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Uptake and Incorporation of Glucose especially into the Glycogen Pools of Preimplantation Mouse Embryos during Culture *in vitro*

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Abstract

Rate of [^{14}C]glucose uptake by mouse embryos during *in vitro* culture in media containing optimal concentrations of lactate and pyruvate increased throughout preimplantation development. A reduction in the glucose concentration of the medium from 5·56 to 0·28 mM resulted in a two- to fivefold decrease in glucose metabolism, suggesting near-saturation of the membrane carrier at the lower concentration. Changes in the level of lactate and pyruvate in the medium had little effect on glucose metabolism after the third cleavage division. However, further evidence of interaction between energy substrates during the initial cleavage was obtained.

Glucose was stored either as desmoglycogen during early cleavage or in a larger acid-soluble glycogen pool in the latter stages of development. The accumulation of glycogen calculated from its production by blastocysts cultured in either 5·56 or 0·28 mM glucose greatly exceeded that determined in blastocysts freshly collected from the uterus. The absence of lactate and pyruvate from the medium had only minimal effects on glycogen accumulation.

Most of the glucose carbon was stored as a form of glycogen. However, considerable amounts were also found in acid-soluble material other than glycogen at all developmental stages, probably as the parent compound and its catabolites. Some was also present in lipids, nucleic acids and proteins.

Introduction

The embryonic glycogen pool increases markedly during culture of two-celled mouse embryos through to the late blastocyst stage in medium containing glucose (5·56 mM), lactate (23 mM) and pyruvate (0·25 mM) (Ozias and Stern 1973). Omission of glucose from this medium results in a decrease in glycogen content of the embryos during culture (Ozias and Stern 1973) suggesting that glucose is the major precursor for glycogen synthesis *in vitro*. Brinster (1969) found incorporation of large amounts of [^{14}C]glucose into glycogen during *in vitro* culture of mouse morulae and early blastocysts in the presence of 5·56 mM glucose. Thus, embryos cultured in 5·56 mM glucose have an adequate supply of glucose and an abundance of glycogen synthetase activity (Stern 1970) to ensure a rapid synthesis of glycogen.

In embryos collected from the reproductive tract there is approximately a 10-fold increase in glycogen content from the one- to the two-celled stage of development (from 0·1 to 1·4 ng per embryo) (Stern and Biggers 1968; Ozias and Stern 1973) but at this time very little glucose is incorporated into glycogen during culture (Brinster 1969). Furthermore, between the two-celled and blastocyst stages, when rapid

synthesis of glycogen occurs *in vitro*, the quantity of glycogen in uterine embryos remains relatively constant at approximately 1–2 ng per embryo (Stern and Biggers 1968; Ozias and Stern 1973). Consequently it has been suggested that the level of glucose in the luminal fluids of the mouse reproductive tract may have a significant effect on the amount of glycogen synthesized by embryos during development *in utero* (Ozias and Stern 1973).

Although no direct measurements have been reported on the concentration of energy substrates such as glucose, lactate and pyruvate in the reproductive tract of the mouse, useful data can be extrapolated from assay of fluid collected by cannulation of the reproductive tract of the rabbit and ewe. Compared with reproductive tract fluid which has an approximate composition of 1–5 mM lactate (Restall and Wales 1966; Iritani *et al.* 1971; Wales 1973*a*), 0·2 mM pyruvate (Holmdahl and Mastroianni 1965) and 0·28 mM glucose (Wales 1973*a*), the most commonly used media for the culture of mouse embryos contains 25 mM lactate, 0·25 mM pyruvate and 5·56 mM glucose (Whitten 1971). This difference in substrate concentration may explain the reported differences in glycogen metabolism between mouse embryos developing *in utero* and those cultured *in vitro*.

As well as resulting in varied patterns of incorporation of glucose into glycogen, substantial changes in the levels of energy substrates in the culture medium could significantly alter other patterns of metabolism during development of the embryo *in vitro*. The present experiments were undertaken to study the uptake and metabolism of glucose by mouse embryos under conditions that have previously been used in *in vitro* experiments and to compare the results with glucose uptake and metabolism when the conditions more closely mimic those expected in the reproductive tract. Of particular interest were changes which might result from a significant reduction in the concentration of glucose in the culture medium from 5·56 to 0·28 mM.

Materials and Methods

General

Embryos were collected from albino Swiss mice, superovulated by intraperitoneal injection of 5–10 i.u. of pregnant mare's serum gonadotrophin (Folligon, Intervet International B.V., Boxmeer, Holland) followed 48 h later by 5–10 i.u. of human chorionic gonadotrophin (hCG) (Chorulon, Intervet International B.V., Boxmeer, Holland). Embryos at the one-, two-, eight-celled and morula–early blastocyst stages were flushed from the reproductive tracts of mated females approximately 24, 48, 72 and 96 h after hCG injection respectively. Single-celled embryos had their cumulus cells removed by incubation for 10 min in medium containing 150 i.u./ml hyaluronidase (hyaluronoglucosaminidase, EC 3.2.1.35, Hyalase, Fisons Pharmaceuticals, Loughborough, England).

