Adenine Nucleotides and the Adenylate Kinase Equilibrium in Livers of Foetal and Newborn Rats

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(Received 7 November 1969)

1. Measurements of ATP, ADP and AMP concentrations in livers of rats that had been delivered by Caesarian section indicate a rapid shift from a low to a high [ATP]/[AMP] ratio. This change is consistent with the cessation of glycolysis and the initiation of gluconeogenesis at birth. 2. When newborn animals are exposed to a 100% nitrogen atmosphere the hepatic ATP concentration falls and AMP increases. 3. Calculations of the $[ATP][AMP]/[ADP]^2$ ratio give values that are close to the equilibrium constant of adenylate kinase except when the ATP concentration is high. 4. This difference cannot be accounted for by the preferential binding of available Mg^{2+} to ATP^{4-} rather than ADP^{3-} . It is concluded that the relative proportions of adenine nucleotides at any level of phosphorylation are only partly regulated by adenylate kinase.

Biochemical changes at birth in mammalian liver are associated with the changeover from a constant source of maternal nutrients to an independent existence. In rats this period is associated with the appearance of gluconeogenesis (Ballard & Oliver, 1963, 1965), the cessation of triglyceride synthesis (Ballard & Hanson, 1967a) and the utilization of carbohydrate and fat reserves as sources of energy (Dawkins, 1966). There is also a change from the relatively anoxic state of the foetus to the highly oxygenated condition in the newborn (Metcalf, Bartels & Moll, 1967). This period of rapidly altering metabolism offers a useful model to test theories of metabolic control that may have fundamental significance.

The adenylate control hypothesis as recently formulated by Atkinson is based on the suggestion that small changes in the 'adenylate charge' ratio $([ATP] + \frac{1}{2}[ADP])/([ATP] + [ADP] + [AMP])$ would have large effects on the activities of enzymes that utilize ATP and are inhibited by ADP or AMP (Atkinson, 1965; Atkinson & Walton, 1967). It is also proposed that the activity of adenylate kinase (EC 2.7.4.3) in cells is sufficient to maintain virtual equilibrium between ATP, ADP and AMP at any 'charge' value (Atkinson & Walton, 1967). To test this second point, the concentrations of ATP, ADP and AMP have been measured in foetal and neonatal liver under conditions where the oxygen partial pressure is changing either as a result of the birth process or by experimentally produced anoxia.

METHODS

Animals. Well-fed pregnant rats were lightly anaesthetized with ether on day 21 of gestation. The uterus was exposed by an abdominal incision and the livers of some foetuses were frozen *in situ* by using Wollenberger clamps cooled in liquid N_2 (Wollenberger, Ristau & Schoffa, 1960). This was accomplished while the foetus was connected to the placental and maternal circulation. Other foetuses were delivered by Caesarian section and kept in a plastic glove-box that contained moist tissue paper and was heated by an i.r. lamp. This technique was used to keep the animals under humid conditions at 35°C. At various times after delivery the livers of these newborn rats were freeze-clamped either without further treatment to the animals or after exposure to an atmosphere of N_2 for up to 30 min at 35°C.

Chemicals. Hexokinase, glucose 6-phosphate dehydrogenase, lactate dehydrogenase, pyruvate kinase, adenylate kinase, phosphoenolpyruvate, ATP, ADP and AMP were obtained from C.F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; NADP⁺ and NADH were from P-L Biochemicals Inc., Milwaukee, Wis., U.S.A.

Extraction of liver samples. The frozen livers were powdered while cooled in liquid N_2 and the frozen powder was extracted with 6% (w/v) $HClO_4$. During this extraction the suspension was heated to room temperature by rapid stirring with a coaxial homogenizer. The liver extracts were cooled to 0°C and centrifuged, and the supernatants adjusted to pH 6.5 with 3M- $KHCO_3$. The precipitated $KClO_4$ was removed by centrifugation after standing in ice for 10 min.

