ACCUMULATION OF CARBOXYLIC ACIDS FROM GLUCOSE BY THE PRE-IMPLANTATION MOUSE EMBRYO

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Summary

The accumulation of carboxylic acids from glucose was measured by incubating pre-implantation mouse embryos in $[U^{.14}C]$ glucose and separating the carboxylic acids by liquid-liquid partition chromatography. Lactic acid was the main carboxylic acid accumulated. The one-cell zygote produced five times as much lactate as the unfertilized ovum, and during subsequent development lactate accumulation increased substantially.

At the later developmental stages, pyruvate and acetate were also identified as products of the metabolism of glucose. Lactate, pyruvate, and acetate were found approximately in the ratio 90:10:1. The metabolism of glucose by blastocysts cultured *in vitro* from two-cell embryos was similar to that by blastocysts removed from the animal prior to incubation.

I. INTRODUCTION

Prior to the third cell division, glucose is unable to support the development of the mouse embryo *in vitro* (Whitten 1957; Brinster 1965*a*) whereas after this stage is reached, it is an effective energy source (Whitten 1956; Brinster and Thomson 1966). In view of these observations, a study of the metabolism of glucose by preimplantation mouse embryos has been initiated. Brinster (1967*a*) has measured ${}^{14}CO_2$ production from glucose by mouse embryos from the stage of fertilization until blastocyst formation and Wales and Brinster (1968) have studied the accumulation of substrate carbon by the two- and eight-cell embryo incubated in the presence of glucose. The present paper describes a method for the measurement of carboxylic acids accumulated from [U- ${}^{14}C$]glucose and presents data on their production by mouse embryos at different stages of development.

II. MATERIALS AND METHODS

Mouse embryos were obtained from randomly bred Swiss mice which had been superovulated by an intraperitoneal injection of 10 i.u. of serum gonadotrophin (Gestyl, Organon), followed 48 hr later by an intraperitoneal injection of 10 i.u. of chorionic gonadotrophin (Pregnyl, Organon) (Brinster 1963). Unfertilized and fertilized ova were removed from the fallopian tubes 20–24 hr

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R. G. WALES

after the second injection and the surrounding cumulus cells were removed by incubation in Dulbecco's phosphate buffer containing hyaluronidase (Brinster 1965b). Later developmental stages of the embryos were flushed from the fallopian tubes and uterus at specific times after injection with human chorionic gonadotrophin. Early blastocysts were obtained 4 days post coitum while late blastocysts were collected 24 hr later. Cultured blastocysts were obtained from two-cell embryos grown *in vitro* for 4 days as described by Brinster (1963).

The basic medium used in the study was Krebs-Ringer bicarbonate containing $1 \cdot 0 \text{ mg/ml}$ of crystalline bovine serum albumin, 100 units/ml of penicillin, and 50 µg/ml of streptomycin (Brinster 1963). For recovery and preliminary handling of the embryos, 25 mm lactate and 0.25 mm pyruvate were included in the medium as an energy source (Brinster 1965*a*). Before incubation the embryos were washed twice in substrate-free medium (Wales and Biggers 1968). For the 3-hr periods of incubation, the embryos were collected in a minimum of substrate-free medium and transferred to a small test tube containing $50 \mu l$ of $[U^{-14C}]$ glucose (5.56 mm; specific activity $2 \cdot 9 \mu \text{Ci}/\mu \text{mole}$) under two drops of light paraffin oil. After gassing with air-carbon dioxide (95:5), the tubes were capped and incubated at 37° C.

Early blastocysts were cultured for 20-24 hr, either in test tubes as described above or in small droplets under paraffin oil as described by Brinster (1963). At the completion of incubation, metabolism was terminated by freezing to -79° C. Samples were then stored at -20° C until assayed.

