METABOLISM OF SPECIFICALLY LABELLED PYRUVATE BY MOUSE EMBRYOS DURING CULTURE FROM THE TWO-CELL STAGE TO THE BLASTOCYST

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Summary

Two-cell mouse embryos were cultured for 24, 48, and 72 hr in media containing 0·5 mM sodium [1-^{14}C]pyruvate or sodium [2-^{14}C]pyruvate. Radioactive lactate was the main metabolite accumulating in the medium when [1-^{14}C]pyruvate was used. Labelled lactate and acetate were identified in the medium at the end of the culture period when [2-^{14}C]pyruvate was the energy source.

Embryos which had developed normally during culture were separated from the medium and the incorporation of label into various intracellular fractions was measured. Accumulation of C2 of pyruvate in the embryo was three to six times that of C1. In both cases, 50–80% of the label was found in the acid-soluble fraction and most of the remainder was present in the protein fraction. There was also a minor labelling of lipids when [2-^{14}C]pyruvate was used.

Between 50 and 80% of the label in the acid-soluble fraction was bound to a cation-exchange resin, indicating the accumulation of large amounts of basic compounds in the embryo from the pyruvate in the medium. Paper chromatography of these compounds indicated the presence of two peaks corresponding to aspartic acid and alanine. Although acidic compounds made up the major part of the remaining label in the acid-soluble fraction no carboxylic acids could be detected in the embryos.

Development of embryos in the medium containing [2-^{14}C]pyruvate was poor, especially between 48 and 72 hr in culture. It is suggested that the large intracellular accumulation of isotope during culture may expose these embryos to harmful amounts of internal radiation.

I. INTRODUCTION

In vitro techniques for the culture of two-cell mouse embryos in a simple chemically defined medium (Whitten 1957; Brinster 1963) have stimulated interest in the metabolism of energy substrates during preimplantation development. This is particularly so in view of the findings that at the two-cell stage only pyruvate, lactate, phosphoenol pyruvate, and oxaloacetate will support further cleavage (Brinster 1965*a*)

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while additional compounds such as glucose, α-ketoglutarate, and to a lesser extent malate and citrate can support further development of eight-cell ova *in vitro* (Whitten 1956; Brinster and Thomson 1966).

Brinster (1967*a*, 1967*b*) measured carbon dioxide production from lactate and pyruvate by mouse embryos and Wales and Whittingham (1967) examined the uptake and utilization of these substrates by the one- and two-cell mouse embryo. However, both of these studies were restricted to short periods of incubation and gave little indication of the overall participation of these energy substrates in the anabolic and catabolic metabolism of the embryo during extended periods in culture. The present studies were undertaken to examine the incorporation of substrate carbon from pyruvate into mouse embryos during culture from the two-cell stage to blastocyst.

II. Materials and Methods

(*a*) *General*

Two-cell mouse embryos were flushed from the fallopian tubes of superovulated random-bred albino mice (Brinster 1963). Modified Krebs–Ringer bicarbonate solution containing 25 mM DL-sodium lactate, 0·25 mM sodium pyruvate, 1 mg/ml bovine serum albumin, 60 μg/ml penicillin, and 50 μg/ml streptomycin sulphate (Brinster 1965*b*) was used as the medium for collection of embryos prior to use. After recovery the embryos were transferred through two changes of a similar medium devoid of energy substrates (2 ml/wash) before incubation in a medium containing isotopically labelled sodium pyruvate.

Specifically labelled sodium pyruvate was obtained from Amersham Laboratories and checked for purity by chromatography. Prior to use it was stored as small dried aliquots at −40°C to avoid decomposition (Wales and Whittingham, unpublished data). Aliquots were reconstructed, as required, with substrate-free medium to give a final concentration of pyruvate of 0·5 μmole/ml. The specific activities of the pyruvate in the culture media were 11·8 μCi/μmole sodium [1-^{14}C]pyruvate and 13·4 μCi/μmole sodium [2-^{14}C]pyruvate.

