THE *IN VITRO* METABOLISM OF [U-^{14}C]GLUCOSE BY THE PREIMPLANTATION RABBIT EMBRYO

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Abstract

The incorporation of glucose carbon into various components of embryos and culture medium was studied throughout the preimplantation period of development in the rabbit embryo.

During the first 3 days after ovulation, embryos were able to develop for 24 hr in a simple chemically defined medium containing glucose and serum albumin. After blastocyst formation, this medium could maintain normal morphological appearance of day 4, day 5, and day 6 blastocysts for at least 3–4 hr.

The uptake of glucose carbon by the embryo increased as development progressed, with substantial increases in the incorporation of glucose occurring after blastocyst formation. A considerable amount of the label incorporated was present in the embryos as the parent substrate, indicating that the uptake of glucose was greater than its conversion to other metabolites. Nevertheless, throughout development glucose carbon was incorporated into a wide variety of the carbon pools of the embryo, such as acidic and basic acid-soluble compounds, proteins, and lipids. Lactate, pyruvate, and acetate also accumulated in the culture medium. The rate of protein synthesis declined in relation to the increase in cell number during development and may reflect the requirements of the rabbit embryo for exogenous protein and amino acids for blastocyst expansion.

A considerable amount of the incorporation of substrate carbon into day 6 blastocysts was due to the accumulation of lactate, pyruvate, acetate, amino acids, and the parent substrate within the blastocoel fluid. The higher concentration of metabolites, such as carboxylic acids and amino acids, in the blastocoel fluid than in the incubation medium indicated that there was some active mechanism for retaining these compounds within the blastocyst. The blastocyst tissue also contained labelled metabolites such as lactate, citrate, and malate as well as the amino acids glutamate, alanine, and aspartate. The accumulation of glucose carbon in these metabolites indicates that glucose is metabolized in the rabbit embryo by the operation of the Embden–Meyerhof pathway and tricarboxylic acid cycle.

I. INTRODUCTION

Although the pattern of energy metabolism in the mouse embryo has been investigated extensively (see review by Brinster 1969*a*; Biggers 1971), little work has been done on this subject in rabbit and other mammalian embryos. Mounib and Chang (1965), Fridhandler *et al.* (1967), and Fridhandler (1968) have studied various aspects of glucose metabolism in the day 6 rabbit blastocyst using calcium-free

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Krebs–Ringer phosphate medium. However, this medium probably does not simulate physiological conditions very precisely. Calcium ions are required for the *in vitro* development of mouse embryos (Wales 1970) and bicarbonate is superior to phosphate-buffered medium in maintaining growth and metabolism of cultured mouse embryos (Quinn and Wales 1973). Thus the use of Krebs–Ringer bicarbonate medium, which contains similar concentrations of calcium and bicarbonate to those found in rabbit fallopian tube fluid (David *et al.* 1969), would probably give results with more physiological significance than those obtained with calcium-free Krebs–Ringer phosphate medium.

The incorporation of energy substrates into the rabbit embryo before blastocyst formation has not previously been investigated. However, Fridhandler (1961) and Brinster (1968*a*) have measured the production of carbon dioxide from glucose at all stages of preimplantation development of the rabbit embryo. From the ratio of carbon dioxide produced from carbon-1 (C1) and carbon-6 (C6) of glucose, these authors concluded that the activity of the pentose phosphate shunt was high until the morula stage, after which the Embden–Meyerhof glycolytic pathway was the major route for glucose oxidation. In the mouse embryo there is little or no pentose phosphate shunt activity at any stage of development (Brinster 1967). Another major difference between the early mouse and rabbit embryo is the energy requirements for *in vitro* culture. Pyruvate or lactate is required by two-cell mouse embryos for development and glucose cannot support development before the eight-cell stage (Brinster 1965; Brinster and Thomson 1966). The two-cell rabbit embryo, however, can develop *in vitro* for 48 hr without an exogenous energy source but with low concentrations of amino nitrogen in the culture medium, the addition of pyruvate or lactate increases the number of embryos developing (Brinster 1970). Nevertheless, cultured one- and two-cell rabbit embryos are unable to develop into expanding blastocysts unless the medium is supplemented with amino acids, vitamins, and extra serum albumin (Kane and Foote 1971).

The studies reported here were undertaken to investigate the incorporation of glucose carbon into various components of rabbit embryos and the incubation medium at all stages of preimplantation development. Standard methods of embryo culture in a simple, chemically defined medium have been used to determine the importance of various metabolic pathways at various stages of development in the embryo and to see if changes in metabolism may explain the differences in the culture requirements of the rabbit embryo before and after blastocyst formation.

