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Measurement of metabolites in single preimplantation embryos; a new means to study metabolic control in early embryos

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SUMMARY

Methods are described for preparing and analyzing single preimplantation mouse embryos for a variety of metabolites and cofactors (glucose-6-P, fructose-6-P, fructose-1,6-bisphosphate, ATP, AMP, P_i, citrate, isocitrate, α-ketoglutarate, and malate). Oil-well and enzymatic cycling techniques are combined to provide the sensitivity needed to measure the amounts present (10^{-12} to 10^{-15} moles). After experimental treatment, embryos are collected on glass slides and freeze-dried. They can then be stored indefinitely under vacuum at −25 °C without deterioration. With these procedures, the embryos were collected at successive stages of development and subjected to starvation and refeeding with glucose, pyruvate or both. The results confirm the existence of a block at early stages at the P-fructokinase step. This may be due to inhibition by the very high citrate levels present. The data suggest that glycolysis is turned on late in preimplantation development by the rise in fructose-6-P, a de-inhibitor of P-fructokinase. In the citrate cycle, no step between citrate and α-ketoglutarate is rate-limiting, but a step between α-ketoglutarate and malate appears to impede the flux at early embryonic stages.

The preimplantation mammalian embryo offers many unique research opportunities. Among these is the possibility of studying the progression of metabolic systems during the transition from the single, free-living, migratory germ cell to the multicellular blastocyst, ready for sessile existence and dependence on second-hand nutrition.

An adequate appraisal of metabolism and of the manner of its control requires not only knowledge of the enzymes concerned but also of the levels of the substrates, products and cofactors of these enzymes. It is particularly useful to

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be able to disturb the metabolism and observe consequent metabolite changes. Controlled disturbances in metabolism can easily be brought about in preimplantation embryos. However, to follow subsequent metabolite changes requires either prohibitive numbers of embryos or the capacity to measure metabolites in single or a few individual embryos.

At first sight the logistics appear formidable. The most sensitive general methods for metabolites are based on the final measurement of the fluorescence of NADH or NADPH. At least 10^{-10} mole are required for reasonable precision (1 ml volume). This is the amount of glucose-6-P in 10000 two-cell mouse embryos. Fortunately, sensitivity can be increased almost without limit by 'enzymic cycling'. This, together with the 'oil-well technique' for carrying out analytical steps in submicrolitre volumes, makes metabolite studies with single embryos completely feasible.

This, therefore, is a detailed description of procedures for measuring a variety of metabolites in single mouse embryos. It also provides data obtained with these procedures which confirm and extend an earlier study (Barbehenn, Wales & Lowry, 1974) of changes in glucose metabolism from 2-cell embryo to blastocyst. Included are some metabolite data for the 1-cell stage and new measurements of embryo volumes.

This project had its origins in the observations that preimplantation mouse embryos cannot use glucose as an energy source for development until they reach the 8-cell stage (Whitten, 1957; Brinster, 1965*a*; Brinster & Thomson, 1966). Instead they rely primarily on pyruvate for their energy needs. However, glucose oxidation, as measured by CO_2 production, does occur at a very low level initially, but increases markedly between the 8-cell and morula stages (Brinster, 1967). Since the enzymes necessary for the glycolytic scheme appear to be present from the time of ovulation (Chapman, Whitten & Ruddle, 1971; Quinn & Kozak, 1975; Biggers & Stern, 1973), we are interested in what other factors determine the functioning of this pathway *in vivo*.

In the previous study (Barbehenn *et al.* 1974), measurements of metabolite levels in refeeding experiments established that there is a block at early developmental stages between glucose-6-P and fructose-1,6-P_2. The presumption that this block is at the P-fructokinase (EC 2.7.1.11) step is now confirmed by determination of fructose-6-P levels during refeeding. It would also appear from the new data that changes in AMP and P_i do not account for the subsequent removal of this block. The earlier study indicated the presence of a relatively slow step between citrate and malate at the 2-cell stage. This is further explored by determining isocitrate and α-ketoglutarate levels during refeeding.

MATERIALS

The chemicals for the media were all reagent grade from Fisher and Mallinckrodt. Other chemicals were from the Sigma Chemical Co. and enzymes were from Sigma or Boehringer and Sons. All water used in tissue culture work was triply distilled – once from glass, twice from quartz.

The embryos were collected as previously described (Barbehenn *et al.* 1974) from 8 to 10 weeks old, random-bred superovulated Swiss mice. The collection medium was a modified Krebs–Ringer bicarbonate Biggers & Stern, 1973), containing 1 mM glucose, 5 mM D,L-lactate, 0·5 mM pyruvate, 1 mg/ml bovine serum albumin, 60 μg/ml penicillin and 50 μg/ml streptomycin. The albumin used in this medium and in the citrate assays was treated with charcoal by the method of Chen (1967) to remove fatty acids and citrate-

After flushing, embryos were transferred into 1 ml of collection medium under oil followed by a second transfer to another 1 ml to free them from blood and debris. (Fertilized and unfertilized one-cell ova were incubated in hyaluronidase solution (0·5 mg/ml) to remove cumulus cells (Brinster, 1965*b*) before transfer to collection medium under oil.)

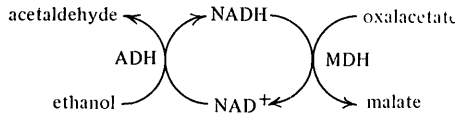
The design of starvation and refeeding experiments has been given (Barbehenn *et al.* 1974). Incubations were done in a constant-temperature bath, maintained at 37 °C, in which was placed a wide-mouth glass jar 8 cm in diameter. This was closed with a two-hole rubber stopper fitted with glass tubing for entrance and exit of the gas mixture. A raised screen platform held the petri dishes over water in the bottom of the jar. Also in the 37 °C bath, and connected to the jar, was a sparger, bubbled continuously with 5 % CO_2:95 % air that had passed through a Millipore filter. The temperature in the jar stayed within 1 °C of the bath temperature.

