FIXATION OF CARBON DIOXIDE BY PREIMPLANTATION RABBIT EMBRYOS *IN VITRO*

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Summary. During the first 3 days of development of the rabbit embryo, carbon fixed from CO₂ accumulated in the embryos and culture medium and increased as development proceeded.

Ultracellularly, incorporation of carbon into acid-soluble material accounted for 60 to 85% of the total fixed carbon, with most of the the remaining carbon entering the protein fraction. In the medium, most of the fixed carbon was found in the acid-soluble fraction. Although a similar proportion of embryos developed in either the presence or absence of exogenous energy substrates, the amount of fixed carbon accumulating in both the embryos and culture medium was greater when energy substrates were included in the medium. There was little difference between the amounts of carbon incorporated into embryos cultured in medium containing either glucose or pyruvate plus lactate. In these media, however, the accumulation of fixed carbon in the glucose medium was lower than that in the medium containing pyruvate and lactate. This difference was less marked at the later stages of embryonic development.

Aspartic and glutamic acids were the major compounds containing fixed carbon in the basic portion of the acid-soluble fraction of the embryos. Fixed carbon accumulating in the medium was found in lactate, malate, citrate, pyruvate and, to a lesser extent, acetate.

INTRODUCTION

The condensation of CO₂ with pyruvate and its entry into the tricarboxylic acid (TCA) cycle as malate or oxaloacetate is an important process in the energy metabolism of the preimplantation mouse embryo (Quinn & Wales, 1971). Although there has been no actual measurement of this process in the preimplantation rabbit embryo, Fridhandler (1968) concluded that, since there was no appreciable accumulation of carbon-1 of pyruvate in glutamate and aspartate in Day-6 rabbit blastocysts, the conversion of pyruvate to a 4-carbon intermediate could not be a major pathway for its utilization.

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The pattern of energy metabolism in mouse and rabbit embryos appears to be similar. The oxidation of pyruvate and lactate is greater than that of glucose in both species (Brinster, 1969). Two-cell mouse embryos develop to the blastocyst stage in medium containing pyruvate or lactate and the addition of either of these substances to medium containing low levels of amino-nitrogen increases the proportion of two-cell rabbit embryos developing to the morula stage (Brinster, 1965a, 1970). In addition, the embryos of both species develop in bicarbonate-buffered medium (Brinster, 1963, 1970). From these similarities, one might expect to find that the fixation of CO_2 occurs in the rabbit as well as the mouse embryo.

The incorporation of fixed carbon from ^{14}C -labelled CO_2 has been measured in cultured rabbit embryos during the first 3 days of preimplantation development. To assess the contributions of energy pools to metabolic turnover, the embryos were cultured in substrate-free medium or medium which contained either glucose or pyruvate plus lactate. Because of the technical difficulties encountered in culturing large embryos in the system used to measure the fixation of CO_2 , no investigations beyond the commencement of blastocoele formation in the rabbit embryo were attempted. The rôle of CO_2 fixation might not be expected to change greatly at these later stages of development as studies of glucose metabolism in preimplantation rabbit embryos have indicated that during blastocyst expansion there is essentially only a quantitative change in metabolism in the embryo (Quinn & Wales, 1973b).

MATERIALS AND METHODS

Collection and culture of embryos

For each replicate, 60 to 100 embryos were obtained from female rabbits of an albino laboratory strain which had been induced to superovulate as previously described (Quinn & Wales, 1973b). Embryos collected on the day after induction of ovulation and insemination (Day 1) were at the one- and two-cell stage. Eight- to sixteen-cell stages and morulae were recovered from donors on Days 2 and 3, respectively.

The basic medium used was a modified Krebs-Ringer bicarbonate solution containing 1 mg bovine serum albumin/ml, 60 μg penicillin/ml and 50 μg streptomycin/ml. When energy substrates were added to this medium, isotonicity was maintained by adjusting the NaCl content. Medium containing 25 mM-DL-sodium lactate and 0.25 mM-sodium pyruvate was used to flush the embryos from the reproductive tracts of the donor animals (Brinster, 1965b). The embryos were washed twice (2 ml/wash) in substrate-free medium and then cultured for 24 hr at 37° C in 20- μl drops of medium (twenty embryos/drop) containing ^{14}C -labelled NaHCO_3 with a gas phase of 5% $^{14}\text{CO}_2$ in air (Wales, Quinn & Murdoch, 1969). The specific activity of the sodium bicarbonate varied between 6.5 and 12.0 $\mu\text{Ci}/\mu\text{mol}$ in different experiments.

