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Adenine nucleotide metabolism and nucleoside transport in human erythrocytes under ATP depletion conditions

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The adenine nucleotides of human red cells were labeled by incubation of the cells with [^{3}H]adenosine. Then, the cells were incubated in Tris-saline with various supplements that cause the loss of cellular ATP, and the degradation products were quantitated as a function of time of incubation at 37°C. Incubation of the cells with 2.5 or 5 mM iodoacetate, iodoacetamide or 1 mM HCHO in combination with 5 mM KF and 50 mM deoxyglucose, 50 mM D-glucose or 10 mM inosine was most efficient in depleting the cells of ATP (100% in 0.5–1 h) without causing cell lysis. In iodoacetate- and iodoacetamide-treated cells practically all catabolism of ATP occurred via ADP → AMP → IMP → inosine → hypoxanthine with hypoxanthine accumulating in the medium. In HCHO-treated cells and in cells incubated in Tris-saline or in Tris-saline with deoxyglucose with and without KF, a substantial proportion of ATP (up to 50%) was catabolized via ADP → AMP → adenosine → inosine → hypoxanthine. Under all conditions, AMP deamination and IMP and AMP hydrolysis were rate-limiting reactions. IMP degradation was more rapid in iodoacetamide- and HCHO-treated than in iodoacetate-treated red cells. It was also more rapid in fresh than in outdated red cells, and it was inhibited by P_i. Treatment with iodoacetamide and HCHO under ATP-depletion conditions resulted in a 60–80% inhibition of uridine transport by the cells. Treatment with iodoacetate or deoxyglucose plus KF had only minor effects on nucleoside transport; thus, cells treated in this manner might be useful for studying the transport of adenosine and deoxyadenosine under conditions were their phosphorylation is prevented.

Introduction

Human erythrocytes lack uridine and thymidine kinases and phosphorylases, and flux of these pyrimidine nucleosides into and out of the cells can therefore readily be measured without ambiguities introduced by metabolic conversion [1,2]. This has allowed detailed characterization of uridine and thymidine transport in human red cells [3–6]. Results from competition experiments have indicated that pyrimidine and purine nucleosides and deoxynucleosides are transported in these cells by a single carrier [3,5,7,8], but direct flux measurements of purine nucleosides have been problematic because of their rapid phosphorylation and deamination in these cells. Deamination can be blocked by treatment of the cells with appropriate adenosine deaminase inhibitors, such as deoxycoformycin [9,10], but phosphorylation, the reaction that seems to predominate at low nucleoside concentrations [11,12], is more difficult to deal with. In cultured cells, we have circumvented this problem by selection of kinase-deficient mutants or in a more general manner by the use of ATP-depleted cells [7,8,13]. The latter method should also be applicable to human red cells. Incubation of cultured cells with 5 mM iodoacetate plus 5 mM KCN for 10–15 min is sufficient

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to deplete them of ATP [7]. ATP in human red cells seems to be considerably more stable, but several treatments have been found to drastically lower ATP levels over a 1–4-h incubation period and have been used to assess the effect of ATP on various properties of these cells [14–18]. Most efficient seems to be incubating the cells with iodoacetamide (which inhibits 3-phosphoglyceraldehyde dehydrogenase and thus ATP generation via glycolysis) plus high concentrations of a substrate whose metabolism consumes ATP such as D-glucose, 2-deoxy-D-glucose, adenosine or inosine [16]. Recently, it has been reported [18] that incubation with HCHO is also very effective in lowering the ATP level of human red cells, but by what mechanism has not been elucidated.

In the present study, we have compared the efficacy of various treatments in depleting human red cells of ATP, with the aim of optimizing the conditions for obtaining ATP-depleted cells with undiminished and unaltered nucleoside-transport capacity. We have also investigated in some detail the pathways of ATP degradation and quantitated the products accumulating intra- and extracellularly as a function of time of treatment. This information was not previously available.

Experimental procedures

Fresh human erythrocytes were obtained from Dr. J. Kersey (Department of Pathology) as a by-product of lymphocytic isolation. Outdated blood was obtained from the Blood Bank, University Hospitals, University of Minnesota and used within 2 weeks after the expiration date. The cells were washed three times in cold saline containing 5 mM Tris-HCl (pH 7.4) (Tris-saline) and then suspended to about $5 \cdot 10^8$ cells/ml of Tris-saline. All experiments were conducted with red cells from freshly drawn blood, except where specifically indiated.