(*b*) *Culture of Embryos*

Between 160 and 600 embryos were cultured in each test. The required number of 20-μl drops of radioactive medium were prepared in a plastic culture dish (Falcon Plastics) containing 10 ml of paraffin oil (Brinster 1963) and 20 embryos were incubated in each drop. Preliminary tests had indicated that under these conditions approximately half of the pyruvate in the drops was decarboxylated during a 72-hr culture. Using air–carbon dioxide (95:5 v/v) as the gas phase, embryos were incubated at 37°C for 24, 48, or 72 hr.

At the completion of the incubation period 10 embryos which had undergone development in the test were separated from the incubation medium by centrifugation through isotonic sucrose (Wales and Biggers 1968) to which 0·5 mM unlabelled pyruvate had been added, and assayed for radioactivity. After the 24-hr incubation, four- and eight-cell embryos were collected separately while after 48- and 72-hr culture, only morulae and blastocysts respectively were considered normal and were collected. It was found that the blastocysts obtained after 72-hr incubation showed a tendency to collect at the interface between the medium and the sucrose rinse during centrifugation. Therefore the isotonic sucrose rinse used in the centrifuge tube was replaced by non-radioactive medium for this stage. The remaining embryos in each test which had developed normally were collected, isolated from the incubation medium as above, recovered from the broken tip of the centrifuge tube in 3–5 μl of fluid, and stored at −20°C until extracted. After the removal of all embryos the drops of media in the culture dish were pooled. A small aliquot of pooled medium was radioassayed to determine the loss of isotope during incubation. The remainder was stored at −20°C until assayed for labelled metabolic products.

(c) *Extraction of Embryos*

All manipulations were carried out at 0–5°C. The frozen embryos were thawed and 5 mg bovine serum albumin in 50 μl water was added to increase the bulk of the protein precipitate during extraction. The embryos were then extracted with 400 μl of 2·4% perchloric acid (PCA) for 30 min and centrifuged to remove the precipitate. The supernatant was neutralized with potassium hydroxide and the precipitate of potassium perchlorate removed. The neutralized PCA extract was designated the acid-soluble fraction and stored at −20°C until required.

The precipitate after PCA extraction was suspended in 400 μl of cold 10% trichloroacetic acid, centrifuged, and the whole of the supernatant radioassayed. The precipitate was washed repeatedly until no radioactivity was present in the supernatant (usually four to six washes). The precipitate was dried in a stream of air and the precipitate extracted twice with 2 ml chloroform–ether (1:1 v/v). The lipid extracts were combined, dried, and dissolved in 1 ml hexane before the addition of scintillator and radioassay.

The washes from the lipid fraction were combined with the PCA precipitate, 100 μl 0·1 N sodium hydroxide was added and the precipitate hydrolysed at 37°C for 16 hr. Finally, the fraction was neutralized with 100 μl 0·1 N hydrochloric acid and transferred to a scintillation vial for radioassay. This lipid-extracted, PCA-insoluble fraction is referred to as the protein fraction and in addition to protein would be expected to contain the bulk of the DNA and RNA of the embryo.

(d) *Fractionation of Metabolites*

Carboxylic acids in the medium were separated by liquid–liquid partition chromatography using silicic acid as support (O'Shea and Wales 1968). For the 13 columns run the mean recovery of isotope from the column was 101% (S.E. = 4%) of that applied. In addition to the peak of added pyruvate, major radioactive peaks corresponding to lactic and acetic acid were also found. The identity of the lactate peak was checked by paper chromatography of the recovered isotope (see Wales 1969) and the identity of the acetate peak was checked by steam distillation at pH 4·0. Approximately 95% of the isotope in the acetate peak was recovered in the distillate. From the radioactivity in the respective peaks and the initial radioactivity in the pyruvate substrate, the loss of radioactive pyruvate and the production of labelled lactate and acetate were calculated.

