FIXATION OF CARBON DIOXIDE BY PRE-IMPLANTATION MOUSE EMBRYOS *IN VITRO* AND THE ACTIVITIES OF ENZYMES INVOLVED IN THE PROCESS

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Abstract

The incorporation of fixed carbon from carbon dioxide into mouse embryos was greatest in eight-cell embryos which developed into blastocysts during a 24-hr culture period and was four to five times greater than the incorporation into two-cell embryos cultured for a similar period. The net accumulation of labelled products in the culture medium was greatest during the culture of the morula stage to the blastocyst over 24 hr.

Acid-soluble compounds accounted for the major part of the labelled materials accumulating in both the embryos and medium. Aspartic and glutamic acids were the main basic compounds identified in the acid-soluble fraction of the embryos and lactate and citrate were the major compounds accumulating in the medium. An increasing proportion of labelled glutamate to aspartate and citrate to lactate was found in later stages of development, suggesting a greater activity of the tricarboxylic acid cycle at the later stages.

The activities of pyruvate carboxylase [E.C.6.4.1.1] and malate dehydrogenase (decarboxylating) (NADP) [E.C.1.1.1.40] were measured in the pre-implantation mouse embryo and the levels detected could account for the amount of carbon dioxide fixed by embryos cultured *in vitro*. No detectable levels of phosphopyruvate carboxylase [E.C.4.1.1.32] were found at any stage of development.

It would appear that the substantial gain of carbon by carbon dioxide fixation in the pre-implantation mouse embryo is accomplished mainly by condensation of carbon dioxide with pyruvate and that the four-carbon moiety thus formed is used mainly to supply intermediates for the tricarboxylic acid cycle. Gluconeogenesis from pyruvate would seem unlikely to occur.

I. INTRODUCTION

The requirement of a bicarbonate buffer system for the development of mouse embryos *in vitro* and the demonstration that these embryos are able to accumulate carbon from carbon dioxide (Wales, Quinn, and Murdoch 1969; Graves and Biggers 1970) has confirmed the suggestion of Biggers, Whittingham, and Donahue (1967) that fixation of carbon dioxide may be an important process in the energy metabolism of the early mouse embryo.

Large amounts of bicarbonate are present in the secretions of the oviduct of the rabbit and ewe (David *et al.* 1969; Restall and Wales 1966). If similar concentrations of bicarbonate are also present in the secretions of the mouse oviduct, they would provide a suitable environment for this process to occur and a considerable gain in carbon by the embryo could result.

Wales, Quinn, and Murdoch (1969) have found that carbon incorporated into the eight-cell mouse embryo by this process is widely distributed in the carbon pools

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of the embryo and the similarity between the amount of this incorporation and that of [1-^{14}C]pyruvate suggests a common route of metabolism for C1 of pyruvate and carbon fixed from carbon dioxide. It would appear, therefore, that the activity of enzymes such as pyruvate carboxylase [pyruvate: carbon dioxide ligase (ADP), E.C.6.4.1.1], malate dehydrogenase (decarboxylating) (NADP) [L-malate: NADP oxidoreductase (decarboxylating), E.C.1.1.1.40], and phosphopyruvate carboxylase [GTP: oxaloacetate carboxy-lyase (transphosphorylating) E.C.4.1.1.32] may determine the rate of entry of pyruvate and carbon dioxide into the metabolic pools of the embryo.

The present experiments were undertaken to extend the earlier observations on carbon dioxide fixation and to attempt an identification of labelled compounds. The activities of the three enzymes mentioned above have also been measured during development of the pre-implantation mouse embryo.

II. Materials and Methods

(*a*) *General*

Mouse embryos were obtained by flushing the reproductive tracts of superovulated random-bred albino mice (Brinster 1963). Successive developmental stages from the one-cell to the late blastocyst were collected from 24 to 120 hr after the injection of chorionic gonadotrophin (HCG) (Brinster 1965*a*). When collections were made 96 hr after HCG injection, approximately 60% of the embryos were early blastocysts and the remainder were morulae. These stages were separated before use. Cumulus cells were removed from fertilized and unfertilized one-cell ova by incubation in hyaluronidase solution (Brinster 1965*a*).

The basic medium used was a 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 (Brinster 1965*b*). The embryos for *in vitro* culture were washed twice (2 ml/wash) in this medium before being cultured in medium containing radioactive bicarbonate. Embryos used for the measurement of enzymic activity were washed three times in substrate-free medium, recovered from the third wash in a minimum volume of medium, and transferred to 10 μl of appropriate buffer solution in small tubes.