The embryos were cultured in substrate-free medium or in media which contained either 5.56 mM-glucose or 0.5 mM-sodium pyruvate plus 10 mM-DL-sodium lactate as substrate sources. The levels of pyruvate and lactate used are

similar to those which have been found to be optimal for the growth and metabolism of the preimplantation rabbit embryo (Brinster, 1969, 1970; P. Quinn & R. G. Wales, unpublished observations).

Recovery of embryos and fractionation of samples

After culture for 24 hr, embryos which had developed were removed from the drops of culture medium and collected by centrifugation (Wales & Biggers, 1968). Embryos up to and including the morula stage were centrifuged through isotonic sucrose whereas non-radioactive medium was used during the collection of early blastocysts (Wales & Whittingham, 1970). The total amount of CO₂ fixed in developing embryos was estimated, using a sample of three to five embryos. The remaining embryos were saved for extraction of metabolic products. The embryos were removed from the broken tip of the centrifuge tube, acidified with 0.1 ml of 2 N-H₂SO₄ and left overnight in a sealed beaker which contained NaOH to absorb any CO₂ liberated by the acidification. The samples were then neutralized with 0.1 ml of 2 N-NaOH and stored at -20° C until extracted.

Aliquots of the media in which the embryos had been cultured were acidified and treated in a similar fashion to the embryos to remove any CO₂ which had not reacted. After neutralization, the amount of radioactivity in a 20- to 25-μl sample was assayed to assess the total incorporation of fixed carbon. The remainder of this medium was saved for fractionation. In an initial trial, one- and two-cell embryos which had been killed by incubation for 30 min in substrate-free medium containing 10% formalin had no significant uptake of label or fixation of label into the medium after incubation at 37° C for 24 hr in radioactive bicarbonate. Therefore, control samples of medium in which no embryos had been cultured were used to correct for residual background radioactivity in the samples of culture medium.

Before fractionation of embryos and media, a 50-μl aliquot of sheep serum was added as carrier. The samples were then fractionated into acid-soluble, protein and lipid fractions by the method described by Wales & Whittingham (1970). Cold 2.5% (v/v) perchloric acid was used to extract the acid-soluble fraction and lipids were removed from the acid-insoluble precipitate ('protein' fraction) with chloroform:ether (1:1, v/v). The acid-soluble fraction of all samples was further divided into acidic, basic and neutral portions by passage through columns of ion-exchange resins (see Wales & Whittingham, 1970). To obtain sufficient material for further identification of labelled compounds, replicate samples of the acid-soluble fraction of medium from each treatment were pooled and the carboxylic acids separated by liquid-liquid partition chromatography (O'Shea & Wales, 1968). Replicate samples of the acid-soluble fraction of embryos from similar treatments were also pooled and the basic compounds, recovered by ion-exchange chromatography, were differentiated by paper chromatography using water-saturated phenol as the developing solvent (see Quinn & Wales, 1971). Identification of the acidic compounds in the acid-soluble fractions of the embryos was not attempted.

Radioactivity in all samples was assayed by liquid scintillation techniques using 5 ml Triton X100-toluene (1:2 v/v) containing 0.4% (w/v) 2,5-diphenyl-

oxazole and 0.01% (w/v) 1,4 bis(4-methyl-5-phenyloxazol-2-yl)benzene for each 0.4 ml of aqueous sample.

Statistical analysis

All data relating to the accumulation of fixed carbon in the embryos and media were transformed to logarithms before analysis and the significance of the results was assessed by analysis of variance.