At appropriate times, embryos were transferred to glass slides in a minimum of fluid, frozen in liquid nitrogen, and freeze-dried at −35 °C as described (Barbehenn *et al.* 1974). The dry embryos were stored under vacuum at −25 °C. Experience with other tissues has shown that metabolites, cofactors and most enzymes are stable for months and even years under these conditions.

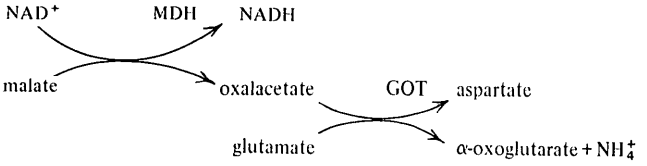
METHODS

All assays were carried out on single embryos except for fructose-6-P at the 2- and 8-cell stage, in which case, three embryos each were used. Enzymatic methods were employed which terminate in the oxidation or reduction of NAD or NADP. The necessary sensitivity was obtained by enzymic cycling.

Enzymic cycling. The principle is illustrated by the NAD cycle (Kato, Berger, Carter & Lowry, 1973) (ADH and MDH represent alcohol dehydrogenase (EC 1.1.1.1) and malic dehydrogenase (EC 1.1.1.37)).



The NADH is alternately oxidized and reduced. In each oxidation reduction cycle 1 mole each of malate and acetaldehyde is produced. Cycling rates up to 30000/h are easily achieved (depending on the concentrations of the two enzymes). After a sufficient number of cycles the enzymes are inactivated with heat (which also destroys the oxalacetate) and the malate is measured by the fluorescence of the NADH generated (from extra NAD^+) as follows (GOT is glutamic-oxalacetate transaminase, EC 2.6.1.1).



The reaction is pulled to completion by the glutamate and its dehydrogenase. Calculations are based on standards in the same rack, and therefore are independent of adventitious variations in enzyme activities, temperature or incubation time. Exact proportionality between readings and NAD concentration is usually achieved because the nucleotide concentrations are kept far below the Michaelis constants for the enzymes. Variability is easily kept below 2 %.

General procedure. Individual embryos were removed from the slides with a hair point, a piece of hair glued into the end of a piece of glass tubing (Lowry & Passonneau, 1972). They were added individually, by means of a quartz-tipped hair point, to a drop of dilute acid or alkali under oil in an 'oil well' in a Teflon block (Lowry & Passonneau, 1972; Matschinsky, Passonneau & Lowry, 1968). Most of the succeeding steps were performed in the wells.

In the *first step* (heating in acid or alkali), enzymes and preformed pyridine nucleotides, which might interfere, are destroyed. In the *second step* the specific reaction sequence takes place, culminating in the oxidation or reduction of a pyridine nucleotide. In the *third step* the excess pyridine nucleotide from the second step is destroyed with acid (if the excess is NADH or NADPH) or with base (if the excess is NAD^+ or $NADP^+$). This step is necessary because the subsequent cycling reaction (*fourth step*) does not distinguish oxidized from reduced forms of the nucleotides. After the cycling reaction is stopped (*fifth step*), one of the cycling products is measured by an enzymatic reaction (*sixth step*) which results in NADH or NADPH formation equivalent to the amount of cycling product. The reduced nucleotides are finally measured either directly by their fluorescence, or, when necessary, after further enhancement of fluorescence with strong alkali (Lowry & Passonneau, 1972; Lowry & Carter,

Table 1. *Flow sheet for analyses**

	Step 1	Step 2	Step 3	Step 4	Step 5	Step 6	Step 7	Step 8
Fructose-6-P	80 nl 20 mM-HCl	80 nl SpR	80 nl 0·15 N-NaOH	1 µl DCR	5 µl 0·15 N-NaOH	3.5 µl to 1 ml DIR	—	—
Malate	80 nl 50 mM-HCl	Same†	80 nl 0·5 N-NaOH	3·5 µl DCR	6 µl 0·1 N-NaOH	6 µl to 1 ml DIR	—	—
Citrate	80 nl 50 mM-NaOH	Same‡	80 nl 0·5 N-HCl	6 µl DCP	3·5 µl 0·2 N-NaOH	Same	—	—
ATP	50 nl 0·1 N-NaOH	300 nl SpR	5 µl 50 mM-NaOH	3·5–50 µl TCR	95°, 5 min	1 ml TIR	—	—
P_i	80 nl 20 mM-NaOH	80 nl SpR	80 nl 0·5 N-NaOH	6 µl TCR	3·5 µl 0·2 N-NaOH	8 µl to 1 ml TIR	—	—
α-oxo-glutarate	80 nl 50 mM-NaOH	Same	80 nl 0·5 N-HCl§	0·3 µl DCR	3·5 µl 0·1 N-NaOH	3·5 µl to DIR	—	—
Glucose-6-P	50 nl 10 mM-HCl	50 nl SpR	50 nl 0·15 N-NaOH	0·5 µl TCR	0·1 µl 0·3 N-NaOH	1 µl TIR	1–50 µl P_i, pH 12	1 ml 6 N-NaOH R**
Isocitrate	70 nl 50 mM-HCl	70 nl† SpR	70 nl 0·5 N-NaOH‖	1 µl TCR	0·5 µl 0·2 N-NaOHπ	5 µl TIR	6–50 µl P_i, pH 12	100 µl 9 N-NaOH R††
Fructose-1,6-P_2	50 nl 20 mM-HCl	50 nl SpR	50 nl 0·3 N-NaOH	2 µl DCR	0·1 µl 1 N-NaOH	1 µl DIR	2–50 µl P_i, pH 12	1 ml 6 N-NaOH R**

