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Hypoxanthine–Guanine Exchange by Intact Human Erythrocytes

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ABSTRACT: The uptake and release of [^{14}C]hypoxanthine by human erythrocytes, suspended in a tris(hydroxymethyl)aminomethane (Tris)–glucose–NaCl isotonic medium (pH 7.4), have been studied at 37 °C. The uptake of hypoxanthine, mediated by its incorporation into inosine 5′-monophosphate (IMP), was markedly stimulated by preincubating the cells in phosphate-buffered saline. After a lag time, [^{14}C]-IMP-enriched erythrocytes released [^{14}C]hypoxanthine in the medium. Formycin B, at concentrations known to inhibit purine nucleoside phosphorylase in intact erythrocytes, affected hypoxanthine uptake and release and led to an increase in the intracellular concentration of inosine, suggesting that the main catabolic path of IMP is the sequential degradation of the nucleotide to inosine and hypoxanthine. The addition of guanine to a suspension of [^{14}C]IMP-enriched erythrocytes led to an increase in the rate of [^{14}C]hypoxanthine release, which was unaffected by the presence of formycin B. During the guanine-induced hypoxanthine release, guanine was taken up by the cells as GMP. These results suggest that the presence of guanine in the incubation medium activates a catabolic path in human erythrocytes leading to IMP degradation without formation of inosine.

Human erythrocytes appear to be important in carrying purines from organs with a purine surplus to organs with a purine requirement (Lowy & Lerner, 1974). De novo purine synthesis does not occur in mature red cells (Lowy et al., 1961); thus, the turnover must result from continual entry and release of purines into the plasma.

Human erythrocytes take up adenine, guanine, hypoxanthine, and xanthine and convert them into nucleotides, but hypoxanthine and xanthine are the only purines released in vitro (Mager et al., 1966). The release of purines by human red cells is mediated by prior conversion of the various purine nucleotides to inosine 5′-monophosphate (IMP), which can be either sequentially degraded to inosine and hypoxanthine or converted to xanthosine 5′-monophosphate (XMP) (Mager et al., 1966). Xanthine formation from the latter mononucleotide does not appear to occur significantly in vivo owing to the conversion of XMP to guanosine 5′-monophosphate (GMP) in the presence of the relatively high glutamine levels prevailing in the blood (Hershko et al., 1967). Therefore, purine catabolism of human erythrocytes in vivo appears to be mainly directed via IMP and inosine by hypoxanthine, which is the chief base released (Murray, 1971). In this paper, hypoxanthine release from mature human red cells has been studied.

Materials and Methods

[8-^{14}C]Hypoxanthine (59 Ci/mol) and [8-^{14}C]guanine (55 Ci/mol) were obtained from Radiochemical Centre, Amersham. All other reagents were high-purity commercial samples from Merck or Sigma.

Human erythrocytes were prepared from blood freshly drawn in heparin and washed twice with ice-cold 0.9% NaCl with removal of white cells by aspiration. The packed erythrocytes were suspended in an equal volume of phosphate-buffered saline containing 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 7.4), 5 mM glucose, 0–12 mM sodium phosphate, and the appropriate amount of NaCl to give an isotonic solution and incubated for 15–60 min at 37 °C. At the end of the incubation period, the cells were washed twice in buffered saline not containing phosphate (50 mM Tris-HCl, 5 mM glucose, and 12.8 mM NaCl, pH 7.4). The packed cells were transferred to an equal volume of the last medium containing cold or ^{14}C-labeled hypoxanthine (0–12 μM), cold or ^{14}C-labeled guanine (0–70 μM), and formycin B (0–8 mM). At regular intervals, samples of the incubation mixture were removed. The medium, separated from the cells by centrifugation, was employed for liquid scintillation counting and paper chromatography (Gerlach et al., 1965). The cell count in the incubation media (either containing or not containing phosphate) ranged from 4.2 to 4.7 million/mm^3.

The radioactive compounds present within the erythrocytes were identified by paper chromatography as previously described (Giacomello & Salerno, 1979). Intracellular concentration of radioactive compounds was determined from the relative amplitude of radioactive peaks obtained by paper chromatography. Intracellular 5-phosphoribosyl 1-pyrophosphate concentration was determined according to published procedures (Hershko et al., 1967).

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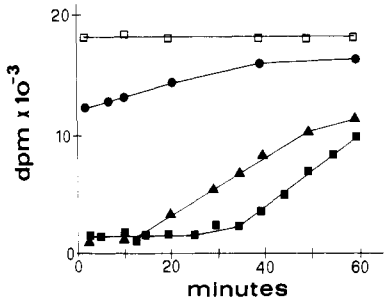

FIGURE 1: Effect of phosphate concentration in the preincubation medium on the release of radioactive material from [^{14}C]IMP-enriched human erythrocytes. Erythrocytes were preincubated for 40 min at 37 °C in buffered saline, pH 7.4, containing phosphate [(□) none, (●) 3 mM, (▲) 9 mM, (■) 12 mM) and washed twice in buffered saline not containing phosphate. Intracellular 5-phosphoribosyl 1-pyrophosphate concentration was unassayable (□), 2 μM (●), 6 μM (▲), and 8 μM (■). The packed cells were transferred to an equal volume of buffered saline not containing phosphate but containing 5 μM [^{14}C]hypoxanthine. At regular intervals, samples of the cell suspension were removed. The medium, separated from the cells by centrifugation, was employed for liquid scintillation counting. The incubation was carried out at 37 °C. All other conditions were as described in the text. (Abscissa) Time of incubation in the medium containing [^{14}C]hypoxanthine.