(*b*) *Culture and Recovery of Embryos for Estimating* in vitro *Fixation of Carbon Dioxide*

For each replicate 50–450 embryos, depending on the stage of development, were cultured for 24 hr at 37°C in modified Krebs–Ringer bicarbonate medium which contained $NaH^{14}CO_3$ with a gas phase of 5% $^{14}CO_2$ in air. The method of culturing embryos under these conditions has been described previously (Wales, Quinn, and Murdoch 1969). In initial experiments, analysis of the $NaH^{14}CO_3$ by precipitation as barium carbonate (Wales and Biggers 1968) was used to determine the actual specific radioactivity of the bicarbonate in both the medium and atmosphere; the specific activity between replicates varied from 4·9 to 9·3 μCi/μmole, the concentration of bicarbonate in the medium being 25 mM. In subsequent experiments, the specific activity of the $NaH^{14}CO_3$ was determined by diluting aliquots in alkaline water and assaying by liquid scintillation techniques. In these later tests sufficient radioactive bicarbonate was used to obtain a constant specific activity of 8·9 μCi/μmole.

At the end of the incubation period, embryos at each developmental stage were separated from the medium and recovered for further fractionation. Prior to the blastocyst stage this was done by centrifuging the embryos through isotonic sucrose (Wales and Biggers 1968). Blastocysts were collected by centrifugation through non-radioactive culture medium (Wales and Whittingham 1970). The embryos were recovered from the broken tip of the centrifuge tube and both the embryos and samples of incubation medium were acidified to remove unfixed carbon dioxide as described previously (Wales, Quinn, and Murdoch 1969). After neutralization of the samples with sodium hydroxide, they were stored at −20°C until metabolic products were extracted and assayed. In addition the total incorporation of fixed carbon in the embryos for each experiment

was also estimated by separating five embryos at each developmental stage from the incubation medium as outlined above, transferring the broken end of the centrifuge tube containing the embryos to a scintillation vial, and estimating the fixed carbon after acidification. The total incorporation of carbon into the medium was estimated on a 20-μl aliquot of the neutralized solution.

(*c*) *Extraction and Identification of Metabolites*

The frozen embryos and media were thawed and 50 μl of sheep serum were added to act as carrier for labelled compounds which were then fractionated into acid-soluble, protein, and lipid portions as described previously (Wales, Quinn, and Murdoch 1969; Wales and Whittingham 1970). A 20-μl aliquot of the acid-soluble fraction was assayed for radioactivity and the remainder stored at -20°C until required. The distribution of radioactivity in the acidic, basic, and neutral compounds of the acid-soluble fractions of the embryos and medium was determined by passing an aliquot (25–200 μl containing radioactivity of between 200–1400 disintegrations/min) first through a cationic and then through an anionic exchange resin as described by Wales and Whittingham (1970). Quantitative recovery of radioactivity applied to the columns was obtained and the proportion of total counts in each fraction was used to determine the distribution of label present in the acid-soluble fraction. The incorporation of radioactivity by the embryos into the various fractions of the medium was corrected for residual background radioactivity in the medium. This was estimated by extracting samples of medium which had not been incubated with embryos.

To obtain sufficient labelled material for further identification the remaining portions of the perchloric extracts of the embryos and of the medium for each stage of development were pooled separately. The carboxylic acids present in aliquots of the pooled acid-soluble fractions of the medium were then separated on a column of silicic acid using hexane–butanol mixtures (O'Shea and Wales 1968).

The basic and acidic compounds in the pooled samples of acid-soluble material from the embryos were separated as before and reduced in volume under a stream of air. The carboxylic acids in the acidic fraction were then separated on a column of silicic acid using hexane–butanol mixtures. Aliquots of the basic fraction containing radioactivity of between 200 and 900 disintegrations/min were chromatographed in a descending system on Whatman No. 1 chromatography paper in each of the following solvent systems: (1) water-saturated phenol; (2) pyridine–acetic acid–water (50:35:15 v/v); (3) ethanol–7·5N ammonia–water (8:1:1 v/v); (4) propanol–16·5N ammonia–water (6:3:1 v/v).

After development the chromatograms were cut into 1-cm strips. Each strip was eluted by soaking in 0·4 ml of water for 30 min and the radioactivity assayed by liquid scintillation techniques. Authentic standards of aspartic acid, glutamic acid, and alanine run in the above systems at the same time had R_F values of 0·19, 0·34, and 0·54 (solvent 1), 0·27, 0·40, and 0·45 (solvent 2), 0·07, 0·10, and 0·45 (solvent 3), and 0·43, 0·44, and 0·60 (solvent 4). Chromatography of embryo extracts using a combination of these solvents allows clear resolution of these compounds from each other and from other amino acids. Since recovery of radioactivity from the embryo extracts applied to the chromatograms was quantitative (99$\pm$10%), the proportion of radioactivity in peaks which corresponded with the amino acid standards was used to estimate the total radioactivity associated with that particular compound. As the limited amount of material had made pooling necessary, only one estimate of activity for a particular compound at any one stage of embryonic development could be obtained.

All samples were radioassayed 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(4-methyl-5-phenyloxazol-2-yl)benzene; 5 ml of this scintillation mixture was used for each 0·4 ml of aqueous sample.