II. Materials and Methods

(*a*) *Collection, Culture and Recovery of Embryos*

Virgin female rabbits of an albino laboratory strain, 6–10 months old, were superovulated by an intramuscular injection of 150 i.u. pregnant mare serum gonadotrophin (PMSG; Primantron, Schering AG) given in 0·5 ml of 0·9% (w/v) saline, followed 90–96 hr later by an intravenous injection of 50 i.u. human chorionic gonadotrophin (HCG; Pregnyl, Organon) given in 0·2 ml of 0·9% saline. At the time of the second injection, the females were artificially inseminated with 0·1–0·2 ml of a pooled sample of good-quality semen collected by means of an artificial vagina (White 1955) from at least two fertile bucks.

Embryos were flushed from the reproductive tracts of the superovulated females at specific times after the injection of HCG and insemination (Brinster 1968*a*), the day after HCG injection

being referred to as day 1. The basic medium used was a modified Krebs–Ringer bicarbonate solution containing 5·56 mM glucose, 1 mg/ml bovine serum albumin, 60 μg/ml penicillin, and 50 μg/ml streptomycin (Brinster 1965).

(i) *Experiment 1*

Embryos were washed twice in substrate-free medium before culture in medium containing radioactive glucose (Wales and Biggers 1968). The embryos collected on the first 3 days after mating were cultured for 24 hr at 37°C in droplets of medium under a layer of paraffin oil contained in plastic Petri dishes (Brinster 1963). For each replicate, 50–80 embryos in groups of 20–25 were placed in drops of medium (20–25 μl per drop) containing [U-^{14}C]glucose at a specific activity of 3 μCi/μmole. For day 4 blastocysts, 10–40 embryos were used for each replicate, 5–10 embryos being incubated for 3–4 hr in 50-μl drops of radioactive medium under oil. Between 10 and 40 day 5 and day 6 blastocysts were incubated for 3–4 hr in 1 ml of medium in glass cavity dishes. The blastocysts collected on day 4, day 5, and day 6 after mating were incubated in medium containing [U-^{14}C]glucose at a specific activity of 1 μCi/μmole. At all stages of development, the embryos were incubated under a humidified atmosphere of 5% CO_2 in air.

Up to and including the day 4 blastocyst stage, embryos which had developed during culture were removed from the medium and collected by centrifugation (Wales and Biggers 1968). Isotonic sucrose containing 5·56 mM glucose was used in the centrifuge tube before the blastocyst stage, whereas non-radioactive glucose medium was used to collect early blastocysts (Wales and Whittingham 1970). The embryos were recovered from the broken tip of the centrifuge tube and stored at −20°C until extracted. Because of their larger size, day 5 and day 6 blastocysts were handled differently from the earlier stages. At the end of the incubation period, the medium was removed from these blastocysts and they were then quickly washed with 1 ml of non-radioactive glucose medium. After the washing, blastocysts which appeared normal were transferred to extraction tubes and stored at −20°C after as much of the non-radioactive wash as possible had been removed from around the blastocysts by means of a drawn-out Pasteur pipette. At all stages of development, three to five embryos were collected as an independent assessment of the total incorporation of radioactivity in the embryos.

(ii) *Experiment 2*

In this experiment, the blastocoel fluid was separated from the trophoblastic tissue of day 6 blastocysts and analysed separately. The glucose and lactate concentrations in the blastocoel fluid of freshly collected embryos and in embryos incubated for up to 3 hr in medium containing radioactive glucose were measured. The incorporation of glucose carbon into various components of the blastocoel fluid and trophoblastic tissue of the incubated embryos was also assessed.

Blastocysts were removed from the uteri of rabbits by opening the uterus along its length and carefully transferring the blastocysts from the endometrium to a cavity dish with a spatula. For analysis of glucose and lactate concentrations in blastocoel fluid of freshly collected embryos, approximately 5–10 blastocysts were placed in a dry glass cavity dish, pierced with a fine Pasteur pipette and the blastocoel fluid removed. The fluid was then stored at −20°C until analysed. For each replicate of the experiment, a further 20–30 blastocysts were collected from donors in a similar manner and placed in medium containing non-radioactive glucose. The embryos were then quickly washed twice (2 ml per wash) in non-radioactive glucose medium and incubated in 1 ml of medium containing [U-^{14}C]glucose (1 μCi/μmole) under similar conditions to those used for day 6 blastocysts in experiment 1.

At 1, 2, and 3 hr after the start of the incubation, approximately one-third of the blastocysts which had remained morphologically normal during the incubation were removed from the radioactive medium in a minimum volume of fluid, with a Pasteur pipette of internal diameter slightly larger than the diameter of the blastocysts. The blastocysts were placed in a cavity dish and washed quickly with 1 ml of non-radioactive glucose medium. This medium was then removed from around the blastocysts which were then pierced and the blastocoel fluid removed. This fluid was stored at −20°C for further analysis. The tissue of the pierced blastocyst was removed from the zona pellucida, given a further wash in 1 ml of non-radioactive glucose medium, and also stored at −20°C.