For labeling the adenine nucleotides of the cells, cell suspensions were supplemented with 2–3 μCi [2-^{3}H]adenosine (20–25 Ci/mmol; Moravek Biochemicals, Brea, CA) per ml and incubated at 37°C for 20–25 min. The absolute concentration of adenosine in the medium (about 0.1 μM) was too low to affect the adenine nucleotide levels of the cells. The cells were collected by centrifugation, washed once in cold Tris-saline and aliquots of cells were suspended to the original density in Tris-saline supplemented with various chemicals as indicated in the appropriate experiments. At various times during incubation at 37°C, the cells from duplicate 0.5-ml suspension were collected by centrifugation through an oil layer and analyzed for radioactivity as described previously [19]. The cells from additional 0.8 ml of suspensions were centrifuged through oil directly into an acid layer which immediately stops cellular metabolism [19,20]. The medium was rapidly aspirated and mixed with 50 μl of 0.5 M trichloroacetic acid. The acid extracts of the cells were further processed and, along with medium samples, analyzed by paper chromatography as described previously [20]. The cell extracts were chromatographed with both solvent 28, which separates ATP and ADP from AMP and IMP, which comigrate, and from adenosine, inosine and hypoxanthine, which comigrate, and solvent 9, which separates adenosine and IMP from hypoxanthine, inosine, ATP and AMP whose migrations overlap. The medium was chromatographed with solvent 9 for quantitation of radioactivity in adenosine, inosine and hypoxanthine.

Inward equilibrium exchange * and zero-*trans* * influx of 2 mM uridine at 25°C were measured as described previously [5,22]. Detailed time-courses of equilibration of [5-^{3}H]uridine (Moravek Biochemicals, Brea, CA) were determined by rapid kinetic techniques, and appropriate integrated rate equations based on the simple carrier model [21] were fitted to the data. For the analysis of the equilibrium exchange data, K^{ee}, the Michaelis-Menten constant for equilibrium exchange, was fixed at 700 μM [5,22]. For the analysis of the zero-*trans* data, R_{ee} was fixed at the value obtained from the analysis of the equilibrium ex-

* As defined by Stein [21], 'zero-*trans*' designates the transport of a substrate from one side of the membrane (the *cis* side) to the other side, where its concentration is initially zero. 'Equilibrium exchange' designates unidirectional flux of radioactively labeled substrate from one side to the other side of the membrane, where substrate is held at equal concentration. We generally follow the nomenclature and definitions of kinetic parameters formulated by Stein [21]. Arbitrarily, we designate the outside and inside faces of the membrane as 1 and 2, respectively.

change data for the same cell population, and K, the limit Michaelis-Menten constant (intrinsic dissociation constant, Ref. 21), was fixed at 100 μM [5,22]. The initial velocities of equilibrium exchange (v^{ee}) and zero-*trans* entry (v_{12}^{zt}) were calculated as the slopes of the substrate equilibration curves at $t = 0$.

Results and Discussion

ATP catabolism in red cells under various experimental conditions

In the following experiment, we have assessed the effect of iodoacetate plus KF in combination with P_i, two metabolizable substrates (D-glucose and inosine) and a nonmetabolizable hexose (2-deoxy-D-glucose) on the ATP turnover in human erythrocytes. The adenine nucleotides of the cells were labeled by incubation of the cells with [^{3}H]adenosine and the cells were then incubated in Tris-saline containing 5 mM iodoacetate plus 5 mM KF and, as indicated, 50 mM D-glucose, 10 mM inosine, or 50 mM deoxyglucose each with or without 20 mM P_i. During incubation at 37°C, the cells were monitored for labeled ATP, ADP, AMP, IMP, adenosine inosine and hypoxanthine and the extracellular fluid for adenosine, inosine and hypoxanthine. Under the labeling conditions used, it is reasonable to assume that the specific radioactivities of ATP and its metabolic products were the same and remained constant during the incubation period, so that the radioactivity values reported are a reflection of absolute concentrations. Representative results are presented in Fig. 1 and can be summarized as follows:

(1) At the beginning of the treatment period, over 85% of the intracellular radioactivity was associated with ATP plus ADP (the latter represented only a minor fraction). Most of the remainder of the radioactivity was associated with AMP (Fig. 1A). The amounts of radioactivity associated with IMP, adenosine, inosine and hypoxanthine were insignificant (< 2% of total). Cellular ATP remained about stable during 1 h of incubation in Tris-saline (Fig. 1A), but thereafter became slowly degraded. The accumulating products were intracellular AMP and extracellular hypoxanthine. As will be documented later, ATP degradation to hypoxanthine seems to occur both via ADP → AMP → IMP → inosine and via ADP → AMP → adenosine → inosine. Thus, the results were consistent with the view that the only rate-limiting steps under these conditions were the degradation of ATP and the deamination or hydrolysis of AMP.