RESULTS

The development of rabbit embryos during culture for 24 hr in the presence of radioactive bicarbonate is given in Table 1. Embryos collected from donors on Day 1 developed from the one- and two-cell stage to the four- and eight-cell stage. Day-2 embryos developed from the eight- to sixteen-cell stage into

Table 1. Fixation of carbon dioxide by rabbit embryos cultured for 24 hr in media containing various energy substrates

Period of development	Energy substrates in medium	% embryos developed	Carbon incorporated from CO ₂ (pg atoms/embryo)			
			Total incorporation		Acid-soluble fraction	Protein fraction
			Estimate A*	Estimate B†		
1- and 2-cell to 4- and 8-cell	None	82 ± 6	0.48 ± 0.05	0.85 ± 0.04	0.53 ± 0.08	0.31 ± 0.11
	Glucose	82 ± 10	0.99 ± 0.28	1.21 ± 0.22	0.86 ± 0.14	0.35 ± 0.09
	Pyruvate + lactate	85 ± 3	1.20 ± 0.18	1.46 ± 0.20	1.03 ± 0.13	0.43 ± 0.07
8- and 16-cell to morula	None	94 ± 1	0.53 ± 0.18	0.84 ± 0.26	0.70 ± 0.26	0.14 ± 0.01
	Glucose	95 ± 1	1.91 ± 0.30	2.07 ± 0.51	1.76 ± 0.48	0.31 ± 0.06
	Pyruvate + lactate	96 ± 1	1.55 ± 0.23	1.87 ± 0.38	1.53 ± 0.37	0.32 ± 0.03
Morula to early blastocyst	None	87 ± 5	3.86 ± 1.25	4.02 ± 0.68	3.04 ± 0.42	0.97 ± 0.26
	Glucose	81 ± 4	8.24 ± 1.52	7.64 ± 1.61	5.71 ± 1.38	1.92 ± 0.24
	Pyruvate + lactate	78 ± 6	6.35 ± 1.05	5.66 ± 0.74	4.33 ± 0.79	1.32 ± 0.25

Mean values of three replicates ± S.E.M. at each stage of development.

* Estimate of total incorporation from five unfractionated embryos.

† Estimate of total incorporation from sum of fractionated carbon in extracted embryos.

morulae and morulae collected on Day 3 developed to the early blastocyst stage. The supply of exogenous energy substrates had no effect on the numbers of embryos developing and 80 to 95% of all embryos developed during culture. The incorporation of carbon from ¹⁴C-labelled NaHCO₃ and ¹⁴CO₂ into the embryos is also given in Table 1. The estimate of total carbon incorporated into the embryos from a sample of unfractionated embryos (Estimate A of Table 1) was in good agreement (86 ± 5%) with the estimate based on the sum of fractionated carbon in the extracted embryos (Estimate B). The embryos cultured in media containing exogenous energy substrates incorporated significantly ($P < 0.01$) more fixed carbon than those cultured in substrate-free medium. A significant ($P < 0.01$) linear increase in the fixation into the embryos occurred as development progressed, but the rate of this increase depended on the provision of an adequate source of energy. Thus, the total incorporation of fixed carbon into embryos cultured in substrate-free medium

did not differ appreciably between Day 1 and Day 2 of development, whereas a substantial increase in incorporation occurred over this period in embryos cultured in the presence of energy substrates. Incorporation of fixed carbon into Day-3 embryos cultured in all three media was three to five times greater than that into cultured Day-2 embryos.

Between 60 and 85% of the total carbon accumulating in the embryos was present in the acid-soluble fraction. The protein fraction was the other major component of the embryos in which fixed carbon accumulated. Embryos cultured in substrate-free medium incorporated less fixed carbon into both of these fractions than those cultured in medium containing energy substrates. In general, the amount of carbon incorporated into the protein fraction of the embryos was slightly lower in Day-2 than Day-1 embryos but increased four- to sevenfold between Days 2 and 3. Incorporation into lipids was small, accounting for 1% or less of the total carbon fixed into the embryos and there were no significant changes over the stages of development studied.

