Mechanisms of elevation of adenosine levels in anoxic hepatocytes

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Previous work has shown that normoxic isolated rat hepatocytes continuously produce adenosine from AMP and that the nucleoside is not catabolized further but immediately rephosphorylated by adenosine kinase [Bontemps, Van den Berghe and Hers (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2829–2833]. We now report the effect of anoxia on adenosine production and on the AMP/adenosine substrate cycle. In cell suspensions incubated in O_2/CO_2 , the adenosine concentration was about $0.4 \mu M$. It increased 30-fold in cells incubated in N_2/CO_2 or with 5 mM KCN, and 20-fold in cells incubated with 2 mM amytal. Adenosine production, measured in hepatocytes in which adenosine kinase and adenosine deaminase were inhibited by 5-iodotubercidin and deoxycoformycin respectively, was about 18 nmol/min per g of cells in normoxia; it increased about 2-fold in anoxia, although AMP increased 8–16-fold in this condition. From studies with inhibitors of membrane 5'-nucleotidase and of S-

adenosylhomocysteine hydrolase, it was deduced that adenosine is produced by the latter enzyme and by cytosolic 5'-nucleotidase in normoxia, and by cytosolic and membrane 5'-nucleotidases in anoxia. Unlike in normoxic hepatocytes, inhibition of adenosine kinase by 5-iodotubercidin neither elevated the adenosine concentration nor enhanced total purine release from adenine nucleotides in cells treated with N_2/CO_2 or KCN; it had only a slight effect in cells treated with amytal. This indicates that recycling of adenosine is suppressed or profoundly inhibited in anoxia. The rate of accumulation of adenosine in anoxia was several-fold lower than the rate of its rephosphorylation upon reoxygenation. It is concluded that the elevation of adenosine in anoxic hepatocytes is much more dependent on decreased recycling of adenosine by adenosine kinase than on increased production by dephosphorylation of AMP.

INTRODUCTION

Over the last 15 years, evidence has accumulated that the endogenous nucleoside, adenosine, plays an important regulatory role in several organs (Arch and Newsholme, 1978a; Fox and Kelley, 1978; Ohisalo, 1987). One of the major functions of adenosine is vasodilation, resulting from its elevation in response to hypoxia and aimed at restoration of the oxygen supply (Berne, 1980). A hypoxia-induced elevation of liver adenosine (Belloni et al., 1985; Arnold and Cysyk, 1986) and the role of adenosine as a regulator of hepatic arterial blood flow (Lautt et al., 1985; Ezzat and Lautt, 1987) have been demonstrated. However, the mechanisms whereby oxygen deprivation increases the concentration of hepatic adenosine have not been investigated in full detail. Adenosine can be produced by dephosphorylation of intracellular AMP by cytosolic 5'-nucleotidase(s), or of extracellularly released AMP by membrane 5'-nucleotidase. It can also be formed by hydrolysis of S-adenosylhomocysteine (S-AdoHcy), a product of the transmethylation pathway. Adenosine can be rephosphorylated into AMP by adenosine kinase, and catabolized into inosine by adenosine deaminase. The latter is followed by conversion into the purine catabolites hypoxanthine, xanthine and uric acid or allantoin, depending on tissue and species. We have previously demonstrated the existence of a substrate cycle between AMP and adenosine in normoxic isolated rat hepatocytes (Bontemps et al., 1983). These cells were found to produce adenosine continuously from AMP, but the nucleoside was shown to be immediately and completely rephosphorylated into AMP by adenosine kinase, rather than being converted into allantoin, the terminal purine catabolite in rat liver. This may be explained by the fact that the affinity of adenosine kinase for

adenosine is more than one order of magnitude higher than that of adenosine deaminase (Arch and Newsholme, 1978b). As postulated by Arch and Newsholme (1978a), an adenosine/AMP cycle would allow a rapid and precise regulation of adenosine concentration. The purpose of the present work was to investigate the effects of anoxia on the various mechanisms of production of adenosine and on its recycling in isolated hepatocytes.

MATERIALS AND METHODS

Chemicals

[U- ^{14}C]Adenine (270 Ci/mol) was purchased from Amersham International (Amersham, Bucks., U.K.). 5-Iodotubercidin (ITu) was from Research Biochemicals Inc. (Natick, MA, U.S.A.) and deoxycoformycin (dCF) was from Warner-Lambert (Detroit, MI, U.S.A.). Adenosine dialdehyde, adenosine 5'-[$\alpha\beta$ -methylene]diphosphate (p[CH $_2$]pA) and BSA (fraction V, essentially fatty-acid free) were purchased from Sigma (St. Louis, MO, U.S.A.). Albumin was dialysed overnight against 25 vol. of Krebs–Ringer bicarbonate buffer (KRB), with two buffer changes. Other compounds were the highest grade commercially available.