(*d*) *Enzyme Assays*

For each assay of activity, between 50 and 150 embryos were used. The tubes containing the washed embryos were stored in dry ice for 2–3 hr until about 30 min before the assays were performed. The activities of the enzymes were measured by radiochemical enzyme assay, the total volume of added reactants being 20 μl.

TABLE 1

FIXATION OF CARBON DIOXIDE BY MOUSE EMBRYOS CULTURED FOR 24 HR IN MEDIUM CONTAINING RADIOACTIVE SODIUM BICARBONATE AND CARBON DIOXIDE

Values are the means of three replicates for each stage of development

Stage of Development during Culture		% Eggs Developed	Carbon Incorporated (pg-atoms/embryo)						
				Acid-soluble Fraction:					
At Start	At Completion		Total	Acidic Compounds	Basic Compounds	Neutral Compounds	Protein Fraction	Lipid Fraction	Total Uptake
Embryos:									
2-cell	4-cell	85	0·67 (89)†	0·32 (48)‡	0·32 (48)‡	0·04 (6)‡	0·08 (11)†	0·00 (0)†	0·75
2-cell	8-cell	85	1·14 (86)	0·46 (40)	0·53 (46)	0·15 (13)	0·18 (14)	0·01 (1)	1·33
8-cell	Morula	94	3·34 (87)	1·34 (40)	1·65 (49)	0·35 (11)	0·48 (13)	0·03 (1)	3·85
8-cell	Blastocyst	94	4·98 (86)	1·94 (39)	2·71 (55)	0·33 (7)	0·76 (13)	0·05 (1)	5·79
Morula	Blastocyst	77	3·56 (79)	1·67 (47)	1·58 (44)	0·32 (9)	0·89 (20)	0·06 (1)	4·51
Early blastocyst	Late blastocyst	96	4·43 (80)	2·39 (54)	1·69 (38)	0·36 (8)	1·06 (19)	0·05 (1)	5·54
Medium:									
2-cell	2-, 4-, 8-cell	85	0·38 (100)	0·29 (77)	0·07 (19)	0·02 (4)	0 (0)	0 (0)	0·38
8-cell	Morula, blastocyst	94	2·52 (98)	1·43 (57)	0·80 (32)	0·25 (10)	0·04 (2)	0 (0)	2·56
Morula	Blastocyst	77	3·58 (98)	2·46 (69)	0·50 (14)	0·63 (17)	0·07 (2)	0 (0)	3·65
Early blastocyst	Late blastocyst	96	2·57 (99)	2·08 (81)	0·47 (18)	0·03 (1)	0·02 (1)	0·01 (<1)	2·60

Summary of the Analyses of Variance for Incorporation into Embryos: Variance Ratios (logarithmic transformation)

Source of Variation	D.F.	Acid-soluble Fraction				Protein Fraction	Lipid Fraction	Total§ Uptake
		Total	Acidic	Basic	Neutral			
Between stages	(5)							
A. Slow *v.* fast development	1	37·48**	17·70**	10·91**	1·51	3·92	0·41	50·00**
Effect of time of collection								
B. Linear	1	403·88**	223·74**	116·06*	14·11**	59·09**	32·07**	574·68**
C. Quadratic	1	144·67**	40·68**	71·36**	2·65	5·72*	2·12	170·01**
Interactions								
A × B	1	4·56	0·01	2·89	2·99	1·26	0·99	8·27*
A × C	1	0·05	0·01	0·64	2·24	0·06	1·27	0·07
Within stages (error variance)	12	*0·0032*	*0·0068*	*0·0094*	*0·0832*	*0·0433*	*0·0028*	*0·0025*

* $P < 0·05$. ** $P < 0·01$.

† Percentage of total carbon fractionated shown in parenthesis.

‡ Percentage of acid-soluble fraction shown in parenthesis.

§ Total uptake obtained by summation of carbon incorporated into the acid-soluble, protein, and lipid fractions.

For the assay of pyruvate carboxylase, the reaction was carried out in 1 by 3 cm tubes using the procedure of Utter and Keech (1963). The reaction mixture contained 2·5 μmoles Tris–HCl buffer (pH 7·4), 0·2 μmole $MgCl_2$, 0·05 μmole ATP, 0·014 μmole acetyl-coenzyme A, 0·2 μmole sodium pyruvate, and 0·6 μmole $NaH^{14}CO_3$. For the assay of malate dehydrogenase and phosphopyruvate carboxylase activities, embryos were placed in 0·5 by 2·5 cm tubes. Malate dehydrogenase was assayed by the method of Slack and Hatch (1967), the reaction mixture being composed of 1·0 μmole Tris–HCl buffer (pH 7·4), 0·067 μmole $MgCl_2$, 0·1 μmole glucose 6-phosphate, 0·0067 μmole NADP, 0·33 μmole sodium pyruvate, and 0·6 μmole $NaH^{14}CO_3$. It was not necessary to add glucose-6-dehydrogenase because of the high levels of this enzyme in the embryos (Brinster 1966). Phosphopyruvate carboxylase activity was assayed by the method of Utter and Kurahashi (1955) using 1·0 μmole Tris–HCl buffer (pH 7·4), 0·04 μmole $MnCl_2$, 0·04 μmole ITP, 0·05 μmole reduced glutathione (neutralized to pH 7·0), 0·04 μmole oxaloacetic acid (neutralized to pH 6·2–6·4), and 0·6 μmole $NaH^{14}CO_3$. Controls for all assays consisted of complete reaction mixtures to which no embryos were added. The amount of oxaloacetic acid employed in the phosphopyruvate carboxylase assay was reduced to 1/30th of the amount used by Utter and Kurahashi (1955) to the level recommended by Chang and Lane (1966). This reduction resulted in a considerable lowering of background counts.

