Regulation of Gluconeogenesis During Exposure of Young Rats to Hypoxic Conditions

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1. Two-day-old rats were exposed at constant temperature to atmospheres containing air and nitrogen with the air content varied in steps from 100 to 0%. By using this system of graded hypoxia a comparison was made between rates of gluconeogenesis from lactate, serine and aspartate in the whole animal and the concentrations of several liver metabolites. 2. Gluconeogenesis, expressed as the percentage incorporation of labelled isotope into glucose plus glycogen, proceeds linearly for 30 min when the animals are incubated in a normal air atmosphere, but is completely suppressed if the atmosphere is 100% nitrogen. 3. Preincubation of animals for between 5 and 30 min under an atmosphere containing 19% air results in the attainment of a new steady state with respect to gluconeogenesis and hepatic concentrations of ATP, ADP, AMP, lactate, pyruvate, β -hydroxybutyrate and acetoacetate. 4. When lactate (100 μ mol), aspartate (20 μ mol) or serine (20 μ mol) was injected, it was shown that the more severe the hypoxia the greater the depression of gluconeogenesis. Under conditions when gluconeogenesis was markedly inhibited there were no changes in the degree of phosphorylation of hepatic adenine nucleotides, but free $[NAD^+]/[NADH]$ ratios fell in both cytosol and mitochondrial compartments of the liver cell. 5. Measurements of total liver NAD^+ and $NADH$ showed that the concentrations of these nucleotide coenzymes changed less with anoxia, in comparison with the concentration ratio of free coenzymes. 6. Calculations showed that the difference in NAD^+ - $NADH$ redox potentials between mitochondrial and cytosol compartments increased with the severity of hypoxia. 7. From the constancy of the concentrations of adenine nucleotides it is concluded that liver of hypoxic rats can conserve ATP by lowering the rate of ATP utilization for gluconeogenesis. Gluconeogenesis may be regulated in turn by the changes in mitochondrial and cytosol redox state.

Most studies on the regulation of gluconeogenesis have involved measurements of the overall rate of the pathway with perfused organ preparations. Since this rate can be altered by the addition of glucagon (Exton & Park, 1968; Williamson, Browning, Thurman & Scholz, 1969c; Ross, Hems, Freedland & Krebs, 1967) or fatty acids (Struck, Ashmore & Wieland, 1965; Söling, Willms, Friedrichs & Kleineke, 1968; Williamson, Browning & Scholz, 1969a) to the preparation, it is possible to follow changes in those tissue intermediates that may play a regulatory role. Philippidis & Ballard (1969, 1970) have utilized the transition from foetus to newborn in rats as a model for studies on the regulation of gluconeogenesis, since this period is characterized by the complete appearance of the pathway rather than an alteration in activity of a

pre-existing sequence. The technique also has the physiological advantage of using intact animals rather than perfused liver. However, the technical difficulties of experimentation on animals *in utero* are a limit to the effectiveness of this particular model.

In the present work these problems have been avoided by taking young rats, with a fully developed gluconeogenic pathway, and progressively inhibiting the pathway by exposure of the animals to hypoxia. One advantage of this technique is that the main effect of oxygen lack should be to lower or prevent oxidative phosphorylation. The relationships of this depression in mitochondrial phosphorylation of ADP to hepatic gluconeogenesis and to changes in redox state, adenine nucleotide proportions and other liver intermediates can then be followed.

METHODS

Animals. The rats were 2-day-old hooded Wistar animals (average wt. 5g) from the Divisional colony or were obtained from the Waite Agricultural Institute of the University of Adelaide. These young rats were kept with their mothers until used.

Chemicals. Lactate dehydrogenase, pyruvate kinase, adenylate kinase, malate dehydrogenase, glucose 6-phosphate dehydrogenase, hexokinase, β -hydroxybutyrate dehydrogenase, alcohol dehydrogenase, phosphoenolpyruvate, ATP, ADP and AMP were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; NAD⁺, NADH and NADP⁺ were from P-L Biochemicals Inc., Milwaukee, Wis., U.S.A.; L-[U-¹⁴C]lactate, L-[U-¹⁴C]aspartate and L-[U-¹⁴C]serine were from The Radiochemical Centre, Amersham, Bucks., U.K.

Hypoxia experiments. Mixtures of air and N₂ were prepared by passing the gases through individual flowmeters. Each gas mixture flowed into a plastic glove box (60 cm $\times$ 30 cm $\times$ 30 cm) that contained moist tissue paper and was maintained at 30°C by an i.r. lamp. After 15 min at a flow rate of approx. 15 litres/min the gas flow was decreased to 7 litres/min and the animals placed in the box to equilibrate for 5 min. At this time each rat was injected intraperitoneally with a gluconeogenic precursor that was dissolved in 50 μ l of 0.9% NaCl and adjusted to pH 6 with HCl or NaOH. The amounts used were 100 μ mol of sodium L-lactate, 20 μ mol of sodium L-aspartate, or 20 μ mol of L-serine. The respective labelled compound (0.5 μ Ci) was added to the injection solution when rates of gluconeogenesis were to be measured. At 15 min after injection the rats were either frozen in liquid N₂ if gluconeogenesis was being measured, or the livers were freeze-clamped *in situ* with Wollenberger clamps cooled in liquid N₂ (Wollenberger, Ristau & Schoffa, 1960).