Preparation and incubation of isolated hepatocytes

Hepatocytes were isolated as previously described (Van den Berghe et al., 1980) from male Wistar rats which were fasted overnight unless indicated otherwise. The cells were resuspended in KRB gassed with O_2/CO_2 (19:1) and supplemented with 10 mM glucose and 1% BSA. The concentration of the hepatocytes (approx. 50 mg of cells/ml) was determined at the beginning

Abbreviations used: p[CH $_2$]pA, adenosine 5'-[$\alpha\beta$ -methylene]diphosphate; dCF, deoxycoformycin; ITu, 5-iodotubercidin; KRB, Krebs–Ringer bicarbonate buffer; S-AdoHcy, S-adenosylhomocysteine.

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of the incubations by weighing the pellet obtained by centrifuging 1 ml of the cell suspension. Cells were preincubated for 15 min before induction of anoxia, either by replacing O_2/CO_2 by N_2/CO_2 (19:1), or by addition of 5 mM KCN or 2 mM amytal at time zero. In some experiments, 1 μM [$U-^{14}C$]adenine was added 5 min before time zero, to label the adenine nucleotide pool as described previously (Van den Berghe et al., 1980). When used, dCF and adenosine dialdehyde, tight-binding inhibitors of adenosine deaminase (Henderson et al., 1977) and of S-AdoHcy hydrolase (Bartel and Borchardt, 1984) respectively, were added 15 min before time zero, whereas ITu, a potent inhibitor of adenosine kinase (Henderson et al., 1972) was added at time zero. Preliminary experiments had shown that 0.05 μM dCF inhibited by more than 90% the deamination of 100 μM [$8-^{14}C$]adenosine added to hepatocyte suspensions, but had no effect on either AMP deaminase activity or the production of allantoin, which in normoxic hepatocytes is derived only from the deamination of AMP (Van den Berghe et al., 1980). Inactivation of S-AdoHcy hydrolase by adenosine dialdehyde was tested as follows. Isolated hepatocytes were incubated for 15 min in the presence of the inhibitor, separated from the incubation medium, frozen, and resuspended in buffer for assay according to Hershfield (1979). The addition of 20 μM adenosine dialdehyde to the hepatocyte suspension was found to inhibit the activity of S-AdoHcy hydrolase by 99%.

Analytical methods

Metabolites were measured as described before (Bontemps et al., 1983) in perchloric acid extracts prepared from the whole-cell suspension or, when indicated, from the extracellular medium after centrifugation to pellet the cells. ATP, ADP and AMP were separated and quantified by h.p.l.c. on a 12.5 cm $\times$ 0.46 cm Partisphere 5 SAX column (Whatman, Maidstone, Kent, U.K.) by the method of Hartwick and Brown (1975). Adenosine was measured by reversed-phase h.p.l.c. on a 12.5 cm $\times$ 0.46 cm Partisphere C18 column (Whatman) eluted at a flow rate of 1.4 ml/min with 10 mM NaH_2PO_4 , pH 5.5, and a 0–10% gradient of methanol over 15 min. When cells had been preincubated with [$U-^{14}C$]adenine, the radioactivity in the adenine nucleotides and in their degradation products was determined by t.l.c. as described previously (Van den Berghe et al., 1980). The amounts of nucleosides and bases produced from the adenine nucleotides were calculated from their specific radioactivities at time zero. Lactate dehydrogenase was measured by the method of Vassault (1983).

Results are given as means $\pm$ S.E.M. Representative experiments, when shown, are typical of at least three studies that gave similar results.