(*b*) *Extraction and Analysis of Samples*

All samples were radioassayed by liquid scintillation techniques using 5 ml of Triton X100–toluene (1 : 2 v/v) containing 0·4% (w/v) 2,5-diphenyloxazole and 0·01% (w/v) 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene for each 0·4 ml of aqueous sample.

(i) *Embryos*

In experiment 1 the whole embryo and in experiment 2 the trophoblastic tissue from day 6 blastocysts were fractionated into acid-soluble, protein, and lipid fractions as described by Wales and Whittingham (1970). The acid-soluble fraction was further divided into acidic, basic, and neutral portions by passage through columns of ion-exchange resins (see Wales and Whittingham 1970). The acid-soluble material of the blastocoel fluid from embryos in experiment 2 was also fractionated on ion-exchange resins. The identity of the radioactive compounds in the acidic and basic portions of the acid-soluble fraction of the blastocyst tissue from experiment 2 was analysed by chromatographic procedures. The amino acids in the basic portion were chromatographed on paper, with water-saturated phenol as the developing solvent (see Quinn and Wales 1971). The acidic compounds were separated by liquid–liquid partition chromatography, on silicic acid columns, with hexane–butanol mixtures (O'Shea and Wales 1968) and their identity further checked by paper chromatography with n-butanol–acetic acid–water (4 : 1 : 5 v/v) as the solvent system. Carboxylic acids in the blastocoel fluid of day 6 blastocysts in experiment 2 were also separated by liquid–liquid partition chromatography on silicic acid columns. The accumulation of substrate carbon in the various fractions was then calculated from the counts accumulating in the respective fractions and from the specific activity of the glucose in the culture medium.

(ii) *Medium*

In both experiments 1 and 2, the radioactive glucose medium was collected at the end of the culture period and stored at −20°C. The radioactive carboxylic acids accumulating in the incubation media were separated by liquid–liquid partition chromatography on columns of silicic acid with hexane–butanol mixtures. The production of the various carboxylic acids was then calculated from the radioactivity in the respective peaks and from the specific activity of the added substrate.

(*c*) *Glucose and Lactate Determinations*

In experiment 2, aliquots of the medium at the end of the 3-hr incubation period and of the blastocoel fluid collected 0, 1, 2, and 3 hr after the start of the experiment were extracted with cold perchloric acid (PCA) so that the final concentration of PCA in the sample was 2·5%. After centrifugation, the concentration of glucose in these extracts was estimated by the glucose oxidase method of Huggett and Nixon (1957) using kit TCM III (Boehringer Mannheim, Germany). The volume of reactants in the assay was reduced so that 5–10 μl of samples was added to 0·4 ml of the reaction mixture and the optical density of the solution was read at 436 nm in microcuvettes which could hold approximately 0·5 ml of the solution.

The amount of L-lactate in aliquots of the extracted samples was assayed by the enzymic method of Barker and Britton (1957) which was modified for microvolumes and fluorimetry. A 10-μl aliquot of the 2·5% PCA extract of the sample was diluted with 100 μl of 2·5% PCA. The amount of lactate in duplicate 50-μl aliquots of this solution was then estimated. The incubation mixture consisted of 1 ml of 0·5M glycine buffer, pH 9·0, containing 0·4M hydrazine hydrate, 0·1 ml of NAD (2 mg/ml), 20 μl of lactate dehydrogenase (1·3 mg protein/ml; 24 mg/ml Sigma stock enzyme diluted with 2·2M ammonium sulphate, pH 7·0), and 50 μl of sample or standard. After 1 hr at room temperature, a further 1 ml of glycine buffer was added to the incubation mixture and the fluorescence of the NADH formed during the 1-hr incubation period was read at an excitation of 340 nm and fluorescence of 465 nm in a Farrand Mark I spectrofluorometer. Standards were prepared from lithium lactate and all solutions were read against reagent blanks.

(*d*) *Statistical Analysis*

All data of the incorporation of substrate carbon were transformed to logarithms prior to analysis and the significance of the results was assessed by analysis of variance. In order to conserve

space, the analyses have not been tabulated and only means ± standard errors of the means for the untransformed data are given in the tables.

III. Results

(a) *Experiment 1*

The incorporation of substrate carbon from [U-^{14}C]glucose into rabbit embryos during culture was measured at all stages of preimplantation development. The proportion of embryos which developed during culture in radioactive glucose and the incorporation of glucose carbon into the various fractions of these embryos are given in Table 1. Embryos collected from the same animal on the day after insemination (day 1) were usually all either one- or two-cell embryos and after 24 hr culture, these embryos developed to the four- or eight-cell stage. In embryos collected on day 2, it was difficult to determine the exact number of blastomeres in wet mounts because some of the individual cells were surrounded by other blastomeres. These embryos are referred to as eight- to 16-cell embryos and developed to the morula stage during the 24-hr culture period. Morulae were collected on day 3, and after 24 hr of culture a well-formed blastocoel cavity had developed in most of these embryos.