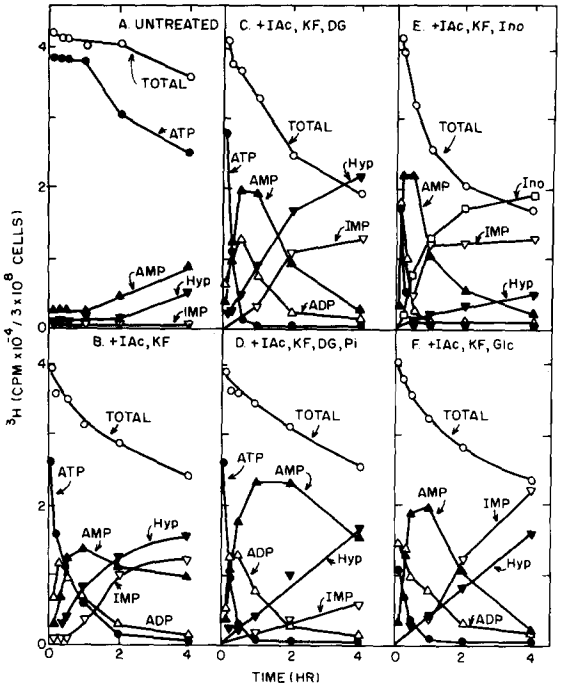

Fig. 1. ATP metabolism in human erythrocytes incubated under various experimental conditions. [^{3}H]Adenosine-labeled red cells were incubated in Tris-saline ($6 \cdot 10^8$ cells/ml) which was supplemented as indicated with 5 mM iodoacetate (IAc), 5 mM KF, 50 mM deoxyglucose (DG), 10 mM inosine (Ino), 50 mM D-glucose (Glc) and 20 mM P_i. At various times of incubation at 37°C, samples of cells from duplicate 0.5-ml samples were collected by centrifugation through oil and analyzed for radioactivity (total, ○———○). The cells from other samples were acid-extracted and the acid extracts and the cell-free medium were chromatographed as described under Experimental procedures. The amounts of radioactivity associated with the various intracellular labeled components were calculated on the basis of the chromatographic separations and the total radioactivity associated with the cells and the medium: intracellular ATP (●———●), ADP (△———△), AMP (▲———▲), IMP (▽———▽) and extracellular hypoxanthine (Hyp, ▼———▼) and inosine (Ino, □———□).

(2) The presence of iodoacetate and KF in the medium greatly accelerated the loss of ATP (Fig. 1B). The intracellular changes of ATP, ADP, AMP and IMP exhibited typical precursor-product relationships. No significant amounts of labeled

adenosine or inosine were detected either intracellularly or extracellularly. The loss of total cellular radioactivity was practically all accounted for by accumulation of hypoxanthine in the medium. If AMP → adenosine → inosine were the route of degradation, one would expect deoxycoformycin to cause an accumulation of adenosine, which was not seen (see later, Fig. 2). Thus, it appears that ring deamination occurred at the nucleotide level, i.e., AMP to IMP, and that the rate of degradation of ATP was sufficiently rapid to cause a transient accumulation of the intermediates ADP, AMP and IMP. On the other hand, any inosine that was formed must have been rapidly phosphorolyzed. The failure of nucleosides or hypoxanthine to accumulate intracellularly was to be expected, because of the high transport activity of these cells, which ensures rapid equilibration of the substrates with the extracellular fluid [5,22]. Since the extracellular aqueous space in the suspension was about 25-times greater than the intracellular water space, the hypoxanthine that was formed accumulated in the medium.

(3) The addition of deoxyglucose, inosine or glucose (Fig. 1C, E and F, respectively) further accelerated the loss of ATP, so that the cells became depleted of ATP within about 30 min of incubation. The subsequent reactions were also slightly accelerated, so that the cells had also become practically depleted of ADP and AMP by 4 h of incubation. But the overall reaction pathway was not altered as indicated by the precursor-product relationships between ATP, ADP, AMP and IMP. The main rate-limiting step in the complete degradation of the nucleotides, under each of the three experimental conditions, was the hydrolysis of IMP. The presence of unlabeled 10 mM inosine inhibited the phosphorolysis of radiolabeled inosine formed from ATP (Fig. 1E), presumably due to radioisotope dilution, but in the presence of deoxyglucose or glucose, hypoxanthine was the only product accumulating in the medium. The results indicate that P_i, required for the phosphorolysis of inosine, was not limiting, even though the cells had been repeatedly washed in P_i-free medium and were incubated in P_i-free medium. Furthermore, the presence of 20 mM P_i had no significant effect on the rate of loss of ATP whether or not the medium contained deoxyglucose, glucose or inosine. The only apparent effect of P_i was an impairment in the deamination of AMP to IMP. This was the same for all treatments and thus the data for only one of them (deoxyglucose) are presented (Fig. 1D). The decrease in AMP deamination resulted in a decreased formation of hypoxanthine (cf. Fig. 1C and D). In the case of the inosine treatment (Fig. 1E), the presence of P_i, not unexpectedly, stimulated the conversion of labeled inosine to hypoxanthine, so that after 4 h of incubation, 80% of the radioactivity in the medium was associated with hypoxanthine and only 18% with inosine (data not shown). This effect has been previously reported [15,16].