The carboxylic acids present in the incubation medium were separated by liquid-liquid partition chromatography on a silicic acid support using hexane-butanol mixtures as solvent (O'Shea and Wales 1967). A mixing device was used to increase progressively the concentration of butanol in the hexane applied to the column (see O'Shea and Wales 1967). Aliquots of $3 \cdot 0$ ml were collected and assayed for radioactivity by liquid scintillation techniques after the addition of 5 ml of xylene containing 0.4% (w/v) 2,5-diphenyloxazole (PPO) and 0.01% (w/v) 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (POPOP). In later experiments, aliquots were counted after the addition of 5 ml of toluene-Triton X100 (2 : 1) containing 0.4% (w/v) PPO and 0.01%(w/v) POPOP. The counts in the peaks corresponding to authentic lactate, pyruvate, and acetate were corrected for efficiency of counting and for background. Production of the various carboxylic acids was then calculated from the radioactivity in the respective peaks and the specific activity of the added substrate.

After 3 hr of incubation all the radioactive peaks were small and it was necessary to radioassay the whole of each fraction to obtain an accurate estimate of the production of each carboxylic acid. With the longer incubation periods, however, the identity of the peaks could be checked by rechromatography in alternative systems. First the peaks were identified and determined quantitatively by counting a portion of the fractions from the column. The remainder of the eluate corresponding to each peak was neutralized and dried under a stream of air. The residue was dissolved in a minimum of water and chromatographed on Whatman No. 1 paper (formic acid washed) in a descending system for 16 hr.

The compound identified tentatively as lactate was characterized using the following solvents: n-butanol-acetic acid-water (4:1:5); n-butanol-propionic acid-water (10:5:7); n-butanol (water saturated)-formic acid (95:5); n-butanol-acetone-water (2:7:1); isopropanol-acetic acid-water (7:1:2); and ethanol-ammonia $(7\cdot5n)$ -water (8:1:1). In all cases a single radioactive peak was obtained with an R_F similar to authentic lactate. A single peak was also obtained using these solvents if the compound was chromatographed with the addition of a similar amount of authentic [¹⁴C]lactate.

The unknown peak identified tentatively as pyruvate was also run in the above systems. Although the smaller production of this compound made radioassay more difficult, small radioactive peaks with R_F values similar to authentic pyruvate were obtained in the first five solvents. However, in alkaline solvents such as ethanol-ammonia-water (8:1:1) or phenol-water-ammonia (160:40:1), pyruvate was found to decarboxylate with the formation of acetate during development of the chromatograms.

Insufficient accumulation of the carboxylic acid identified as acetate occurred to allow rechromatography in alternative systems. However, this peak was not seen if the incubation mixture was chromatographed after drying at acid pH.

III. Results

The lactate production by mouse embryos at various periods during the first 5 days of development is shown in Table 1. Unfertilized ova produced very little lactic acid and, even with 250 ova per incubation, only 100 counts/min were present in the lactate peak, representing a conversion to lactate of 0.007% of the glucose in the 50-µl incubation. The fertilized ovum produced five times as much lactate as the unfertilized cell and there was another threefold rise following first cleavage. Lactate production doubled during the next 24 hr, and then at morula and blastocyst formation there was again a steep increase. Production of lactate in blastocysts was 10 times that in the eight-cell embryo. With the rise in production at the later stages of development, it was possible to estimate it effectively with fewer embryos.

TABLE 1

accumulation of lactic acid from glucose by mouse embryos at various stages of development during a 3-hr incubation period at $37^\circ\mathrm{C}$

Stage of	No. of	Mean No.	Mean No.Counts/minof Embryosin LactateIncubatedPeak	Lactate Accumulated	
Development of Embryo	Observ- ations	of Embryos Incubated		$\overbrace{(\text{mean}\pm\text{S.E.})}^{\text{A}}$	B (mean)
One-cell					
(unfertilized)	3	248	107	$0 \cdot 05 \pm 0 \cdot 013$	1.8
One-cell					
(fertilized)	3	273	508	$0 \cdot 24 \pm 0 \cdot 02$	$8 \cdot 6$
Two-cell	3	153	855	0.70 ± 0.08	$26 \cdot 8$
Eight-cell	3	140	1363	$1 \cdot 25 \pm 0 \cdot 07$	$53 \cdot 4$
Morula	3	37	2479	$9\cdot 32 \pm 0\cdot 65$	$452 \cdot 4$
Blastocyst					
(early)	2	44	3960	$11 \cdot 10 \pm 1 \cdot 18$	$465 \cdot 4$
Blastocyst					
(late)	2	14	1480	$14 \cdot 10 \pm 1 \cdot 36$	$645 \cdot 3$

A: p-moles/embryo/hr. B: µmoles/gram of protein*/hr

* Protein values from Brinster (1967).