Table 2. Fixation of carbon dioxide into the acidic, basic and neutral compounds of the acid-soluble fraction of rabbit embryos cultured for 24 hr in various media

Period of development	Energy substrates in medium	Carbon incorporated from CO ₂ (μg atoms/embryo)		
		Acidic compounds	Basic compounds	Neutral compounds
1- and 2-cell to 4- and 8-cell	None	0.17 ± 0.04 (32)	0.31 ± 0.11 (59)	0.05 ± 0.02 (9)
	Glucose	0.21 ± 0.02 (24)	0.58 ± 0.13 (67)	0.07 ± 0.02 (9)
	Pyruvate + lactate	0.32 ± 0.07 (31)	0.56 ± 0.11 (54)	0.15 ± 0.05 (14)
8- and 16-cell to morula	None	0.25 ± 0.13 (36)	0.29 ± 0.08 (42)	0.15 ± 0.07 (22)
	Glucose	0.62 ± 0.14 (35)	0.96 ± 0.41 (55)	0.18 ± 0.03 (10)
	Pyruvate + lactate	0.78 ± 0.14 (51)	0.63 ± 0.21 (41)	0.13 ± 0.06 (8)
Morula to early blastocyst	None	1.05 ± 0.13 (35)	1.65 ± 0.38 (54)	0.33 ± 0.13 (11)
	Glucose	1.76 ± 0.51 (31)	3.33 ± 1.13 (58)	0.62 ± 0.20 (11)
	Pyruvate + lactate	1.67 ± 0.27 (39)	2.44 ± 1.04 (56)	0.22 ± 0.11 (5)

Mean values ± S.E.M. for three replicates at each stage of development are given with the percentage of the acid-soluble fraction shown in parentheses.

The distribution of fixed carbon in the acid-soluble fraction of the embryos is given in Table 2. Between 40 to 65% and 25 to 50% of the acid-soluble carbon was present in basic and acidic compounds, respectively, and similar trends to those occurring for incorporation into the acid-soluble fraction were evident with these compounds. The neutral portion of the acid-soluble fraction accounted for only 5 to 20% of the acid-soluble carbon fixed during the incubation. As with the basic and acidic portions, the amount of acid-soluble carbon accumulating in the neutral compounds increased as the age of the embryos increased.

A tentative identification of the labelled amino acids present in the basic portion of the acid-soluble fraction of the embryos is given in Table 3. Overall, quantitative recovery (100 ± 11%) of radioactivity applied to the chromatograms was achieved and peaks with *R_F* values corresponding to aspartic and glutamic acids accounted for the majority of the radioactivity in the fraction. Alanine

Table 3. Incorporation of fixed carbon into the amino acids of the acid-soluble fraction of rabbit embryos cultured for 24 hr in media containing various energy substrates and radioactive bicarbonate and carbon dioxide

Period of development	Energy substrates in medium	Carbon incorporated from CO ₂ (pg atoms/embryo)		
		Aspartate	Glutamate	Alanine
1- and 2-cell to 4- and 8-cell	None	0.13 (41)	0.02 (5)	< 0.01 (2)
	Glucose	0.54 (94)	0.01 (2)	< 0.01 (1)
	Pyruvate + lactate	0.30 (54)	0.09 (17)	0.02 (3)
8- and 16-cell to morula	None	0.18 (60)	0.07 (24)	0
	Glucose	0.70 (72)	0.25 (26)	0.02 (2)
	Pyruvate + lactate	0.38 (60)	0.15 (24)	0.01 (2)
Morula to early blastocyst	None	1.00 (60)	0.45 (27)	0.12 (7)
	Glucose	2.31 (69)	1.00 (30)	0.02 (1)
	Pyruvate + lactate	1.35 (56)	0.62 (26)	0.24 (10)

Values for three pooled replicates within each treatment are given. The percentage of the total basic acid-soluble compounds that each compound represents is shown in parentheses.

represented a small proportion of the carbon fixed into the basic compounds of the embryos.