RESULTS

Effects of N_2/CO_2 , KCN and amytal on the concentration of adenosine

When hepatocytes isolated from fasted rats were incubated in O_2/CO_2 , the concentration of adenosine in the cell suspension was about 0.4 nmol/ml (Table 1). When O_2/CO_2 was replaced by N_2/CO_2 , adenosine increased 30-fold, to about 12 nmol/ml after 20 min of anoxia. Addition of 5 mM KCN or 2 mM amytal, which inhibit mitochondrial ATP synthesis at sites III and I respectively, also induced an elevation of adenosine, which was less pronounced with amytal than with KCN. With all three experimental conditions, elevation of adenosine was associated

with a decrease in ATP and an increase in AMP. Both changes were more marked with N_2/CO_2 and KCN than with amytal. The concentrations of adenosine measured in the extracellular medium were not significantly different from those in the total cell suspension, except in the control condition in which adenosine was about 2-fold lower in the extracellular medium than in the total suspension (results not shown). This can be explained by partial binding of adenosine inside the hepatocytes, as reported by Ueland and Helland (1983).

In suspensions of hepatocytes isolated from fed rats, an increase in adenosine was also recorded upon incubation with N_2/CO_2 , KCN or amytal. In the fed condition, however, this elevation was 2–5-fold lower than in cells from fasted rats and, as already shown previously (Vincent et al., 1982), the decrease in ATP and the increase in AMP were also less pronounced (results not shown). The better maintenance of ATP levels in the fed state may be explained by the presence of glycogen stores, allowing the generation of ATP by glycolysis.

Rates and sources of adenosine production

Absolute rates of production of adenosine can be measured by inhibiting both the catabolism of the nucleoside by adenosine deaminase and its recycling by adenosine kinase. Results from experiments performed in the presence of ITu, a potent inhibitor of adenosine kinase (Henderson et al., 1972), and dCF, a tight-binding inhibitor of adenosine deaminase (Henderson et al., 1977), showed that adenosine accumulated nearly linearly in this condition, at a rate of 18.0 ± 0.9 nmol/min per g of cells in O_2/CO_2 ($n = 10$). Strikingly, the marked 8–16-fold elevations in AMP recorded under anoxic conditions (Table 1) resulted in much less pronounced increases in the rate of production of adenosine, namely 35.4 ± 2.0 nmol/min per g of cells in the presence of N_2/CO_2 ($n = 10$), 33.1 ± 1.6 nmol/min per g in the presence of KCN ($n = 3$) and 23.9 ± 0.5 nmol/min per g in the presence of amytal ($n = 3$). Rates were similar whether measured in the total cell suspension or in the extracellular medium, indicating that adenosine exited easily from the cells under all conditions.

To assess the contribution of the transmethylation pathway to the production of adenosine, hepatocytes were incubated in the presence of dCF, ITu and adenosine dialdehyde, a potent tight-binding inhibitor of S-AdoHcy hydrolase (Bartel and Borchardt, 1984). As shown in Figure 1(a), the production of adenosine measured in the presence of dCF and ITu was nearly halved by adenosine dialdehyde in normoxic hepatocytes. In contrast, the inhibitor had no significant effect in anoxic cells (Figure 1b), notwithstanding the complete inhibition of S-AdoHcy hydrolase,

Table 1 Influence of anoxia and inhibitors of mitochondrial ATP synthesis on the concentrations of adenosine and adenine nucleotides in suspensions of hepatocytes isolated from fasted rats

Samples were taken 20 min after additions. Adenine nucleotides (expressed per g of cells) and adenosine (expressed per ml of suspension) were determined by h.p.l.c. Values are means $\pm$ S.E.M. for $n = 5$.

Conditions	Adenosine (nmol/ml)	ATP (μ mol/g)	ADP (μ mol/g)	AMP (μ mol/g)
O_2/CO_2	0.4 ± 0.1	2.26 ± 0.15	0.68 ± 0.03	0.12 ± 0.01
N_2/CO_2	11.8 ± 2.1	0.03 ± 0.01	0.18 ± 0.02	2.01 ± 0.11
5 mM KCN	12.3 ± 2.9	0.04 ± 0.01	0.27 ± 0.02	1.99 ± 0.05
2 mM Amytal	7.5 ± 1.4	0.46 ± 0.11	0.92 ± 0.07	0.99 ± 0.05

Figure 1 Effect of adenosine dialdehyde on the production of adenosine in O₂/CO₂ (○, ●) or in N₂/CO₂ (▲, ■)

Hepatocytes were incubated in the presence of 0.05 μM dCF and 100 μM ITu, without (○, △) or with (●, ▲) 20 μM adenosine dialdehyde. Adenosine dialdehyde was added 15 min before zero time. Adenosine was measured by h.p.l.c. Results are means ± S.E.M. of three separate experiments.

as evidenced by its assay 15 min after addition of N₂/CO₂ (results not shown). This indicates that the transmethylation pathway contributed about 40% of the production of adenosine in normoxia, but not at all to the production of the nucleoside in anoxia.