Table 1

GLUCOSE CARBON ACCUMULATED BY RABBIT EMBRYOS DURING CULTURE IN MEDIUM CONTAINING 5·56 mM [U-^{14}C]GLUCOSE

Mean values of three replicates ± standard error of means at each stage of development are given with percentage of total carbon fractionated shown in parentheses

Stage of development	Embryos developing (%)	Glucose carbon incorporated				
		Total (Est. A)*	Total (Est. B)†	Acid-soluble fraction	Protein fraction	Lipid fraction
Days 1–3; 24-hr culture		Values in pg-atoms per embryo per 24 hr				
1-cell, 2-cell to 4-cell, 8-cell	68 ±6	38 ±5	43 ±8	28 (65) ±6	15 (34) ±3	0·3 (<1) ±0·1
8-cell, 16-cell to morula	83 ±7	99 ±14	100 ±4	58 (58) ±4	41 (41) ±3	0·9 (<1) ±0·3
Morula to early blastocyst	89 ±3	132 ±19	134 ±18	76 (56) ±21	58 (43) ±3	0·7 (<1) ±0·3
Days 4–6; 3–4-hr culture		Values in pg-atoms per embryo per hour				
Day 4 blastocyst	93 ±6‡	74 ±14	77 ±8	59 (77) ±10	17 (32) ±4	0·9 (1) ±0·4
Day 5 blastocyst	95 ±5	1,980 ±690	1,780 ±600	1,580 (89) ±610	175 (10) ±52	18 (1) ±2
Day 6 blastocyst	98 ±2	30,400 ±5,400	20,800 ±3,400	20,200 (97) ±3,200	560 (3) ±180	80 (<1) ±20

* Estimate of total incorporation from three to five unfractionated embryos.
† Estimate of total incorporation from sum of fractionated carbon in extracted embryos.
‡ Percentage of blastocysts remaining normal during incubation.

The estimates of total carbon accumulated in the embryos, derived from the total incorporation of radioactivity into three to five unfractionated embryos (estimate A of Table 1) and from the sum of carbon accumulated in the various fractions of

the extracted embryos (estimate B), were in close agreement during the first 4 days of development but became more divergent as the stage of development progressed. The incorporation of glucose carbon into the acid-soluble and protein fractions of the embryos increased as development progressed. The proportion of the total carbon present in the protein fraction increased from 34 to 43% over days 1–3, while the proportion in the acid-soluble fraction of the embryos fell from 65 to 56%. A small but variable amount of label accumulated in the lipid fraction of the embryos and accounted for less than 1% of the total glucose carbon accumulated.

As in the day 1 to day 3 embryos, the incorporation of glucose carbon into the various fractions of the day 4 to day 6 embryos increased as development progressed. However, the rate of increase was more marked at the later stages than between days 1 and 3. In all fractions, the relative rate of increase in incorporation was greater between days 4 and 5 than between days 5 and 6. The contributions of substrate carbon accumulated in the protein and lipid fractions of the embryos to total incorporation became relatively less at the later stages of development when there was a large accumulation of acid-soluble products.

TABLE 2

CARBON ACCUMULATED IN ACID-SOLUBLE COMPOUNDS OF RABBIT EMBRYOS DURING CULTURE IN MEDIUM CONTAINING 5·56 mM [U-^{14}C]GLUCOSE

Mean values of three replicates±standard error of means at each stage of development are given with percentage of acid-soluble fraction shown in parentheses

Stage of development	Glucose carbon incorporated into		
	Neutral compounds	Basic compounds	Acidic compounds
Days 1–3; 24-hr culture	(pg-atoms per embryo per 24 hr)		
1-cell, 2-cell to 4-cell, 8-cell	15·3±2·1 (55)	7·8±2·9 (28)	4·9±1·0 (18)
8-cell, 16-cell to morula	31·9±1·1 (55)	13·3±0·3 (23)	13·0±1·0 (22)
Morula to early blastocyst	34·8±12·5 (46)	18·0±5·2 (24)	22·7±4·7 (30)
Days 4–6; 3–4 hr incubation	(pg-atoms per embryo per hour)		
Day 4 blastocyst	38±11 (63)	12±1 (20)	10±1 (17)
Day 5 blastocyst	910±490 (57)	300±65 (19)	380±130 (24)
Day 6 blastocyst	14,900±1600 (74)	1400±300 (7)	3800±1400 (19)

Analysis of the distribution of glucose carbon in the acid-soluble fraction of the embryos (Table 2) showed that a large proportion of the material in this fraction was neutral in character. Between days 1 and 3, the most rapid increase in the accumulation of substrate carbon in the acid-soluble fraction occurred with the acidic compounds. In day 4 to day 6 blastocysts, however, the accumulation of substrate in the neutral portion of the acid-soluble fraction of the embryos was mainly responsible for the increased incorporation as the age of the embryos increased.