Following blastocyst formation 10-20 embryos were sufficient. Table 1 also shows the production of lactate per gram of protein. As there are only small differences in the protein content between the various stages of development (Brinster 1967b), the changes in lactate production per gram of protein are similar to those per embryo.

The production of pyruvate by mouse embryos during 3 hr of incubation is shown in Table 2. At the stages of development prior to morula formation, no radioactive peak corresponding to pyruvate could be detected. After morula formation, pyruvate represented between 3 and 10% of the total carboxylic acids measured.

The lactate production by blastocysts developed from two-cell embryos after 4 days culture *in vitro* in a medium containing lactate and pyruvate is shown in Table 3. In this experiment, two-cell embryos which had been cooled to and stored for 1 hr at 5°C (as described by Wales and Biggers 1968) prior to culture were compared with two-cell embryos from the same groups of mice cultured without prior

treatment. The blastocysts were washed free of their culture media by two transfers through 2 ml of substrate-free medium before incubation for 3 hr in medium containing [U-¹⁴C]glucose. Cooling to 5°C did not affect lactate accumulation, and overall the production of lactate by cultured blastocysts was similar to that by blastocysts removed from mice 5 days post coitum.

	TABLE 2			
ACCUMULATION OF PYE	RUVIC ACID BY MOUSE	E EMBRYOS DURING 37° C	a 3-hr	
A: p-moles/embryo/hr	r. B: as percentag accumulated	e of total carboxy	ylic acid	
Stage of	No. of	Pyruvate Accumulated		
Development	Observations	Á	в	
_		$(\text{mean}\pm\text{S.E.})$	(%)	
Morula	3	0.75 ± 0.13	7.0	
Blastocyst (early)	2	$1 \cdot 26 \pm 0 \cdot 11$	$10 \cdot 2$	
Blastocyst (late)	2	$0 \cdot 48 \pm 0 \cdot 21$		

The production of lactate, pyruvate, and acetate by early blastocysts incubated 20–24 hr *in vitro* is shown in Table 4. In this test, carboxylic acid production by blastocysts incubated in test tubes, as in the previous experiments, was compared with that of blastocysts incubated in culture dishes as described by Brinster (1963).

TABLE 3

CARBOXYLIC ACIDS ACCUMULATED BY BLASTOCYSTS CULTURED FROM TWO-CELL EMBRYOS Numbers in parentheses refer to replicate runs. In the first and second replicate, half of the two-cell embryos were cooled to 5° C and stored at this temperature for 1 hr prior to culture

Pretreatment		Carboxylie A	icid Accumu	ulated (p-mole	es/embryo/h	r)
	Lactic (1)	Pyruvic (1)	Lactic (2)	Pyruvic (2)	Lactic (3)	Pyruvie (3)
Nil	$12 \cdot 9$	0.73	15.7	1.00	16.4	1.28
Cooled to 5°C	$14 \cdot 5$	0.70	14.7	0.89		<u> </u>

Analysis of the results after logarithmic transformation, to equalize variances, indicated that changes in the conditions of incubation had no significant effect either on overall production of carboxylic acid or on the relative amounts of each carboxylic acid produced. Acetate constituted 1% of the carboxylic acids accumulating, pyruvate 8%, and the remaining 91% was lactate. No other organic acids were detected in the incubation medium.