In all assays the specific activity of the radioactive bicarbonate was 7·8 μCi/μmole. The assay mixtures were incubated for 30 min at 30°C in capped tubes and the reaction was terminated by the addition of 10 μl 3N H_2SO_4. The reaction tubes were left overnight in a sealed container containing 6N NaOH to remove any unreacted bicarbonate. They were then placed in a scintillation vial with 0·8 ml of water and the level of fixed radioactivity assessed by liquid scintillation counting using 10 ml of Triton X100–toluene scintillation fluid.

(*e*) *Statistical Analysis*

For the studies of *in vitro* fixation, all data were transformed to logarithms prior to analysis and the significance of the results was assessed by analysis of variance. Since embryos were collected from the two-cell stage onwards at 24-hr intervals, linear and quadratic partitioning of the effect of the time of collection of the embryos on the fixation of carbon were possible.

Where equal numbers of replicates were used for the estimation of enzyme activity at different stages of development, the results were assessed by standard analysis of variance. With unequal numbers of replicates, results were analysed by the method of unweighted means for disproportionate subclass numbers (Snedecor 1957). All variances were tested for homogeneity using Bartlett's test (Snedecor 1957).

III. Results

(*a*) In vitro *Fixation of Carbon Dioxide*

The percentage of embryos reaching various stages of development during culture for 24 hr in the presence of radioactive bicarbonate is given in Table 1. Overall, approximately 90% of embryos cultured in radioactive medium for 24 hr continued to develop. This degree of development of embryos is as great as or greater than that of control embryos cultured in medium containing no radioactive compounds. At the completion of culture, approximately two-thirds of the two-cell embryos had developed to the four-cell stage, 20% had developed to eight cells, and the remainder were still at the two-cell stage. At the eight-cell stage, about twice as many embryos had developed into morulae than into blastocysts during the 24-hr period of culture.

The amount of carbon fixed into the acid-soluble, protein, and lipid fractions of the embryos is also given in Table 1. The acid-soluble fraction accounted for 90% of the total uptake of carbon by cultured two-cell embryos. The percentage of carbon

in this fraction fell to 80% in cultured morulae and blastocysts due to an increase in the proportion of carbon entering the protein fraction. Statistical analysis of the data (Table 1) showed that there was a significantly greater accumulation of label in the acid-soluble fraction of those embryos which showed a faster rate of development. This difference was most notable at the two-cell stage where the accumulation of label in embryos developing to eight cells was almost double that in embryos only reaching the four-cell stage during 24 hr of culture. The greatest accumulation in the acid-soluble fraction occurred in eight-cell embryos which developed into blastocysts and there was little difference between the uptake of embryos collected 72 or 96 hr after HCG injection. As with total incorporation, accumulation of carbon in the acid-soluble fraction in these later stages of development was 4–5 times that in cultured two-cell embryos.

The proportion of fixed carbon found in the protein fraction rose from a little more than 10% in cultured two-cell and eight-cell embryos to 20% in cultured morulae and blastocysts. At all stages, entry of fixed carbon was greater into the protein fraction of embryos which had a faster rate of development but on statistical analysis (Table 1) this effect failed to reach the 5% level of significance. Unlike the results for accumulation of carbon in the acid-soluble fraction, there was a greater incorporation into the protein fraction of cultured morulae and blastocysts than into that of cultured eight-cell embryos. However, where there was a fivefold increase between the two-cell and the eight-cell stage, there was only a doubling between eight-cell embryos and the morulae or blastocysts. This caused a significant quadratic response, as well as linear response, in the analysis of the effects of time of collection on label incorporated into the protein fraction of the embryos (Table 1). Incorporation of fixed carbon into lipids was small and averaged 1% of the total incorporation in the embryos. There was a significant linear increase with time of collection in the accumulation of label into this fraction (Table 1).