The accumulation of radioactive carboxylic acids in the medium in which the embryos were cultured is given in Table 3. Lactate was the major acid accumulating from glucose at all stages of development. Pyruvate and acetate were present in small quantities, the relative contributions of these two compounds to the total

amount of acids in the medium being greater on days 1–3 of development than on days 4–6. The accumulation of all acids increased as development progressed except in the case of acetate at day 6, when its rate of production fell to that found in day 4 blastocysts. The identity of the material which was eluted from silicic acid column

TABLE 3

ACCUMULATION OF RADIOACTIVE CARBOXYLIC ACIDS IN THE MEDIUM DURING CULTURE OF RABBIT EMBRYOS IN 5·56 mM [U-^{14}C]GLUCOSE

Values are the means ± standard error of means of three replicates at each stage of development

Stage of development	Carboxylic acids accumulated from glucose		
	Acetate	Pyruvate	Lactate
Days 1–3	(pmoles per embryo per 24 hr)		
1-cell, 2-cell to 4-cell, 8-cell	6±2	11±2	22±6
8-cell, 16-cell to morula	10±3	20±5	26±8
Morula to early blastocyst	15±2	40±3	273±62
Days 4–6	(pmoles per embryo per hour)		
Day 4 blastocyst	5±2	6±2	39±24
Day 5 blastocyst	10±2	38±10	246±23
Day 6 blastocyst	5±5	440±107	4830±410

chromatograms as radioactive peaks at the positions of authentic lactate and pyruvate was checked further by paper chromatography as described by Wales (1969). The radioactive compounds in these peaks migrated with mobilities similar to authentic lactate and pyruvate run in the same solvent systems. The compound identified as acetate was found to be steam-volatile at pH 4 (O'Shea and Wales 1968).

(*b*) *Experiment 2*

In view of the enormous increase in the accumulation of glucose carbon in the rabbit blastocyst, which parallels the increase in the size of the blastocoel cavity, separate assessments of the accumulation of glucose carbon in the blastocoel fluid and in the trophoblastic tissue of day 6 blastocysts were made.

The diameter of the day 6 blastocysts used was estimated with an eyepiece micrometer and found to be 2·6±1 mm (mean±standard error of 12 groups of blastocysts). This estimate gives a blastocyst volume of 10·6±1·6 μl. The changes in the concentrations and specific activities of glucose and lactate in the blastocoel fluid at hourly intervals for up to 3 hr after incubation in medium containing radioactive glucose are given in Table 4. When radioactive medium was added to the blastocysts, a dilution in the specific activity of the added glucose occurred, since the blastocysts were washed in medium containing non-radioactive glucose prior to incubation and it was impossible to remove all of this medium from around the blastocysts before the addition of the radioactive medium. At the end of the 3-hr incubation period, the specific activity of the glucose in the incubation medium was measured and the value was in good agreement with that expected from the estimated amount of dilution at the commencement of incubation.

The concentration of glucose in the blastocoel fluid increased significantly ($P < 0{\cdot}01$) during the incubation period. The specific activity of the glucose in the fluid approached that of the glucose in the medium within the first hour of the incubation period, and from this time on was not significantly different from that measured

TABLE 4

CONCENTRATION AND SPECIFIC ACTIVITY OF GLUCOSE AND LACTATE IN BLASTOCOEL FLUID OF DAY 6 RABBIT BLASTOCYSTS INCUBATED FOR 1, 2, AND 3 HR IN 5·56 MM [U-^{14}C]GLUCOSE

Values are the means±standard error of means of three replicates

Period of incubation (hr)	Glucose		L-Lactate	
	(mM)	(μCi/μmole)	(mM)	(μCi/μmole)
0	0·61±0·07	—	2·71±0·54	—
1	2·29±0·21	0·74±0·03	1·84±0·29	0·05±0·01
2	3·84±0·38	0·71±0·07	1·75±0·42	0·14±0·02
3	4·48±0·31	0·71±0·05	1·83±0·35	0·29±0·11
Medium after 3 hr	4·70±0·16	0·84±0·05	0·61±0·12	0·07±0·02

in the incubation medium. The concentration of lactate in the blastocoel fluid decreased by approximately one-third during the first hour of the incubation period and then remained constant. The change in its specific activity indicated that new

TABLE 5

GLUCOSE CARBON INCORPORATED INTO THE ACID-SOLUBLE FRACTION OF THE BLASTOCOEL FLUID OF DAY 6 RABBIT BLASTOCYSTS INCUBATED FOR 1, 2, AND 3 HR IN 5·56 MM [U-^{14}C]GLUCOSE