The basic medium used in these studies for collecting embryos and embryo culture was Krebs–Ringer bicarbonate solution supplemented with 1 mg/ml bovine serum albumin (Cohn Fraction V, Commonwealth Serum Laboratories, Parkville, Vic.), 100 i.u./ml sodium penicillin G and 50 i.u./ml dihydrostreptomycin sulfate (Glaxo-Australia, Boronia, Vic.). The medium was further supplemented with a variety of energy substrates as the design of the experiment required. The embryos were flushed from the reproductive tract in medium containing 25 mM sodium lactate and 0·25 mM sodium pyruvate and washed (2 ml per wash) through two changes of culture medium devoid of energy substrate immediately prior to culture. Culture of embryos was carried out in radioactive medium prepared by diluting [U-^{14}C]glucose (The Radiochemical Centre, Amersham, England) with non-radioactive glucose-containing medium to give a final concentration of either 5·56 or 0·28 mM glucose and specific activities of 3 or 30 μCi/μmol (0·1 and 1·1 MBq) respectively. The [^{14}C]glucose was purified by paper chromatography prior to use because poor development was obtained if the isotope was used as supplied (see Murdoch and Wales 1973). In some cases unlabelled lactate and

pyruvate were added as alternative substrates. Droplets of the radioactive medium, 20 μl in volume, were placed in culture dishes (Falcon Plastics, Oxnard, California, U.S.A.) under low specific gravity paraffin oil (BDH Chemicals Ltd., Poole, England). Embryos were added to each droplet (30–50 embryos per droplet) and the dish incubated at 37°C in an humidified atmosphere of 5% CO_2 : 95% air.

Uptake of Glucose Carbon

Total accumulation of label by embryos during culture in [U-^{14}C]glucose for 0·5 and 25 h was estimated. Embryos were recovered after culture and separated from the radioactive medium by centrifugation through isotonic sucrose plus non-labelled glucose using the method previously described by Wales and Biggers (1968) and Brinster (1969). At the blastocyst stage, the change in specific gravity of the embryos prevented their centrifugation through isotonic sucrose so the sucrose was replaced with non-radioactive culture medium for the centrifugation.

Fractionation of Incorporated Glucose Carbon

The following procedures were used to study the incorporation of glucose carbon into biochemical fractions of embryos cultured in [^{14}C]glucose. Embryos showing normal development were collected after culture and washed through two changes (2 ml per wash) of non-radioactive culture medium which contained unlabelled glucose at a concentration identical to that used in culture. Following the wash, embryos were collected in a small volume (approximately 5 μl) for storage.

The washed embryos were mixed with 0·3 ml of fresh mouse liver homogenate, prepared by homogenizing 1 g of mouse liver in 9 ml of 0·25 M sucrose at 4°C and straining through cheese-cloth. The liver homogenate acted as a 'cold' carrier during extraction. Acid-insoluble material was immediately precipitated with 0·1 ml of ice-cold 25·6% (v/v) perchloric acid (PCA). Following thorough mixing, the tube was placed in an ice-bath for 15 min. The acid-soluble and acid-insoluble fractions were separated by centrifugation at 1000 *g* for 10 min at 4°C. The acid-soluble supernatant was removed and the insoluble pellet washed four times by resuspension in 0·5 ml of 5% (v/v) PCA at 4°C. The washes were added to the initial acid-soluble fraction and the pooled sample taken for radioassay. The remaining acid-insoluble material was dissolved in 0·5 ml of 0·8 M NaOH, transferred to a scintillation vial and neutralized with 0·5 ml of 0·8 M HCl in preparation for radioassay.

A preliminary experiment revealed considerable difficulty in the extraction of acid-soluble compounds from the acid-insoluble pellet when the embryos were stored at −20°C prior to the extraction. In all subsequent experiments the embryos were washed after culture, plunged into solid carbon dioxide and passed through two cycles of freezing and thawing before addition of mouse liver homogenate and PCA.

Experiment 1

The acid-soluble fraction was further extracted in this experiment to determine the incorporation of glucose into acid-soluble glycogen as well as into other acid-soluble and acid-insoluble material. Glycogen was precipitated from the pooled acid-soluble fraction by the addition of 1 vol. (2·4 ml) of ethanol. The sample was stored at 4°C overnight and centrifuged at 1000 *g* for 15 min. The pellet was washed with 1 ml ice-cold ethanol and dissolved in 0·5 ml distilled water.

Because the glycogen precipitated from the acid-soluble fraction by ethanol may contain peptides, nucleic acid precursors and other polysaccharides, the fraction was further characterized by passage through a mixed-bed column containing Zeo-Carb 225 (H^+) cationic exchange resin and Deacidite FF formate anionic exchange resin (2 ml packed volume of each resin) (Permutit, Sydney, N.S.W.). The column was precharged with 10 ml of 12 M formic acid and washed with freshly boiled distilled water until the pH of the elute was identical to the distilled water.

