectively.

The basic medium used to collect, wash and culture embryos was the modified Krebs–Ringer bicarbonate solution used in previous studies (Pike and Wales 1982). During collection, this medium was supplemented with sodium lactate (25 mM) and sodium pyruvate (0·25 mM). After the embryos were collected they were washed through one change of flushing medium, two changes of substrate-free medium (2 ml per wash) and transferred to droplets (vol. 25 μl) of culture medium (80–150 embryos per droplet) containing [U-^{14}C]glucose (The Radiochemical Centre, Amersham, England) as the sole energy substrate. Depending on the requirements of the experiment the [U-^{14}C]glucose was diluted with medium containing non-radioactive glucose to give concentrations of either 5·56 mM or 0·28 mM glucose and specific activities of 3 or 28 μCi/μmol (0·1 and 1·0 MBq) respectively. Culture was carried out as previously described (Pike and Wales 1982).

After pulse culture in radioactive glucose for 1 or 2 h the labelled embryos were recovered and rapidly washed through two changes of medium (2 ml per wash) containing non-radioactive glucose at a concentration identical to that used during the pulse-labelling. A sample of approximately one-third of the embryos was selected at random and stored at −70°C for later extraction. The remaining embryos were returned to *in vitro* culture (20–30 embryos per 25 μl droplet) for a 24 h chase period. The medium used for chase culture was of identical composition to that used for the pulse with the exception that the glucose was non-radioactive and the concentrations of the energy substrates glucose, lactate and pyruvate were altered with the requirements of the experiments.

Embryos which underwent normal development during the 24 h chase culture were washed through two changes (2 ml per wash) of fresh chase culture medium and stored at −70°C prior to fractionation.

Extraction of Embryos

The extraction procedure is based on that previously described (Pike and Wales 1982). The embryos were passed through two cycles of freezing in solid CO_2 and fresh mouse liver homogenate (0·3 ml) was added. The tissue was immediately partitioned into acid-soluble and acid-insoluble material by the addition of 0·1 ml of ice-cold 25·6% (v/v) perchloric acid (PCA) followed by centrifugation at 1000 *g* for 10 min at 4°C. The acid-soluble supernatant was removed and the insoluble pellet washed four times with PCA, the washes being added to the initial acid-soluble supernatant. Glycogen was precipitated from the acid-soluble material by the addition of one volume of ethanol. Following overnight storage at 4°C, the pellet was isolated by centrifugation, washed with ice-cold ethanol, dissolved in 0·5 ml distilled water and taken for assay of radioactivity. Samples of the material which was soluble in both PCA and ethanol were dried in a scintillation vial prior to radioassay.

Lipid was extracted from the acid-insoluble pellet using a modification to the method used by Shibko *et al.* (1967). The insoluble material was suspended in turn in (*a*) 0·8 ml of 0·35% PCA in ethanol, (*b*) 0·4 ml of ethanol–chloroform (3 : 1 v/v), (*c*) 0·4 ml of ethanol–diethyl ether (3 : 1 v/v), followed by the addition of 0·2 ml light petroleum after incubation for 15 min at 37°C, and (*d*) 0·8 ml diethyl ether. After each treatment the material was centrifuged for 10 min at 4°C and 1000 *g* and the supernatant removed and pooled in a 20-ml sealed tube. The pooled fractions were finally mixed and 2 ml light petroleum (l.p. 60–80°C) plus 4 ml of distilled water added. Following vigorous shaking, the organic and aqueous phases were allowed to separate. The volumes of each phase were determined and 1-ml samples taken for assay of radioactivity. Solvents present in the organic phase were evaporated under a stream of air before addition of scintillation fluid. Label in the organic phase was classified as incorporation into lipid while label isolated in the aqueous back-wash was added to that incorporated into the other acid-insoluble fraction as suggested by Shibko *et al.* (1967).

The pellet of acid-insoluble material remaining after lipid extraction was dried under a stream of air and solubilized by incubation in 0·2 ml of 0·8 M NaOH overnight. The resultant sample was neutralized with HCl prior to assay of radioactivity. Label in this fraction represented [^{14}C]glucose carbon incorporated into macromolecules other than lipid and includes proteins, nucleic acids and protein bound glycogen (desmoglycogen).