Values are the means±standard error of means of three replicates

Period of incubation (hr)	Glucose carbon (μg-atoms/ml fluid) incorporated into:		
	Acidic compounds	Basic compounds	Neutral compounds
1	1·06±0·12	0·41±0·04	12·23±1·20
2	2·07±0·37	0·86±0·13	19·61±0·33
3	3·14±0·33	1·40±0·13	23·09±0·49
Medium after 3 hr	0·52±0·06	0·16±0·01	29·28±1·42

lactate was being synthesized from glucose carbon through the 3-hr period. The amount of lactate accumulating in the medium at the end of the incubation period was approximately equivalent to the estimates of the amount lost from the blastocoel

fluid during the first hour of incubation, plus the amount of new lactate produced by the blastocyst over the 3-hr period as calculated from the changes in specific activity.

The accumulation of glucose carbon in the acidic, basic, and neutral compounds of the acid-soluble material in the blastocoel fluid of the embryos and in the medium at the end of the 3-hr incubation period is given in Table 5. Incorporation into neutral compounds accounted for 85–90% of the total substrate carbon accumulating in the blastocoel fluid. The rate of accumulation in the acidic and basic compounds of the blastocoel fluid was relatively constant over the 3-hr period, whereas the rate of accumulation in the neutral compounds began to plateau during the second and third hour. At the end of the 3-hr incubation, the concentration of substrate carbon found in the acidic and basic compounds of the incubation medium was lower than that in these compounds in the blastocoel fluid. The amount of glucose carbon found in the acidic compounds in the incubation medium was approximately three times greater than the amount in the basic compounds, while in the blastocoel fluid there was a twofold difference.

TABLE 6

ACCUMULATION OF RADIOACTIVE CARBOXYLIC ACIDS IN THE BLASTOCOEL FLUID OF DAY 6 RABBIT BLASTOCYSTS INCUBATED FOR 1, 2, AND 3 HR IN 5·56 mM [U-^{14}C]GLUCOSE

Values are the means ± standard error of means of three replicates

Period of incubation (hr)	Carboxylic acids (nmoles/ml fluid) accumulated from glucose		
	Acetate	Pyruvate	Lactate
1	12±2	66±7	218±62
2	33±5	109±10	589±103
3	34±8	200±85	1270±500
Medium after 3 hr	4±4	28±2	102±13

The accumulation of carboxylic acids in the blastocoel fluid of the embryos from [U-^{14}C]glucose during the 3-hr incubation period and in the medium at the end of the experiment is given in Table 6. Overall, the amount of glucose accumulating in the carboxylic acids of the blastocoel fluid and incubation medium amounted to 101±13% (mean ± standard error for 12 samples) of that in the acidic compounds separated on ion-exchange columns. The production of lactate accounted for 75–85% of the total carboxylic acids accumulating in the blastocoel fluid and incubation medium, pyruvate 13–23%, and acetate for only 3% or less of the total production of acids from the exogenous glucose. The production of all three carboxylic acids in the blastocoel fluid increased during the incubation, with the production of lactate from exogenous glucose increasing more rapidly than that of either pyruvate or acetate over the 3-hr period. At the end of this period, a greater concentration of labelled carboxylic acids had accumulated in the blastocoel fluid than in the incubation medium.

The distribution of glucose carbon accumulated in the acid-soluble, protein, and lipid fractions of the embryonic tissue during the 3-hr incubation period is given

in Table 7. The total amount of substrate carbon accumulating in the blastocyst tissue and in the various fractions of the tissue increased significantly ($P < 0{\cdot}05$) during the 3-hr period but, whereas accumulation in the protein and lipid fractions increased at a constant rate, the rate of accumulation in the acid-soluble material during the second and third hour of the incubation was only about half of that during the first hour. Incorporation into the protein and lipid fractions of the embryos was equivalent to that found in the day 6 blastocyst in experiment 1 (Table 1). No direct comparison between the amount of glucose carbon in the acid-soluble material of the blastocysts in the two experiments could be made since the volumes of the day 6 blastocysts in experiment 1 were not measured.