Measurement of adenine nucleotides. ATP was measured spectrophotometrically with hexokinase and glucose 6-phosphate dehydrogenase (Lamprecht & Trautschold,

1965). ADP and AMP were measured sequentially with lactate dehydrogenase, pyruvate kinase and adenylate kinase (Adam, 1965). For these measurements a high sensitivity was obtained by using spectrophotometer cells with a volume of 2.0 ml and a 5 cm light-path. It is implicit in the calculations of this paper that these methods are specific for the adenine nucleotides concerned. UTP and GTP, the principal nucleoside triphosphates other than ATP in liver, do not react in the hexokinase-glucose 6-phosphate dehydrogenase assay. Similarly, adenylate kinase is specific for AMP. But pyruvate kinase catalyses the formation of pyruvate from phosphoenolpyruvate with virtually all nucleoside diphosphates, although the maximum velocity with ADP is several times greater than with GDP or UDP. This difference in velocity has been used to correct for nucleotides other than ADP in a manner similar to that reported by Goldberg, Passonneau & Lowry (1966).

Effectiveness of tissue extraction. To test whether the single HClO_4 extraction of liver powder was sufficient to extract the adenine nucleotides quantitatively, second extractions were carried out and ATP, ADP and AMP measured in both extracts. In four experiments averages of 1.39, 0.429 and $0.110 \mu\text{mol}$ of tissue were found for ATP, ADP and AMP in the initial tissue extract, and the corresponding concentrations in the second extract were 0.093, 0.025 and $0.0068 \mu\text{mol/g}$. It is evident that not only do these extracts contain a small fraction of the nucleotides in the first extract, but that the relative rate of extraction of the different nucleotides is constant.

RESULTS AND DISCUSSION

The increase in hepatic oxygen partial pressure that accompanies the transition from foetus to newborn results in a shift in the proportion of adenine nucleotides towards a large preponderance of ATP (Table 1). Changes in adenylate charge, the $[\text{ATP}]/[\text{ADP}]$ ratio and the $[\text{ATP}]/[\text{AMP}]$ ratio are all statistically significant. The changes are somewhat greater than those found in a comparison between livers of foetal and 1-day-old rats (Mäenpää, 1967).

Atkinson (1966) has proposed that changes in the relative proportions of adenine nucleotides are sufficient to alter both the magnitude and the

direction of cellular pathways. In particular, the stimulation by AMP and the inhibition by ATP of phosphofructokinase are well established, as are the opposite actions of these nucleotides on fructose diphosphatase. Like phosphofructokinase, pyruvate kinase and citrate synthase are also inhibited by ATP (Atkinson, 1966). Thus an increase in the $[\text{ATP}]/[\text{AMP}]$ ratio can inhibit glycolysis and the citric acid cycle. Since pyruvate carboxylase and phosphoenolpyruvate carboxykinase both require nucleoside triphosphates for catalytic activity and are inhibited by nucleoside diphosphates (Keech & Utter, 1963; Chang & Lane, 1966), a preponderance of ATP should also stimulate gluconeogenesis. The increase in the $[\text{ATP}]/[\text{AMP}]$ ratio from 4.48 in foetal liver to 29.6 by 6 h after birth (Table 1) would thus favour a shift from glycolysis to gluconeogenesis. That glycolysis is active in foetal rat liver and gluconeogenesis is absent from this tissue has been well established by experiments both *in vitro* (Ballard & Oliver, 1963; Ballard & Hanson, 1967b; Dawkins, 1966) and *in vivo* (Philippidis & Ballard, 1969).

The range between nucleotides fully phosphorylated and less phosphorylated than is found after birth can be extended by exposing the newborn animals to N_2 . At this age rats can live under a total nitrogen atmosphere for about 30 min, after which time the hepatic $[\text{ATP}]/[\text{AMP}]$ ratio is decreased from 30 or more in newborns kept under O_2 to 1 or sometimes less. This large range affords an opportunity to study the effects of different nucleotide proportions on cellular processes, and to test directly whether the relative amounts of nucleotides are governed by the adenylate kinase equilibrium. This second proposal is supported by the high activities of adenylate kinase that occur in most tissues. ATP, ADP and AMP concentrations in livers from untreated or nitrogen-treated animals are plotted in Fig. 1 as a function of adenylate charge (compare Fig. 2a in Atkinson & Walton, 1967). The solid line in each figure represents the predicted proportion of each nucleotide that would

Table 1. Adenine nucleotides in livers of foetal and newborn rats

Concentrations are expressed as $\mu\text{mol/g}$ wet wt. Adenylate charge was calculated by the method of Atkinson & Walton (1967). Values are the means $\pm$ s.e.m. with the numbers of determinations in parentheses.