IV. DISCUSSION

The present paper describes a method by which the production of carboxylic acids by mouse embryos from radioactive glucose can be measured. There appears to be only one other report of the measurement of lactate production by mouse embryos (Popp 1958). In that study, mouse embryos were incubated for 22 days on a plasma clot and the data are unlikely to have much physiological significance. On a number of occasions, lactate production by rabbit blastocysts has been measured using conventional microtechniques (Mounib and Chang 1965; Fridhandler, Wastila, and Palmer 1967; Fridhandler 1968). The present results indicate that in the mouse blastocyst lactate production per gram of protein is similar to that in the

TABLE 4

PRODUCTION OF CA	ARBOXYLIC ACIDS	BY BLAST	OCYSTS DURING A	
20–24-HR I Results	s are means from	four observ	ations	
Method of	Carboxylic Acid Accumulated (p-moles/embryo/hr)			
Culture	Lactic	Pyruv	ic Acetic	
Culture dish	$12 \cdot 50$	1.04 0.		
Test tube	$9 \cdot 94$	0.87 0.1		
Summary of the	Analysis of Vari	ance $(\log_{10} $	transformation)	
Source of Variation		D.F.	Variance Ratios	
Differences between compounds		2	136.16**	
Differences between methods		1	$3 \cdot 24$	
Interaction		2	$0 \cdot 24$	
Error variance		18	0.053	

** Significant at P < 0.01.

rabbit blastocyst when a similar concentration of glucose is present in the medium (Fridhandler, Wastila, and Palmer 1967). However, the protein content of the mouse blastocyst is smaller than that of the rabbit and therefore total production of lactate is smaller and below that which could be measured conveniently by the techniques used in the rabbit studies.

Brinster (1967*a*) measured the production of radioactive carbon dioxide from uniformly labelled glucose by mouse embryos collected at the same stages of development and incubated under similar conditions. Therefore an estimate of the amount of the glucose utilized to that which is oxidized can be gained by comparing the present results with those of Brinster (1967*a*). At the one-cell stage, approximately 50% of the glucose, broken down, is oxidized. At later stages, even though total oxidation and utilization has increased, the proportion of glucose utilized which is oxidized falls to 25% at the morula stage and thereafter stays constant at this level.

R. G. WALES

Assuming that in the mouse embryo glucose is metabolized via the Embden-Meyerhof pathway, these observations indicate that insufficient glycolytic activity occurs at the early developmental stages to maintain adequate levels of intermediates in the cell for entry into the tricarboxylic acid (T.C.A.) cycle. Wales and Whittingham (1967) have shown that, when sufficient pyruvate or lactate is present, substrate turnover via the T.C.A. cycle in both one- and two-cell mouse embryos is several times greater than that estimated where only glucose is present. Only at later stages does the oxidative turnover of glucose approach that of pyruvate in oneand two-cell embryos. Thus the rate of glycolysis in the early embryo may be the important step in limiting the utilization of this substrate as an energy source. Although Wales and Brinster (1968) have shown a difference in the accumulation of substrate carbon from glucose by embryos at different stages of development, more work is needed to decide whether the inability of glucose to support development at the two-cell stage is due to impermeability to the substrate or to inhibition of enzymes of the Embden-Meyerhof pathway at the early stages of development. The work of Brinster (1965b, 1966) has shown that there are marked increases in the total activity of some enzymes as the embryo develops.

Apparently, the rate of glycolysis in mouse embryos is not greatly affected by changes in experimental conditions. As cultured and freshly collected blastocysts have similar glycolytic rates, replacement of the natural environment by a simple synthetic medium for a considerable period of time does not appear to modify metabolic pathways in the zygote. In addition, two-cell mouse embryos withstand cooling to 5°C without derangement of their subsequent metabolic activity, and the storage of mouse embryos rather than unfertilized ova (Sherman and Lin 1958, 1959) at low temperature may be worthy of investigation.

Unlike the mouse blastocyst, rabbit blastocysts do not appear to accumulate substantial amounts of pyruvate during incubation in the presence of glucose (Fridhandler 1968). This difference may be more apparent than real. As mentioned in Section II, pyruvate was found to be unstable during chromatography in alkaline solvent systems, and in the rabbit studies it may have been lost during preparation of samples. The small production of acetate in the present experiments probably arises from the incomplete oxidation of pyruvate. Wales and Whittingham (1967) found a similar production in one- and two-cell mouse embryos incubated in the presence of 0.4 mm pyruvate. This production of acetate by embryos may be important as the first step in a number of synthetic reactions (Huff and Eik-Nes 1966; Fridhandler 1968).

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