The acid-soluble but ethanol-insoluble material was applied to the column and eluted with 20 ml of boiled distilled water to obtain the neutral (uncharged) compounds, followed by 10 ml of 2 M HCl, 10 ml of distilled water, 6 ml of 12 M formic acid and finally 10 ml of distilled water to remove the acidic and basic (charged) compounds. The eluate was collected in 2-ml fractions for radioassay. Labelled compounds in the uncharged portion were classified as acid-soluble glycogen. Label in the acid and basic portion was added to that found in the acid-soluble and ethanol-soluble portion and

termed 'other acid-soluble material'. The pellet of acid-insoluble material was dissolved in NaOH and taken for radioassay as described previously.

Experiment 2

The effect of treatment of embryos cultured in glucose containing medium with amyloglucosidase (exo-1, 4-α-D-glucosidase, EC 3.2.1.3) prior to extraction was investigated in this experiment. The embryos were washed after incubation, divided into two equal groups and stored for extraction. The first group of embryos was pretreated with amyloglucosidase before extraction. These embryos were mixed with 1·0 ml of 0·04 M acetate buffer (pH 4·4) containing 0·1 mg/ml amyloglucosidase (Grade II, Sigma Chemical Co., St Louis, Missouri, U.S.A.) and incubated at 50°C. After 10 min incubation, 0·15 ml of 70% (v/v) PCA was added immediately followed by 0·6 ml of fresh mouse liver homogenate. The second group was incubated in acetate buffer lacking amyloglucosidase to act as a control. The suspensions were incubated for 15 min in an ice-bath and separated into acid-soluble and acid-insoluble fractions by centrifugation. The acid-insoluble pellet was washed three times with 1 ml of 5% (v/v) PCA. Both the acid-soluble and acid-insoluble material was taken for assay of radiation as previously described.

Experiment 3

In this final experiment glucose polymers in both the acid-soluble and acid-insoluble fractions of embryos were extracted. The washed embryos were extracted after culture into acid-soluble and acid-insoluble fractions as previously described. Glycogen was precipitated from the acid-soluble fraction as described in experiment 1. The remaining PCA in the acid-insoluble pellet was washed free using 1 ml cold ethanol. The pellet was dried and suspended in 1·5 ml of 0·1 M acetate buffer (pH 4·4) containing 4 μg/ml of the ribonuclease inhibitor, polyvinylsulfate, plus 0·1 mg/ml of both α-amylase (Type IIIA, Sigma Chemical Co., St Louis, Missouri, U.S.A.) and amyloglucosidase to hydrolyse any glucose polymers in the acid-insoluble material. The remaining acid-insoluble macromolecules were reprecipitated after incubation for 1 h at 37°C by the addition of 0·5 ml of ice-cold 25·6% (v/v) PCA. The supernatant was collected by centrifugation, removed and the pellet washed twice with 0·5 ml of 5% (v/v) PCA. The initial supernatant and the washes were pooled, neutralized with KOH and passed through ion-exchange columns to separate any labelled charged compounds from the neutral glucose monomers released by enzyme action. All samples from the ion-exchange columns were radioassayed. The residual acid-insoluble pellet was dissolved in NaOH and taken for assay as described previously.

Determination of Radioactivity

Samples were assayed by liquid scintillation techniques using Triton X100–toluene (1:2 v/v) containing 0·4% (w/v) 2,5-diphenyloxazole and 0·01% (w/v) 1,4-bis(5-phenyloxazol-2-yl)benzene. The scintillation mixture was added in the ratio of 5 ml scintillator for each 0·7 ml aqueous sample and a Searle Isocap 300 liquid scintillation spectrometer was used for counting. Disintegrations per minute were calculated after allowing for quench by use of a channels ratio, quench-correction curve. The correction curve was prepared by adding increasing quantities of chloroform to a standardized [U-^{14}C]glucose scintillation sample and plotting % efficiency of counting against the channels ratio.

During the extraction experiments, samples were taken at all major steps to ensure high recovery of label during subsequent extraction. The uptake and incorporation of glucose carbon was calculated (in pg-atoms) from the radioactivity detected in the samples and the specific activity of the [^{14}C]glucose.

Statistical Analyses

Results were obtained from three or more replicates and recorded as mean ± standard errors of the mean. When necessary, the significance of data in experiments with the factorial design was tested by standard analysis of variance. For comparison of results between two means the data were analysed by *t*-tests. In all cases, data obtained for incorporation of radioisotope were transformed to logarithms prior to analysis to reduce the heterogeneity of variance.

Results

Uptake of Glucose Carbon

Data for the uptake of glucose by preimplantation mouse embryos during incubation in a variety of culture media containing either 5·56 or 0·28 mM [U-^{14}C] glucose are shown in Table 1. Total uptake of glucose increased with increasing developmental age of the embryos especially between the eight-celled and morula–early blastocyst stage ($P<0·001$). During culture for 0·5 h in medium containing 5·56 mM glucose, morulae–early blastocysts accumulated considerably more glucose carbon than eight-celled embryos which, in turn, accumulated significantly ($P<0·05$) more glucose carbon than two-celled embryos. However, when the medium contained 0·28 mM glucose the difference between the 0·5 h uptake of glucose by two- and eight-celled embryos was not significant.