Age ...	Foetus	2h	6h
ATP	0.996 ± 0.093 (7)	1.59 ± 0.11 (11)	1.99 ± 0.14 (9)
ADP	0.495 ± 0.044 (7)	0.289 ± 0.047 (11)	0.248 ± 0.028 (9)
AMP	0.265 ± 0.035 (7)	0.142 ± 0.022 (11)	0.072 ± 0.010 (9)
$[\text{ATP}]/[\text{ADP}]$	2.34 ± 0.36 (7)	6.45 ± 0.92 (11)	8.53 ± 0.85 (9)
$[\text{ATP}]/[\text{AMP}]$	4.48 ± 1.02 (7)	13.8 ± 2.1 (11)	29.6 ± 3.8 (9)
Adenylate charge	0.711 ± 0.028 (7)	0.860 ± 0.014 (11)	0.913 ± 0.010 (9)

Fig. 1. Percentage of each adenine nucleotide present in freeze-clamped liver samples from foetal and newborn rats: (a) ATP; (b) ADP; (c) AMP. Some animals had been exposed to a N_2 atmosphere. Each point represents a determination on a single animal. Values are plotted as a function of adenylyte charge. The continuous line is the theoretical proportion of each nucleotide that would be present if the adenylate kinase equilibrium $[ATP][AMP]/[ADP]^2 = 0.8$ was controlling the relative amounts of nucleotide.

be present at any fixed 'adenylyte charge' if it is assumed that the proportion of nucleotides are regulated by the adenylate kinase equilibrium:

$$[ATP][AMP]/[ADP]^2 = K \quad (1)$$

Although the fit of experimental points to $K = 0.8$ (Markland & Wadkins, 1966) is not always close, it is obvious that in general the nucleotide concentrations change with adenylyte charge in such a way as to implicate adenylate kinase as an important factor in the regulation of nucleotide proportions.

To test whether the proportions of adenine nucleotides better fit the adenylate kinase equilibrium at one region of the adenylyte charge than at another, equilibrium constants have been calculated

Table 2. Effect of adenylyte charge on adenylate kinase equilibrium constants calculated from measurements *in vivo*

The apparent equilibrium constant (K) was calculated from the relationship: $[ATP][AMP]/[ADP]^2 = K$, by using determinations of ATP, ADP and AMP in liver *in vivo*. Values are the means $\pm$ s.e.m. with the number of determinations in parentheses.

Adenylyte charge	Apparent equilibrium constant
<0.5	1.30 ± 0.14 (6)
0.5-0.6	1.14 ± 0.24 (9)
0.6-0.7	1.22 ± 0.11 (11)
0.7-0.8	1.52 ± 0.23 (8)
0.8-0.9	2.27 ± 0.22 (22)
>0.9	3.87 ± 0.51 (13)

for each liver sample according to eq. (1) and are shown in Table 2. Livers from animals kept longest in O_2 show both the greatest 'adenylyte charge' and also the greatest deviation from the adenylate kinase equilibrium. In these animals the ADP concentrations are substantially lower and the AMP and ATP concentrations higher than expected from consideration of the adenylate kinase equilibrium. Although careful measurements of adenine nucleotides in liver have not been previously carried out over a wide range of relative nucleotide proportions, there are many reports of ATP, ADP and AMP determinations in adult rat liver (see Bücher, Krejci, Rüssmann, Schnitger & Wesemann, 1964). Normally an $[ATP]/[ADP]$ ratio about 4-5 has been found, and when the adenylate kinase equilibrium is calculated values in excess of 1 are obtained.

One explanation of the increase in apparent equilibrium constant when the adenylyte charge is high is that errors occurred in the ADP or AMP measurements. This possibility has been tested by the addition of small amounts of nucleotides to tissue extracts and measurement of the recovery of these added nucleotides. In all cases these recoveries have been quantitative, even for AMP contents below $0.1 \mu\text{mol/g}$ of tissue. It is also possible that the ADP measurements were either under- or over-estimated owing to the correction made for the amount of nucleoside diphosphates other than ADP in the tissue extracts. However, under no circumstances did those nucleotides contribute more than 15% of the NADH oxidation produced by the addition of pyruvate kinase for the determination of ADP, and consequently any error would be small.