Samples of the acid-soluble fraction from the embryos were separated into basic, acidic, and neutral compounds on ion-exchange resins. A 200-μl sample of each extract was applied to a column of Zeo-Carb 225 (H^+) cation-exchange resin, (Permutit Co.) of 2 ml bed volume. Acidic and neutral compounds were eluted with six bed volumes of water. This was followed by elution with six bed volumes of 10% ammonium hydroxide to recover basic compounds including amino acids. After a wash with 4 ml of water the resin was finally eluted with 2N hydrochloric acid to remove any tenaciously bound compounds. The three fractions were dried in a stream of air, dissolved in 0·4 ml water, and a suitable aliquot (usually the total fraction) was taken for radioassay. Although the total activity applied to the Zeo-Carb 225 was low and varied from only 300 to 5000 counts/min, recovery of isotope was good and averaged 93% for all samples. Neutral and acidic compounds were separated by applying the water eluate from the Zeo-Carb 225 column to a column of deacidite FF (formate) anion-exchange resin (Permutit Co.) of 2 ml bed volume. The column was eluted with 12 ml carbon dioxide–free water followed by 12 ml of 6N formic acid. The fractions were radioassayed after drying and dissolving in 0·4 ml of water.

All samples were radioassayed by liquid scintillation techniques using 5 ml of Triton X100–toluene mixture (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 and the counts corrected for differences in efficiency. The accumulation of substrate carbon from the labelled site in the various fractions was calculated from the counts accumulating in the fraction and the specific activity of the pyruvate added to the culture medium.

(e) *Statistical Analysis*

Results were analysed by the method of unweighted means for disproportionate subclass numbers (Snedecor 1957). To equalize variances, data for the intracellular accumulation of metabolites were transformed to logarithms prior to analysis.

III. Results

Table 1 shows the stages to which two-cell mouse embryos developed during varying periods of culture in the presence of specifically labelled sodium pyruvate. When 0·5 mM [1-^{14}C]pyruvate was added to the medium, the percentage of embryos undergoing normal development was high and approximately 70% of embryos developed into blastocysts after 72 hr in culture. In the experiments using [2-^{14}C]pyruvate, development was delayed and although 60% of embryos had reached the four-cell stage after 24 hr in culture no eight-cell embryos were collected. After a 48-hr period of culture, 50% of two-cell embryos had developed into morulae. However, in the cultures incubated for 72 hr only 10–12% of embryos developed

Table 1

Number of two-cell mouse embryos developing in media containing 0·5 mM sodium [1-^{14}C]pyruvate or sodium [2-^{14}C]pyruvate during culture for varying periods *in vitro*

Period of Culture (hr)	No. of Embryos	Stage of Development				
		Four-cell	Eight-cell	Morula	Blastocyst	Other*
		Label: [1-^{14}C]pyruvate				
24	380	196	173	—	—	11
	600	268	301	—	—	31
48	280	12	5	245	5	13
	266	16	11	224	7	8
72	180	—	—	15	135	30
	160	—	—	10	122	28
	180	—	—	25	120	35
	160	—	—	18	107	35
		Label: [2-^{14}C]pyruvate				
24	570	324	—	—	—	236
	550	321	—	—	—	229
48	280	—	—	166	9	105
	320	—	—	135	9	176
72	260	—	—	—	32	228
	320	—	—	—	36	284

* Undeveloped, abnormally developed, or degenerated cells at time of harvesting.

into blastocysts despite the fact that when the embryos used in this experiment were examined at 48 hr, a large proportion had developed to the morula stage. During the last 24 hr in culture many of these morulae showed degenerative changes or developed into abnormal blastocysts. In the subsequent study of substrate accumulation in the embryo, only those with normal morphology were collected and fractionated.

The average decrease in radioactivity in the droplets of culture medium after incubation of embryos was assessed by assaying a 5-μl sample of the pooled medium and comparing the radioactivity with that of unincubated medium. The results for

the different periods of culture are shown in Table 2, and the analyses of variance in Table 4. Approximately 15% of the C1 label was lost for each 24 hr of culture. Loss of radioactivity was less with [2-^{14}C]pyruvate than with [1-^{14}C]pyruvate and, with [2-^{14}C]pyruvate, averaged 9% for each 24 hr in culture.