Measurement of gluconeogenesis. Animals injected with radioactive aspartate, lactate or serine and frozen in liquid N₂ were ground in a percussion mortar cooled by liquid N₂. The powder from each animal was extracted by homogenization with 20 ml of 5% (w/v) HClO₄. This mixture was centrifuged (2000g for 10 min) and the supernatant retained. The precipitate was re-extracted with 5 ml of 5% (w/v) HClO₄, and after centrifugation the two supernatants were combined. The pH of this solution was adjusted to pH 6 with 2M-KHCO₃ and the KClO₄ was removed by centrifugation. A portion of neutralized extract was applied to a column containing Dowex 1 (formate form) of 2 ml bed vol. layered over a similar amount of Dowex 50 (H⁺ form), and the column was eluted with water to give a volume of 20 ml. The radioactivity in a sample of this eluate was measured in a liquid-scintillation spectrometer with 10 ml of a scintillation mixture containing 500 ml of Triton X-100, 1 litre of toluene, 4g of 2,5-diphenyloxazole and 0.15g of 1,4-bis-(5-phenyloxazol-2-yl)benzene. Columns of the size used were satisfactory for the complete removal of radioactive serine or aspartate from 5 ml of neutralized extract. In these experiments 1 ml of eluate was taken for radioactivity determinations. When lactate was the injected precursor, only 1 ml of neutralized extract could be passed through the columns for complete removal of lactate. The radioactivity in 5 ml of column eluate was measured in these experiments.

It is important that the radioactivity passed through the columns is only glucose or glycogen and that these two compounds are not retained on the columns. To test the first assumption neutralized extracts of animals injected with each precursor and left for 15 min in an air atmosphere were passed through columns as described above to give an eluate volume of 20 ml. The eluates were concentrated by freeze-drying and made up to a volume of 2.5 ml with water. Glucose oxidase was added to 1 ml portions of these solutions to convert the free glucose into gluconate. This gluconate was adsorbed to columns of Dowex 1 (Cl⁻ form) and eluted with 0.2M-NaCl. Glycogen was precipitated from other 1 ml portions of the concentrated solutions by the addition of 2 vol. of ethanol. Measurements of radioactivity in fractions from animals with active gluconeogenesis showed that essentially all the radioactivity that had passed through the Dowex 1-Dowex 50 column was either converted into gluconate by glucose oxidase or precipitated by two volumes of ethanol. It is concluded that glucose and glycogen were the only significant compounds labelled and that the radioactivity of the material passed through the Dowex 1-Dowex 50 column could be counted directly as an index of gluconeogenesis. It was also shown that neither glucose nor glycogen were retained by this column.

The gluconeogenic rates are expressed as the percentage of injected radioactivity recovered in glucose plus glycogen. Zero-time controls were carried out in each experiment whereby rats were injected with precursor and immediately frozen in liquid N₂. Radioactivity in 'glucose plus glycogen' in these controls represented 0.40, 0.31 and 1.05% of the injected serine, aspartate and lactate respectively and was presumably due to uncharged impurities in the labelled compounds. The percentage incorporation in these controls was subtracted from test values.

Extraction of liver samples. The frozen livers were powdered while cooled in liquid N₂, weighed and extracted by homogenization with 2 ml of 6% (w/v) HClO₄. During this extraction the temperature was raised to room temperature to ensure complete extraction of tissue metabolites. Measurements of the effectiveness of tissue extraction have been reported previously (Ballard, 1970a). The homogenate was centrifuged at 0°C and the supernatant adjusted to pH 6.5 with 2M-KHCO₃. The precipitated KClO₄ was removed by centrifugation and the supernatant used for the measurement of all intermediates except NADH. For the measurement of NADH powdered liver was extracted with hot ethanolic KOH as described by Klingenberg (1965b).

Measurements of liver metabolites. All compounds were determined spectrophotometrically by coupled enzymic assays. Owing to the small amount of liver obtained in the freeze-clamping procedure (approx. 100 mg), the sensitivity of each assay was increased by using cuvettes with 5 cm light-paths and with a usable volume of 1.5 ml. Changes in the extinction due to NADH or NADPH at 340 nm corresponding to a tissue content of 15 nmol/g wet wt. could be determined with good reproducibility without using fluorimetric methods.

Tissue intermediates were assayed by established methods: lactate (Hohorst, 1965a), pyruvate (Bücher, Czok, Lamprecht & Latzko, 1965), malate (Hohorst, 1965b), acetoacetate (Mellanby & Williamson, 1965), β -hydroxybutyrate (Williamson & Mellanby, 1965),

NAD⁺ (Klingenberg, 1965a), NADH (Klingenberg, 1965b), ATP (Lamprecht & Trautschold, 1965) and ADP and AMP (Adam, 1965).

β-Hydroxybutyrate dehydrogenase assay. This enzyme was assayed in the direction of β -hydroxybutyrate oxidation in samples of freeze-dried homogenates or liver fractions prepared by differential centrifugation (Lehninger, Sudduth & Wise, 1960). One unit of activity is defined as the formation of 1 μ mol of NADH/min at 37°C.