The estimate of the total incorporation of carbon into embryos, based on the summation of incorporation of label into the acid-soluble, protein, and lipid fractions is also given in Table 1 together with the summary of the analysis of variance. There were significant effects of the time of collection of embryos and of the rate of development on total incorporation of carbon. A second estimate of total incorporation in each replicate was made on a small sample of embryos as described in the materials and methods. Although this estimate showed similar trends to those described above, the results using this method were higher and more variable within stages than those obtained by summing the fractions. On analysis of variance, this greater variability resulted in a 14-fold increase in the within-stage variance when compared with the estimate made by summing the fractions.

The distribution of label in the acid-soluble fraction of the embryos and associated analyses of variance are also included in Table 1. Acidic and basic compounds made up approximately 90% of the radioactivity present in this fraction, the distribution between these two components being about equal at all stages. Consequently, analysis of the data for carbon incorporated into both the acidic and basic compounds (Table 1) indicated similar changes to those that occurred in the total acid-soluble fraction. Thus embryos which developed at a faster rate incorporated more carbon into both fractions and the incorporation into cultured two-cell embryos

was much lower than that into later stages that were cultured. Incorporation into neutral compounds represented 10% of the total label in the acid-soluble fraction. However, there was a considerable degree of variation in the results for incorporation into this fraction and although there was no difference in the incorporation into embryos collected 72 and 96 hr after HCG, the quadratic component of the effects of time of collection did not test as significant.

On paper chromatography of the basic fraction, compounds with R_F values similar to glutamic and aspartic acids represented 50–75% of the total basic compounds present and the proportion of glutamate to aspartate gradually increased with increasing developmental age of the embryos (Table 2). In a sample of the acidic

TABLE 2

CARBON ACCUMULATED IN BASIC COMPOUNDS OF THE ACID-SOLUBLE FRACTION OF MOUSE EMBRYOS CULTURED IN RADIOACTIVE SODIUM BICARBONATE AND CARBON DIOXIDE

Stage of Development at Commencement of Culture	Carbon Accumulated (pg-atom/embryo)		Ratio of Glutamate to Aspartate
	Glutamate	Aspartate	
Early and late 2-cell	0·27	0·16	1·7
8-cell	1·47	0·67	2·2
Morula and blastocyst	0·62	0·22	2·8

compounds from the acid-soluble fraction of the embryos, labelled carboxylic acids were eluted from a silicic acid column with hexane–butanol mixtures. The major peak corresponded to either citrate or malate but insufficient material was available to identify the compound or compounds further. The low levels of radioactivity accumulated in the neutral portion of the acid-soluble fraction of the embryos did not allow any further identification to be undertaken.

The accumulation of fixed carbon in the acid-soluble, protein, and lipid fractions of the medium is shown in Table 1. Almost all of the label was found in the acid-soluble fraction and acidic compounds made up 60–80% of the label in this fraction at all stages. The amounts of labelled acidic and basic compounds in the acid-soluble fraction of medium in which two-cell embryos were cultured was significantly less ($P<0·01$) than that at later stages. No significant differences in the accumulation of label in the acid-soluble fraction, as well as the acidic and basic compounds of this fraction, was found between the eight-cell, morula, and blastocyst stages. The accumulation of labelled neutral compounds in the acid-soluble fraction of medium in which two-cell embryos and early blastocysts were cultured was significantly less than that at the eight-cell and morula stages. Summation of the carbon incorporated into the acid-soluble, protein, and lipid fractions gave a value for total incorporation into the medium which closely approximated ($96\pm2\%$) the estimate of total incorporation based on a 20-μl aliquot of neutralized medium.

The acidic compounds in the medium were separated on a silicic acid column as described in Section II. Quantitative recovery of the acidic compounds was achieved and the elution patterns of the carboxylic acids at various stages of embryo development are shown in Figure 1. During the culture of two-cell embryos, lactate was the major compound accumulated but with subsequent stages of development, other peaks of radioactivity, the major ones of which ran at positions corresponding to