Table 1. Uptake of glucose carbon by mouse embryos during short- and long-term incubation in medium containing either 0·28 or 5·56 mM [U-^{14}C] glucose

Values are means ± s.e.m. *Statistically different $P < 0·05$. **Statistically different $P < 0·005$

Alternative energy substrates in medium	Incubation period (h)	Glucose uptake (pg-atoms per embryo) At 2-cell stage	At 8-cell stage	At morula–early blastocyst stage
		Glucose concn 5·56 mM (three replicates)		
None	0·5	2·31±0·23	3·29± 0·19	11·3 ± 1·6
	25	No data	70·0 ±19·7	385·4 ±30·3
25 mM lactate +	0·5	2·40± 0·21	3·02± 0·46	11·2 ± 2·6
0·25 mM pyruvate	25	64·3 ±12·6**	72·2 ±13·1	440·6 ± 2·9
5 mM lactate +	0·5	2·31± 0·60	3·65± 0·11	10·7 ± 2·0
0·5 mM pyruvate	25	37·1 ± 6·9**	106·3 ±17·3	422·8 ±29·2
		Glucose concn 0·28 mM (five replicates)		
None	0·5	0·37±0·07	0·66± 0·09	4·52± 1·0
	25	No data	33·9 ± 5·8	218·5 ±22·4
25 mM lactate +	0·5	0·51±0·15	0·67± 0·08	4·01± 0·56
0·25 mM pyruvate	25	14·7 ±1·5*	45·9 ± 5·1	232·7 ±23·8
5 mM lactate +	0·5	0·49±0·08	0·63± 0·12	4·70± 0·65
0·5 mM pyruvate	25	8·8 ±1·3*	46·0 ± 3·0	234·6 ±49·6

The accumulation of glucose carbon by embryos was greatly increased by increasing the length of time of incubation from 0·5 to 25 h. However, the initial rate of uptake during the first incubation of 0·5 h was not maintained throughout the 25-h incubation period. The uptake of substrate carbon was also dependant upon the concentration of glucose in the medium. A 20-fold decrease in glucose concentration from 5·56 to 0·28 mM resulted in a two- to sixfold decrease in the accumulation of substrate carbon within the embryos. The decrease was greatest at earlier stages of embryonic development.

At the two-celled stage of development, mouse embryos do not survive culture in medium containing glucose as the sole energy substrate and thus no data are given for 25 h culture in this medium. During short-term incubation of two-celled embryos in 5·56 mM glucose, the accumulation of substrate carbon was not significantly

affected by the presence or absence of the alternative energy substrates lactate and pyruvate. A marked increase in the accumulation of label was found when two-celled embryos were cultured for 25 h in 25 mM lactate, 0·25 mM pyruvate plus glucose over that in the 5 mM lactate, 0·5 mM pyruvate plus glucose (in 5·56 mM glucose, $t_2 = 15·8$, $P < 0·005$; and in 0·28 mM glucose, $t_4 = 2·92$, $P < 0·05$). At later stages, the presence or absence of alternative energy sources had no significant effect on the uptake of glucose label.

Table 2. Incorporation of glucose carbon into biochemical fractions of mouse embryos during 24 h

Embryos cultured *in vitro* in medium containing 0·28 mM [U-^{14}C] glucose and in the presence or absence of lactate (5 mM) and pyruvate (0·5 mM). Values are mean ± s.e.m. The percentage of the total incorporated label is shown in parentheses

Stage of development	Presence of lactate + pyruvate	No. of replicates	Mean No. of embryos per replicate	Glucose carbon (pg-atoms per embryo) accumulated in 24 h into: Acid-soluble glycogen	Other acid-soluble material	Acid-insoluble material
1-cell→2-cell	+	4	320	0·14± 0·07(2)	2·67± 1·01(38)	4·25± 1·32(60)
2-cell→8-cell	+	4	403	0·26± 0·09(2)	5·45± 0·65(37)	9·07± 2·61(61)
8-cell→morula	+	4	230	9·21± 1·04(19)	13·45± 1·64(27)	26·54± 2·42(54)
Morula–early blastocyst→ late blastocyst	+	3	377	171·9 ± 40·1(58)	34·9 ± 2·4(12)	90·6 ± 7·7(30)
Morula–early blastocyst→ late blastocyst	−	5	238	96·1 ± 10·4(40)	52·2 ± 2·6(22)	89·1 ± 10·5(38)

Fractionation of Incorporated Glucose Carbon

The incorporation of glucose into various biochemical fractions of embryos was studied in the next three experiments and the results are summarized in Tables 2–4.

Table 2 shows the results obtained in experiment 1 when embryos were cultured in medium thought to mimic conditions *in vivo*. The accumulation of glucose carbon into all fractions increased with increasing developmental age of the embryos. The pattern of incorporation in embryos cultured from the one- and two-celled stages was very similar with 60% of the label extracted in the acid–insoluble fraction. Very little label (2%) was incorporated into acid-soluble glycogen at this time. From the eight-celled stage onwards there was a rapid increase in the amount of glucose carbon incorporated into this pool of glycogen. There was a significant reduction in the amount of label entering the glycogen pool at the blastocyst stage when the culture medium lacked lactate and pyruvate as alternative energy substrates.