Redox calculations. Calculations of [NAD⁺]/[NADH] ratios from the [lactate]/[pyruvate] or [β -hydroxybutyrate]/[acetoacetate] ratios are described by Williamson, Lund & Krebs (1967). Similar equilibrium constants have been employed, and it is assumed that the pH of the liver cell was 7.0. It is recognized that hypoxic conditions may cause a fall in intracellular pH, and thus an error in the [NAD⁺]/[NADH] ratio calculated by using an incorrect equilibrium constant. However, as large amounts of slightly acidic glucose precursor were injected, it is assumed that the error in [NAD⁺]/[NADH] ratios will be relatively constant throughout the various hypoxic states.

RESULTS

Results presented by Philippidis & Ballard (1970) show that under conditions of prolonged ether anaesthesia foetal rats do not convert radioactive pyruvate into glycogen. It was demonstrated that the depressed gluconeogenesis was caused in part by hypoxic conditions produced by the anaesthesia. Experiments in which gluconeogenesis is measured in 2-day-old animals under normal conditions and during exposure to a total N₂ atmosphere are shown in Fig. 1. These experiments, with either lactate (Fig. 1a) or aspartate (Fig. 1b) as gluconeogenic precursor, indicate that gluconeogenesis is prevented by anoxia. With lactate as precursor 23% of the injected isotope was recovered as radioactive glucose plus glycogen 30 min after injection into normal animals. If it is assumed that this gluconeogenesis is confined to the liver, rates of glucose labelling of approx. 2.5 μ mol of lactate/min per g of liver are apparent, values that are comparable with net gluconeogenesis in perfused livers of starved rats (Williamson *et al.* 1969a; Exton & Park, 1968; Ross *et al.* 1967). The observed values are probably underestimates of true gluconeogenic flux, since they do not take into account leakage of radioactivity at the site of injection, contribution of unlabelled lactate to the injected pool or breakdown of newly synthesized hexose. The results in Fig. 1 clearly show that incorporation of radioactivity into glucose plus glycogen in the oxygenated animal proceeds at a linear rate with time. In subsequent experiments incorporation rates were measured 15 min after the injection of precursor.

Variation between experiments was minimized by taking the animals from their mothers just before

Fig. 1. Incorporation of (a) lactate or (b) aspartate into glucose plus glycogen in the intact 2-day-old rat. Incorporation, expressed as the percentage of injected radioactivity in glucose plus glycogen, was measured at different times after injection of precursors. Animals were either exposed to a normal air atmosphere (●) or were preincubated under a total N₂ atmosphere for 5 min and maintained under these anoxic conditions for the duration of the experiment (○). Details of the experimental procedure are given in the Methods section. Each point is the average of three determinations. Values at zero time indicate the blank incorporation. The second vertical axis represents the radioactivity (c.p.m.) actually measured in the 1 ml portion of column eluate in the aspartate experiments or in the 5 ml portions of column eluate in the lactate experiments.

experimentation, and also by doing experiments at the same time of day. Although the rate of glucose synthesis is closely dependent on nutritional factors, which are continually changing in the growing animal, it is assumed for the purpose of these experiments that the young animal is in a steady state. Although this assumption may hold for normal oxygenated animals, it cannot be true for animals exposed to anoxia, for this condition is eventually fatal. To check the attainment of an approximate steady state in animals exposed to hypoxic conditions, both liver metabolites and gluconeogenic rates were determined at different intervals after transfer of the animals to the hypoxic atmosphere. The animals were preincubated in the 19% air atmosphere for between 5 and 30 min,

Table 1. *Effects of different times of preincubation in an atmosphere containing 19% air on concentrations of liver metabolites and on gluconeogenesis from aspartate*

Animals were exposed to an atmosphere containing 19% air for 5, 10, 20 or 30 min before injection of 20 μ mol of aspartate containing 0.5 μ Ci of [U- 14 C]aspartate. After an additional 15 min the livers were freeze-clamped or the whole animal was frozen in liquid N₂. Intermediates were extracted and measured as described in the Methods section. Gluconeogenesis is expressed as the percentage of injected radioactivity in glucose plus glycogen. Values are the average of determinations on two animals and concentrations are expressed as μ mol/g wet wt. of tissue.

Preincubation ...	5 min	10 min	20 min	30 min
[ATP]	1.52	1.37	1.68	1.27
[ADP]	0.35	0.32	0.37	0.26
[AMP]	0.15	0.12	0.14	0.10
[Lactate]	3.16	3.40	2.99	4.72
[Pyruvate]	0.120	0.114	0.114	0.114
[β -Hydroxybutyrate]	1.15	0.50	0.35	1.43
[Acetoacetate]	0.120	0.099	0.067	0.206
Gluconeogenesis	0.93	1.26	1.02	0.90

Table 2. *β -Hydroxybutyrate dehydrogenase activity in livers of developing rats*

Activities were measured in freeze-dried homogenates and are expressed as μ mol of NADH formed/min per g of liver. Values are the means $\pm$ S.E.M. for the numbers of animals in parentheses.