Formation and recycling of adenosine from prelabelled adenine nucleotides

Since the results reported in the previous section showed that the production of adenosine in anoxia was derived exclusively from the dephosphorylation of AMP, further studies were aimed at assessing the AMP/adenosine cycle under this condition. For this purpose, experiments were performed in which the hepatocyte adenine nucleotide pool was prelabelled with [¹⁴C]adenine. Previous studies have shown that, owing to the high activity of

adenylate kinase, this procedure results in identical specific radioactivities of ATP, ADP and AMP (Van den Berghe et al., 1980; Bontemps et al., 1983). Labelling of the adenine nucleotide pool allows one to determine the concentrations of all its catabolites, including allantoin, which is not detectable by u.v. absorption. In addition, it permits measurement of the production of xanthine, uric acid and allantoin from adenylates, without interference by catabolites of the guanylates, which remain unlabelled.

Experiments with N₂/CO₂

Figure 2, depicting a representative experiment, shows a time course of the effect of the replacement of O₂/CO₂ by N₂/CO₂ on the accumulation of [¹⁴C]adenosine and of the other ¹⁴C-labelled catabolites derived from the labelled adenine nucleotides. In the absence of inhibitors, labelled adenosine reached a steady state after 10–20 min (Figure 2a). The other catabolites continued to build up over the whole duration of the experiment (Figure 2b). The prominent catabolites which accumulated in the anoxic cell suspension were hypoxanthine and uric acid, which were produced at about equal rates. As shown previously (Vincent et al., 1982), uric acid accumulated instead of allantoin, the terminal product of purine catabolism in rat liver, owing to the oxygen-dependence of urate oxidase (Keilin and Hartree, 1936). That hypoxanthine rather than xanthine accumulated is probably explained by the anoxia-induced increase in the NADH/NAD⁺ ratio, which is known to inhibit xanthine dehydrogenase activity (Kaminski and Jezewska, 1981). In accordance with this interpretation, addition of 10 mM pyruvate to the anoxic cell suspension resulted in the conversion of hypoxanthine into uric acid (results not shown). The production of the sum of the labelled adenine nucleotide catabolites was increased about 10-fold by anoxia, from 8.3 ± 4.2 nmol/45 min per ml of suspension (*n* = 4) in O₂/CO₂ (not shown) to 97.4 ± 9.7 nmol/45 min per ml of suspension (*n* = 8) in N₂/CO₂ (Figure 2c).

When dCF had been added to the suspension prior to induction of anoxia, [¹⁴C]adenosine did not reach a steady state but accumulated over the whole duration of the experiment (Figure 2a). After the first 10 min of anoxia, the production of the other

Figure 2 Effect of dCF and ITu on the production of [¹⁴C]nucleosides and bases from [¹⁴C]adenine nucleotides in the presence of N₂/CO₂

Hepatocytes were incubated without inhibitors (○), or with 100 μM ITu (●), 0.05 μM dCF (▲) or both (■). Catabolites were determined by t.l.c. as described in the Materials and methods section. Ado, adenosine; Ino, inosine; HX, hypoxanthine; X, xanthine; UA, uric acid. Panel (c) represents the sum of the catabolites of panels (a) and (b).

Figure 3 Effect of dCF and ITu on the production of [^{14}C]nucleosides and bases from [^{14}C]adenine nucleotides in the presence of 2 mM amytal

Hepatocytes were incubated without inhibitors (○), or with 100 μM ITu (●), 0.05 μM dCF (▲) or both (■). Catabolites were determined by t.l.c. as described in the Materials and methods section. Ado, adenosine; Ino, inosine; HX, hypoxanthine; X, xanthine; UA, uric acid; AL, allantoin. Panel (c) represents the sum of the catabolites of panels (a) and (b).

labelled purine catabolites was equivalently decreased (Figure 2b). Accordingly, the sum of all catabolites was not modified (Figure 2c). In normoxic hepatocytes, inhibition of adenosine deaminase does not provoke an accumulation of adenosine (Van den Berghe et al., 1980), owing to recycling of the nucleoside by adenosine kinase (Bontemps et al., 1983). It was thus suspected that anoxia decreased the activity of the latter enzyme.