The difference between predicted and experimental values may lie in the concentration of Mg^{2+} ion in liver, as it has been reported (Morrison &

James, 1965) that the reaction catalysed by adenylate kinase is:

Direct measurements *in vitro* of the adenylate kinase equilibrium have been carried out with purified muscle adenylate kinase. This enzyme is reported to show a specificity towards the various nucleotide-Mg²⁺ complexes identical with that of the liver enzyme (Markland & Wadkins, 1966). The two experiments described (Table 3) were designed to test whether the apparent equilibrium constant of adenylate kinase (eqn. 1) was increased in a manner similar to the results found *in vivo* (Table 2) under conditions when the [ATP]/[AMP] ratio was changed from a low (Expt. 1) to a high (Expt. 2) value. It is apparent that the equilibrium constant was unaffected by the [ATP]/[AMP] ratio or the adenylate charge and reached a maximum value when the [nucleotide]/[Mg²⁺] ratio was unity. These findings are consistent with eqn. (2) and are probably the result of the preferential complexing of free Mg²⁺ ion with ATP⁴⁻ rather than with ADP³⁻. This is indicated by the stability constants for MgATP²⁻ and MgADP⁻ of 70 000 and 4000 M⁻¹ respectively (Morrison & James, 1965). At low total magnesium concentrations the concentration of MgADP⁻ would be low relative to that of MgATP²⁻, thus resulting in an equilibrium shift

towards more ADP³⁻ and MgADP⁻. At high total magnesium concentrations the concentration of ADP³⁻ would be low, resulting also in a shift towards more ADP³⁻ and MgADP⁻. Nonetheless these effects cannot explain the experimental findings of equilibrium constants greater than 1.0.

An alternative explanation for the high apparent equilibrium constants from the measurements *in vivo* would be that adenine nucleotide proportions are not in equilibrium throughout the liver cell. This is quite likely as, although adenylate kinase is present at high activity in both cytosol and mitochondrial fractions of liver (Adelman, Lo & Weinhouse, 1968), it has been reported that the enzyme is absent from the mitochondrial matrix (Heldt & Schwalbach, 1967). An increase in AMP in this compartment such as that caused by the activation of fatty acids is sufficient to alter markedly the value of *K* (eqn. 1) in experiments on isolated mitochondria (Williamson, Olson, Herczeg & Coles, 1968). It is also possible that an increase in AMP in the mitochondrial matrix would cause the displacement of the adenylate kinase equilibrium in the whole tissue.

This work was performed with the technical assistance of Mr S. Knowles. Financial assistance from the Queen Elizabeth II Fellowship Committee is gratefully acknowledged.

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Table 3. *Determinations of adenylate kinase equilibrium constants with muscle adenylate kinase*

The adenylate kinase was from muscle. In Expt. 1, 1.1 μmol each of ATP and AMP were mixed with adenylate kinase (1 unit), 0.1 M-tris-HCl buffer, pH 7.5, and various concentrations of MgSO₄ in a final volume of 1 ml. Samples were taken after 15, 30 and 90 min, and, after inactivation with HClO₄, neutralization and removal of the KClO₄ precipitate, ATP, ADP and AMP were measured. As values at the three times were within 5% of each other, averages are given. The equilibrium constant (*K*) was calculated according to the relationship [ATP][AMP]/[ADP]² = *K*. Expt. 2 was performed in an identical manner but with initially 1.9 μmol of ATP and 0.3 μmol of AMP.

Expt. no.	MgCl ₂ (mm)	ATP (mm)	ADP (mm)	AMP (mm)	<i>K</i>
1	0.5	0.655	0.714	0.596	0.76
	1.0	0.685	0.681	0.653	0.96
	2.0	0.720	0.671	0.645	1.03
	4.0	0.660	0.725	0.632	0.80
	6.0	0.650	0.801	0.582	0.59
2	0.5	1.55	0.448	0.060	0.52
	1.0	1.61	0.396	0.067	0.69
	2.0	1.63	0.355	0.081	1.04
	4.0	1.59	0.376	0.081	0.90
	6.0	1.66	0.398	0.070	0.74

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