The incorporation of glucose carbon into other acid-soluble compounds increased with developmental age of the embryos and significantly more label was found in this fraction when morulae–early blastocysts were cultured in the absence of lactate and pyruvate. The presence or absence of the alternative energy substrates had no effect on the accumulation of glucose carbon into the acid-insoluble fraction.

The effect of pretreatment of cultured embryos with amyloglucosidase prior to their fractionation into acid-soluble and acid-insoluble material is shown in Table 3. The results clearly demonstrate that during culture a large quantity of glucose polymer is synthesized which precipitates with other acid-insoluble components. At the two-celled stage, amyloglucosidase treatment increased the proportion of the label in the acid-soluble fraction from 34 to 93% with a concomitant reduction in label in the acid-insoluble material. A smaller but significant increase in the acid-soluble label was found at the later developmental stage. Statistically, this interaction of stages of development and treatment with amyloglucosidase was highly significant ($P<0{\cdot}001$).

Table 3. Effect of amyloglucosidase treatment prior to extraction on the distribution of glucose carbon into acid-soluble and acid-insoluble fractions of embryos cultured for 24 h in vitro in [U-^{14}C]glucose medium

Values are mean ± s.e.m. for three replicates. Data in parentheses are percentage of total label incorporated

Stage of development	Amyloglucosidase treatment	Mean No. of embryos per replicate	Glucose carbon (pg-atoms per embryo) incorporated in 24 h into: Acid-soluble material	Acid-insoluble material
2-cell→8-cell	−	494	27·1± 4·4(34)	52·0± 6·8(66)
	+		74·4± 4·9(93)	5·4± 0·2(7)
Morula–early blastocyst→late blastocyst	−	454	386·9±12·9(89)	49·2±10·1(11)
	+		533·3± 9·4(93)	37·1± 0·9(7)

The results of the final experiment where the energy substrates were added at concentrations most commonly used for successful *in vitro* culture of mouse embryos are shown in Table 4. As previously found, the quantity of glucose label incorporated into the acid-soluble and acid-insoluble fractions increased with developmental age of the embryos. There was a general sixfold increase in the accumulation of glucose into acid-soluble glycogen between all stages of development. The amount of label incorporated into the remainder of the acid-soluble fraction increased approximately twofold at each stage studied.

During early cleavage stages a large proportion (43%) of the total label accumulated by the embryos was found in acid-insoluble glycogen. However, with formation of the blastocoele only 3% of the accumulated carbon was isolated in this fraction. No statistically significant difference was found in the overall rate of incorporation of label into acid-insoluble glycogen at all developmental stages studied as there was large variability between replicates. The rate of accumulation of substrate carbon into other acid-insoluble components of the embryos was constant prior to blastocyst formation and increased at this later stage.

Although the absence of lactate and pyruvate from the culture medium used to incubate morulae–early blastocysts had no significant effect on the total accumulation of glucose carbon during culture, it did cause some redistribution of label between the various fractions isolated. Less label was found in the other acid-soluble material of embryos cultured in medium containing glucose as sole energy source while the amount of label accumulated in the other acid-insoluble fraction was increased under these conditions.

Characterization of Glycogen Fractions

The identity of the uncharged but radioactively labelled compounds referred to in the above experiments as acid-soluble glycogen and acid-insoluble glycogen was further investigated. Samples of the acid-soluble glycogen were acid hydrolysed in 1 M HCl at 100°C for 12 h, neutralized and desalted by ion-exchange chromatography.

Table 4. Incorporation of glucose carbon into the acid-soluble and acid-insoluble glycogen and other biochemical fractions of mouse embryos during 24 h

Embryos cultured *in vitro* in medium containing 5·56 mM [U-^{14}C]glucose and in the presence and absence of lactate (25 mM) and pyruvate (0·25 mM). Values are mean ± s.e.m. for three replicates. Data in parentheses are percentage of total label incorporated

Stage of development	Presence of lactate + pyruvate	Mean No. of embryos per replicate	Glucose carbon (pg-atoms per embryo) incorporated during 24 h into: Acid-soluble glycogen	Other acid-soluble material	Acid-insoluble glycogen	Other acid-insoluble material
1-cell→2-cell	+	194	0·9± 0·2(2)	10·3± 0·6(22)	20·1± 1·3(43)	15·6± 0·8(33)
2-cell→8-cell	+	265	5·6± 2·9(7)	21·5± 2·4(27)	34·4± 7·9(44)	17·6± 4·7(22)
8-cell→morula	+	327	43·5± 11·3(31)	44·0± 5·4(31)	34·1± 11·8(24)	19·1± 4·3(14)
Morula–early blastocyst→late blastocyst	+	279	285·0± 4·9(65)	102·2± 8·9(23)	21·4± 6·0(5)	27·9± 5·0(7)
Morula–early blastocyst→ late blastocyst	−	228	331·0± 26·4(69)	79·3± 5·4(16)	14·8± 1·6(3)	57·5± 13·2(12)

The neutral fraction was then evaporated to dryness. Neutral acid-insoluble material which was hydrolysable with carbohydrase was collected from cultured two-cell embryos, desalted and dried. Both of these neutral fractions were subjected to descending paper chromatography in various solvent systems. Most of the labelled products migrated with an R_f value identical to [U-^{14}C]glucose standard. No other peaks of labelled products were obtained.