* At *Step* 1, samples were heated 20 min at 80 °C, except that for ATP this was increased to 30 min at 95 °C to destroy glucose-6-P. At *Step* 2, incubation times with the specific reagent (SpR) were 20–30 min, except that for fructose-6-P this was 10 min (see text). At *Step* 3, samples were heated 20 min at 80 °C. At *Step* 4, incubations were for 1 h. This was at 20–25 °C for samples with NAD cycling reagent (DCR) and 37 °C with NADP cycling reagent (TCR). At *Step* 5, cycling was stopped by adding alkali and heating 20 min at 80 °C. In the case of ATP, because samples were in fluorometer tubes instead of oil wells. cycling was stopped by simply heating 5 min at 95–100 °C. At *Step* 6, the indicator reactions were carried out either in the fluorometer tubes (the first 5 cases) or in the oil wells. Incubation times were 20–30 min at room temperature. (DIR and TIR represent indicator reagents for the NAD and NADP cycles). At *Step* 7, samples (in fluorometer tubes) were heated 10 min at 60 °C, with a solution of 0·25 M-K_2HPO_4:0 25 M-Na_3PO_4 ('P_i, pH 12'). This destroyed excess NAD^+ or $NADP^+$ from the indicator steps, and permitted enhancement of the NADH or NADPH fluorescence with strong alkali ('NaOH R') at *Step* 8 (10 min at 60 °C). The composition of the various specific reagents is given in the text.

† After Step 1 an equal volume of 0 1 M Tris base was added before the specific reagent.

‡ After Step 1 an equal volume of 0·1 M Tris-HCl (no base) was added before the specific reagent.

§ After this step, 80 nl of 1 M Tris base were added.

‖ After this step, 70 nl of 1 M Tris-acetate (no base) were added.

π After this step, 0·2 µl of 1 M Tris-HCl (no base) were added.

** 6 N-NaOH plus 0·03 % H_2O_2 and 10 mM imidazole, the last to prevent light sensitivity (12).

†† 9 N-NaOH plus 0·045 % H_2O_2; after heating 15 min at 60 °C, 1 ml of H_2O was added before reading.

1974). The final fluorescence readings were made in 1 ml volumes in 10 × 75 mm fluorometer tubes in a Farrand fluorometer (Model A). The flow sheet for each assay is given in Table 1. Additional information is supplied below.

Most of the assays have been described in principle before (Lowry & Passonneau, 1972), but as a rule some modification was needed to achieve enough sensitivity for single embryo analysis. The fructose-6-P method is believed to be new. The AMP method is described elsewhere (Barbehenn, Law, Brown & Lowry, 1976).

Standards. These were incorporated in the acid or alkali used at *Step 1* (Table 1) at three or more levels to cover the embryo range.

Cycling reagents. The basic composition has been given for the NADP (Lowry & Passonneau, 1972) and NAD (Kato *et al.* 1973) cycling reagents. The standard enzyme concentrations for NADP cycling were 200 μg/ml of glutamic dehydrogenase (EC 1.4.1.2) and 50 μg/ml of baker's yeast glucose-6-P dehydrogenase (EC 1.1.1.49) (about 30 % pure). The standard enzyme concentrations for NAD cycling were 300 μg/ml of yeast alcohol dehydrogenase and 30 μg/ml of beef heart malic dehydrogenase. Departures from these standard concentrations will be indicated.

Indicator reagents. The indicator reagent for measuring 6-P-gluconate after the NADP cycle has been described (Lowry & Passonneau, 1972). It would be possible to reduce the necessary 6-P-gluconate dehydrogenase (EC 1.1.1.44) by a factor of 3 or 4 if the buffer were changed from Tris-HCl, pH 8·1, to imidazole HCl, pH 7. The composition of the indicator reagent was modified in the case of glucose-6-P (see below).

The indicator reagent for measuring malate after the NAD cycle is the second of the three choices originally given (Kato *et al.* 1973). It consists of 50 mM 2-NH_2-2-methyl-1-propanol buffer, pH 9·9, with 200 μM NAD^+, 10 mM glutamate, 5 μg/ml pig-heart malic dehydrogenase and 2 μg/ml beef liver glutamic-oxalacetic transaminase. The composition was modified in the case of fructose-1,6-P_2 measurement (see below).

Fructose-6-P. The metabolite is converted to 2 mole of 3-P-glycerate with P-fructokinase (EC 2.7.1.11), aldolase (EC 4.1.2.7), triose-P isomerase (EC 5.3.1.1), and glyceraldehyde-3-P dehydrogenase (EC 1.2.1.12) with the formation of 2 mole of NADH. After destroying excess NAD^+ with alkali, the NADH is amplified by enzymic cycling.

The alternative method would have been to convert fructose-6-P to 6-P-gluconate with glucose phosphate isomerase (EC 5.3.1.9) and glucose-6-P dehydrogenase. In addition to giving only half as much final product, this would require prior removal of glucose-6-P, which is always present at much higher levels than fructose-6-P.

The only difficulty encountered was a small contamination of the aldolase with glucose phosphate isomerase. This was overcome by keeping aldolase to a minimum and limiting the reaction time to 10 min. This was shown to be

sufficient by a direct check in the fluorometer with comparable concentrations, but in a volume of 1 ml, i.e. 6000 times that used in the oil-well assay. As an additional precaution, glucose-6-P was tested directly in the reagent at concentrations known to exist in embryos to be sure it did not contribute a blank. Separate blanks with added glucose-6-P were included in the oil-well assays.

The method includes fructose-1,6-P_2 and triose phosphates in the measurement, but these metabolites are ordinarily present at levels much lower than those of fructose-6-P, and a valid correction can be made. In most instances, the correction necessary for fructose-1,6-P_2 plus triose phosphates was less than 10 %. Only in the case of starvation, when fructose-1,6-P_2 rises and fructose-6-P falls, was this correction a large factor (15–50 % of the total). Starvation values therefore are estimates only; more exact levels must await simultaneous analyses for these metabolites on the same embryo.

The overall blank was equivalent to 5 fmole of fructose-6-P. The assay was linear up to at least 40 fmole.