Age	Activity (units/g of tissue)
Foetus, 21 days	6.7 $\pm$ 0.7 (7)
Young, 2 days	13.5 $\pm$ 0.3 (5)
Young, 7 days	30.1 $\pm$ 1.0 (5)
Adult, both sexes	34.7 $\pm$ 2.6 (7)
Lactating female	17.5 $\pm$ 2.5 (5)

injected with aspartate and then maintained for an additional 15 min before freeze-clamping of the liver or measurement of gluconeogenesis. There is no relationship between time of preincubation and concentrations of lactate, pyruvate, acetoacetate, β -hydroxybutyrate, ATP, ADP or AMP in the liver (Table 1). Similarly the rate of glucose synthesis is relatively uniform. The variations in acetoacetate and β -hydroxybutyrate concentrations are caused by the variable developmental appearance of ketogenesis, since the animals in this experiment were from two litters. It is concluded that, although this hypoxic condition is severe, the young animal is in a relatively steady state.

In the following experiments rates of gluconeogenesis in whole animals from lactate, aspartate or serine have been compared with the degree of phosphorylation of hepatic adenine nucleotides (Atkinson & Walton, 1967); the redox state of the cytosol as calculated from the [lactate]/[pyruvate] ratio (Williamson *et al.* 1967); and the redox state of the mitochondria, which was in turn derived

from the [β -hydroxybutyrate]/[acetoacetate] ratio (Williamson *et al.* 1967). Since preliminary experiments indicated that this [β -hydroxybutyrate]/[acetoacetate] ratio did not change in foetal liver under conditions when the cytosol redox state was markedly altered, the activity of β -hydroxybutyrate dehydrogenase has been measured to see if foetal liver contains a low activity of this enzyme. Table 2 shows that the enzyme is present at a substantial activity in the foetus, and that this activity increases 100% in the 2-day-old animal and to five times the foetal activity in the adult. β -Hydroxybutyrate dehydrogenase activity is also lower in livers of lactating animals. Intracellular distribution studies confirmed that the enzyme activity in livers from animals of all ages was localized in the mitochondria.

Measurements of hepatic intermediates after lactate injection are presented in Table 3. With increasing severity of hypoxia there are decreases in the concentrations of both ATP and ADP, so that the [ATP]/[ADP] ratio remains at approx. 4 under all conditions except complete anoxia. AMP concentrations increase with increasing hypoxia, but as with the concentrations of the other adenine nucleotides the major change was observed when the atmosphere was changed from 12 to 0% air. In these experiments with lactate as glucose precursor measurements of lactate concentrations in liver were not attempted, since no satisfactory estimate could be made for extracellular lactate. Pyruvate concentrations show no trend with hypoxia, but when anoxia is attained the pyruvate concentration is lowered to less than 20% of that found under the other conditions. Since it is likely that the intracellular lactate in these experiments is derived mainly from the large injected dose rather than from glycolysis, it is probable that the constancy of

Table 3. *Changes in the concentrations of liver metabolites after injection of lactate into young rats exposed to atmospheres containing different proportions of air and nitrogen*

The experimental procedure is described in the Methods section. Lactate (100 μmol) was injected into each animal and the measurements were made on liver that was freeze-clamped 15 min later. Values are expressed as $\mu\text{mol/g}$ of liver and are presented as the means $\pm$ S.E.M. for from six to eight determinations.

Air (%)	[ATP]	[ADP]	[AMP]	[Pyruvate]	[β -Hydroxybutyrate]	[Acetoacetate]
100	2.28 $\pm$ 0.25	0.48 $\pm$ 0.06	0.10 $\pm$ 0.01	0.275 $\pm$ 0.069	0.92 $\pm$ 0.19	0.459 $\pm$ 0.173
50	1.70 $\pm$ 0.13	0.48 $\pm$ 0.03	0.12 $\pm$ 0.02	0.193 $\pm$ 0.019	0.38 $\pm$ 0.03	0.354 $\pm$ 0.166
30	1.72 $\pm$ 0.10	0.35 $\pm$ 0.04	0.11 $\pm$ 0.01	0.246 $\pm$ 0.022	0.84 $\pm$ 0.14	0.354 $\pm$ 0.131
19	1.71 $\pm$ 0.16	0.43 $\pm$ 0.04	0.14 $\pm$ 0.01	0.259 $\pm$ 0.007	1.02 $\pm$ 0.16	0.202 $\pm$ 0.091
12	1.41 $\pm$ 0.01	0.38 $\pm$ 0.07	0.17 $\pm$ 0.01	0.236 $\pm$ 0.009	1.08 $\pm$ 0.11	0.114 $\pm$ 0.063
0	0.58 $\pm$ 0.05	0.44 $\pm$ 0.06	0.81 $\pm$ 0.07	0.039 $\pm$ 0.010	0.44 $\pm$ 0.05	0.045 $\pm$ 0.026

Table 4. *Effects of exposure of young rats to atmospheres containing different proportions of air and nitrogen on the concentrations of liver metabolites after aspartate injection*

The experimental procedure is described in the Methods section. Aspartate (20 μmol) was injected into each animal and the measurements were made on liver that was freeze-clamped 15 min later. Concentrations are expressed as $\mu\text{mol/g}$ of liver and are presented as means $\pm$ S.E.M. for from six to eight determinations.