Assay of Radioactivity

Radioactivity in various aqueous fractions isolated was assessed by liquid scintillation spectrometry as previously described by Pike and Wales (1982). Scintillation cocktail was added to the samples in the ratio of 10 ml of scintillation fluid for every 0·8 ml aqueous sample. In the case of the dried lipid material, the sample was dissolved in 5 ml of ethanol : toluene (1 : 2 v/v) which contained the scintillation fluors.

Results

The incorporation of glucose carbon by eight-celled and morula–early blastocyst stage mouse embryos during 1 h pulse culture and the turnover of the accumulated glucose carbon during a further 24 h *in vitro* when the culture medium contained 5·56 mM glucose as sole energy substrate was examined in the initial experiment. The results are shown in Table 1. The total uptake of glucose during 1 h culture in the [^{14}C]glucose medium increased three- to fourfold between the eight-celled and morula–early blastocyst stages of development and this was reflected in significantly increased incorporation into all biochemical fractions. During the subsequent 24 h chase in non-labelled glucose medium, approximately 70% of the glucose carbon accumulated during the pulse was lost from the embryos. Immediately

following the pulse 80% of the total substrate carbon incorporated was found in the acid-soluble fraction of the embryos at both stages of development. However, some of the substrate had been utilized for synthesis of macromolecules during the first hour of culture and significant amounts of glucose carbon were found in the acid-insoluble macromolecules. Furthermore, at the later stage of development approximately 20% of the label incorporated into the embryos was present as acid-soluble glycogen.

Table 1. Glucose carbon accumulated in mouse embryos during a 1-h pulse and following 24-h chase culture in medium containing 5·56 mM glucose

Values are the means of four replicates. Data in parenthesis are the percentage of the total carbon incorporated. * $P < 0{\cdot}05$. ** $P < 0{\cdot}01$

Stage of development at pulse	Treatment	N[A]	Glucose carbon (pg-atoms per embryo) accumulated in: Acid-soluble fraction Glycogen	Acid-soluble fraction Non-glycogen	Acid-insoluble fraction Lipid	Acid-insoluble fraction Non-lipid
8-cell	Pulse	267	0·06 (1)	3·04 (80)	0·12 (3)	0·60 (16)
	Chase	545	0·05 (4)	0·47 (35)	0·04 (3)	0·77 (58)
Morula–early blastocyst	Pulse	277	2·72 (19)	8·66 (60)	0·21 (1)	2·96 (20)
	Chase	517	2·61 (51)	1·07 (21)	0·10 (2)	1·35 (26)

Summary of the analyses of variance

Source of variation	D.f.	Variance ratio Glycogen	Non-glycogen	Lipid	Non-lipid
Stage of development	1	43·65**	13·63**	10·86**	12·18**
Pulse v. chase	1	0·01	36·35**	17·33**	2·92
Interaction	1	0·05	8·86*	0·19	4·46
Error variance	12	*0·16*	*0·71*	*0·005*	*0·18*

[A] N, mean number of embryos per replicate.

During the chase, the loss of substrate carbon previously incorporated within the biochemical fractions of the embryos was predominantly due to a substantial and significant decrease in the level of glucose carbon in the non-glycogen acid-soluble material. At the eight-celled stage, 2·57 pg-atoms of glucose carbon were lost from a total of 3·04 pg-atoms in this fraction while a decrease of 7·59 pg-atoms of glucose carbon from a total of 8·66 pg-atoms was found at the later stage studied. In contrast to the eight-celled embryos a large amount of glycogen was synthesized during 1 h *in vitro* culture of mouse morulae–early blastocysts. This glycogen was not utilized during the chase in the presence of non-radioactive glucose and represented 51% of the total label retained by cultured blastocysts during the chase. Lipid material accounted for only a small proportion of the total carbon incorporated at both stages of development and carbon accumulated in the lipid fraction during the pulse consistently decreased during incubation in non-radioactive medium. The amount of label in the non-lipid, acid-insoluble fraction showed an increase during the chase culture of the eight-celled embryos but decreased by 50% during the chase of the later

stage embryos. However, this effect varied between replicates and the interaction term in the analysis of variance, which would have demonstrated the statistical significance of this difference between stages of development approached but failed to reach the 5% level of significance.