Discussion

Uptake of Glucose Carbon

The transfer of glucose across the cell membrane from the fluid environment to the intracellular space of the mouse embryo has previously been examined by Wales and Brinster (1968). Data obtained in the present study for the rate of uptake of glucose carbon by two- and eight-celled embryos during culture for 30 min in 5·56 mM glucose medium are in agreement with those in the above paper. In mouse embryos, glucose uptake involves a membrane carrier system which remains close to saturated when the glucose level in the surrounding environment is reduced from 5·0 mM (equivalent to circulating blood plasma) to 0·5 mM (Wales and Brinster 1968; Brinster 1969). Moreover, a high rate of glucose uptake by the carrier is maintained when the glucose

concentration is lowered to 0·28 mM, a concentration which might be expected to bathe the embryo in the reproductive tract of the mouse.

The addition of the alternative energy substrates lactate and pyruvate at either levels expected in oviducal fluid (Wales and Whittingham 1973) or found to result in optimal growth of mouse embryos *in vitro* (Cross and Brinster 1973) did not significantly affect the uptake of glucose during culture from the eight-celled stage of development onwards. However, these substrates influenced the accumulation of glucose carbon during culture of two-celled embryos. In view of the well-documented interaction between lactate and pyruvate during early *in vitro* development of the mouse embryo (Wales 1973*b*) the present result supports the hypothesis that a change in the intracellular redox state, as caused by alterations to the lactate/pyruvate ratio, plays a significant role in the regulation of metabolic pathways (Brinster 1965; Wales 1973*b*). Within the apparent physiological range of concentrations of lactate and pyruvate measured in the reproductive tract fluid and used by Wales and Whittingham (1973) (5 mM lactate and 0·5 mM pyruvate) glucose uptake is less than optimal at the two-celled stage. Furthermore, significant changes in long-term glucose carbon accumulation result from varying the lactate and pyruvate concentration and possibly reflect a significant alteration to the turnover of glucose pools within the early embryo.

Incorporation of Glucose Carbon into Glycogen

Glucose metabolism in mammalian tissues is extremely diverse and there are numerous possible fates of carbon originally incorporated by the embryo in the form of glucose. Catabolically, some of the substrate is used as a source of energy and is metabolised to carboxylic acids (Wales 1969) and CO_2 (Brinster 1967) during *in vitro* culture. Anabolically, routes exist within the embryo via which glucose carbon is incorporated into lipids (Flynn and Hillman 1978), nucleic acids (Pike *et al.* 1977) and the amino acid moiety of proteins (Schneider *et al.* 1976). The present experiments and those of Brinster (1969) show that the most important pathway of glucose metabolism in the mouse embryo is the synthesis of glycogen. Most significantly it was observed in the present study that the glucose polymer is synthesized in two distinct forms, one of which is freely soluble in dilute PCA and another which is precipitated by PCA presumably due to its covalent binding to protein. Although previously thought to be an artefact of acid extraction procedures (Roe *et al.* 1961), the existence of the protein-bound form of glycogen, originally described by Willstatter and Rhodewald (1934) and termed desmoglycogen, has been more recently investigated by Bartley and Dean (1968) and reviewed by Whelan (1976).

Previously anomalous results regarding embryonic glycogen metabolism have been clarified in this observation. The large synthesis of glycogen between the one- and two-celled stage of development (Stern and Biggers 1968; Ozias and Stern 1973) employing a relatively large pool of uridine diphosphate glucose (UDPG) (Young *et al.* 1978) results exclusively from the synthesis of desmoglycogen which was not measured in the previous study of Brinster (1969). The synthesis of protein-bound glycogen during the earliest stages of development of the mouse zygote and the later synthesis of the more usual acid-soluble form of glycogen between the eight-celled and blastocyst stages provides further evidence for the hypothesis of Krisman and Barengo (1975) and Barengo and Krisman (1978) on the initiation of glycogen synthesis. The initial synthesis is proposed to take place by covalent attachment of

glucose residues to a specific polypeptide via utilization of a glycogen initiator synthetase enzyme. This protein primer with attached oligosaccharide is believed to represent the core for synthesis, catalysed by glycogen synthetase and branching enzyme, of the branched glycogen polymer referred to as desmoglycogen. Intracellular glycogen hydrolases then catalyse the release of some oligosaccharide fragments from the desmoglycogen which, in turn, act as a primer for the synthesis of the acid-soluble form of glycogen again involving glycogen synthetase, branching enzyme and UDPG.