The accumulation of fixed carbon in the culture medium is given in Table 4. The estimates of total incorporation based on aliquots of unfractionated media (Estimate A of Table 4) were in good agreement ($100 \pm 2\%$) with the estimates based on the sum of fractionated carbon in the extracted samples (Estimate B). Nearly all of the fixed carbon in the culture medium was present in the acid-soluble fraction. There was a significant ($P < 0.01$) linear increase

Table 4. Accumulation of fixed carbon in the medium during culture of rabbit embryos for 24 hr in the presence of various energy substrates and radioactive bicarbonate and carbon dioxide

Period of development	Energy substrates in medium	Carbon incorporated from CO ₂ (pg atoms/embryo)		
		Total incorporation		Acid-soluble fraction
		Estimate A*	Estimate B†	
1- and 2-cell to 4- and 8-cell	None	0.29 ± 0.11	0.34 ± 0.16	0.34 ± 0.16
	Glucose	0.48 ± 0.13	0.51 ± 0.17	0.51 ± 0.17
	Pyruvate + lactate	2.17 ± 0.73	2.21 ± 0.79	2.21 ± 0.78
8- and 16-cell to morula	None	0.44 ± 0.09	0.48 ± 0.09	0.46 ± 0.08
	Glucose	0.98 ± 0.15	0.94 ± 0.15	0.93 ± 0.16
	Pyruvate + lactate	2.22 ± 0.66	2.04 ± 0.52	2.00 ± 0.53
Morula to early blastocyst	None	3.51 ± 0.73	3.36 ± 0.72	3.21 ± 0.66
	Glucose	9.95 ± 4.22	9.56 ± 2.87	9.40 ± 2.86
	Pyruvate + lactate	12.44 ± 2.77	12.17 ± 2.52	12.07 ± 2.52

Mean values of three replicates ± S.E.M. are given.

* Estimate of total incorporation from an aliquot of unfractionated medium.

† Estimate of total incorporation from sum of fractionated carbon in extracted medium.

Table 5. Carbon fixed into the acidic, basic and neutral compounds of the acid-soluble fraction of media in which rabbit embryos were cultured for 24 hr in the presence of various energy substrates and radioactive bicarbonate and carbon dioxide

Period of development	Energy substrates in medium	Carbon incorporated from CO ₂ (pg atoms/embryo)		
		Acidic compounds	Basic compounds	Neutral compounds
1- and 2-cell to 4- and 8-cell	None	0.18 ± 0.07 (53)	0.13 ± 0.08 (39)	0.03 ± 0.02 (8)
	Glucose	0.15 ± 0.07 (30)	0.33 ± 0.07 (64)	0.03 ± 0.03 (6)
	Pyruvate + lactate	1.50 ± 0.20 (68)	0.71 ± 0.17 (32)	0
8- and 16-cell to morula	None	0.21 ± 0.03 (46)	0.19 ± 0.07 (41)	0.06 ± 0.03 (13)
	Glucose	0.59 ± 0.12 (63)	0.34 ± 0.07 (37)	0.01 ± 0.01 (1)
	Pyruvate + lactate	1.31 ± 0.36 (66)	0.54 ± 0.15 (27)	0.14 ± 0.07 (7)
Morula to early blastocyst	None	1.31 ± 0.26 (41)	1.20 ± 0.47 (37)	0.69 ± 0.08 (22)
	Glucose	7.05 ± 1.88 (75)	1.32 ± 0.30 (14)	1.03 ± 0.72 (11)
	Pyruvate + lactate	9.17 ± 2.22 (76)	2.09 ± 0.20 (17)	0.81 ± 0.22 (7)

Mean values ± S.E.M. of three replicates at each stage of development with percentage of acid-soluble fraction are shown in parentheses.

in the amount of carbon accumulating as the age of the embryos increased. Significantly ($P < 0.01$) more fixed carbon accumulated in the media containing energy compounds than in substrate-free medium. Accumulation in medium containing pyruvate and lactate was significantly ($P < 0.01$) greater than that in the medium containing glucose. The relative difference in the incorporation into these media became less as embryonic development progressed.