The finding that deoxyglucose and glucose had about the same effect on ATP metabolism suggests that the acceleration of ATP degradation by both is mainly mediated through the use of ATP in their phosphorylation. On the other hand, inosine (and adenosine) probably accelerates the loss of ATP in iodoacetate-treated cells not by its own phosphorylation but by giving rise to fructose 6-phosphate, which is phosphorylated at the expense of ATP [15].

Results similar to those shown in Fig. 1 were obtained in other experiments involving these treatments, except that the rate of conversion of IMP to hypoxanthine varied somewhat when different populations of cells were subjected to the same treatment (cf. Figs. 1C and 2A). We have no explanation for these variations but find that the rate of IMP degradation is also one of the main variables between different treatments inducing a loss of ATP as, for example, illustrated in the experiment presented in Fig. 2. In this experiment, we compared the effects of iodoacetate, iodoacetamide and HCHO in the presence of deoxyglucose or glucose on ATP metabolism and examined as well the effect of deoxycoformycin. The most conspicuous difference was a much more rapid loss of total cellular radioactivity from HCHO-treated and iodoacetamide-treated than from iodoacetate-treated cells (Fig. 2A, C and E). The more detailed analyses of the cellular pools and the medium showed that the more rapid loss of total radioactivity from the cells reflected a more rapid degradation of IMP to inosine, which resulted in an increased accumulation of hypoxanthine in the medium. In fact, only low con-

centrations of IMP were found in HCHO-treated cells (Fig. 2E). On the other hand, the treatments did not differ significantly in effecting the conversion of ATP to IMP.

The inhibition of adenosine deamination by treatment of the cells with deoxycoformycin had no effect on ATP catabolism, whether it was induced by iodoacetate (Fig. 2A and B), or iodoacetamide (Fig. 2C and D). Deoxycoformycin has been found to inhibit the AMP deaminase of human erythrocytes ($K_i = 50$ nM) [9], but under the conditions of our experiments, it had no effect

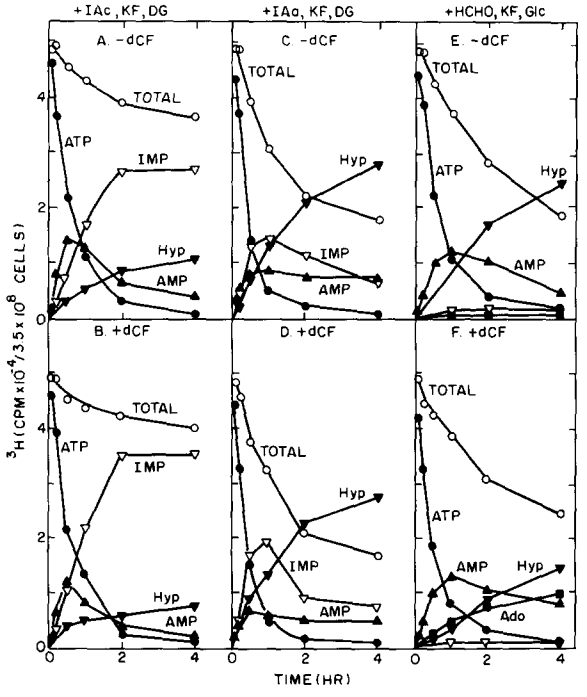

Fig. 2. Comparison of the effects of iodoacetate, iodoacetamide and HCHO in the absence and presence of deoxycoformycin on ATP metabolism in human erythrocytes. [^{3}H]Adenosine-labeled red cells were incubated in Tris-saline (about $7 \cdot 10^8$ cells/ml) supplemented with 5 mM KF and 50 mM deoxyglucose (DG) or D-glucose (Glc) and as indicated with 5 mM iodoacetate (IAc), 6 mM iodoacetamide (IAa) or 1 mM HCHO (plus 200 μM $CaCl_2$) and 25 μM deoxycoformycin (dCF) and at various times of incubation at 37°C, duplicate 0.5-ml samples of suspension were analyzed for radioactivity in total cell material (○——○). Other samples of suspension were analyzed for radioactivity in various extracellular and intracellular components as described under Experimental procedures and the legend to Fig. 1: intracellular ATP plus ADP (●——●), AMP (▲——▲), IMP (▽——▽) and extracellular hypoxanthine (Hyp, ▼——▼) and adenosine (Ado, ■——■).

on AMP deamination in whole cells, whereas it completely inhibited adenosine deamination (Fig. 3; the K_i for the inhibition of adenosine deamination by deoxycoformycin is about 2.5 pM [9]). Therefore, the finding that only little adenosine accumulated in deoxycoformycin-treated cells or in the extracellular fluid indicates that hydrolysis of AMP to adenosine played a negligible role in ATP catabolism in iodoacetate- or iodoacetamide-treated red cells.