Air (%)	[ATP]	[ADP]	[AMP]	[Lactate]	[Pyruvate]	[β -Hydroxybutyrate]	[Acetoacetate]
100	1.92 $\pm$ 0.17	0.31 $\pm$ 0.02	0.09 $\pm$ 0.01	0.64 $\pm$ 0.09	0.41 $\pm$ 0.003	0.42 $\pm$ 0.04	0.315 $\pm$ 0.075
50	1.78 $\pm$ 0.06	0.47 $\pm$ 0.09	0.14 $\pm$ 0.02	0.59 $\pm$ 0.12	0.042 $\pm$ 0.005	0.80 $\pm$ 0.12	0.425 $\pm$ 0.101
30	1.82 $\pm$ 0.11	0.48 $\pm$ 0.08	0.13 $\pm$ 0.02	0.96 $\pm$ 0.19	0.057 $\pm$ 0.008	0.76 $\pm$ 0.09	0.364 $\pm$ 0.101
19	1.68 $\pm$ 0.09	0.52 $\pm$ 0.07	0.16 $\pm$ 0.01	3.58 $\pm$ 0.61	0.123 $\pm$ 0.020	1.00 $\pm$ 0.15	0.197 $\pm$ 0.033
12	1.44 $\pm$ 0.15	0.61 $\pm$ 0.06	0.22 $\pm$ 0.02	5.61 $\pm$ 0.94	0.151 $\pm$ 0.021	0.84 $\pm$ 0.08	0.091 $\pm$ 0.037
0	0.96 $\pm$ 0.02	0.63 $\pm$ 0.06	0.56 $\pm$ 0.08	6.80 $\pm$ 0.97	0.068 $\pm$ 0.008	0.71 $\pm$ 0.14	0.035 $\pm$ 0.014

the pyruvate concentration with changing hypoxia reflects a fairly constant [lactate]/[pyruvate] ratio. If true, this would suggest that the cytosol redox state does not change until anoxia is attained. No trend is evident in the β -hydroxybutyrate concentration, except perhaps a fall under anoxic conditions. However, the acetoacetate concentration falls when the fraction of air in the atmosphere is lowered below 30%, so that under anoxic conditions the acetoacetate concentration is only 10% of that found in livers from normal animals.

When the [β -hydroxybutyrate]/[acetoacetate] ratio is used to calculate the [NAD⁺]/[NADH] ratio in mitochondria, and these values are plotted against the decreasing proportion of air in the atmosphere, a gradual change towards a more reduced state can be seen (Fig. 2b). No explanation can be given for the lower [NAD⁺]/[NADH] ratio in normal animals as compared with animals exposed to 50% air. Nevertheless gluconeogenesis shows a parallel trend under all conditions tested (Fig. 2c), although the adenylate charge was constant until the animals were exposed to complete anoxia (Fig. 2a).

With aspartate as injected precursor the changes in the concentrations of liver metabolites with increasing hypoxia are generally similar to those

found when lactate was used. The proportions of adenine nucleotides show a more pronounced change with hypoxia towards less ATP and more AMP, but as with lactate injection the most pronounced changes were evident between livers of animals exposed to atmospheres containing 12 and 0% air (Table 4). Hepatic lactate concentrations increase as the proportion of air in the atmosphere is lowered, a result also found with pyruvate measurements, except when anoxia is attained. β -Hydroxybutyrate and acetoacetate concentrations show similar changes with hypoxia as those shown for the lactate injection experiments in Table 3. A comparison between the results given in Tables 4 and 1 shows that the steady state reached with 5 min exposure to an atmosphere containing 19% air before aspartate injection is indeed close to a true steady state. It might be expected that with longer times of preincubation the concentrations of liver metabolites would change towards the pattern found in more severe hypoxia. However, it is clear that even after 30 min preincubation in 19% air the relative phosphorylation of adenine nucleotides and the concentrations of other metabolites do not show the pattern found with 5 min preincubation under the atmosphere containing 12% air.

Fig. 2. Effects of changing the atmosphere from one containing 100% air to one containing 0% air on (a) the adenylate charge ratio ($[\text{ATP} + \frac{1}{2}\text{ADP}]/[\text{ATP} + \text{ADP} + \text{AMP}]$) in liver, (b) the $[\text{NAD}^+]/[\text{NADH}]$ ratio in mitochondria as calculated from the $[\beta\text{-hydroxybutyrate}]/[\text{acetoacetate}]$ ratio in liver, and (c) the percentage incorporation of ^{14}C from $[\text{U-}^{14}\text{C}]\text{lactate}$ into glucose plus glycogen in the whole animal. In all experiments animals were exposed to the particular atmosphere for 5 min before injection and 15 min after injection of $100\mu\text{mol}$ of lactate. Other experimental details are given in the Methods section. Values shown for adenylate charge and percentage incorporation of ^{14}C into glucose are the means $\pm$ S.E.M. for six to eight determinations. The mitochondrial redox state is obtained from the mean concentrations of acetoacetate and β -hydroxybutyrate in Table 3 as described by Williamson *et al.* (1967).