Estimates of the accumulation of carbon from CO₂ in the acid-insoluble protein fraction of the media were also attempted. Day-1 embryos did not accumulate carbon from CO₂ in this fraction. In some of the media in which Day-2 embryos were cultured, counts of up to 20% above background were measured in this fraction. The variation between samples was such that, overall, the counts were not significantly greater than background. When Day-3

Table 6. Carbon accumulated in carboxylic acids of media in which rabbit embryos were cultured for 24 hr in presence of various energy substrates and radioactive bicarbonate and carbon dioxide

Period of development	Energy substrates in medium	Carbon incorporated from CO ₂ (pg atoms/embryo)				
		Acetate	Pyruvate	Lactate	Malate	Citrate
1- and 2-cell to 4- and 8-cell	None	0.0	0.0	0.0	0.0	0.0
	Glucose	0.02 (4)	0.03 (5)	0.03 (6)	0.12 (25)	0.0
	Pyruvate + lactate	0.03 (1)	0.11 (5)	0.61 (28)	0.05 (2)	0.14 (6)
8- and 16-cell to morula	None	0.03 (7)	0.07 (15)	0.03 (7)	*	*
	Glucose	0.06 (6)	0.07 (7)	0.11 (12)	0.12 (12)	0.08 (9)
	Pyruvate + lactate	0.10 (5)	0.15 (7)	0.70 (35)	0.16 (8)	0.07 (4)
Morula to early blastocyst	None	0.22 (7)	0.19 (6)	0.55 (17)	0.83 (26)	0.34 (10)
	Glucose	0.35 (4)	0.53 (6)	2.49 (27)	1.87 (20)	1.18 (13)
	Pyruvate + lactate	0.10 (1)	1.23 (10)	4.19 (35)	2.08 (17)	1.56 (13)

Values for three pooled replicates within each treatment are given. The percentage of the total acid-soluble fraction that each compound represents is shown in parentheses.

* Sample lost.

embryos were cultured, however, an average of 63% more counts than background accumulated in the protein fraction of the media. This gave values of 0.16 ± 0.07 , 0.16 ± 0.02 and 0.10 ± 0.01 pg atoms/embryo for the accumulation of fixed carbon in the protein fraction of substrate-free medium, glucose medium and pyruvate plus lactate medium, respectively. These values represent only 5% or less of the total carbon accumulating in the media during culture of Day-3 embryos. There was no accumulation of label in the lipid fraction of the media at any stage of development.

The distribution of carbon fixed in the acid-soluble fraction of media is given in Table 5. The amount of fixed carbon accumulating in the acidic, basic and neutral compounds increased with embryonic development. Most of this increase during culture of Day-3 embryos was due to incorporation of carbon into the acidic compounds. Large differences in the accumulation of carbon in the acidic and basic compounds were found between substrate-free medium and media containing energy substrates. Incorporation into the neutral compounds represented 20% or less of the total carbon accumulated and there were no overall significant differences between the different media in the fixation of CO_2 into these neutral compounds.

The acid-soluble fractions of pooled replicates of culture media were chromatographed on columns of silicic acid to separate and identify carboxylic acids. The results are given in Table 6. Although more than half of the acid-soluble carbon fixed into substrate-free medium following culture of one- and two-cell embryos was acidic in nature (Table 5), labelled carboxylic acids could not be detected in this medium. In other media analysed, the percentage of acid-soluble carbon accumulating in the carboxylic acids amounted to $104 \pm 12\%$ of the label designated as acidic after separation by ion-exchange resins. In most of these media, lactate was the major acid identified while pyruvate comprised 5 to 15% of the labelled acid-soluble material. At all stages of development, the accumulation of fixed carbon in both lactate and pyruvate was greater in medium containing lactate and pyruvate than in the other media. Labelled malate and citrate were also present in most media, the amounts increasing when Day-3 embryos were cultured. A small amount of radioactivity having a chromatographic mobility equivalent to acetate was also detected in the extracts of the media.

DISCUSSION

The results obtained in this study demonstrate that the fixation of carbon dioxide is an active metabolic process in the rabbit embryo during the first 3 days of preimplantation development. Fridhandler (1968) has suggested that the fixation reaction does not play a very important rôle in the metabolism of the Day-6 rabbit blastocyst but his use of a phosphate-buffered medium with air as the gas phase may have been unfavourable for the fixation of CO_2 . A non-carboxylic buffered medium with air as the gas phase favours the reversal of the reactions of CO_2 fixation resulting in the decarboxylation of intermediates such as oxaloacetate (see Paul, 1965). The concentration of bicarbonate found in rabbit oviduct secretions (David, Brackett, Garcia & Mastroianni,

1969) is similar to that used in modified Krebs–Ringer bicarbonate medium for the culture of mouse and rabbit embryos (Brinster, 1963, 1970). This would suggest that the fixation of CO₂ could operate as well *in vivo* as it does *in vitro* in both the mouse and rabbit embryo and, as a result, benefit the energy metabolism of these embryos by maintaining the levels of intermediates in the TCA cycle during development.