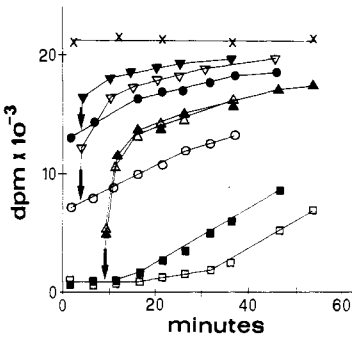

FIGURE 2: Effect of guanine and formycin B on the release of radioactive material from [^{14}C]IMP-enriched human erythrocytes. The cells were preincubated for 0 (×), 20 (○, ●, ▽, ▼), or 60 min (□, ■, △, ▲) in buffered saline containing 9 mM phosphate, washed twice in buffered saline not containing phosphate, and transferred to an equal volume of the last medium containing 5 μM [^{14}C]hypoxanthine and formycin B [(×, ●, ▼, ■, ▲) none; ○, □, △, ▽) 8 mM]. Arrows indicate the time of addition of 0.03 mL of isotonic NaCl containing 1.4 mM guanine to 1.2 mL of the incubation mixture. (×, ○, ●, □, ■) Time course of the release of radioactive material in the absence of guanine; (▼, ▽, ▲, △) time course of the release of radioactive material in the presence of guanine. All other experimental conditions were as described in Figure 1. (Abscissa) Time of incubation in the medium containing [^{14}C]hypoxanthine.

At the end of the incubation period, the hematocrits were within 1% of the initial values, and hemolysis was very low [hemoglobin in the suspending medium, determined as cyanomethemoglobin (Wintrobe, 1961), was less than 1.5% of the total hemoglobin content of the incubation mixture].

RESULTS

Human erythrocytes were incubated for 15–60 min in 3–12 mM phosphate buffered saline in order to enrich the cells with 5-phosphoribosyl 1-pyrophosphate (Hershko et al., 1967; Giacomello & Salerno, 1979). The erythrocytes were washed twice in buffered saline not containing phosphate. The packed cells, whose intracellular 5-phosphoribosyl 1-pyrophosphate content (ranging from 1.5 to 10 μM) was a direct function of phosphate concentration and of the time of incubation in the phosphate medium, were transferred in an equal volume of buffered saline not containing phosphate but containing 5 μM ^{14}C hypoxanthine. The labeled purine base was rapidly (within 2 min) taken up by the cells in good agreement with previous observations (Giacomello & Salerno, 1979). The amount of [^{14}C]hypoxanthine incorporated within the erythrocytes (ranging from 1.5 to 5 nmol/mL of packed cells) was a direct function of intracellular 5-phosphoribosyl 1-pyrophosphate concentration (Figure 1). When the amount of [^{14}C]hypoxanthine added to the suspending medium was greater than the intracellular 5-phosphoribosyl 1-pyrophosphate content, the amount of the labeled purine base incorporated was roughly proportional to the intracellular 5-phosphoribosyl 1-pyrophosphate concentration, which, within 2 min, decreased to unassayable levels.

After a lag time, particularly evident when the 5-phosphoribosyl 1-pyrophosphate content in the 5-phosphoribosyl 1-pyrophosphate enriched erythrocytes was in excess with respect to the amount of [^{14}C]hypoxanthine added to the suspending medium, radioactive material was released from erythrocytes in the extracellular buffer (Figures 1 and 2). At the end of the lag time, intracellular 5-phosphoribosyl 1-pyrophosphate concentration was unassayable.

If, after the preincubation in the phosphate-buffered saline, erythrocytes were transferred into buffered saline containing not only 5 μM [^{14}C]hypoxanthine but also 8 mM formycin B, then the amount of the labeled base taken up by the cells increased (Figure 2). When intracellular 5-phosphoribosyl 1-pyrophosphate concentration was sufficient to allow the incorporation of all [^{14}C]hypoxanthine, the presence of formycin B led to an increase in the lag time preceding the release of radioactive material from erythrocytes (Figure 2). After the lag time, the rate of release of ^{14}C-labeled material was about the same in the presence or absence of formycin B.