The *specific reagent* was 100 mM imidazole-HCl, pH 7·5, with 50 μM NAD^+, 2 mM-$Na_2HA_sO_4$, 2 mM EDTA, 4 mM β-mercaptoethanol, 4 mM-$MgCl_2$, 100 mM ATP, 0·04 % bovine plasma albumin, 1 μg/ml aldolase, 25 μg/ml glyceraldehyde-P dehydrogenase, 2 μg/ml triose-P isomerase, and 1 μg/ml P-fructokinase (all rabbit muscle enzymes).

Malate. The specific reagent was 150 mM 2-amino-2-methyl-1-propanol buffer, pH 9·9, 150 μM NAD^+, 3 mM glutamate, 0·075 % bovine plasma albumin, 40 μg/ml pig-heart malic dehydrogenase and 8 μg/ml beef liver glutamic-oxalacetic transaminase. The cycling reagent contained 25 mM additional Tris-HCl to compensate for the extra alkali present.

Citrate. The specific reagent was 200 mM Tris-HCl, pH 7·6, 4 mM ascorbic acid (fresh), 100 μM-$ZnCl_2$, 50 μM NADH, 9 μg/ml citrate lyase (EC 4.1.3.6) (from *Aerobacter aerogenes*), and 1 μg/ml, malic dehydrogenase. The cycling enzymes were reduced to one third standard concentrations and the oxalacetate level trebled.

ATP. The specific reagent consisted of 50 mM Tris-HCl, pH 7·5, 1 mM-$MgCl_2$, 0·5 mM dithiothreitol, 100 μM glucose, 15 μM $NADP^+$, 0·02 % bovine plasma albumin, 3 μg/ml yeast hexokinase (EC 2.7.1.1) and 1·5 μg/ml baker's yeast glucose-6-P dehydrogenase (30 % pure). The initial heating in alkali at 95 °C (Table 1) causes some destruction of ATP, but the standards, which are exposed to the same treatment, compensate for this.

Inorganic P. The specific reagent consisted of 100 mM imidazole-HCl buffer, pH 6·6, 60 μM $NADP^+$, 15 μM AMP, 0·5 μM glucose-1,6-P_2, 10 mM glycogen (as glucosyl residues), 1 mM magnesium acetate, 1 mM dithiothreitol, 2 mM EDTA, 0·05 % bovine plasma albumin, 6 μg/ml muscle P-glucomutase (EC 2.7.5.1), 5 μg/ml baker's yeast glucose-6-P dehydrogenase (30 % purity), and 90 μg/ml muscle phosphorylase *a* (EC 2.4.1.1).

α-Oxoglutarate. The specific reagent was composed of 200 mM imidazole-HCl

buffer, pH 6·9, 50 mM ammonium acetate, 200 μM ADP, 2 μM NADH, 4 mM ascorbic acid, 0·05 % bovine plasma albumin, and 20 μg/ml of glutamic dehydrogenase. The time of cycling was extended to 90 min.

Glucose-6-P. The specific reagent was 100 mM Tris-HCl, pH 8·1, with 0·08 % bovine plasma albumin, 30 μM $NADP^+$, 0·2 mM dithiothreitol and 0·3 μg/ml baker's yeast glucose-6-P dehydrogenase (about 30 % pure). The enzymes of the cycling reagent were doubled in concentration.

The overall blank was equivalent to about 1 fmole of glucose-6-P and the assay was linear up to 12 fmole. With larger samples, the volume of cycling reagent was increased and the strong alkali enhancement step omitted. The composition of the *indicator* reagent was 100 mM Tris-HCl, pH 7·2, 0·25 mM EDTA, 75 mM ammonium acetate, 12 mM-$MgCl_2$, 200 μM $NADP^+$, 0·03 % bovine plasma albumin and 5 μg/ml yeast 6-P-gluconate dehydrogenase (30 % purity).

Isocitrate. The specific reagent was 150 mM Tris-HCl, pH 8, with 300 μM-$MnCl_2$, 30 μM $NADP^+$, 0·08 % bovine plasma albumin, and 6 μg/ml pig heart, NADP-linked, isocitrate dehydrogenase (EC 1.1.1.42). All components of the *indicator* reagent were increased 50 % in strength.

Fructose-1,6-P_2 (plus triose phosphates). The specific reagent was the same as for fructose-6-P except that $MgCl_2$, ATP and P-fructokinase were omitted, aldolase was 20 μg/ml and glyceraldehyde-P dehydrogenase 100 μg/ml. The *indicator* reagent was modified by increasing the glutamate 10-fold and all other components about 5-fold. This was to compensate for the volume and the alkalinity of the reagents already present.

Vitellus volumes. Volumes of the vitellus were determined by making a wet mount of the embryo with the cover-slip supported on paraffin. The diameter was measured under high power with a calibrated eye-piece micrometer. Two measurements were taken at right angles, averaged, and volumes calculated assuming a spherical shape.

RESULTS

The embryo volumes used in calculations of metabolite levels are given in Table 2. The Lewis & Wright (1935) value of .60 picolitres was used for 2- and 8-cell embryos.

Because the distribution of metabolites between blastocoele cavity and tissue is unknown, we have given basal blastocyst values two ways: (*a*) assuming uniform distribution and (*b*) assuming distribution confined to the cells (Table 3). However, in the figures we have adopted the second assumption.

Basal metabolite levels

ATP, AMP and P_i. ATP fell 50 % overall in concentration during preimplantation development with the largest drop (30 %) between morula and blastocyst (Table 3). These values are in good agreement with those of Quinn & Wales

Table 2. *Volumes of mouse embryos*

(Values are given ± S.E.M. for the number of embryos in parentheses.)

Stage	Picolitres
Unfertilized	227 ± 4 (20)
Fertilized 1-cell	205 ± 2 (16)
Morula	173 ± 9 (10)
Blastocyst	
Total volume	354 ± 29 (10)
Cavity	174 ± 13 (10)
Cells*	180 ± 12 (10)

* By difference.

(1971). Other workers have obtained values considerably higher although the overall pattern is similar (Epstein & Daentl, 1971; Ginsberg & Hillman, 1973). AMP, in contrast, was low and showed little change. Only four embryos were analysed at the earliest stages, too few to be statistically significant, but they are included because they tend to show that levels at all stages are maintained within fairly narrow limits.