On the other hand, when the cells were incubated with HCHO in the presence of deoxycoformycin, substantial amounts of labeled adenosine accumulated in the medium (Fig. 2F). Particularly during the first 1 h of incubation, the main labeled component in the medium was adenosine, whereas in the absence of deoxycoformycin, the amounts of labeled adenosine present were insignificant. The results indicate that under these conditions, ATP became catabolized both via AMP → IMP → inosine → hypoxanthine and AMP → adenosine → inosine → hypoxanthine and that the contribution of each pathway varied as a function of time of incubation. The difference between the iodoacetate and iodoacetamide treatments and the HCHO treatment was not due to the nature of the metabolizable substrate which was also present, since adenosine was not formed whether the cells were incubated with iodoacetate plus deoxyglucose or glucose (Fig. 1). The additional finding that little IMP accumulated in HCHO-treated cells whether or not deoxycoformycin was also present indicates that, under these conditions, IMP was hydrolyzed as rapidly as it was formed.

A situation similar to that observed during incubation with HCHO plus KF, glucose and deoxycoformycin held for cells incubated with deoxycoformycin alone, except that the loss of ATP was far less rapid (Fig. 4A). Although the amount of ATP degraded beyond the monophosphate level was very low, by 2 h of incubation about 50% of the radioactivity in the medium was associated with adenosine and significant amounts of IMP did not accumulate intracellularly. Similarly, Henderson et al. [17] reported that incubation of red cells with deoxyglucose plus deoxycoformycin resulted in the formation of adenosine, whereas in the absence of deoxycoformycin hypoxanthine was the main endproduct. Furthermore, the authors

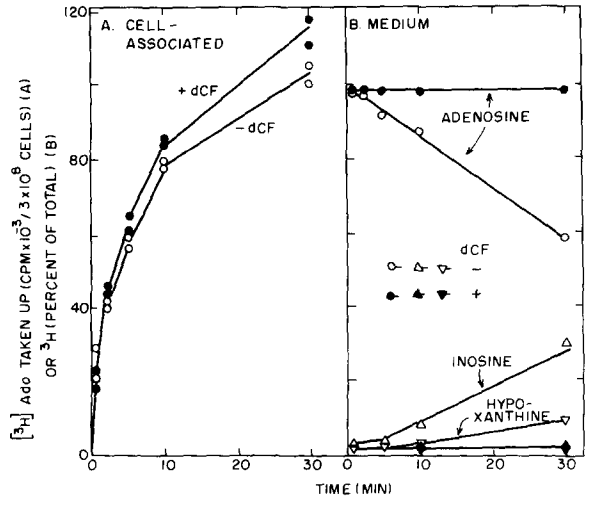

Fig. 3. Effect of deoxycoformycin on adenosine deamination in human erythrocytes. One sample of suspension of washed red cells in Tris-saline ($6 \cdot 10^8$ cells/ml) was supplemented with 20 μM deoxycoformycin (dCF) and another sample remained untreated. After 10 min of incubation at 25°C, [^{3}H]adenosine (Ado) was added to 1 mM (0.4 cpm/pmol). At various times of further incubation at 25°C, the cells from duplicate 0.5-ml of suspension were collected by centrifugation through oil and analyzed for radioactivity (A). The medium was chromatographed with solvent 9 to determine the proportion of radioactivity associated with adenosine, inosine and hypoxanthine (B).

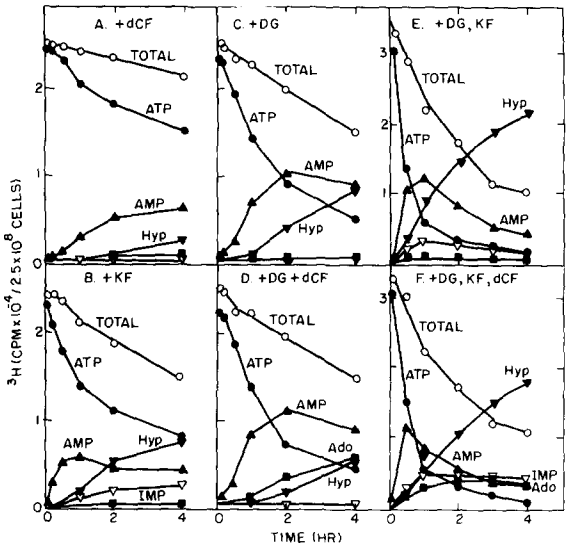

Fig. 4. Effect of deoxyglucose and KF alone and in combination in the absence and presence of deoxycoformycin on ATP metabolism in human erythrocytes. [3H]Adenosine-labeled red cells were incubated in Tris-saline ($5 \cdot 10^8$ cells/ml) supplemented as indicated with 50 mM deoxyglucose (DG), 5 mM KF and 25 μM deoxycoformycin (dCF) and at various times of incubation at 37°C duplicate 0.5-ml samples of suspension were analyzed for radioactivity in total cell material (○——○). Other samples of suspension were analyzed for radioactivity in various extracellular and intracellular components as described under Experimental procedures and the legend to Fig. 1: intracellular ATP plus ADP (●——●), AMP (▲——▲), IMP (▽——▽), and extracellular hypoxanthine (Hyp, ▼——▼) and adenosine (Ado, ■——■). The results in A–D and E and F are from two different experiments.