The two-cell rabbit embryo does not require exogenous energy substrates to develop to the morula stage *in vitro* (Brinster, 1970) whereas the mouse embryo cannot accomplish this until the eight-cell stage is reached (Brinster & Thomson, 1966; Wales & Whittingham, 1973). Thus, the rabbit embryo is capable either of relying more on endogenous reserves or of utilizing the amino-nitrogen in the medium more effectively than the mouse embryo. Either of these processes would lessen the dependence of the rabbit embryo on CO₂ fixation for the formation of TCA cycle intermediates. Although the rabbit embryo is approximately three times the size of the mouse embryo before the blastocyst stage (Brinster, 1968a), the amount of fixed carbon accumulating from CO₂ during culture in media containing pyruvate and lactate is approximately the same, at similar stages of development (Quinn & Wales, 1971). This observation also suggests that the rabbit embryo relies less on the fixation of CO₂ than does the mouse embryo.

The accumulation of fixed carbon from CO₂ into the amino acids, aspartate and glutamate, is similar to that which occurs in the mouse embryo (Quinn & Wales, 1971). The relatively constant rate of accumulation of fixed carbon in these amino acids during the first 2 days of development is reflected in the rate of accumulation into the protein fraction of the embryos. After the morula stage, accumulation in both the amino acids and the protein fraction of the embryos increases several-fold over that at the preceding stages of development. By the fractionation procedures used in the present study, the protein fraction would also include macromolecules such as RNA and DNA. Manes (1969) and Manes & Daniel (1969), using the incorporation of labelled nucleosides and amino acids, report that during development of the rabbit embryo, the synthesis of RNA and DNA remains relatively constant during the first 2 days and then begins to accelerate just before blastocyst formation. This is followed some 35 hr later by an acceleration of protein synthesis. This pattern of macromolecular synthesis is similar to that found in the present study.

Even though a similar proportion of rabbit embryos develop in either substrate-free medium or media containing exogenous energy substrates, the amount of fixed carbon accumulating in both the embryos and culture medium is greater when energy substrates are present. This may be due to an increased metabolic turnover in the embryo in the presence of exogenous energy substrates, as suggested by Brinster (1970). Another likely explanation for the increased amount of CO₂ fixed by the embryo in the presence of exogenous energy substrates is the trapping of radioactive compounds in unlabelled metabolic pools. Such a process could account for the high proportion of fixed carbon accumulated in medium containing pyruvate and lactate.

The accumulation of fixed carbon from CO₂ in acetate and pyruvate in the medium during culture of the rabbit but not the mouse embryo (Quinn &

Wales, 1971) may reflect the higher levels of lactate dehydrogenase (E.C.1.1.1.27) in the mouse as compared to the rabbit embryo (Brinster, 1968b). This would tend to divert more carbon into lactate than either pyruvate or acetate in the mouse embryo. The increasing amount of fixed carbon found in both malate and citrate as development of the rabbit embryo progresses is similar to the situation found in the mouse embryo (Quinn & Wales, 1971) and suggests that the activity of the TCA cycle increases as embryonic development proceeds. The ratio of ATP to ADP in the rabbit embryo decreases with increasing embryonic development (Brooks & Lutwak-Mann, 1971; P. Quinn and R. G. Wales, unpublished data). As suggested for the mouse embryo (Quinn & Wales, 1973a), this probably also indicates an increase in the activity of the TCA cycle in the rabbit embryo as development of the embryo progresses.

The accumulation of carbon from CO₂ in the protein fraction of the media in which Day-3 embryos were cultured may be due to the accumulation of labelled macromolecules in the blastocoele fluid of these embryos and subsequent diffusion of these compounds into the culture medium.

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