Inorganic P was measured in embryos collected in medium containing 10 μM instead of the usual 1 mM phosphate. This lower level has been shown to allow normal *in vitro* development (Wales, 1970), yet is low enough to eliminate significant interference from P_i in adherent collection medium. Although it had been expected that P_i concentration might parallel the increase in glycolytic activity with development, such was not the case. Concentrations were maintained between 5·6 and 6·6 mmole/kg at all preimplantation stages examined.

Glucose-6-P. Glucose-6-P had previously been shown to increase 4-fold between the 2-cell and blastocyst stage in embryos isolated in the usual collection medium, i.e. with 1 mM glucose (plus pyruvate and lactate) present (Barbehenn *et al.* 1974) (Table 3, 'glucose-6-P A'). However, when embryos were collected in medium with the glucose omitted, a much more striking increase was seen (glucose-6-P B). In this medium and over the same developmental span, glucose-6-P levels rose 14-fold, although they never reached more than 65 % of the level in cells isolated in 1 mM glucose. Blastocysts, which had developed *in vitro* from the 2-cell stage in pyruvate/lactate medium (without glucose), had glucose-6-P levels which were almost undetectable (footnote to Table 3).

Fructose-6-P and fructose-1,6-P_2. Fructose-6-P levels in freshly collected embryos increased 3-fold between the 2-cell and blastocyst stage, with the biggest increase occurring between the 8-cell and the morula. The changes are reasonably parallel to those shown by glucose-6-P, and indicate near equilibrium at the P-glucoisomerase step. This is in contrast to fructose bisphosphate, which remains low at all stages from 2-cell to blastocyst.

Table 3. *Metabolites and related substances in freshly isolated embryos**

	1-cell (UF)	1-cell (F)	2-cell	8-cell	Morula	Blastocyst Vol. A	Blastocyst Vol. B
ATP	4700 ± 150 (5)	4600 ± 200 -(4)	4400 ± 30 (5)	3700 ± 300 (6)	3600 ± 200 (5)	2500 ± 400 (4)	1300
AMP	87,170†	145,150†	83 ± 12 (4)	150 ± 15 (6)	115 ± 18 (4)	109 ± 12 (4)	51
P_i	—	—	5560 ± 500 (12)	5620 ± 430 (12)	6290 ± 700 (12)	5330 ± 880 (6)	2710
Glucose-6-P A	—	—	53 ± 4 (9)	81 ± 13 (11)	188 ± 13 (12)	196 ± 16 (9)	101
Glucose-6-P B	—	—	10 ± 1 (5)	14 ± 3 (5)	81 ± 19 (5)	126 ± 20‡ (3)	65
Fructose-6-P	—	—	28 ± 6§	14 ± 4§	48 ± 7 (8)	82 ± 7 (10)	42
Fructose-1,6-P_2	—	—	4·4 ± 0·6 (7)	2·0 ± 0·3 (8)	3·6 ± 0·6 (12)	5·1 ± 1·1 (9)	2·6
Citrate	2400 ± 220 (10)	2200 ± 300 (6)	1600 ± 150 (14)	1700 ± 130 (21)	1800 ± 180 (9)	5300 ± 1300 (10)	2700
Isocitrate	75 ± 4 (3)	55 ± 5 (14)	47 ± 3 (14)	54 ± 3 (12)	48 ± 5 (14)	212 ± 29 (7)	108
α-oxoglutarate	172 ± 31 (4)	178 ± 14 (22)	72 ± 11 (11)	120 ± 12 (13)	114 ± 11 (11)	188 ± 26 (10)	90
Malate	226 ± 22 (15)	366 ± 24 (12)	390 ± 31 (15)	390 ± 30 (12)	360 ± 40 (12)	410 ± 29 (11)	260

* Embryos were collected in the glucose/pyruvate/lactate medium described in *Materials*, except that glucose was omitted for 'glucose-6-P B', and P_i was reduced from 1 to 0·01 mM for embryos analysed for P_i (see text). The blastocyst data are calculated two ways: on the basis of cell volume ('Vol. A') and on the basis of total volume ('Vol. B'). UF and F refer to unfertilized and fertilized embryos. Values are μmole/kg wet weight ± S.E.M. for the numbers of embryos given in parentheses.

† Only these two values available.

‡ The level was only 5 μmole/kg in blastocysts grown from the 2-cell stage (72 hrs) in this glucose free medium.

§ Four analyses of 3 embryos each.

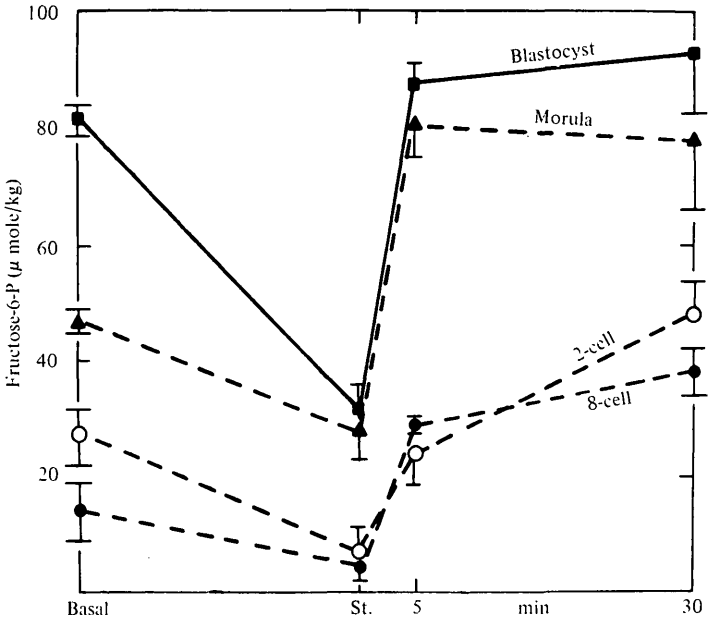


Fig. 1. Fructose-6-P levels before (Basal) and after 60 min starvation (St) followed by refeeding 5 and 30 min in medium containing 5·5 mM glucose. Basal and starvation levels are averages from several experiments with a total of 8–12 embryos per point. Other points are the averages of 5–9 embryos. Bars indicate ± S.E.M. Very similar results were obtained by refeeding with 5.5 mM glucose plus 0.5 mM pyruvate.