observed that large amounts of AMP accumulated in the cells, but no IMP. We have confirmed and extended these results (Fig. 4C and D). However, upon incubation with deoxyglucose in absence of inhibitors of glycolysis, there were still substantial amounts of ATP(ADP) left even after 4 h of incubation. The same was the case for incubation with KF alone (Fig. 4B), but the effects of KF and deoxyglucose were additive (Fig. 4E). Little, if any, ATP was left after 4 h of incubation with 5 mM KF plus 50 mM deoxyglucose. However, in the presence of KF, whether or not deoxyglucose was also present, considerable amounts of IMP accumulated intracellularly, but less than in iodoacetate- or iodoacetamide-treated cells. Hypoxanthine was the only extracellular product, but when deoxycoformycin was present, some adenosine accumulated in the medium of cells incubated with KF plus deoxyglucose (Fig. 4F).

We also compared the effect of iodoacetamide (plus KF) on ATP metabolism in red cells from freshly drawn and outdated blood (Fig. 5). There was a marked difference in the degradation of IMP, which was considerably slower in outdated than fresh red cells. Thus, instead of a rapid accumulation of hypoxanthine in the medium, outdated cells accumulated IMP. The rate of loss of ATP, on the other hand, was slightly greater in outdated than fresh cells. ATP catabolism in the presence of iodoacetate (plus KF), on the other hand, was about the same in fresh and outdated red cells (data not shown), but it should be recalled that, in any case, treatment with iodoacetate results in the accumulation of IMP as the main product (see Fig. 2B).

Overall, our data indicate that the contribution of the two competing pathways in the degradation of AMP to the catabolism of ATP differ under different experimental conditions and that different steps may be rate-limiting. When the cells are

incubated in Tris-saline or with deoxyglucose, a large proportion of the ATP that is utilized is catabolized via AMP → adenosine → inosine → hypoxanthine. AMP degradation is clearly rate-limiting, since large concentrations of AMP (up to 500 μM based on the radioactivity values and an ATP content of untreated red cells of about 1 mM) accumulate in deoxyglucose-treated cells. Relatively little AMP is deaminated, but such catabolism as does occur must be via AMP → IMP, since the inhibition of adenosine deaminase with deoxycoformycin does not block the appearance of hypoxanthine. However, IMP does not accumulate under these conditions.

On the other hand, upon treatment with iodoacetate or iodoacetamide, ATP becomes catabolized mainly via AMP → IMP → inosine → hypoxanthine, which most likely reflects an activation of AMP deaminase as an indirect result of the inhibition of glycolysis by these treatments. AMP deaminase has been found to be strongly inhibited by 2,3-diphosphoglycerate (as well as by P_i and PP_i) but is activated by ATP [23,24]. Inhibition of glycolysis as a result of the inactivation of glyceraldehyde-3-phosphate dehydrogenase by iodoacetate and iodoacetamide would be expected to result in a rapid loss of 2,3-diphosphoglycerate and thus the activation of AMP deaminase. In the absence of inhibitors of glycolysis, even in the presence of deoxyglucose, the level of 2,3-diphosphoglycerate may be more stable and AMP deaminase may remain mainly inhibited. Under these conditions, the concentration of AMP does not seem to be the main factor in determining whether AMP is deaminated or hydrolyzed as postulated for physiological conditions [25,26]. Henderson et al. [17] reported that tubercidin treatment of human erythrocytes also results in a loss of ATP with the accumulation of IMP and hypoxanthine, but not of adenosine, even in the presence of deoxycoformycin. Thus, its effect resembles that induced by iodoacetate and iodoacetamide, but whether it functions in a similar manner is not known.

In cells treated with HCHO or deoxyglucose plus KF, ATP is catabolized both via AMP → adenosine and AMP → IMP → inosine, but the contributions of the two pathways vary as a function of time of incubation. Initially, AMP hydrolysis seems to predominate, but in later stages, AMP deamination becomes the predominant reaction, probably reflecting an activation of AMP deamination as a result of a depletion of 2,3-diphosphoglycerate, since the shift correlates with a loss in ATP. Furthermore, in these cells, IMP hydrolysis seems to be very efficient, since little accumulated intracellularly.