To evaluate the activity of adenosine kinase, experiments were performed with ITu. When this adenosine kinase inhibitor was present alone, the pattern of production of [^{14}C]adenosine (Figure 2a) and the other purine catabolites (Figure 2b) evolved exactly as in its absence. Furthermore, ITu neither accelerated the decrease in ATP nor diminished the sum of the adenine nucleotides (results not shown), as it does in normoxic hepatocytes (Bontemps et al., 1983). The absence of an effect of ITu on the catabolism of adenine nucleotides induced by N_2/CO_2 could not be explained by a decreased penetration of ITu into the anoxic hepatocytes. Indeed, h.p.l.c. analyses performed after separation of the cells from the extracellular medium showed that the intracellular concentration of ITu was identical in cells incubated in O_2/CO_2 and in N_2/CO_2 (results not shown). The absence of an effect of ITu thus demonstrated that adenosine kinase is inactive under anoxic conditions. As a consequence, adenosine is not recycled into AMP, but undergoes deamination by adenosine deaminase followed by conversion into uric acid.

In the presence of both ITu and dCF, both [^{14}C]adenosine (Figure 2a) and the other ^{14}C -labelled purine catabolites (Figure 2b) were produced as with dCF alone. Under all conditions, rates of adenosine production calculated from the accumulation of [^{14}C]adenosine were in good agreement with those measured by h.p.l.c. in the same experiment.

Experiments with KCN

Experiments identical to that described in Figure 2 but performed with 5 mM KCN gave results similar to those obtained with N_2/CO_2 (results not shown). Inhibition of adenosine deaminase by dCF provoked an accumulation of [^{14}C]adenosine and an equivalent decrease in the production of hypoxanthine, the terminal product of adenine nucleotide catabolism in the presence

of KCN, owing to the inhibitory effect of KCN on xanthine dehydrogenase (Fridovich and Handler, 1958). On the other hand ITu, alone or together with dCF, influenced neither the accumulation of [^{14}C]adenosine nor that of the other labelled purine catabolites.

Experiments with amytal

Whereas ATP nearly completely disappeared in the presence of N_2/CO_2 or KCN in hepatocytes isolated from fasted rats, it remained at about 0.5 mM in the presence of 2 mM amytal (Table 1). Complete inactivity of adenosine kinase would thus not be expected under this condition. Accordingly, as shown in Figure 3 (depicting a representative experiment), ITu slightly increased the steady-state level of adenosine (Figure 3a) as well as the production of the other catabolites (Figure 3b). An ITu-induced loss of about 30% of the adenine nucleotide pool was also recorded (results not shown). When comparing different experiments, the increases in adenosine levels and catabolite production induced by ITu were higher when ATP was closer to 0.6 than to 0.4 mM, indicating that the activity of adenosine kinase was critically dependent on ATP in this concentration range. In contrast to what occurred in the presence of N_2/CO_2 or KCN, dCF had very little effect on the level of [^{14}C]adenosine (Figure 3a) whereas, like N_2/CO_2 and KCN, it decreased the production of the other catabolites, mainly (> 90%) allantoin (Figure 3b), and consequently decreased total [^{14}C]purine release (Figure 3c). If ITu was added together with dCF, [^{14}C]adenosine accumulated to much higher levels than with dCF alone (Figure 3a), whereas the production of allantoin was not modified (Figure 3b). This result indicated that adenosine did not accumulate in the presence of dCF alone, owing to its rephosphorylation by adenosine kinase. Taken together, the effects of ITu on adenine nucleotide catabolism in the presence of amytal indicate that adenosine kinase remains sufficiently active in this condition to allow some phosphorylation of adenosine to proceed. However, recycling becomes insufficient to salvage all of the adenosine produced, so that a portion of it is deaminated, as demonstrated by the results obtained in the presence of dCF.

Figure 4 Effect of p[CH₂]pA and β-glycerophosphate on the production of adenosine and on the accumulation of extracellular AMP in O₂/CO₂ (○, ●) or in N₂/CO₂ (Δ, ▲)

Hepatocytes were incubated in the presence of 0.05 μM dCF and 100 μM ITu, without (○, △) or with (●, ▲) a mixture containing 2.5 mM p[CH₂]pA and 10 mM β-glycerophosphate. Adenosine (a) and AMP (b) were determined in the extracellular medium by h.p.l.c. AMP was undetectable in the absence of p[CH₂]pA. Lactate dehydrogenase (LDH) was measured in the total suspension and in the extracellular medium. Results are means ± S.E.M. of three separate experiments.