A comparison after aspartate injection of $[\text{NAD}^+]/[\text{NADH}]$ ratios calculated from the concentrations of liver metabolites is presented in Fig. 3. A sharp fall in gluconeogenesis was found when the fraction of air in the atmosphere was lowered from 30 to 19%. This was accompanied by changes in both mitochondrial and cytosol redox state towards a more reduced state. The change in adenylate charge

Fig. 3. Effects of changing the atmosphere from 100 to 0% air on measurements in liver and in the whole rat. (a) Liver adenylate charge ratio ($[\text{ATP} + \frac{1}{2}\text{ADP}]/[\text{ATP} + \text{ADP} + \text{AMP}]$); (b) $[\text{NAD}^+]/[\text{NADH}]$ ratio in the liver cytosol, calculated from the $[\text{lactate}]/[\text{pyruvate}]$ ratio; (c) $[\text{NAD}^+]/[\text{NADH}]$ ratio in the mitochondrial fraction of liver, calculated from the $[\beta\text{-hydroxybutyrate}]/[\text{acetoacetate}]$ ratio; (d) percentage incorporation of ^{14}C from $[\text{U-}^{14}\text{C}]\text{aspartate}$ into glucose plus glycogen in the whole animal 15 min after the injection of $20\mu\text{mol}$ ($0.5\mu\text{Ci}$) of L-aspartate. Values for adenylate charge and gluconeogenesis are means $\pm$ S.E.M. for six to eight determinations. Redox values were calculated from the means of determinations in Table 4 as described by Williamson *et al.* (1967).

Table 5. *Effects of exposure of young rats to atmospheres containing different proportions of air and nitrogen on the concentrations of liver metabolites after serine injection*

The experimental procedure is described in the Methods section. Serine (20 μ mol) was injected into each animal and the measurements were made on liver that was freeze-clamped 15min later. Concentrations are expressed as μ mol/g of liver and are presented as means $\pm$ S.E.M. for six determinations.

Air (%)	[ATP]	[ADP]	[AMP]	[Lactate]	[Pyruvate]	[β -Hydroxybutyrate]	[Acetoacetate]
100	1.74 $\pm$ 0.09	0.56 $\pm$ 0.05	0.14 $\pm$ 0.01	0.64 $\pm$ 0.10	0.046 $\pm$ 0.005	0.31 $\pm$ 0.05	0.341 $\pm$ 0.051
50	1.68 $\pm$ 0.09	0.53 $\pm$ 0.03	0.13 $\pm$ 0.01	0.95 $\pm$ 0.05	0.071 $\pm$ 0.009	0.32 $\pm$ 0.08	0.370 $\pm$ 0.087
30	1.71 $\pm$ 0.14	0.50 $\pm$ 0.03	0.13 $\pm$ 0.01	1.10 $\pm$ 0.14	0.079 $\pm$ 0.005	0.35 $\pm$ 0.04	0.305 $\pm$ 0.037
19	1.46 $\pm$ 0.14	0.40 $\pm$ 0.02	0.12 $\pm$ 0.01	2.24 $\pm$ 0.25	0.107 $\pm$ 0.008	0.60 $\pm$ 0.03	0.275 $\pm$ 0.046
12	1.42 $\pm$ 0.18	0.45 $\pm$ 0.02	0.13 $\pm$ 0.01	3.23 $\pm$ 0.48	0.135 $\pm$ 0.019	0.66 $\pm$ 0.09	0.222 $\pm$ 0.045
0	0.94 $\pm$ 0.15	0.55 $\pm$ 0.03	0.48 $\pm$ 0.05	4.86 $\pm$ 0.36	0.089 $\pm$ 0.014	0.44 $\pm$ 0.05	0.065 $\pm$ 0.011

Table 6. *Concentrations of malate in livers of animals exposed to hypoxia*

Animals were treated and injected with glucose precursors as described in the legends to Tables 3, 4 and 5. Values are the means $\pm$ S.E.M. for from six to eight animals and are expressed as μ mol/g of liver 15min after injection of precursor.

Air (%)	Injected precursor	...	Serine	Lactate	Aspartate
100			0.138 $\pm$ 0.024	0.214 $\pm$ 0.051	0.088 $\pm$ 0.005
50			0.126 $\pm$ 0.030	0.149 $\pm$ 0.027	0.101 $\pm$ 0.008
30			0.131 $\pm$ 0.032	0.247 $\pm$ 0.124	0.118 $\pm$ 0.009
19			0.150 $\pm$ 0.026	0.223 $\pm$ 0.085	0.144 $\pm$ 0.014
12			0.145 $\pm$ 0.022	0.217 $\pm$ 0.092	0.157 $\pm$ 0.018
0			0.266 $\pm$ 0.047	0.204 $\pm$ 0.066	0.376 $\pm$ 0.031

between these two atmospheres was from 0.836 to 0.822, and was not significant at the 5% probability level. When the air content was lowered to 12% substantial changes were apparent in adenylate charge, cytosol redox state, mitochondrial redox state and rate of gluconeogenesis. Measurements in animals exposed to 50% and 19% air showed that whereas gluconeogenesis, the mitochondrial redox state and the cytosol redox state were lowered to 25, 36 and 50% respectively at the lower air content, the adenylate charge was unaffected.

With serine as glucose precursor the proportions of adenine nucleotides in liver show even less change with increasing hypoxia than is found when either lactate or aspartate is injected (Table 5). Although ATP concentrations showed a tendency to fall with greater hypoxia, neither the [ATP]/[ADP] ratio nor the [ADP]/[AMP] ratio fell until complete anoxia was attained. The changes in lactate, pyruvate, β -hydroxybutyrate and acetoacetate concentrations were similar to those found after aspartate injection, except that acetoacetate concentrations were somewhat greater under the 12% air atmosphere. Unlike the aspartate or lactate experiments, gluconeogenesis from serine was not substantially depressed until the air content was lowered to 12% (Fig. 4).