The enzymes responsible for the hydrolysis of AMP and IMP have not been identified, and it is not clear whether they are regulated. Several nucleotidase activities have been recognized in human erythrocytes, some of which may primarily function in the degradation of RNA and DNA nucleotides during differentiation of the red cells [27,28]. Red cells possess at least one deoxyribonucleotidase and one pyrimidine nucleotidase which differ in substrate specificities [27,28]. One of these also efficiently hydrolyzes IMP [28]. Cells of other tissues also possess cytoplasmic 5′-nucleotidase activity [29,30]. Its activity with AMP and IMP varies in a complex manner as a function

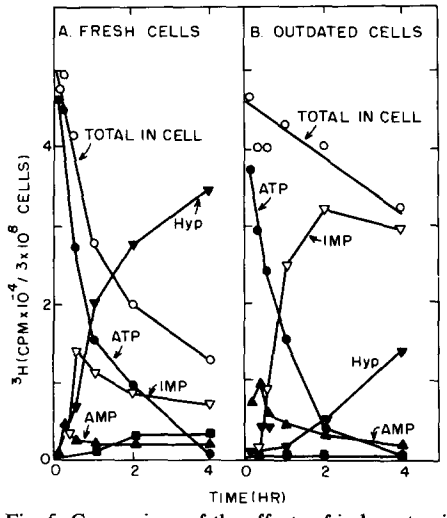

Fig. 5. Comparison of the effects of iodoacetamide plus KF on ATP metabolism in red cells from freshly drawn (A) and outdated blood (B). The cells were labeled with [^{3}H]adenosine and then incubated in Tris-saline ($6 \cdot 10^8$ cells/ml) containing 5 mM iodoacetamide and 5 mM KF at 37°C. At various times, samples of cells were analyzed for radioactivity in total cell material (○——○) and for radioactivity in various intracellular and extracellular components as described in Experimental procedures and the legend to Fig. 1: intracellular ATP plus ADP (●——●), AMP (▲——▲), IMP (▽——▽) and extracellular hypoxanthine (Hyp, ▼——▼).

of the adenylate charge, but under most conditions IMP is hydrolyzed more efficiently than AMP [29]. Whether such activity operates in human erythrocytes has been difficult to ascertain [26]. We have no explanation for the differences in IMP degradation between iodoacetate-, iodoacetamide- and HCHO-treated cells (Fig. 2) and between fresh and outdated red cells (Fig. 5). One possibility is that iodoacetate, and to a lesser extent iodoacetamide, cause an inactivation of IMP nucleotidase and that its activity is lost during blood storage. On the other hand, differences in effects on the regulation of the enzymes may be involved, since some of the nucleotidases seem to be subject to such controls. On the other hand, the inhibition of IMP hydrolysis by P_i may simply be the result of product inhibition or a mass-action effect. In contrast to AMP deamination and AMP and IMP hydrolysis, adenosine deaminase and purine nucleoside phosphorylase activities are not rate-limiting in adenine nucleotide catabolism, since neither adenosine nor inosine accumulate to any extent during ATP degradation.

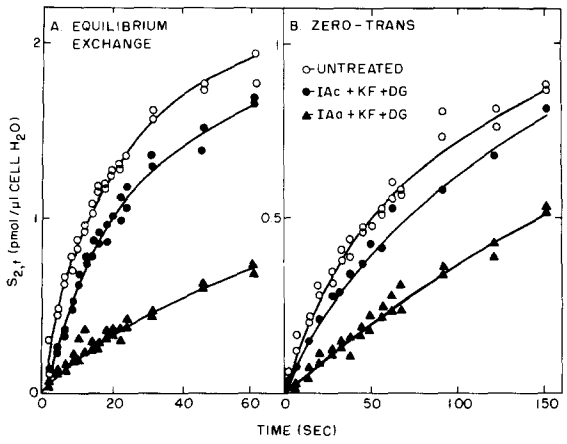

Fig. 6. Inward equilibrium exchange and zero-*trans* flux of 2 mM uridine in untreated and ATP-depleted red cells. Samples of a suspension of red cells ($5 \cdot 10^8$ cells/ml) in Tris-saline or in Tris-saline supplemented with 5 mM iodoacetate (IAc) or 5 mM iodoacetamide (IAa) plus 5 mM KF and 50 mM deoxyglucose (DG) were incubated at 37°C for 2 h. Then, the accumulation of 2 mM [^{3}H]uridine (0.25 cpm/pmol) was measured under equilibrium exchange and zero-*trans* conditions as described under Experimental procedures. The measured initial velocities are listed in Table I (Expt. 1).