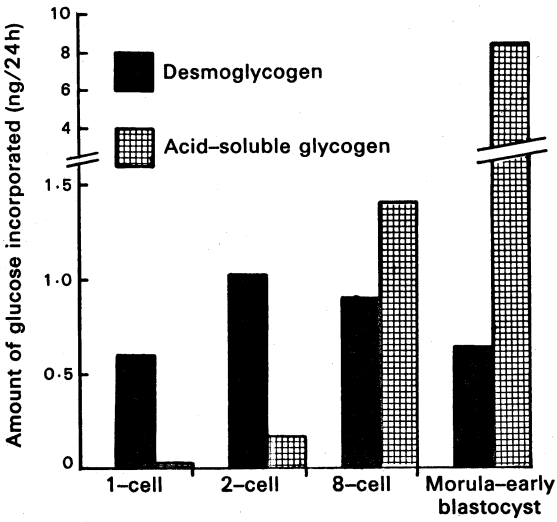

Fig. 1. Estimates of the rate of synthesis of glycogen by preimplantation mouse embryos during *in vitro* culture in medium containing lactate, pyruvate and 5·56 mM glucose.

Assuming that, during long-term (24 h) *in vitro* culture of mouse embryos under the conditions of the experiments, the specific activity of the intracellular glucose pool equilibrates with that initially set up in the culture medium, a quantitative estimate of glycogen synthesis during the culture period can be calculated. The results of such a calculation (Fig. 1) correlate closely with estimates of glycogen content in cultured embryos using the direct enzyme assay technique. Approximately 1 ng of glycogen is stored in each mouse embryo between the one- and two-celled stages of development (Stern and Biggers 1968; Ozias and Stern 1973) while during culture over this period approximately 0·6 ng of glucose per embryo was incorporated into desmoglycogen. Furthermore, at the time of blastocyst formation approximately 9 ng glucose was incorporated into glycogen during culture in 5·56 mM glucose. The total glycogen content of mouse embryos cultured in medium containing 5·56 mM increases from around 1 ng per embryo at the two-cell stage to 10 ng per embryo by the late blastocyst stage of development (Ozias and Stern 1973). However, the situation *in vivo* seems to differ significantly from that observed during *in vitro* culture as the glycogen content of freshly collected two-celled, eight-celled and morula stage embryos is relatively constant at around 1–2 ng per embryo (Stern and Biggers 1968; Ozias and Stern 1973). A limited synthesis of glycogen *in utero* cannot be explained by assuming a glucose concentration of 0·28 mM in uterine fluid as culture in the presence of this low concentration resulted in 5 ng glucose incorporated into glycogen over 24 h. Alternatively, the glycogen pool of embryos represents a balance between glycogen synthesis and degradation and *in vivo* a greater rate of degradation may occur as a

result of regulation by specific components such as hormones within the luminal fluid of the reproductive tract.

Incorporation of Glucose Carbon into other Molecules

Apart from the accumulation of glucose carbon into the glycogen fractions there are a large number of other possible compounds into which glucose carbon could be metabolized. The labelled acid-soluble pool, other than glycogen, most likely represents low molecular weight molecules such as metabolic intermediates, precursors of macromolecules such as amino acids and the unmetabolized parent compound. A large proportion of the total glucose carbon incorporated was found in this fraction and the level increased with the development of the embryo. The increase probably reflects the exponential increase in cell number within the embryo rather than a marked increase in intracellular metabolism. Without further characterization of the labelled molecules in the other acid-soluble fraction the degree of involvement of particular metabolic pathways through which the glucose carbon is being transferred is unknown.

It is also difficult to comment on the synthesis of macromolecules which are extracted in the acid-insoluble fraction, but resistant to hydrolysis by carbohydrases, without identification of the labelled products. A considerable proportion of the glucose label incorporated into this fraction may be located in glycoproteins, especially the terminal carbohydrate moiety of desmoglycogen which could not be removed by amylase or amyloglucosidase treatment. Concanavalin A, a lectin which specifically binds to α-D-mannose and α-D-glucose groups, binds to the surface of mouse embryos at all stages of preimplantation development indicating the presence of considerable amounts of glycoproteins containing these groups (Wartiovaara *et al.* 1978; Wu and Chang 1978). The observations of incorporation of glucose carbon into lipids (Pike and Wales 1972; Flynn and Hillman 1978), nucleic acids (Murdoch and Wales 1973; Pike *et al.* 1977) and proteins (Schneider *et al.* 1976) during *in vitro* culture suggest that glucose may play a significant role in the synthesis of cellular machinery required for early embryonic growth and differentiation but the relative importance of this role, compared to the synthesis of energy stores, appears to wane as cell number increases.

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References

Barengo, R., and Krisman, C. R. (1978). Initiation of glycogen biosynthesis in *E. coli*. Studies of the properties of the enzymes involved. *Biochim. Biophys. Acta* **540**, 190–6.

Bartley, W., and Dean, B. (1968). Extraction and estimation of glycogen and oligosaccharides from rat heart. *Anal. Biochem.* **25**, 99–108.

Brinster, R. L. (1965). Studies on the development of mouse embryos *in vitro*. IV. Interaction of energy source. *J. Reprod. Fertil.* **10**, 227–40.

Brinster, R. L. (1967). Carbon dioxide production from glucose by the preimplantation mouse embryo. *Expl Cell Res.* **47**, 271–7.