Citrate cycle intermediates. Citrate levels are strikingly high. For example, at the 1-cell stage the level is about 6 times that in adult rat brain (Goldberg, Passonneau & Lowry, 1966) and 14 times that in adult rat liver (Burch, Lowry, Bradley & Max, 1970). Isocitrate is also high (about 3 times that of rat brain (Goldberg *et al.* 1966). The ratio of citrate to isocitrate averaged 33:1 for all ages combined, with no significant difference among age groups. In contradistinction, α-oxoglutarate and malate are not remarkably high, being about the same as in rat brain (Goldberg *et al.* 1966).

Citrate, isocitrate and α-oxoglutarate levels all dropped between the unfertilized 1-cell stage and the 2-cell stage (33, 37 and 58 % respectively) while malate rose 42 %. Values for all these metabolites remained fairly constant until the blastocyst stage when, with the exception of malate, they rose and became quite variable. We postulate that this variability is due to the presence of a major fraction of these metabolites in the blastocele. Some evidence to this effect was obtained in the case of citrate by a few analyses of blastocysts that had been cut in two, one containing most of the blastocele. (For technique of free-hand dissection of objects of this size, see Lowry & Passonneau, 1972, chap. 11.) Before starvation, both halves contained similar high levels of citrate,

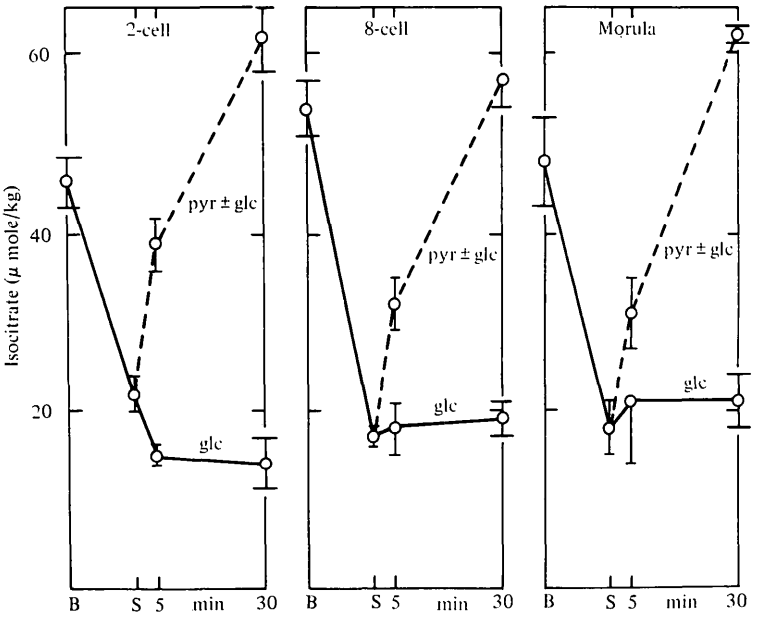


Fig. 2. Isocitrate before (B) and after 60 min starvation (S) followed by refeeding in medium containing either 5·5 mM glucose (glc) or 0·5 mM pyruvate plus 5·5 mM glucose (pyr + glc). In the case of the 2-cell and 8-cell stages some embryos received only pyruvate. This omission of glucose did not appear to change the results and the data were combined (pyr ± glc). Each point represents 6 embryos in the case of morulas refed with glucose. The other points represent about 12 embryos.

but after starvation for 30–60 min the blastocele-rich half retained the larger proportion of the citrate. (This may constitute a useful energy reserve, available during longer periods of starvation.)

Starvation and refeeding

Fructose-6-P. The patterns shown previously for glucose-6-P on starvation and realimentation with glucose (Barbehenn *et al.* 1974) were essentially duplicated by fructose-6-P except for the lower levels (Fig. 1). There was a drop during starvation, after which basal levels were regained or exceeded within 5 min after refeeding. These higher values were maintained throughout the remainder of the test period, as was also true for glucose-6-P. Similarly, as in the case of glucose-6-P, there was no difference whether refeeding was with glucose alone or glucose plus pyruvate (not shown). The data show that at no stage is there an obstacle to glycolysis at the glucose phosphate isomerase step.

Citrate cycle intermediates. Isocitrate fell during starvation and remained unchanged at the resultant low levels during the 30 min period of refeeding with glucose, regardless of the stage of development (Fig. 2). On the other