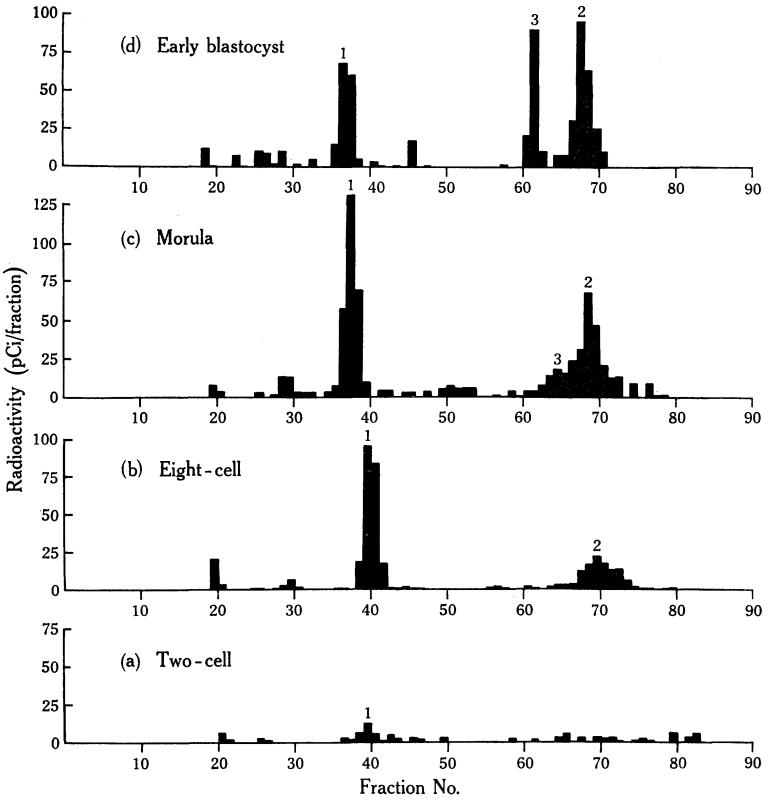

Fig. 1.—Elution patterns of radioactive carboxylic acids in acid-soluble fractions of medium in which mouse embryos were cultured in the presence of radioactive sodium bicarbonate and carbon dioxide. The equivalent of 25 μl of medium in which 50 embryos were cultured was applied to each column. Peaks 1, 2, and 3 are lactic, citric, and malic acids respectively. (*a*)–(*d*) Stages at commencement of culture.

malate and citrate, were also eluted. At the blastocyst stage nearly twice as much citrate as lactate or malate was detected in the acid-soluble fraction of the medium. Radioactive peaks with R_F values similar to lactate, citrate, and malate were also obtained by paper chromatography of the acid-soluble fraction of the media using butanol–acetic acid–water (4:1:5 v/v) as solvent.

During the course of these experiments, fixation of carbon dioxide by other stages of development of the mouse embryo during culture for 24 hr was also investigated. The results for late two-cell and four-cell embryos are given in Table 3.

These two-cell embryos were collected from mice 5 hr later than collections were usually made. At this time about 12% of the embryos were at the four-cell stage and these were cultured separately. The levels of carbon accumulated in the various fractions of these embryos were intermediate between those found in the normal two-cell and eight-cell embryos.

TABLE 3

FIXATION OF CARBON DIOXIDE BY LATE TWO-CELL AND FOUR-CELL MOUSE EMBRYOS CULTURED FOR 24 HR IN MEDIUM CONTAINING RADIOACTIVE SODIUM BICARBONATE AND CARBON DIOXIDE

Mean values ± standard errors of the means are given. Number of observations at each stage given in parenthesis

Stage of Development during Culture		% Eggs Developed	Carbon Incorporated (pg-atoms/embryo)					
				Acid-soluble Compounds:				
At Start	At Completion		Total	Acidic	Basic	Neutral	Protein Fraction	Lipid Fraction
Embryos								
Late 2-cell	4-cell (3)	96	1·22±0·05	0·52±0·05	0·58±0·02	0·12±0·03	0·16±0·05	0·01
Late 2-cell	8-cell (4)		1·55±0·14	0·66±0·08	0·72±0·11	0·18±0·04	0·24±0·08	0·01
4-cell	Morula (2)	91	3·16±0·36	1·35±0·19	1·28±0·09	0·52±0·08	0·45±0·19	0·02
Medium								
Late 2-cell	4- and 8-cell	96	0·49±0·13	0·26±0·04	0·14±0·04	0·09±0·05	0·02	0
4-cell	Morula	91	1·28±0·20	0·42±0·15	0·50±0·27	0·37±0·09	0·09±0·06	0·01

(*b*) *Enzyme Assays*

The activities of pyruvate carboxylase and malate dehydrogenase in the pre-implantation mouse embryo at various stages of development are given in Table 4. In both cases the total activity per assay was proportional to the numbers of embryos used and the level of activity shown is the amount of bicarbonate fixed in the presence of pyruvate in excess of the fixation in the absence of pyruvate.

TABLE 4

ACTIVITIES OF PYRUVATE CARBOXYLASE AND MALATE DEHYDROGENASE IN VARIOUS STAGES OF THE PRE-IMPLANTATION MOUSE EMBRYO

Mean values ± standard errors with number of replicates shown in parenthesis are given

Stage of Development	Time After HCG (hr)	Pyruvate Carboxylase Activity (pmole HCO_3^- fixed/hr/embryo)	Malate Dehydrogenase Activity (pmole HCO_3^- fixed/hr/embryo)
1-cell (unfertilized)	24	0·282±0·044 (3)	0·079±0·021 (4)
1-cell (fertilized)	24	0·254±0·021 (3)	0·120±0·025 (4)
2-cell	48	0·256±0·039 (6)	0·112±0·009 (4)
8-cell	72	0·180±0·011 (8)	0·245±0·027 (4)
Morula	96	0·133±0·020 (5)	0·247±0·020 (4)
Blastocyst (early)	96	0·141±0·011 (5)	0·349±0·035 (4)
Blastocyst (late)	120	0·089±0·017 (3)	0·297±0·066 (4)

The levels of pyruvate carboxylase showed a significant linear decrease ($P < 0·01$) with increasing age of the embryos. All other comparisons did not test as significant. Malate dehydrogenase, on the other hand, showed a significant linear

increase ($P<0\cdot01$) with increasing development of the embryos and there was a significant difference ($P<0\cdot05$) between the activities of this enzyme in morulae and early blastocysts.