Table 2. Glucose carbon accumulated in mouse morulae–early blastocysts during 1-h pulse culture in medium containing 5·56 mM [U-^{14}C]glucose and following chase culture in medium with and without glucose

Values are the means of five replicates and data in parenthesis are the percentage of total carbon accumulated. Chase culture medium contained 5 mM lactate; 0·5 mM pyruvate ± 0·28 mM glucose. * $P < 0·05$. ** $P < 0·01$

Incubation	N[A]	Glucose carbon (pg-atoms per embryo) accumulated in: Acid-soluble fraction		Acid-insoluble fraction	
		Glycogen	Non-glycogen	Lipid	Non-lipid
Pulse	211	2·21 (17)	7·41 (58)	0·28 (2)	2·97 (23)
Chase + glucose	263	2·63 (47)	1·16 (20)	0·13 (2)	1·73 (31)
Chase − glucose	243	3·15 (37)	1·92 (22)	0·18 (2)	3·38 (39)

Summary of the analyses of variance

Source of variation	D.f.	Variance ratio			
		Glycogen	Non-glycogen	Lipid	Non-lipid
Pulse *v.* chase	1	0·74	47·1**	5·02*	0·43
Effect of glucose	1	0·32	0·59	0·60	5·11*
Error variance	12	*0·42*	*0·49*	*0·002*	*0·27*

[A] N, mean number of embryos per replicate.

The effect of chase culture in medium which contained alternative simple energy substrates and a reduced glucose concentration was examined in the second experiment. Morula–early blastocyst stage embryos were pulse-labelled for 1 h in the presence of 5·56 mM [U-^{14}C]glucose as in experiment 1. After this pulse, the embryos were chase-cultured for 24 h in medium containing 5 mM sodium lactate and 0·5 mM sodium pyruvate either in the presence or absence of 0·28 mM non-radioactive glucose. The results of this pulse–chase experiment are shown in Table 2. During the pulse, glucose carbon accumulated in all fractions of the embryos and the results agree closely with those obtained in experiment 1 (see Table 1). The total loss of labelled glucose and the changes in the pattern of its incorporation into the embryonic fractions during chase culture in lactate–pyruvate–glucose medium were also similar to those obtained during chase culture in medium containing 5·56 mM glucose as sole energy substrate (see Table 1). However, considerably more label was retained by the embryos when the chase was conducted in medium containing lactate and pyruvate but lacking glucose (8·63 pg-atoms per embryo compared with 5·65 pg-atoms per embryo in the presence of glucose). This increase in total retention of substrate carbon during the chase in medium devoid of glucose was reflected in a general increased retention of glucose carbon in each of the embryonic fractions. However, a statistically significant increase in retention of glucose carbon during

chase culture in the absence of glucose compared to retention in its presence occurred only in the case of the non-lipid, acid-insoluble fraction.

During chase culture of morulae–early blastocysts in the presence of any of the simple energy substrates, label in the acid-soluble glycogen fraction was maintained at levels at least as great as those found immediately after pulse. As a result, this fraction contained 40–50% of the label remaining in the embryos after the 24 h chase while the level in the non-glycogen acid-soluble fraction had fallen from approximately 60% of the label in the embryos at the end of the pulse to 20% at the end of the chase.

In a final experiment, an estimate of the turnover of glucose carbon within the blastocyst was made during chase culture in medium devoid of any simple energy substrate. Morulae–early blastocysts were pulse-labelled in medium containing 5 mM lactate, 0·5 mM pyruvate and 0·28 mM [U-^{14}C]glucose. The intention of the experiment was to maximize the labelling of the biochemical fractions during pulse to ensure the presence of sufficient label for radioassay after chase in a substrate-free medium. As previous experiments (Pike and Wales 1982) have indicated that a 20-fold reduction in glucose concentration causes relatively minor changes in the incorporation of glucose, maximum labelling was achieved by pulse culture in a low concentration of glucose (0·28 mM) but a high specific activity (1·0 MBq/μmol) for a period of 2 h and chase-culturing all embryos in substrate-free medium. The distribution of label within the embryo after the 24 h chase is shown in the following tabulation (values are means ± s.e.m. for two replicates; data in parenthesis are the percentage of the total carbon accumulated):

Fraction assayed	Glucose carbon remaining after chase (pg-atoms/embryo)
Acid-soluble glycogen	0·33 ± 0·15 (4)
Non-glycogen acid-soluble	1·74 ± 0·15 (24)
Lipid	0·38 ± 0·00 (5)
Non-lipid acid-insoluble	4·94 ± 0·64 (67)

By contrast with the earlier experiments in which acid-soluble glycogen constituted a major pool within which glucose carbon was conserved, the glycogen fraction in this experiment contained only a minor portion of the label retained during the chase in substrate-free medium. After taking into consideration the difference in the length of the pulse period and the lower concentration of glucose during pulse culture, the incorporation of label into the other embryonic fractions was comparable to that found in the earlier experiments.