Role of membrane 5'-nucleotidase and aspecific phosphatases

Adenosine can be produced from AMP by cytosolic 5'-nucleotidase(s) acting on intracellular AMP, but also by membrane ecto-5'-nucleotidase and by various phosphatases acting on nucleotides released extracellularly from broken cells. This extracellular formation of adenosine can be inhibited, as already described (Bontemps et al., 1983), by a mixture of β-glycerophosphate, a competitive inhibitor of aspecific phosphatases, and p[CH₂]pA, a potent inhibitor of membrane 5'-nucleotidase (Burger and Lowenstein, 1975). Figure 4(a) depicts the effect of a combination of both inhibitors on the accumulation of adenosine in the extracellular medium in the presence of dCF and ITu. In normoxic conditions, the inhibitory mixture decreased the rate of accumulation of adenosine by 15–20%. In contrast, a 40% reduction was recorded in N₂/CO₂. The same results were recorded if adenosine was measured in the total cell suspension (not shown). In both normoxic and anoxic hepatocytes, extracellular AMP was undetectable in the absence of p[CH₂]pA.

Figure 5 Influence of anoxia followed by reoxygenation on the concentrations of adenosine and adenine nucleotides

Hepatocytes were incubated in the presence of N₂/CO₂ without inhibitors (○), with 100 μM ITu (●) or with 0.05 μM dCF (▲). After 30 min of anoxia, N₂/CO₂ was replaced by O₂/CO₂. Metabolites were determined by h.p.l.c. AMP concentrations are represented by broken lines.

However, in the presence of the latter, AMP did accumulate (Figure 4b). This accumulation was 3–4-fold higher in anoxic conditions than in normoxia. As shown in Figure 4(c) this greater build-up of AMP was associated with a greater release of lactate dehydrogenase into the extracellular medium. Inhibition of mitochondrial ATP synthesis by amyltal or KCN was also found to be associated with 50% and 300–400% increases respectively in the release of lactate dehydrogenase into the extracellular medium (results not shown).

Effects of reoxygenation

Accumulated AMP is promptly reconverted into ATP when anoxic hepatocytes are reoxygenated (Vincent et al., 1982). The effect of reoxygenation on the fate of adenosine was therefore investigated. In the representative experiment depicted in Figure 5, hepatocytes isolated from a fasted rat were first incubated in the presence of N₂/CO₂, without inhibitors, or with ITu or dCF. When N₂/CO₂ was replaced by O₂/CO₂ after 30 min, adenosine which had accumulated during anoxia nearly completely disappeared in a few minutes, except in the presence of ITu (Figure 5a). The disappearance of adenosine following reoxygenation

was associated, in the presence of dCF, with a higher recovery of ATP (Figure 5b) and of the adenine nucleotide pool (Figure 5c). That this higher recovery of ATP and of the adenine nucleotides was not observed in the presence of ITu indicates that it results from the phosphorylation of adenosine by adenosine kinase. In the presence of dCF, a comparison of the rates of adenosine accumulation in N_2/CO_2 and of adenosine disappearance after reoxygenation (Figure 5a) clearly demonstrated that the rate at which hepatocytes can phosphorylate adenosine is several-fold higher than their capacity to produce this nucleoside.

DISCUSSION

In accordance with experiments by others using isolated rat hepatocytes (Belloni et al., 1985) and in perfused rat liver (Arnold and Cysyk, 1986), we found that the concentration of adenosine, which remains constant at about $0.4 \mu M$ in normoxic hepatocyte suspensions, increases up to $12 \mu M$ in cells made anoxic (Table 1). In the present study, we have identified the enzyme(s) involved in the hepatic formation of adenosine under anoxic conditions and investigated the influence of anoxia on the substrate cycle operating between AMP and adenosine. These two points will be discussed separately.

Enzymes involved in the anoxic formation of adenosine

As quoted in the Introduction, adenosine can be produced intracellularly by cytosolic 5'-nucleotidase(s) and by S-AdoHcy hydrolase, or extracellularly by membrane 5'-nucleotidase acting on extracellularly released nucleotides. The participation of each of these enzymes can be assessed with the use of specific inhibitors, i.e. in cells in which the utilization of adenosine by adenosine deaminase and adenosine kinase has been blocked by dCF and ITu respectively, as reported by others (Newby et al., 1983; Worku and Newby, 1983; Meghji et al., 1988).