To demonstrate pyruvate carboxylase activity in two-cell mouse embryos required the presence of pyruvate, acetyl-coenzyme A, and ATP, whereas malate dehydrogenase activity in eight-cell embryos depended upon the presence of pyruvate and a system for generating NADPH (Table 5). In the case of both enzymes, the requirement of magnesium ions was not tested because of the presence of this ion in culture medium fluid in which the embryos were transferred to the reaction tubes before freezing.

TABLE 5

EFFECT OF OMISSIONS OF VARIOUS COMPONENTS OF ASSAY MIXTURE ON ACTIVITY OF PYRUVATE CARBOXYLASE AND MALATE DEHYDROGENASE IN MOUSE EMBRYOS

Pyruvate Carboxylase†		Malate Dehydrogenase‡	
Conditions of Assay	Activity (pmole HCO_3^-/hr/embryo)	Conditions of Assay	Activity (pmole HCO_3^-/hr/embryo)
Complete reaction mixture	$0\cdot229\pm0\cdot040$	Complete reaction mixture	$0\cdot169\pm0\cdot020$
Pyruvate omitted	$0\cdot047\pm0\cdot018$*	Pyruvate omitted	$0\cdot023\pm0\cdot010$*
Acetyl-CoA omitted	$0\cdot032\pm0\cdot005$*	Glucose 6-phosphate omitted	$0\cdot032\pm0\cdot012$*
ATP omitted	$0\cdot030\pm0\cdot009$*	NADP omitted	$0\cdot067\pm0\cdot024$*

* Significantly less ($P<0\cdot05$) than complete mixture—Tukey's test (Snedecor 1957).

† Pyruvate carboxylase measured in two-cell embryos. Mean values of three replicates ± S.E.M. given.

‡ Malate dehydrogenase measured in eight-cell embryos. Mean values of four replicates ± S.E.M. given.

Phosphopyruvate carboxylase activity could not be detected in any stages of pre-implantation development. With the assay method used, activity could be detected in mitochondrial-free preparations of adult mouse liver prepared by the method of Schneider (1948) using 5 μg wet weight of tissue. This is one-quarter of the amount of tissue used for the assay of this enzyme in the pre-implantation mouse embryo. For this comparison the estimates of Loewenstein and Cohen (1964) and Lewis and Wright (1935) were used as the basis for the wet weight of the embryonic tissue.

IV. DISCUSSION

The relative changes observed in the fixation of carbon dioxide by various stages of the pre-implantation mouse embryo in the present study are similar to those reported by Graves and Biggers (1970). The incorporation of bicarbonate carbon increases from the two-cell stage and reaches a peak during early blastocyst formation before declining slightly during later blastocyst development. The distribution of carbon in the various fractions of the embryos is somewhat different in the two

studies, especially in relation to the proportion of label entering the protein and lipid fractions. However, these variations may be due to the different culture and extraction procedures used. In the present study, the differences in incorporation of fixed carbon by embryos with different rates of development may reflect the range of developmental ages of embryos at the time of collection as there is a 3–4 hr range in the time of ovulation after the injection of HCG (Edwards and Gates 1959).

The distribution of label between the various fractions of the embryos indicates the wide range of utilization of carbon assimilated during carbon dioxide fixation. The labelling of aspartate and glutamate in the basic protion of the acid-soluble fraction of the embryos is similar to that found in many bacteria which assimilate carbon by carbon dioxide fixation and the production of these compounds probably occurs by transamination of the tricarboxylic acid (TCA) cycle intermediates, oxaloacetate and α-ketoglutarate (see review by Lachica 1968). The increasing proportion of label found in glutamate relative to aspartate with increasing age of development of the embryos probably indicates a greater turnover of label in the TCA cycle. In mammalian mitochondria, the TCA cycle is regulated in part by the levels of adenosine triphosphate (Atkinson 1968) and the steady decline in the level of this compound which occurs during pre-implantational development of the mouse embryo (Quinn and Wales 1971) would also suggest greater activity of the TCA cycle over this time. The labelled amino acids which are formed during carbon dioxide fixation can then be incorporated into proteins, accounting, in part at least, for the accumulation of label in the protein fraction of the embryos. Since, in these experiments, nucleic acids were not separated from proteins, another route for the accumulation of label in this fraction would be by incorporation into nucleic acids, which has been shown to occur by Graves and Biggers (1970) and Murdoch and Wales (1971).