TABLE 7

CARBON ACCUMULATED IN EMBRYONIC TISSUE FROM DAY 6 RABBIT BLASTOCYSTS INCUBATED FOR 1, 2, AND 3 HR IN 5·56 MM [U-^{14}C]GLUCOSE

Values are the means±standard error of means of three replicates

Period of incubation (hr)	Glucose carbon (pg-atoms per embryo) incorporated into:			
	Total	Acid-soluble fraction	Protein fraction	Lipid fraction
1	4160±820	3380±700	690±140	90±10
2	4880±490	3470±390	1240±160	170±30
3	6980±1330	4740±990	1900±320	340±90

The accumulation of substrate carbon in the acidic, basic, and neutral portions of the acid-soluble material in the blastocyst tissue is given in Table 8. Although 30–40% of the acid-soluble material in the blastocyst tissue was neutral in character,

TABLE 8

CARBON ACCUMULATED IN ACID-SOLUBLE FRACTION OF EMBRYONIC TISSUE FROM DAY 6 RABBIT BLASTOCYSTS INCUBATED FOR 1, 2, AND 3 HR IN 5·56 MM [U-^{14}C]GLUCOSE

Values are the means±standard error of means of three replicates with percentage of total acid-soluble fraction shown in parentheses

Period of incubation (hr)	Glucose carbon (pg-atoms per embryo) incorporated into:		
	Acidic compounds	Basic compounds	Neutral compounds
1	580±130 (17)	1520±340 (45)	1290±490 (38)
2	630±60 (18)	1840±220 (53)	1010±150 (29)
3	980±180 (21)	2420±500 (51)	1350±350 (28)

there was no significant change in the accumulation of glucose carbon in this portion of the acid-soluble fraction over the 3-hr incubation period. The increased amount of substrate carbon found in the acid-soluble material of the blastocyst tissue during

the incubation period was due mainly to an increasing incorporation into the basic, and to a lesser extent the acidic, portions of this fraction. Accumulation of the amino acid identified as glutamic acid was largely responsible for the increased incorporation into the basic compounds; lactate was the most abundant compound in the acidic portion of the acid-soluble embryonic material (Table 9). Alanine and an unidentified peak of radioactivity were also present in the basic compounds, with trace amounts of aspartate. Two compounds with chromatographic properties similar to citric and malic acids were the only other compounds identified in the acidic portion; the accumulation of radioactivity in both of these compounds increased by approximately 35–45% between the first and third hour of the incubation period.

TABLE 9

CARBON ACCUMULATED IN ACIDIC AND BASIC COMPOUNDS OF THE ACID-SOLUBLE FRACTION OF EMBRYONIC TISSUE FROM DAY 6 RABBIT BLASTOCYSTS INCUBATED FOR 1, 2, AND 3 HR IN 5·56 mM [U-^{14}C]GLUCOSE

Values for three pooled replicates are given

Period of incubation (hr)	Glucose carbon (pg-atoms per embryo) incorporated into:						
	Lactate	Citrate	Malate	Glutamate	Alanine	Aspartate	Unidentified*
1	323	188	38	920	328	32	162
2	286	127	30	1100	311	23	258
3	434	252	56	1487	422	34	305

* Compound with $R_F = 0·88$ on paper chromatography using water-saturated phenol as solvent.

IV. DISCUSSION

The limited information available on the energy metabolism of the rabbit embryo during preimplantation development has been due in part to the lack of a suitable culture system to maintain development of the embryo, but this problem has recently been overcome (Kane and Foote 1970*a*, 1971; Brinster 1970). The results presented here also show that a simple medium containing glucose and bovine serum albumin is able to support development of the rabbit embryo for 24-hr periods between the one-cell and morula stage, and that after the blastocyst stage this medium is capable of maintaining the normal morphological appearance of the embryo for at least 3–4 hr.

The increased incorporation of glucose carbon into most fractions of the embryos and culture medium with increasing embryonic development in the present study reflects the large increase in the cell number and size of the rabbit embryo as development progresses (Daniel 1964). A similar increase also occurs in the production of carbon dioxide from glucose, pyruvate, and lactate during preimplantation development of the rabbit embryo (Brinster 1968*a*, 1969*b*). The incorporation of substrate carbon into the neutral compounds of the acid-soluble fraction of the embryos probably represents the accumulation of glucose itself (Hollinger and Davis 1968) or glycogen. The large amount of radioactivity present in this neutral material would therefore indicate that the rate of accumulation of glucose, either in its original

form or in a storage form such as glycogen, exceeds its utilization in the metabolic pathways of the embryo.

A study by Manes and Daniel (1969) of the incorporation of amino acids into protein by the preimplantation rabbit embryo indicated that, although total protein synthesis increases with development, this increase is not directly proportional to the increase in cell number and up to the early stage of blastocyst formation actually declines on a per embryonic cell basis, but subsequently increases tenfold. Using the data of Daniel (1964) for the number of cells per embryo, the present experiments indicated that a decline in the rate of protein synthesis on a per embryonic cell basis continues to occur after blastocyst formation. These differences in results may reflect the need to supplement culture medium with amino acids and serum albumin to obtain development of rabbit embryos beyond the morula stage (Kane and Foote 1970*a*; 1970*b*; 1971), and suggest that the rate of formation of amino acids from glucose is not sufficient to meet all the requirements for the continuing development of the rabbit embryo beyond the early blastocyst stage. The accumulation of label in the lipid fraction of the embryos probably arises from the diversion of glucose carbon into acetate and glycerol. The synthesis of lipids from radioactive acetate in day 6 rabbit blastocysts has been reported by Huff and Eik-Nes (1966).