TABLE 2

LOSS OF RADIOISOTOPE DURING CULTURE OF 20 TWO-CELL MOUSE EMBRYOS IN 20-μl DROPLETS OF MEDIUM CONTAINING 0·01 μMOLE OF [1-^{14}C]PYRUVATE OR [2-^{14}C]PYRUVATE

Number of observations given in parenthesis

Culture Period (hr)	Percentage of Added Isotope Lost	
	[1-^{14}C]Pyruvate	[2-^{14}C]Pyruvate
24	14·2 (2)	5·7 (1)
48	33·0 (2)	18·2 (2)
72	45·1 (4)	25·9 (2)

The loss of labelled pyruvate and the production of other labelled carboxylic acids in the incubation medium during culture were measured and the results are shown in Table 3, and the analyses of variance in Table 4. Lactic acid was the main labelled metabolite produced in the medium from either [1-^{14}C]- or [2-^{14}C]pyruvate.

TABLE 3

ACCUMULATION OF LABELLED CARBOXYLIC ACIDS DURING INCUBATION OF TWO-CELL MOUSE EMBRYOS IN 0·5 mM [1-^{14}C]PYRUVATE OR [2-^{14}C]PYRUVATE

Values are the total amounts of acid produced over the period of culture

Period of Culture (hr)	No. of Obser-vations	Pyruvate Utilized (nmoles)	Labelled Carboxylic Acids Produced (nmoles)		
			Lactic Acid	Acetic Acid	Other Acids
		Label: [1-^{14}C]pyruvate			
24	2	2·9	0·8	0·0	0·5
48	2	5·3	1·4	0·0	0·4
72	4	6·9	2·0	0·0	0·3
		Label: [2-^{14}C]pyruvate			
24	1	2·3	1·3	0·3	0·1
48	2	4·4	2·0	0·4	0·1
72	2	6·1	2·9	0·4	0·1

Although there was a small but significant difference in the estimate of pyruvate utilization using the different labels, the estimates of lactate production did not differ significantly. However, there was a linear increase in the production of lactate with increasing periods of incubation. Labelled acetic acid accumulated only when [2-^{14}C]pyruvate was the substrate. Small amounts of other labelled carboxylic acids accumulated during culture in both [1-^{14}C]- and [2-^{14}C]pyruvate and the total

accumulation of these compounds is given in the table. This total includes trace amounts of a number of unidentified acids eluted as minor radioactive peaks from

TABLE 4

ANALYSES OF VARIANCE OF DATA IN TABLES 2 AND 3

Source of Variation	D.F.	Isotope Lost	Variance Ratios	
			Pyruvate Utilized	Lactate Produced
Effect of label	1	5·47	5·77*	5·19
Effect of time				
Linear	1	37·79**	90·18**	14·00**
Quadratic	1	0·58	0·66	0·02
Interaction	2	0·95	0·08	0·21
Error	7	*17·2*	*0·17*	*0·14*

* $P < 0·05$. ** $P < 0·01$.

the silicic acid column (see Fig. 1). However, insufficient amounts of these compounds were present to allow positive identification.