Hepatic concentrations of malate after injection of serine, lactate or aspartate into rats exposed to hypoxic conditions are shown in Table 6. With injected aspartate the malate concentration increased gradually as the hypoxia was made more severe, but in the animals exposed to an atmosphere containing no air the hepatic malate concentration was much higher than under all hypoxic conditions. These results are in accordance with the changes in cytosol and mitochondrial redox states (Fig. 3) with malate concentrations increasing with an increase in the [NADH]/[NAD⁺] ratio. The changes in malate concentration after serine injection are not comparable with the redox changes in Fig. 4 and only with complete anoxia was an increase in malate noted. With lactate injection the malate concentrations in livers from oxygenated or hypoxic animals were extremely variable. There was no change in malate with increased hypoxia and no correlation with changes in mitochondrial redox state (Fig. 2) or pyruvate concentration (Table 3).

[Lactate]/[pyruvate] and [β -hydroxybutyrate]/[acetoacetate] ratios were used to calculate [NAD⁺]/[NADH] ratios in the cytosol and mitochondria respectively. Since these ratios in liver are altered substantially by exposure of the animals to hypoxic

Fig. 4. Effects of changing the atmosphere from 100 to 0% air on measurements in liver and in the whole rat. (a) Hepatic adenylate charge ratio ($[ATP + \frac{1}{2}ADP]/[ATP + ADP + AMP]$); (b) $[NAD^+]/[NADH]$ ratio in the liver cytosol, calculated from the $[lactate]/[pyruvate]$ ratio; (c) $[NAD^+]/[NADH]$ ratio in the mitochondrial compartment of liver, calculated from the $[\beta\text{-hydroxybutyrate}]/[acetoacetate]$ ratio; (d) percentage incorporation of ^{14}C from $[U\text{-}^{14}C]$ serine into glucose plus glycogen in the whole rat 15 min after injection of $20\mu\text{mol}$ ($0.5\mu\text{Ci}$) of L-serine. Values for adenylate charge and percentage incorporation are means $\pm$ S.E.M. for six determinations. Redox values were calculated from data in Table 5 as described by Williamson *et al.* (1967).

conditions, it was necessary to determine whether these changes applied to the total cellular concentrations of NAD^+ and $NADH$ or were confined to the free nucleotides. Measurements of concentrations of tissue NAD^+ and $NADH$ in livers of animals injected with each precursor are shown in Table 7. Experiments in which animals are either exposed to a normal air atmosphere or to a total N_2 atmosphere indicate only slight changes in the concentrations of the nucleotide coenzymes.

DISCUSSION

Effects of oxygen lack on gluconeogenesis. Philippidis & Ballard (1969, 1970) demonstrated only slight incorporation *in vivo* of radioactive pyruvate, lactate or aspartate into the glucose and glycogen of foetal rat liver. However, if careful precautions were taken to prevent foetal hypoxia the gluconeogenic pathway became functional, but at rates much lower than observed in postnatal animals. The relationship between hypoxia and gluconeogenesis is more obvious with the experimental approach used in this paper, in that under anoxic conditions the synthesis of glucose is almost completely suppressed. This inhibition was evident with aspartate, serine or lactate as glucose precursor, and, although there were quantitative differences in the effects of oxygen lack, gluconeogenesis was always suppressed when the air content of the atmosphere was lowered below 30%. Further, the exposure of animals to an atmosphere containing 19% air showed that labelling in glucose plus glycogen was rapidly lowered to a new rate that was constant during 30 min incubation (Table 1). The attainment of a steady state with respect to gluconeogenesis permits the use of this technique to study the effects of regulatory factors on the overall pathway.

Changes in concentrations of adenine nucleotides. Both ischaemia (Burch, 1965) and anoxia (Mäenpää, 1967) decrease the phosphorylation of the hepatic adenylate pool. Similar results were found in the present study when rats were exposed to complete anoxia, but the air content of the atmosphere could be lowered to 19% without any significant changes in the proportions of adenine nucleotides. Rats preincubated for up to 30 min under a 19% air atmosphere and then left for an additional 15 min, after injection of aspartate had hepatic proportions of adenine nucleotides that were similar to those found in normal animals. Evidently the animal can adapt to this severe hypoxia, presumably by decreasing the expenditure of ATP. An effective way to lower ATP utilization would be to decrease the gluconeogenic flux since this pathway can account for a large fraction of the ATP generated (Williamson, Scholz & Browning, 1969b). It is likely

Table 7. *Concentration of NAD⁺ and NADH in livers of normal and anoxic animals*

Animals were treated and injected with glucose precursors as described in the legends to Tables 3, 4 and 5. Values are expressed as $\mu\text{mol/g}$ of liver and are presented as the means $\pm$ S.E.M for determinations on six to eight animals.