As indicated by the experiments in which S-AdoHcy hydrolase was inactivated with adenosine dialdehyde (Figure 1), this enzyme contributes to the production of adenosine in normoxic, but not in anoxic, hepatocytes. The importance of S-AdoHcy hydrolase for the normoxic production of adenosine has already been shown in heart (Achterberg et al., 1985; Lloyd et al., 1988). From the data depicted in Figure 1(a), one can calculate that this enzyme contributes close to 7 nmol/min per g, or about 40%, of the total adenosine production in normoxic liver cells. The rate of production is of the same order of magnitude as the rate of transmethylation measured in mouse liver *in situ* (Hoffman, 1980). On the other hand, the lack of inhibition of the production of adenosine by adenosine dialdehyde in anoxic cells (Figure 1b) shows that hydrolysis of S-AdoHcy is not a source of adenosine under these conditions. This indicates that transmethylation is arrested in anoxia, as expected from the ATP-dependence of the activation of methionine.

The finding that inhibition of membrane ecto-5'-nucleotidase and of aspecific phosphatases decreased the production of adenosine by 15–20% in normoxia (Figure 4a) indicates that there is a small contribution of extracellularly released AMP to this production, as suggested by the accumulation of the latter under the same conditions (Figure 4b). Hepatocytes (Van Schaftingen et al., 1987), similarly to other cell types (Kather, 1988; van Waeg and Van den Berghe, 1991), are indeed known to release small amounts of nucleotides extracellularly. This is most probably due to unavoidable cell breakage during the incubations, as shown by leakage of lactate dehydrogenase from the cells (Figure 4c). The contribution of extracellularly released AMP to the production of adenosine increased to 30–40% in anoxia, in

accordance with the more marked elevation of the concentrations of AMP and lactate dehydrogenase in the extracellular medium, reflecting increased cell breakage under these conditions.

Despite a sizeable contribution of S-AdoHcy hydrolase to the production of adenosine in normoxia, and of ecto-5'-nucleotidase in anoxia, our studies show that, under both conditions, a major proportion of the adenosine produced is derived from the action of cytosolic 5'-nucleotidase(s) on intracellular AMP. Two cytosolic purine 5'-nucleotidases are found in mammalian and avian tissues: the IMP-GMP 5'-nucleotidase, which exhibits a preference for the indicated substrates (Van den Berghe et al., 1977; Itoh et al., 1978; Itoh, 1981; Bontemps et al., 1988), and an AMP-specific cytosolic 5'-nucleotidase described in rat (Truong et al., 1988) and pigeon (Skladanowski and Newby, 1990) heart. When compared with the 20-fold elevation of intracellular AMP provoked by N_2/CO_2 and KCN, and with the 10-fold elevation induced by amytal (Table 1), the 2-fold increases in absolute adenosine production recorded under anoxic conditions (from 18 nmol/min per g of cells in normoxia, to about 35 nmol/min per g of cells in N_2/CO_2) are strikingly low. This could be explained by the kinetic properties of the cytosolic 5'-nucleotidases. Both have low affinity for AMP, with K_m values in the millimolar range, but in addition are subjected to stimulation by ATP and inhibition by P_i , although the latter effect is much more pronounced with the IMP-GMP 5'-nucleotidase than with the AMP-specific 5'-nucleotidase. The effect of the elevation of AMP on the activity of the cytosolic 5'-nucleotidases could thus be counteracted by the anoxia-induced decrease of ATP and elevation of P_i (Vincent et al., 1982). The presence of the AMP-specific 5'-nucleotidase in rat liver, and its participation in the hepatic production of adenosine, remain to be investigated.

Influence of anoxia on the AMP/adenosine cycle

As postulated in the Introduction, the 30-fold elevation in the steady-state level of adenosine in anoxic hepatocytes may be due to an increase in its production and/or to a decrease in its recycling into AMP. As discussed in the previous paragraph, anoxia induces a barely 2-fold increase in the production of adenosine, which is derived exclusively from AMP, in major part via cytosolic 5'-nucleotidase(s). This small increase suggested that the elevation of adenosine induced by anoxia or by conditions which impair oxidative phosphorylation is mainly caused by a decreased recycling of adenosine by adenosine kinase. Indeed, in normoxic cells, inhibition of adenosine kinase elevates the concentration of adenosine, because it suppresses its recycling (Bontemps et al., 1983).