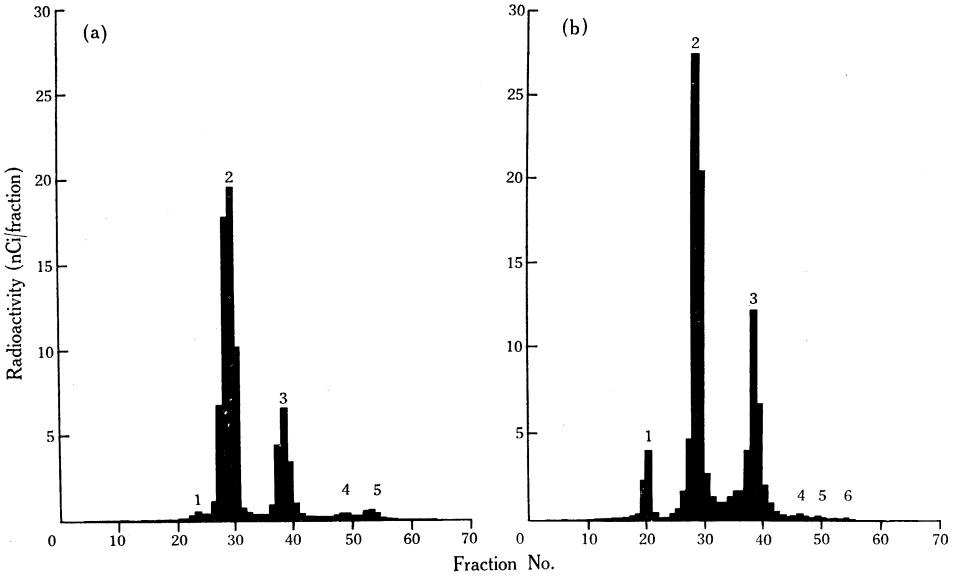

Fig. 1.—Elution pattern of radioactive carboxylic acids from a 20-μl drop of medium in which 20 mouse embryos have been cultured for 48 hr in the presence of (*a*) 0·5 mM sodium [1-^{14}C]pyruvate (11·8 μCi/μmole), (*b*) 0·5 mM sodium (2-^{14}C]pyruvate (13·4 μCi/μmole). In (*a*), peaks 2 and 3 are pyruvic and lactic acids, respectively, and peaks 1, 4, and 5 are unidentified metabolites. In (*b*), peaks 1, 2, and 3 are acetic, pyruvic, and lactic acids, respectively, and peaks 4, 5, and 6 are unidentified metabolites.

The total accumulation of substrate carbon from C1 and C2 of pyruvate during culture is shown in Table 5. More substrate carbon accumulated from C2 than from

C1 of pyruvate and there was a linear increase in the accumulation of both carbons as the period of culture was increased. At the same time, there was some suggestion

TABLE 5

TOTAL ACCUMULATION OF SUBSTRATE CARBON BY TWO-CELL MOUSE EMBRYOS CULTURED IN [1-^{14}C]PYRUVATE OR [2-^{14}C]PYRUVATE FOR VARYING PERIODS

Number of replicates given in parenthesis

Culture Period (hr)	Stage of Development Reached	Accumulation of Substrate Carbon from Position Labelled (pg-atoms/embryo)		Summary of the Analysis of Variance (logarithmic transformation)		
		[1-^{14}C]Pyruvate	[2-^{14}C]Pyruvate	Source of Variation	D.F.	Variance Ratio
24	4- to 8-cell	1·04 (4)	6·31 (2)	Effect of position labelled	1	20·40**
48	Morula	3·91 (2)	12·48 (2)	Effect of time		
				Linear	1	7·40*
72	Blastocyst	4·09 (3)	11·48 (5)	Quadratic	1	2·94
				Interaction	2	0·80
				Error	12	*0·025*

* $P < 0·05$. ** $P < 0·01$.

TABLE 6

SUBSTRATE CARBON ACCUMULATED IN ACID-SOLUBLE, PROTEIN, AND LIPID FRACTIONS OF MOUSE EMBRYOS CULTURED IN RADIOACTIVE SODIUM PYRUVATE FOR VARYING PERIODS

Percentage of total substrate carbon accumulated shown in parenthesis

Culture Period (hr)	Stage of Development Reached	Replicate No.	Substrate Carbon Accumulated from Position Labelled (pg-atoms/embryo)		
			Acid-soluble Fraction	Protein Fraction	Lipid Fraction
			Label: [1-^{14}C]pyruvate		
24	4-cell	1	0·57 (60)	0·37 (40)	0·002 (1)
		2	0·40 (52)	0·37 (48)	0·000 (0)
	8-cell	1	0·75 (64)	0·41 (35)	0·008 (1)
		2	0·56 (52)	0·52 (48)	0·003 (1)
48	Morula	1	3·03 (67)	1·49 (33)	0·005 (1)
		2	2·33 (71)	0·97 (29)	0·007 (1)
72	Blastocysts	1	2·46 (63)	1·44 (37)	0·000 (0)
		2	2·84 (70)	1·35 (30)	0·008 (1)
		3	2·23 (57)	1·68 (43)	0·000 (0)
		4	2·83 (63)	1·63 (37)	0·000 (0)
			Label: [2-^{14}C]pyruvate		
24	4-cell	1	4·72 (76)	1·42 (23)	0·11 (2)
		2	5·03 (79)	1·25 (20)	0·08 (1)
48	Morula	1	7·80 (65)	3·51 (29)	0·67 (6)
		2	8·67 (67)	3·54 (27)	0·86 (7)
72	Blastocysts	1	3·60 (55)	2·34 (36)	0·56 (9)
		2	4·42 (45)	4·62 (47)	0·89 (9)

that after 48 hr in culture, there was little increase in the accumulation of substrate carbon even though the quadratic component of the effect of time was not significant.