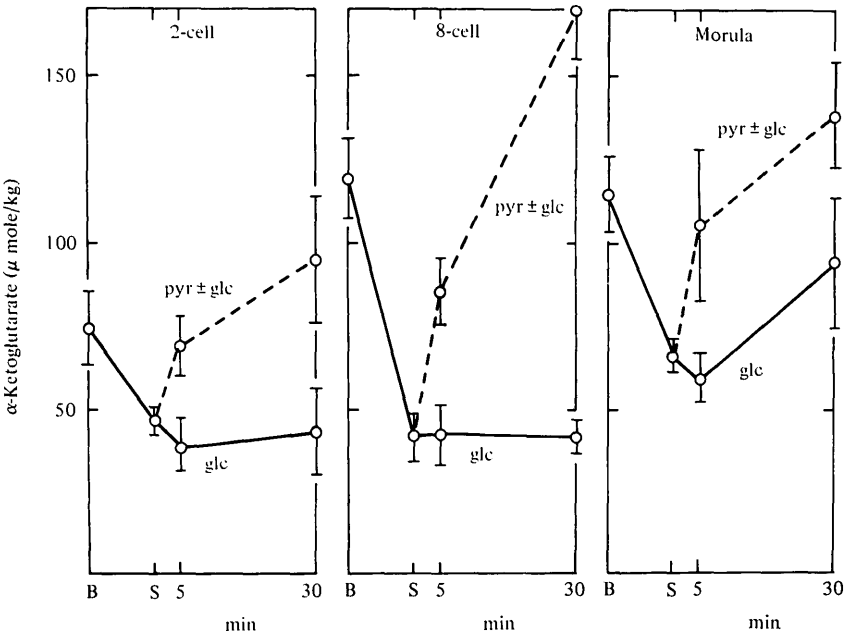


Fig. 3. α-Oxoglutarate before (B) and after 60 min starvation (S) followed by refeeding with either 5·5 mM glucose (glc) or 0·5 mM pyruvate plus 5·5 mM glucose (pyr + glc). Some of the embryos at the 2-cell and 8-cell stages received only pyruvate. The glucose omission did not appear to influence the results and the data were combined (pyr ± glc). In the case of refeeding with glucose at all stages and with pyruvate at the morula stage, each point is the average for 6 embryos; the other points are the average for about 12 embryos.

hand, when pyruvate was included, either alone or with glucose, isocitrate rose to at least 50 % of the basal level by 5 min and by 30 min had recovered or exceeded pre-starvation levels.

The changes in isocitrate are essentially parallel to those previously reported for citrate (Barbehenn *et al.* 1974) and indicate that whereas pyruvate can be converted to citrate and isocitrate rapidly enough at all stages to repair the starvation deficit in perhaps 10 min, glucose cannot begin to do this at any stage. The average ratio of citrate to isocitrate was 34:1 for 2-cell, 8-cell and morula stages when isolated directly from collection medium. During starvation the ratio fell to 24:1, but because of the exceedingly low isocitrate levels the change cannot be regarded as significant. During realimentation with glucose the ratio averaged 28:1. When realimentation was with pyruvate, or pyruvate plus glucose, the ratio averaged 31:1. These ratios are all higher than the equilibrium value obtained *in vitro* (about 16:1). Nevertheless, the absence of significant shift during the realimentation with pyruvate suggests that there is no material impedance at the aconitase step.

α-oxoglutarate. The patterns of response of α-oxoglutarate to starvation and

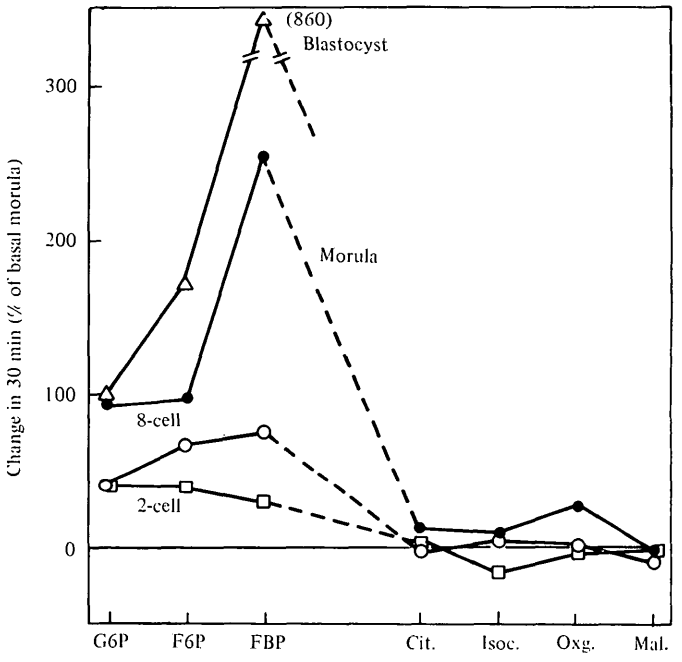


Fig. 4. Changes in seven metabolites after refeeding glucose. Embryos were starved 60 min and then refed for 30 min with medium containing 5.5 mM glucose. The changes are calculated as a percentage of the levels in freshly collected morulas (Table 3). G6P, glucose-6-P; F6P, fructose-6-P; FBP, fructose-1,6-P_2; Cit, citrate; Isoc, isocitrate; Oxg, α-oxoglutarate; Mal, malate.

refeeding were more or less parallel to those of isocitrate (Fig. 3). However, there were two differences. At the 2-cell stage the levels were lower than in older embryos both initially and after refeeding with pyruvate. Conversely, at the morula stage α-oxoglutarate did not fall as low during starvation as in the younger embryos, and there was some recovery after refeeding with glucose alone.

DISCUSSION

Changes in all metabolites during refeeding

To obtain an overview of the changes which occur when starved embryos are refed, the increases for all the metabolites measured here and earlier (Barbehenn *et al.* 1974) have been plotted relative to the respective levels for morulas (Table 3) in the original collection medium. After *glucose* refeeding (Fig. 4) all three glycolytic intermediates rise. The increases become greater with advancing age. This is especially true for fructose bisphosphate, which increases 30 % at the 2-cell stage and 860 % in the blastocyst. Of at least as much importance is the fact that the ratio of the increase in fructose bisphosphate to that of fructose-6-phosphate is 0·75, 1·11, 2·6 and 5·0 at the 2-cell,