When mouse embryos are cultured in media containing glucose, the carboxylic acids, lactate, and, after the morula stage, pyruvate and acetate accumulate in the medium (Wales 1969). Up to the third day of preimplantation development in the rabbit, the amount of glucose carbon accumulating in lactate is very similar to that which occurs in the mouse, despite the fact that the rabbit embryo is approximately three times as large as the mouse embryo at this time (Brinster 1968*a*). Unlike mouse embryos, however, rabbit embryos produced substantial amounts of both pyruvate and acetate throughout preimplantation development when incubated in medium containing glucose. During glycolysis, the high lactate dehydrogenase (E.C. 1.1.1.27) activity in mouse embryos probably diverts most of the glucose carbon utilized into lactate, whereas the lower activity of this enzyme in rabbit embryos would allow more glucose carbon to accumulate in pyruvate and acetate (Brinster 1968*b*). Nevertheless, the production of both pyruvate and acetate relative to lactate declines after the formation of the blastocoel cavity in the rabbit embryo. Similarly, in the mouse embryo the production of pyruvate from glucose is lower in late blastocysts than in the morula and early blastocyst stages (Wales 1969).

The concentrations of glucose and lactate found in the blastocoel fluid of freshly collected day 6 rabbit blastocysts in the present study are similar to those reported previously for blastocoel fluid and uterine secretions of the rabbit (Mastroianni and Wallach 1961; Lutwak-Mann 1962; Holmdahl and Mastroianni 1965).

During incubation of the day 6 blastocyst in lactate-free medium, it appears that lactate diffuses from the blastocoel fluid into the medium during the first hour of the incubation period, after which an equilibrium is reached when the accumulation of newly synthesized lactate in the blastocoel fluid equals its rate of diffusion into the incubation medium. From changes in the concentration and specific activity of the lactate, it can be calculated that approximately 70% of the original lactate in the blastocoel fluid was replaced with newly synthesized lactate during the 3-hr incubation period.

The greater accumulation of glucose carbon in lactate, pyruvate, and acetate, as well as base-containing compounds, in the blastocoel fluid of day 6 rabbit blastocysts compared to that in the incubation medium at the end of the 3-hr incubation period, suggests the operation of some active process which retains metabolites within the blastocyst. Recent studies by Cross and Brinster (1970) indicate that energy-requiring processes are involved in maintaining ionic gradients across the trophoblast membrane of the rabbit blastocyst. Similar mechanisms are probably responsible for the differences in the concentration of metabolites between the blastocoel fluid and incubation medium in the present study. The build-up in the reserve of substrates within the blastocyst may be beneficial for development during implantation or it could ensure an adequate supply of energy substrate for the blastocyst during periods of maternal deprivation.

Although the work of Fridhandler and his associates (Fridhandler 1961, 1968; Fridhandler *et al.* 1967) on the incorporation and oxidation of glucose by the preimplantation rabbit embryo is relevant to the present study, there are basic differences in the culture media, viz. omission of calcium and bicarbonate in Fridhandler's studies, which may explain many of the discrepancies in the results. In the presence of bicarbonate buffer, significant amounts of carbon dioxide are fixed by the rabbit embryo, mainly through the formation of four-carbon intermediates. This process stimulates the activity of the tricarboxylic acid (TCA) cycle and results in the accumulation of malate and citrate in the medium in which the embryos are cultured (P. Quinn and R. G. Wales, unpublished data). In phosphate-buffered medium, a decrease in the activity of the TCA cycle could be expected due to a reduction in carbon dioxide fixation. This may explain the failure of Fridhandler (1968) to find any evidence of a large scale conversion of [1-^{14}C]pyruvate to four-carbon intermediates in day 6 rabbit blastocysts incubated in calcium-free Krebs–Ringer phosphate medium. Stimulated activity of the TCA cycle in embryos cultured in bicarbonate buffered medium would probably also account for the intracellular accumulation of glucose carbon in malate and citrate observed in the present study but not detected by Fridhandler (1968).

In a study of the production of carbon dioxide from glucose by the preimplantation rabbit embryo, Fridhandler (1961) concluded that little, if any, glucose is oxidized via the Embden–Meyerhof glycolytic pathway before the blastocyst stage. However, since glucose carbon was detected in lactate, pyruvate, and acetate in the incubation medium in the present studies, glycolysis must occur throughout preimplantation development in the rabbit. The failure of Fridhandler (1968) to detect any accumulation of glucose carbon in pyruvate and acetate may have been due to the chromatographic procedures used. As pointed out by Wales (1969) pyruvate is decarboxylated when chromatographed in ethanol–ammonia–water. Acetate is lost when extracts are chromatographed in n-butanol–acetic acid–water.

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