Table 6 shows the amounts of substrate carbon accumulated into the acid-soluble, protein, and lipid fractions of embryos following incubation for varying periods with pyruvate as the sole substrate. Approximately 60% of the total carbon accumulated from position one was found in the acid-soluble fraction and the remaining 40% in the protein fraction. Negligible amounts of C1 of pyruvate were incorporated into the lipid fraction. Total incorporation of C1 of pyruvate into both the acid-soluble and protein fractions increased with time, especially during the first 48 hr, and there was no change in the percentage of counts entering either of these fractions. In the experiments using [2-^{14}C]pyruvate, accumulation of isotope in the acid-soluble fraction reached a peak after 48 hr culture and at 72 hr values in the blastocyst were half those found at the morula stage. Incorporation of C2 into protein remained constant over this period and, as a result, the ratio of incorporation in the acid-soluble fraction to that in the protein fraction changed from 3:1 following 24 hr incubation to 1:1 after 72 hr in culture. Using [2-^{14}C]pyruvate, there was also a substantial accumulation of label in the lipid fraction of the embryo and the proportion of the total counts found in this fraction increased as the time of culture was increased. In all these experiments the sum of the substrate carbon accumulated in the various fractions showed good agreement with the estimate of total incorporation using a small sample of embryos (Table 5).

Samples of the acid-soluble fraction were applied to a cation-exchange column to separate labelled basic compounds from acidic plus neutral compounds (Table 7).

TABLE 7

FRACTIONATION ON CATION-EXCHANGE RESIN OF THE ISOTOPICALLY LABELLED COMPOUNDS IN THE ACID-SOLUBLE EXTRACTS OF EMBRYOS

Amounts as percentages of acid-soluble fraction shown in parenthesis

Culture Period (hr)	Replicate No.	Substrate Carbon Accumulated from [1-^{14}C]Pyruvate (pg-atoms/embryo)		Substrate Carbon Accumulated from [2-^{14}C]Pyruvate (pg-atoms/embryo)	
		Acid + Neutral Compounds	Basic Compounds	Acid + Neutral Compounds	Basic Compounds
24	1	0·25 (38)	0·41 (63)	1·75 (37)	2·97 (63)
	2	0·20 (41)	0·28 (60)	3·02 (60)	2·01 (40)
48	1	1·33 (44)	1·70 (56)	3·12 (40)	4·68 (60)
	2	0·58 (25)	1·72 (74)	2·69 (31)	5·98 (69)
72	1	1·15 (47)	1·30 (53)	0·83 (23)	2·77 (77)
	2	1·42 (50)	1·42 (50)	1·06 (24)	3·36 (76)
	3	1·09 (49)	1·14 (51)	— —	— —
	4	1·42 (50)	1·42 (50)	— —	— —

The former made up at least 50% of the label in the acid-soluble fraction when either [1-^{14}C]- or [2-^{14}C]pyruvate was used and no significant change occurred in the fraction of the total acid-soluble label found in these basic compounds. The greatest accumulation of labelled basic compounds occurred at the morula stage and decreased at the formation of the blastocyst, especially when [2-^{14}C]pyruvate

was used as substrate. In samples where substantial incorporation into this fraction occurred, total incorporation was measured by assaying a small aliquot of the eluate from the column. The remainder was reduced in volume and chromatographed in a descending system on Whatman No. 1 chromatography paper using one of the following solvent systems: (1) n-butanol–acetic acid–water (4:1:5 v/v); (2) pyridine–acetic acid–water (50:35:15 v/v); (3) ethanol–7·5N ammonia–water (8:1:1 v/v); (4) water-saturated phenol. Radioactive peaks were obtained at R_F 0·22 (solvent 1), 0·34 and 0·45 (solvent 2), 0·08 and 0·38 (solvent 3), 0·24 and 0·57 (solvent 4). These R_F values correspond to those of authentic aspartic acid and alanine run in these systems at the same time.