Injected precursor	Air (%)	[NAD ⁺]	[NADH]	[NAD ⁺]/[NADH] ratio
Serine	100	0.268 $\pm$ 0.012	0.082 $\pm$ 0.009	3.50 $\pm$ 0.37
	0	0.257 $\pm$ 0.029	0.085 $\pm$ 0.005	3.09 $\pm$ 0.41
Lactate	100	0.299 $\pm$ 0.031	0.078 $\pm$ 0.010	4.45 $\pm$ 0.19
	0	0.278 $\pm$ 0.023	0.095 $\pm$ 0.006	2.76 $\pm$ 0.23
Aspartate	100	0.286 $\pm$ 0.013	0.065 $\pm$ 0.004	3.98 $\pm$ 0.40
	0	0.262 $\pm$ 0.013	0.099 $\pm$ 0.012	2.93 $\pm$ 0.18

Table 8. *Effects of hypoxic conditions on NAD⁺-NADH redox potentials in liver*

Animals were treated and injected with glucose precursors as described in the legends to Tables 3 and 4. The redox potentials given by Williamson, Browning, Scholz, Kreisberg & Fritz (1968) and the assumptions and equilibrium constants of Williamson *et al.* (1967) were used. Potentials are expressed in mV.

NAD ⁺ –NADH redox potentials									
Precursor	from:	Air (%)	...	100	50	30	19	12	0
Serine	[Lactate]/[pyruvate] ratio			–250	–250	–250	–256	–258	–269
	[β -Hydroxybutyrate]/[acetoacetate] ratio			–296	–295	–299	–308	–312	–323
	Difference			46	45	49	52	54	54
Aspartate	[Lactate]/[pyruvate] ratio			–252	–250	–253	–260	–264	–277
	[β -Hydroxybutyrate]/[acetoacetate] ratio			–301	–305	–307	–319	–327	–338
	Difference			49	55	54	59	63	61

that gluconeogenesis uses much of the ATP formed in livers of 2-day-old animals because the diet is rich in protein and fat so that much of the glucose needed must be synthesized *de novo*. Further, measurements with liver slices from rats of different ages indicate a maximum gluconeogenic potential at about this age (Ballard & Oliver, 1963, 1965). Obviously the suppression of gluconeogenesis could help maintain a highly phosphorylated pool of adenine nucleotides.

It is also apparent that the degree of phosphorylation of adenine nucleotides in whole liver is not regulating gluconeogenesis. Possibly the hypoxic conditions might cause a decrease in the mitochondrial pool of ATP if this pool is small relative to the content of whole cells. Such a change might not be detectable in measurements on whole tissue, especially if it were countered by a small increase in ATP in the other cell compartments. Indeed it is difficult to imagine how hypoxic conditions could depress gluconeogenesis or any other process unless oxidative phosphorylation were impaired. A decrease in the degree of adenine nucleotide phosphorylation, if confined to mitochondria, could explain the inhibition of glucose synthesis from lactate or serine. However, with injected aspartate a fall in the concentration of mitochondrial ATP could not

itself cause a decrease in gluconeogenesis, since no mitochondrial ATP-utilizing reactions are involved in glucose synthesis from this precursor.

Redox measurements. With all three precursors the use of hypoxic conditions that were severe enough to inhibit gluconeogenesis also caused marked falls in the mitochondrial [NAD⁺]/[NADH] ratio. Presumably this change was the result of a lower rate of oxidative phosphorylation and thus a restricted mitochondrial reoxidation of NADH. An accumulation of NADH in mitochondria could hinder gluconeogenesis in several ways, but not all would be applicable to the three substrates used in the present study. First, an increase in the [NADH]/[NAD⁺] ratio may depress the activity of the tricarboxylic acid cycle by inhibition of dehydrogenase reactions. Secondly, transamination of aspartate in the cytosol via aspartate aminotransferase will generate glutamate. A highly reduced mitochondrial redox state may prevent the oxidative deamination of glutamate to α -oxoglutarate so that α -oxoglutarate would not be formed for transamination of additional aspartate in the cytosol. Thirdly, an increase in mitochondrial redox state may cause an increase in the [NADH]/[NAD⁺] ratio outside the mitochondria.

One or all of the above hypotheses may account

for the correlation between $[NADH]/[NAD^+]$ ratios and decreased gluconeogenesis. Certainly the third suggestion is supported by the experimental findings as changes in cytosol redox state do indeed accompany the alterations in mitochondrial redox state. When expressed as NAD^+ - $NADH$ redox potentials (Table 8) it can be seen that the NAD pool in both cellular compartments becomes more reduced when the degree of hypoxia is increased. The difference in NAD^+ - $NADH$ redox potential between mitochondria and cytosol is about 45–50 mV under oxygenated conditions. It increases with hypoxia to 54 mV when serine was precursor and over 60 mV with injected aspartate. Evidently the more severe the hypoxic condition the more reduced the mitochondrial NAD pool becomes relative to the cytosol pool. This is consistent with mitochondrial oxidative phosphorylation being depressed under the various hypoxic states to produce a secondary change in the cytosol.

Inhibition of gluconeogenesis by a more reduced redox state could occur directly, via reactions inhibited by $NADH$, or indirectly if the changes in redox state caused alterations in the concentrations of key intermediates in the gluconeogenic sequence. Such changes must be applicable to glucose synthesis from aspartate, serine and lactate, since hypoxia lowered gluconeogenesis from all three precursors. A fall in the oxaloacetate concentration may fulfill these conditions as this is the first unique intermediate in glucose synthesis from all precursors and the concentration of oxaloacetate in liver is below the Michaelis constant for phosphoenolpyruvate carboxykinase (Williamson, Veloso, Ellington & Krebs, 1969; Ballard, 1970b).

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