The extracellular accumulation of citrate, lactate, and malate during carbon dioxide fixation by the mouse embryo is similar to that reported in ram spermatozoa (O'Shea and Wales 1970) and these compounds probably accumulate extracellularly due to a low steady-state turnover within the cell. The increasing amount of citrate found extracellularly is probably further evidence for a greater turnover of the TCA cycle as development of the embryos proceeds.

In the present study there was a discrepancy between estimates of total incorporation of fixed carbon dioxide in the embryos based on the total radioactivity in a small number of embryos and the summation of label found in the various fractions of the extracted embryos. In the estimate of total incorporation based on a few embryos, the embryos were not removed from the broken tip of the centrifuge tube before acidification. This procedure possibly results in a less efficient removal of unfixed carbon dioxide than occurs during fractionation and therefore an elevated and more variable response is obtained. Thus, to obtain an accurate estimate of total carbon dioxide fixation using a small sample of embryos, it would appear necessary to remove the embryos from the end of the centrifuge tube prior to acidification and radioassay.

There are many similarities between carbon dioxide fixation and pyruvate metabolism in the pre-implantation mouse embryo. The total gain in carbon by carbon dioxide fixation over the various stages studied is comparable to that from C1 of pyruvate and aspartate is one of the major basic compounds labelled in embryos

grown in medium containing labelled pyruvate (Wales and Whittingham 1970). In view of these similarities, the most likely routes of carbon dioxide fixation in the mouse embryo are either pyruvate carboxylase or malate dehydrogenase, both of which were detected in the present study. Although the sum of the activities is relatively constant throughout the period of pre-implantation development, the levels of activity of these enzymes show quite different patterns, and malate dehydrogenase follows more closely the quantitative changes which occur in the *in vitro* fixation of carbon dioxide by the mouse embryo.

After the eight-cell stage of development, the total activity of both pyruvate carboxylase and malate dehydrogenase is just sufficient to account for the amount of carbon dioxide fixed by the embryos *in vitro*, suggesting that these activities may be rate-limiting for this reaction after the eight-cell stage and could thus determine the rate of entry of C1 of pyruvate into the TCA cycle. However, the greater incorporation of C2 than C1 of pyruvate by the mouse embryo during pre-implantation development (Wales and Whittingham 1970) shows that the oxidation of pyruvate to citrate is a more important site of entry of pyruvate into the TCA cycle than its carboxylation to oxaloacetate or malate. The amount of carbon dioxide fixed by two-cell embryos cultured to the eight-cell stage is considerably less than the theoretical amount of carbon dioxide which could be fixed if both pyruvate carboxylase and malate dehydrogenase are fully active. It may be possible, therefore, that in the intact embryo the activities of these two enzymes before the eight-cell stage are inhibited by factors which become less important after the eight-cell stage. One of the important regulatory factors of pyruvate carboxylase activity is the ATP/ADP ratio in the tissue (Brech, Shrago, and Wilken 1970). However, it is unlikely that a low ATP/ADP ratio is the inhibitory factor before the eight-cell stage as the levels of ATP have been found to be higher in the two-cell mouse embryo than later in pre-implantation development (Quinn and Wales 1971). The requirement of acetyl-coenzyme A for the activity of pyruvate carboxylase is similar to the situation found in other mammalian and in avian sources of this enzyme (Keech and Utter 1963) and levels of acetyl-coenzyme A in the developing embryo could play a part in its regulation.

Gluconeogenesis from pyruvate would require the formation of phosphoenolpyruvate. The formation of phosphoenolpyruvate by reversal of the pyruvate kinase reaction is unlikely because of the unfavourable kinetics of this reaction and therefore the dicarboxylic acid shuttle involving phosphopyruvate carboxylase would be required (Wood and Utter 1965). In the case of liver, the appearance of this enzyme has been implicated as the initiator of gluconeogenesis (Ballard and Hanson 1967). The absence of any detectable activity of phosphopyruvate carboxylase in the embryos would indicate that gluconeogenesis from pyruvate, as suggested by Biggers, Whittingham, and Donahue (1967), does not occur to any great extent in the pre-implantation mouse embryo. However Brinster (1969) has shown that glycogen formation from pyruvate between the one- and eight-cell stages of development of the mouse embryo amounts to approximately one-fifth of that from glucose over the same period. This low incorporation of pyruvate into glycogen could be due to levels of phosphopyruvate carboxylase unable to be detected in the assay system used here, reversal of the pyruvate kinase reaction, or to the formation of phosphoenolpyruvate from dicarboxylic acids by a reaction not involving phosphopyruvate carboxylase

(Bartley and Dean 1969). Therefore, the substantial gain in glycogen which occurs between the one- and eight-cell mouse embryo (Stern and Biggers 1968) is probably derived either from glucose and other carbohydrates present in the uterine secretions or from endogenous sources within the embryo itself. Pyruvate, on the other hand, appears to be used mainly to provide intermediates for the TCA cycle, as is evidenced by the greater oxidation of pyruvate by the mouse embryo than its incorporation when compared to glucose (Brinster 1969).

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