By pooling extracts from similar treatments, sufficient of the neutral plus acidic fractions was obtained for fractionation into acidic and neutral compounds on an anion-exchange column. In all cases tried, acidic compounds made up 70–95% of this fraction. However, no radioactive carboxylic acids were detected when PCA extracts of embryos were applied to a silicic acid column and eluted with hexane–butanol mixtures.

IV. Discussion

Because of the limited amount of biological material available and the restricted range over which energy substrates can be tolerated by the mouse embryo during early development (Brinster 1965*a*) it is necessary in any study of their metabolism to utilize isotopically labelled substrates at relatively high specific activities to obtain the desired experimental results. In the present experiment, development of two-cell embryos in a medium containing [1-^{14}C]pyruvate compared well with that obtained by Brinster (1965*a*) and Whittingham and Wales (unpublished data) using unlabelled pyruvate as sole energy source. The specific activity of the [2-^{14}C]pyruvate was similar to that of the [1-^{14}C]pyruvate. However, the poor development of embryos, especially towards the end of the period of culture in the medium containing [2-^{14}C]pyruvate, may be related to the large intracellular accumulation of isotope exposing these embryos to harmful amounts of internal radiation. In view of this, caution must be exercised in interpreting changes in the products of the metabolism of [2-^{14}C]pyruvate, especially towards the end of the incubation period. However, the data for the loss of isotope from the medium (Table 2), for the utilization of pyruvate (Table 3), and for the accumulation of lactate during culture (Table 3) indicate that the embryos cultured in both [1-^{14}C]pyruvate and [2-^{14}C]pyruvate were active metabolically during the whole of the period of culture.

The loss of isotope from the medium during incubation presumably is due to the oxidation of pyruvate and loss of radioactive carbon dioxide to the atmosphere. Using the data in Table 2, the rates of carbon dioxide production from C1 and C2 of pyruvate were calculated to be approximately 2·9 and 1·2 p-mole/embryo initially cultured/hr respectively and the ratio of carbon dioxide produced from C1 to carbon dioxide from C2 to be approximately 2·0 at all stages. These values are similar to the earlier values of Wales and Whittingham (1967) for two-cell embryos incubated over 3 hr, and suggest that in the present experiments catabolic metabolism of both [1-^{14}C]- and [2-^{14}C]pyruvate was unimpaired and remained constant through all stages of development.

The constant rate of production of carbon dioxide from pyruvate indicated by the present experiments is in marked contrast to the initially low but increasing rate of carbon dioxide production from glucose during development (Brinster 1967*a*, 1967*b*). This observation strengthens the suggestion made by Wales (1969) that when glucose alone is available as an energy source to the two-cell embryo, insufficient glycolytic activity occurs to maintain potential tricarboxylic acid cycle activity and development fails. When metabolic intermediates such as pyruvate are provided, tricarboxylic acid cycle activity is high and development proceeds.

An increasing amount of lactate was found to accumulate from pyruvate during the 72 hr in culture. It is puzzling, therefore, that in an earlier study (Wales and Whittingham 1967) no lactate was formed from pyruvate during incubation for 3 hr of two-cell mouse embryos even though in the present studies a substantial amount of acetate (a minor metabolite) was identified. Possibly, acetate is produced as an initial product of metabolism when pyruvate is the sole substrate in the medium and reaches a steady level (see Table 3) before substantial quantities of lactate are formed.

In general, the accumulation of intracellular isotope increases markedly during the first 2 days of culture but between morula and blastocyst formation its level remains steady or declines. The greatest decline occurs in the acid-soluble products accumulated from [2-^{14}C]pyruvate. Although only blastocysts of normal appearance were separated and used to measure intracellular constituents, the above result may reflect some derangement to metabolism caused by the high level of intracellular isotope. This may not be the only reason for the decline as accumulation of the acid-soluble products fell during the same period when [1-^{14}C]pyruvate was used and development was apparently normal.