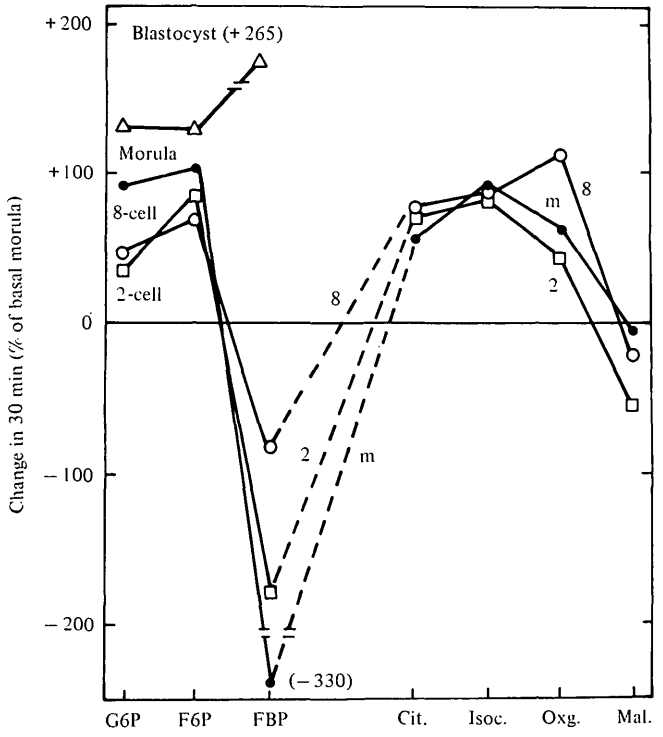


Fig. 5. Changes in seven metabolites after refeeding pyruvate and glucose. Embryos were starved 60 min and then refed for 30 min with medium containing 0·5 mM pyruvate plus 5·5 mM glucose. The data for the citrate cycle metabolites are averages of the results with these embryos combined with results from other animals which had been refed with pyruvate alone. The glucose omission was apparently without effect on the changes in these four metabolites. The changes are calculated as in Fig. 4 and the same abbreviations are used.

8-cell, morula and blastocyst stages. This is strong evidence of progressive facilitation with age at the P-fructokinase step (see below). None of the citrate cycle intermediates are significantly increased by glucose refeeding, with the exception of α-oxoglutarate in the morula.

In sharp contrast, when refeeding is with both glucose and pyruvate, although glucose-6-P and fructose-6-P rise as before, fructose bisphosphate falls precipitously at all stages prior to the blastocyst (Fig. 5). The final fructose bisphosphate concentrations, except for the blastocyst, were below the level of detection. Clearly, the addition of pyruvate has introduced a block at the P-fructokinase step. Now, the first three of the measured citrate cycle intermediates rise (40–110 %), whereas the fourth in the sequence, malate, falls at the 2- and 8-cell stages and does not change in the morula. One possibility for the fall in malate is that the rapid synthesis of citrate removes oxalacetate and hence malate faster than it can be regenerated through the cycle. If this is the correct

explanation, the limiting step must be between α-oxoglutarate and malate. (An alternative possibility is that a flood of pyruvate lowers the NADH:NAD^+ ratio and therefore the malate: oxalacetate ratio.)

The cause of the glycolysis block

It seems inescapable from the above that when pyruvate is present in the medium, glycolysis is blocked at the P-fructokinase step until the blastocyst stage is reached. Citrate is a potent inhibitor of P-fructokinase. Therefore, the rise in citrate induced by pyruvate probably accounts for the result. The very low fructose bisphosphate levels in all freshly collected specimens (Table 3) can be explained on the same basis. The collection medium contained pyruvate (and less glucose than in the above experiment) and tissue citrate concentrations were consistently high. Even in the blastocyst, pyruvate appears to be the preferred energy source.

The evidence for a significant early block in P-fructokinase in the absence of pyruvate is more complicated. If P-fructokinase were the only variable factor, one would expect realimentation with glucose to cause the greatest increase in fructose-6-P when blockade in glycolysis is greatest, i.e. at the 2-cell stage. Actually the opposite is true. However, the increase with age in the response to glucose is much greater for fructose bisphosphate than for its precursor (Fig. 5). Although this does not constitute a 'crossover', it is an equally good indication of facilitation at this step. The changes are in fact those expected if both the *formation* of fructose-6-P as well as its conversion to fructose bisphosphate are facilitated with age.

We believe that the best explanation for these age changes is that in the embryo, as in many other biological systems, glucose utilization is controlled by coordination between glucose uptake, hexokinase and P-fructokinase. Hexokinase is inhibited by glucose-6-P; P-fructokinase is powerfully inhibited by ATP as well as by citrate, and deinhibited by many metabolites, one of the most potent of which is fructose-6-P. Our thesis is that for some unknown reason glucose phosphorylation at the 2- and 8-cell stages is shut off at a low level of glucose-6-P. This means that only a low level of fructose-6-P is attained, a level too low to deinhibit P-fructokinase. Later when glucose phosphorylation increases, glucose-6-P levels (and hence fructose-6-P levels) rise. This deinhibits P-fructokinase and glycolysis proceeds.

If this thesis is correct, the true cause of the low glycolytic rate at early stages is some hindrance in either glucose uptake or hexokinase action, although the consequent block of the P-fructokinase step is a necessary part of the mechanism. This explanation does not require that there be any change in the nature or amount of P-fructokinase, and Brinster (1971) found ample activity of this enzyme, as measured *in vitro*, with little change with age. In contrast, hexokinase is low in activity in preimplantation embryos and increases 3-fold during the critical period (Brinster, 1968).

The advantage to the early embryo of a block in glycolysis is not clear. Glycogen is high in early embryos (Ozias & Stern, 1973). Perhaps the evolutionary 'purpose' of the block at the P-fructokinase step is to make sure that available glucose is stored as glycogen for future needs. The block is probably no disadvantage since the oviduct fluid has adequate pyruvate levels to meet the energy requirements of preimplantation development (Hohmdahl & Mastrioianni, 1965). This glycogen store may be essential for a critical period during implantation when contact with uterine fluids is being lost and full connexion may not have been established with the maternal blood supply.

Single embryo assays

We believe this report demonstrates the practicability of metabolite assays of single preimplantation embryos, and suggest that the general methodology described could be useful in many kinds of early embryo studies. The ability to analyse individual embryos not only reduces the necessary amount of experimental material but makes possible the assessment of individual differences. For example, Wudl & Chapman (1976) have shown that useful genetic studies can be made by examining enzyme differences among individual preimplantation mouse embryos of mixed parentage. The analytical system described here, because of its generality, should make it possible to extend such genetic studies to a wide variety of enzymes and other cell constituents.

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