Uridine transport in ATP-depleted human erythrocytes

In the following experiments, we have assayed equilibrium exchange and zero-*trans* influx of 2 mM uridine in red cells that had been ATP-depleted by various treatments in order to assess the general utility of ATP depletion as means of rendering nucleoside-transport substrates metabolically inert in red cells. Inosine at effective concentrations cannot be used in any preincubations because it would interfere with subsequent nucleoside transport measurements. We have therefore chosen to deplete the cells of ATP by incubation with iodoacetate, iodoacetamide or HCHO in combination with KF and deoxyglucose, or with deoxyglucose and KF alone. The results in Fig. 6 and Table I show that incubation of the cells in media containing iodoacetamide or HCHO resulted in a 60–80% reduction in uridine transport by the cells, but that equilibrium exchange was affected more than zero-*trans* influx. Since the difference in magnitude between v^{ee} and v_{12}^{zt} is a reflection of the differential mobility of substrate-loaded and empty carrier [5,22], the results may suggest that the treatments not only inhibited the function of the transporter, but did so differentially with respect to loaded and empty carrier. However, a complete kinetic analysis would be required to ascertain whether this suggestion is correct. The iodoacetate-deoxyglucose-KF treatment was also inhibitory (Table I), but its effect was relatively minor, and it thus might be appropriate for rendering human red cells suitable for purine nucleoside transport measurements. We have previously demonstrated that deoxycoformycin at concentrations required in such transport assays, practically completely inhibits adenosine and deoxyadenosine deamination, and even at substantially higher concentrations, has no significant effect on nucleoside transport [31]. The deoxyglucose-KF treatment was also only slightly inhibitory for transport (Table I) and thus might be suitable for preparing transport-active, non-phosphorylating red cells, but it is far less efficient in depleting the red cells of adenine nucleotides and prolonged incubation will be required to reduce the ATP level sufficiently to prevent adenosine and deoxyadenosine phosphorylation.

The mechanism by which iodoacetamide, HCHO and iodoacetate treatment inhibit nucleo-

TABLE I

INWARD EQUILIBRIUM EXCHANGE AND ZERO-*TRANS* INFLUX OF 2 mM URIDINE IN UNTREATED AND ATP-DEPLETED CELLS

Washed, fresh human red cells were incubated at a density of about $5 \cdot 10^8$ cells/ml in Tris-saline supplemented where indicated with 5 mM (Expt. 1) or 2.5 mM (Expt. 2) iodoacetate (IAc), 5 mM iodoacetamide (IAa), 50 mM 2-deoxy-D-glucose (DG), 1 mM HCHO (plus 200 μM $CaCl_2$) and 5 mM KF at 37°C for 2 h in Expts. 1 and 2 and for 4 h in Expt. 3. For equilibrium exchange measurements, the suspensions were equilibrated at 25°C and inward equilibrium exchange and zero-*trans* influx at 2 mM uridine (0.25 cpm/pmol) were measured in duplicate by rapid kinetic techniques as described under Experimental procedures and illustrated in Fig. 6 for Expt. 1. In Expt. 4, one-half of a cell suspension was supplemented with 500 μM unlabeled uridine (equilibrium exchange), the other half remained without uridine (zero-*trans*). The suspensions were incubated at 37°C for 1 h, and then portions of each were incubated with *p*-hydroxymercuribenzenesulfonate (pHMBS) at 37°C for 20 min. Thereafter, the suspensions were equilibrated at 25°C and the inward equilibrium exchange and zero-*trans* influx of 500 μM [^{3}H]uridine (1 cpm/pmol) were measured. Transport velocity values are presented ± S.E. of the estimate.

Expt.	Treatment	v^{ee} (μM/s)	v_{12}^{zt} μM/s)
1	none	99.4 ± 3.4	16.5 ± 0.7
	IAc + KF + DG	67.5 ± 2.1	12.8 ± 0.5
	IAa + KF + DG	16.3 ± 0.6	6.5 ± 0.3
2	none	71.1 ± 2.1	11.0 ± 0.4
	IAc + KF + DG	55.0 ± 2.6	8.9 ± 0.4
	HCHO + KF + DG	10.3 ± 0.5	3.2 ± 0.2
3	none	74.0 ± 3.4	13.0 ± 0.8
	KF + DG	60.0 ± 2.3	8.4 ± 0.5
4	none	20.9 ± 1.2	3.0 ± 0.2
	pHMBS (100 μm)	10.3 ± 0.7	2.2 ± 0.1
	pHMBS (200 μM)	4.3 ± 0.2	1.6 ± 0.1

side transport has not been elucidated. It does not reflect outright damage to the cells, since there was no significant hemolysis during 4 h of incubation with iodoacetamide or HCHO plus KF and deoxyglucose, inosine or glucose and the volume of the cells as measured by ^3H_2O space or with a Coulter counter was not significantly altered (data not shown). It is known, however, that iodoacetamide and iodoacetate interact with sulfhydryl groups and that sulfhydryl groups play an important function in nucleoside transport in various types of mammalian cells [32], including human red cells. For example, as shown in Table I, incubation of red cells with *p*-hydroxymercuribenzenesulfonate caused a similar inactivation of uridine transport as incubation with iodoacetamide or HCHO. The results illustrate that caution is needed in causally relating changes in various properties of red cells to alterations in intracellular ATP concentrations induced by incubation with iodoacetamide or HCHO. There was clearly no correlation between the degree of ATP depletion and inactivation of uridine transport induced by the various treatments.

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