It is obvious from the results that both C1 and C2 of pyruvate are distributed widely in the embryo and there is substantial incorporation of both carbon atoms into protein. One explanation of the incorporation of C1 of pyruvate into protein is its direct conversion to alanine as occurs in the rabbit blastocyst (Fridhandler 1968). The evidence that alanine was one of the labelled basic compounds in the mouse embryo supports this view. However, Wales, Quinn, and Murdoch (1969) have established that substantial amounts of carbon dioxide are fixed by the mouse embryo. The amount of carbon fixed in their experiment and the amount of C1 of pyruvate incorporated in the present experiment were similar. This suggests that the conversion of pyruvate to a four-carbon intermediate is the most important route of entry of C1 of pyruvate into the carbon pool of the mouse embryo, a reaction that Fridhandler (1968) considers unimportant in the rabbit blastocyst. The aspartic acid present in the labelled basic compounds probably arises by the conversion from oxaloacetate. Unlike the rabbit blastocyst (Fridhandler 1968) no evidence of substantial glutamate accumulation was found in the mouse embryo under these conditions and if formed is rapidly converted to other compounds.

There was no detectable accumulation of carboxylic acids in embryos, indicating a low steady state of these compounds in the rapidly metabolizing tissue. As a result, the identity of the labelled acidic compounds in the acid-soluble fraction is unknown and further work will be required to decide the role of these compounds in the metabolism of the zygote.

The amount of label in the lipid fraction of embryos incubated in [1-^{14}C]pyruvate was never more than 50% above background (30 counts/min) and probably reflects a slight contamination from the protein fraction which was not completely removed by back washing the lipid extract. On the other hand, substantial incorporation of isotope into lipid occurred when [2-^{14}C]pyruvate was used. No doubt, the first step in this incorporation is the decarboxylation of pyruvate to acetyl-CoA which can then enter into fatty acid synthesis. This reaction has been demonstrated in the rabbit blastocyst (Fridhandler 1968).

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VI. References

Brinster, R. L. (1963).—A method for *in vitro* cultivation of mouse ova from two-cell to blastocyst. *Expl Cell Res.* **32**, 205.

Brinster, R. L. (1965*a*).—Studies on the development of mouse embryos *in vitro*. II. The effect of energy source. *J. exp. Zool.* **158**, 59.

Brinster, R. L. (1965*b*).—Studies on the development of mouse embryos *in vitro*. IV. Interaction of energy sources. *J. Reprod. Fert.* **10**, 227.

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

Brinster, R. L. (1967*b*).—Carbon dioxide production from lactate and pyruvate by the preimplantation mouse embryo. *Expl Cell Res.* **47**, 634.

Brinster, R. L., and Thomson, J. L. (1966).—Development of eight-cell mouse embryos *in vitro*. *Expl Cell Res.* **42**, 308.

Fridhandler, L. (1968).—Intermediary metabolic pathways in preimplantation rabbit blastocysts. *Fert. Steril.* **19**, 424.

O'Shea, T., and Wales, R. G. (1968).—Metabolism of [1-^{14}C] sodium lactate and [2-^{14}C] sodium lactate by ram spermatozoa. *J. Reprod. Fert.* **15**, 337.

Snedecor, G. W. (1957).—"Statistical Methods Applied to Experiments in Agriculture and Biology." 5th Ed. (Iowa State College Press: Ames, Iowa).

Wales, R. G. (1969).—Accumulation of carboxylic acids from glucose by the pre-implantation mouse embryo. *Aust. J. biol. Sci.* **22**, 701.

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

Wales, R. G., Quinn, P., and Murdoch, R. N. (1969).—The fixation of carbon dioxide by the eight-cell mouse embryo. *J. Reprod. Fert.* **20**, 541.

Wales, R. G., and Whittingham, D. G. (1967).—A comparison of the uptake and utilization of lactate and pyruvate by one- and two-cell mouse embryos. *Biochim. biophys. Acta* **148**, 703.

Whitten, W. K. (1956).—Culture of tubal mouse ova. *Nature, Lond.* **177**, 96.

Whitten, W. K. (1957).—Culture of tubal ova. *Nature, Lond.* **179**, 1081.