Discussion

In the previous study of Pike *et al.* (1973) the feasibility of utilizing the pulse–chase technique to investigate glucose metabolism by mouse embryos was reported. Sufficient radioactivity was accumulated by mouse morulae during a 1-h culture period in [^{14}C]glucose for easy detection of label in both the acid-soluble and acid-insoluble fractions of the embryos. In the present work it has been possible to extend the use of the method to the eight-celled stage of development.

During pulse-culture most of the glucose carbon entered the non-glycogen, acid-soluble pool presumably in the form of glucose or glucose-6-phosphate. The

degree of labelling of this fraction decreased markedly during the chase, indicating that compounds in this fraction were metabolized during the ensuing 24-h chase and the end products of their metabolism lost into the surrounding medium. Exchange diffusion of intracellular with extracellular glucose and the utilization of components of this fraction in anabolic pathways could also explain some of the loss of label from this pool during the chase. In view of (1) the high rate of glycogen synthesis using glucose units, (2) the apparent lack of glycogen degradation during culture, and (3) the high proportion of free glucose expected in the acid-soluble fraction during a pulse, it is surprising that the decrease in label in the non-glycogen, acid-soluble fraction was not accompanied by a more significant rise in label in the glycogen fraction of morulae–early blastocysts. This should have been especially significant when the chase medium contained lactate and pyruvate as under these conditions glycolysis would be more inhibited (Barbehenn *et al.* 1974) resulting in a larger pool of glucose for polymerization. However, only a slight and non-significant increase in glucose incorporation into glycogen was found under these conditions.

Some substrate carbon remained in the non-glycogen, acid-soluble fraction after the 24-h chase. Without further characterization of the constituent compounds it is difficult to postulate the major reason for retention of label in this fraction. A few macromolecules, such as histones, are soluble in acid and could be responsible for the residual radioactivity in this fraction.

Mouse morulae–early blastocysts are capable of synthesizing up to 9 ng of glycogen per day from glucose *in vitro* at a rate that is relatively independent of glucose concentration (Ozias and Stern 1973; Pike and Wales 1982). The present experiments indicate that this glycogen is stable during subsequent culture provided that a simple energy substrate is present in the medium. Recent evidence (Edirisinghe and Wales, unpublished data) indicates that substrate concentration is not a limiting factor to glycogen metabolism in the mouse uterus and thus the relatively low levels of glycogen found *in vivo* must be due to control by other, as yet unknown, components of the uterine environment modifying the turnover of this compound.

A considerable amount of glucose carbon was found to be incorporated into the acid-insoluble macromolecular fraction of embryos. This fraction would contain such macromolecules as lipids, nucleic acids and proteins. In order for glucose carbon to enter the carbon skeleton of the fatty acid, nucleotide and amino acid components of these macromolecules, the glucose must be initially catabolized into glycolytic and TCA cycle intermediates which are then used in the biosynthetic pathways. With glycolysis tightly controlled in mouse embryos up to the late blastocyst stage of development and tightly inhibited in the presence of pyruvate (Barbehenn *et al.* 1978) it is not surprising that only a small proportion of the total glucose carbon incorporated by the embryo is found in the carbon backbone of these macromolecules (Schneider *et al.* 1976; Pike *et al.* 1977; Flynn and Hillman 1978). Thus, most of the label isolated in the non-lipid, acid-insoluble fraction of embryos at both the eight-celled and morula–early blastocyst stages is probably present in the carbohydrate moieties of various glycoproteins. Most of the label in this fraction has been found in the form of a glucose polymer hydrolysable from the protein by amyloglucosidase action, indicating the presence of desmoglycogen (Pike and Wales 1982).

As well as glycoprotein in the form of desmoglycogen, the non-lipid, acid-insoluble fraction may contain label in carbohydrate moieties through the conversion of glucose

to other hexoses and their subsequent incorporation into cell surface glycoproteins. An increasing number of recent reports suggest considerable synthesis of glycoproteins during preimplantation development of mouse embryos (Wartiovaara *et al.* 1978; Wu and Chang 1978; Surani 1979) but further characterization of the labelled material isolated in the acid-insoluble fractions is necessary before further comment is justified.

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