Experiments with ITu in anoxic cells confirmed this suggestion. In N_2/CO_2 and with KCN, the absence of an effect of ITu on the accumulation of adenosine, on the release of other purine catabolites and on the loss of adenine nucleotides, indicates that the phosphorylation and thus recycling of adenosine were completely suppressed under these conditions. This is most likely explained by the near-complete loss of ATP (Table 1), the substrate of adenosine kinase, and by the increase in AMP, which is known to inhibit the enzyme (Palella et al., 1980; Rotllan and Miras Portugal, 1985; Hawkins and Bagnara, 1987). As a consequence of its elevation, adenosine is deaminated into inosine by adenosine deaminase. Deamination of adenosine occurs in anoxic cells, as demonstrated by the fact that inhibition of adenosine deaminase by dCF induces, after 10–15 min, an enhancement of the accumulation of adenosine (Figure 2a) and a proportional decrease, up to near-complete suppression, of the release of the other purine catabolites (Figure 2b). The observation that $0.05 \mu M$ dCF had a more pronounced effect on the

accumulation of adenosine in the present work (Figure 2a) than did 0.1 μ M coformycin in our previous studies (Figure 2 in Vincent et al., 1982) suggests that adenosine deaminase was not sufficiently inhibited by the latter inhibitor. As described by Deuticke and Gerlach (1966) and Vincent et al. (1982), and as indicated by the 10 min latency of the effect of dCF (Figure 2b), the catabolism of liver AMP induced by anoxia occurs mainly by way of AMP deaminase in the first few minutes. The present work shows that, subsequently, catabolism proceeds nearly exclusively by means of dephosphorylation of AMP.

In the presence of amytal some recycling of adenosine may still occur, as shown by the slight increase induced by ITu of both the steady-state level of adenosine and the release of the other purine catabolites (Figures 3a and 3b). Nevertheless, recycling of adenosine by adenosine kinase is not complete, as indicated by the elevation of the nucleoside and by the inhibitory effect of dCF on the release of other purine catabolites (Figure 3b and 3c). Adenosine produced by the hepatocytes in the presence of amytal is thus partly phosphorylated and partly deaminated. The response to amytal probably reflects what would occur in anoxic hepatocytes from fed rats, in which ATP is decreased to a lesser extent than in hepatocytes from fasted animals (Vincent et al., 1982).

The reoxygenation experiment (Figure 5) shows that adenosine promptly disappears when N_2/CO_2 is replaced by O_2/CO_2 , except if the adenosine kinase inhibitor, ITu, is present. In the latter condition, despite the return of AMP levels to a 'normal' value (Figure 5b), the steady-state level of adenosine is barely modified (Figure 5a), demonstrating the crucial role of adenosine kinase in the removal of adenosine. The better recovery of ATP and of the total adenine nucleotide pool after reoxygenation in the absence of ITu, particularly in the presence of dCF, suggests, moreover, that adenosine may be a good precursor for the post-ischaemic resynthesis of ATP in the liver, as also observed in canine heart (Henrichs et al., 1988) and in renal tubular cells (Cadnapaphornchai et al., 1991). On the other hand, a comparison of the rate of accumulation of adenosine in the presence of N_2/CO_2 and dCF with the rate of its disappearance after reoxygenation clearly shows that hepatocytes can phosphorylate adenosine much more rapidly than they produce it. This observation implies that the increase in the production of adenosine has to be very large to exceed the rate of its phosphorylation and to bring about an increase in the concentration of the nucleoside in the absence of a simultaneous decrease in adenosine kinase activity. Our results thus agree with the hypothesis of Arch and Newsholme (1978a) that decreased recycling of adenosine into AMP allows amplification of the increase in adenosine concentration resulting from an increased AMP hydrolysis.

Taken together, our results allow us to conclude that the 20–30-fold elevation in the adenosine concentration induced by anoxia in isolated hepatocytes is mainly explained by the profound decrease in the activity of adenosine kinase. However, the residual activity of this enzyme could not be measured, at least with radioactive adenosine, owing to the unexpected discovery that the nucleoside can be phosphorylated by an exchange reaction with AMP, as described in the accompanying paper (Bontemps et al., 1993). Suppression of adenosine kinase activity was expected in N_2/CO_2 , but it is less clear why a large decrease was recorded in 2 mM amytal; indeed, under this condition, the concentration of ATP is still about 2-fold higher than the K_m of

rat liver adenosine kinase for this nucleotide (Ogasawara et al., 1980). Further studies of adenosine kinase are required in order to understand its kinetic behaviour in anoxic conditions.

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