Brinster, R. L. (1969). Incorporation of carbon from glucose and pyruvate into the preimplantation mouse embryo. *Expl Cell Res.* **58**, 153–8.

Cross, P. C., and Brinster, R. L. (1973). The sensitivity of one-cell mouse embryos to pyruvate and lactate. *Expl Cell Res.* **77**, 57–62.

Flynn, T. J., and Hillman, N. (1978). Lipid synthesis from [U-^{14}C]glucose in preimplantation mouse embryos in culture. *Biol. Reprod.* **19**, 922–6.

Holmdahl, F. H., and Mastroianni, L. (1965). Continuous collection of rabbit oviduct secretions at low temperature. *Fertil. Steril.* **16**, 587–95.

Iritani, A., Nishikawa, Y., Gomes, W. R., and Van Demark, N.L. (1971). Secretion rates and chemical composition of oviduct and uterine fluids in rabbits. *J. Anim. Sci.* **33**, 829–35.

Krisman, C. R., and Barengo, R. (1975). A precursor of glycogen biosynthesis: α-1,4-glucan-protein. *Eur. J. Biochem.* **52**, 117–23.

Murdoch, R. N., and Wales, R. G. (1973). Incorporation of [^{14}C]glucose and [^{3}H]uridine into the major classes of RNA in mouse embryos during preimplantation development. *Aust. J. Biol. Sci.* **26**, 889–901.

Ozias, C. B., and Stern, S. (1973). Glycogen levels of preimplantation mouse embryos developing *in vitro*. *Biol. Reprod.* **8**, 467–72.

Pike, I. L., Kaye, P.L., and Wales, R. G. (1977). Glucose incorporation into nucleic acids of mouse morulae. *Proc. Aust. Biochem. Soc.* **10**, 75 (Abstr.).

Pike, I. L., and Wales, R. G. (1972). The incorporation of [U-^{14}C]glucose into the preimplantation mouse embryo. *J. Reprod. Fertil.* **28**, 140 (Abstr.).

Restall, B. J., and Wales, R. G. (1966). The fallopian tube of the sheep. III. The chemical composition of the fluid from the fallopian tube. *Aust. J. Biol. Sci.* **19**, 687–98.

Roe, J. H., Bailey, J. M., Gray, R., and Robinson, J. N. (1961). Complete removal of glycogen from tissues by extraction with cold trichloroacetic acid solution. *J. Biol. Chem.* **236**, 1244–6.

Schneider, J. H. (Jr), Olds, D., Tucker, R. E., and Mitchell, G. E. (Jr) (1976). Mouse embryos use glucose in protein synthesis. *J. Anim. Sci.* **42**, 271 (Abstr.).

Stern, S. (1970). The activity of glycogen synthetase in the cleaving mouse embryo. Proc. Soc. Stud. Reprod. Third Annual Meeting, Columbus, Ohio. (Abstr.).

Stern, S., and Biggers, J. D. (1968). Enzymatic estimation of glycogen in the cleaving mouse embryo. *J. Exp. Zool.* **168**, 61–6.

Wales, R. G. (1969). Accumulation of carboxylic acids from glucose by the preimplantation mouse embryo. *Aust. J. Biol. Sci.* **22**, 701–7.

Wales, R. G. (1973*a*). The uterus of the ewe. II. Chemical analysis of uterine fluid collected by cannulation. *Aust. J. Biol. Sci.* **26**, 947–59.

Wales, R. G. (1973*b*). Biochemistry of the developing embryo. *J. Reprod. Fertil. Suppl.* **18**, 117–25.

Wales, R. G., and Biggers, J. D. (1968). The permeability of two- and eight-cell mouse embryos to L-malic acid. *J. Reprod. Fertil.* **15** 103–11.

Wales, R. G., and Brinster, R. L. (1968). The uptake of hexoses by preimplantation mouse embryos *in vitro*. *J. Reprod. Fertil.* **15**, 415–22.

Wales, R. G., and Whittingham, D. G. (1973). The metabolism of specifically labelled lactate and pyruvate by two-cell mouse embryos. *J. Reprod. Fertil.* **33**, 207–22.

Wartiovaara, J., Lehtonen, E., and Virtanen, I. (1978). Lectin binding in early mouse embryos. *Med. Biol.* **56**, 393–7.

Whelan, W. J. (1976). On the origin of primer for glycogen synthesis. *Trends Biochem. Sci.* **1**, 13–15.

Whitten, W. K. (1971). Nutrient requirements for the culture of preimplantation embryos. *Adv. Biosci.* **6**, 129–89.

Willstatter, R., and Rhodewald, M. (1934). Uber den Zustand des Glykogens in der Leber-im Muskel und in Leukocyten. *Z. Physiol. Chem.* **225**, 103–24.

Wu, J. T., and Chang, M. C. (1978). Increase in concanavalin A binding sites in mouse blastocysts during implantation. *J. Exp. Zool.* **205**, 447–53.

Young, R. J., Sweeney, K., and Bedford, J. M. (1978). Uridine and guanosine incorporation by the mouse one-cell embryo. *J. Embryol. Exp. Morphol.* **44**, 133–48.

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