[^{14}C]Hypoxanthine was taken up by erythrocytes as [^{14}C]IMP, which, in the absence of formycin B, was the only radioactive compound present in appreciable amount within the cells. In the presence of 8 mM formycin B, [^{14}C]inosine was detected within erythrocytes, but [^{14}C]IMP was always the chief radioactive intracellular compound, accounting for up to 90% of the total labeled material present within the cells. The radioactive material released by the cells either in the presence or in the absence of formycin B was identified as hypoxanthine by paper chromatography.

The addition of 0.03 mL of 1.4 mM guanine to 1.2 mL of a suspension of [^{14}C]IMP-enriched erythrocytes in buffered saline led to an increase of the rate of release of the radioactive material (Figure 2) identified as hypoxanthine by paper chromatography. Hypoxanthine was released from [^{14}C]-IMP-enriched erythrocytes as soon as guanine was added. The time course of [^{14}C]hypoxanthine release from [^{14}C]IMP-enriched erythrocytes after the addition of guanine was not appreciably affected by the presence of formycin B up to 8 mM (Figure 2).

Erythrocytes not preincubated in phosphate-buffered saline incorporated very little [^{14}C]guanine from the suspending medium (Figure 3). The labeled purine base was incorporated within the cells when erythrocytes were preincubated in phosphate-buffered saline in order to enrich them with 5-phosphoribosyl 1-pyrophosphate, in good agreement with previous observations (Hershko et al., 1967).

Erythrocytes enriched with cold IMP took up [^{14}C]guanine (Figure 3). Under the experimental conditions employed, during the time of incubation of IMP-enriched erythrocytes with [^{14}C]guanine (around 40 min) GMP was the only radioactive compound formed in an appreciable amount within the cells. In the experiments shown in Figure 3, 5-phosphoribosyl 1-pyrophosphate enriched erythrocytes have been incubated with an excess of cold hypoxanthine (14 nmol/mL

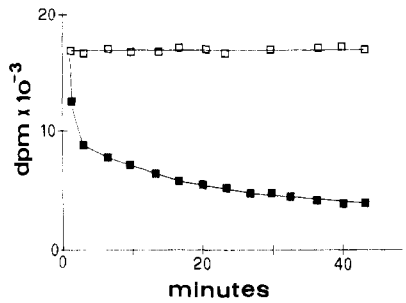

FIGURE 3: [^{14}C]Guanine uptake by IMP-enriched human erythrocytes. The cells were preincubated for 50 min at 37 °C in buffered saline containing 9 mM phosphate (■) or not containing phosphate (□), washed twice in buffered saline not containing phosphate, and transferred to an equal volume of the last medium containing 14 μM hypoxanthine. After 4 min of incubation, 0.05 mL of isotonic NaCl containing 0.15 mM [^{14}C]guanine was added to 2 mL of the incubation mixture. At regular intervals, samples of the incubation mixtures were removed, and the separated medium was employed for liquid scintillation counting. The incubation was carried out at 37 °C. All other conditions were as described in the text. (Abscissa) Time of incubation in the medium containing [^{14}C]guanine.

of suspending medium) to consume all the available intracellular 5-phosphoribosyl 1-pyrophosphate (around 7 nmol/mL of packed cells) through the hypoxanthine–guanine phosphoribosyl transferase catalyzed reaction with formation of IMP (Hershko et al., 1967). Indeed, 2 min after the addition of hypoxanthine to the incubation mixture, the intracellular 5-phosphoribosyl 1-pyrophosphate concentration was very low (undetectable). By use of [^{14}C]hypoxanthine instead of cold hypoxanthine, it was possible to demonstrate that at the concentration of hypoxanthine employed only half of the labeled purine base was incorporated.

Discussion

Hypoxanthine appears to be the main purine base released from human erythrocytes (Mager et al., 1966; Hershko et al., 1967; Murray, 1971). It has been suggested (Mager et al., 1966) that purine catabolism in these cells in vivo is entirely directed via IMP to the corresponding purine base. The only catabolic pathway so far accepted (Murray, 1971) for IMP degradation involves nucleotidase and purine nucleoside phosphorylase. In this pathway, IMP should be successively split to inosine and hypoxanthine.

Some of the results obtained in this paper could be taken to support this hypothesis. In fact, in the absence of guanine in the suspending medium formycin B, at the concentrations that are known to inhibit purine nucleoside phosphorylase in intact human erythrocytes but not appreciably affect purine transport (Sheen et al., 1968), leads to the appearance of detectable amounts of inosine within the cells and to a delay in hypoxanthine release. Assuming that IMP degradation occurs through the metabolic path previously described, since after the lag time formycin B does not appreciably affect the rate of hypoxanthine release, it seems likely to suggest (McLure, 1969; Easterby, 1973) that the reaction catalyzed by purine nucleoside phosphorylase is not the rate-limiting step in this pathway. This interpretation is in line with the observation that in the absence of formycin B in the suspending medium inosine is not present in an appreciable amount within the erythrocytes.

The results concerning the lag phase preceding hypoxanthine release are not against the hypothesis that IMP is successively split to inosine and to the purine base. However, the interpretation of the results is complex and does not lead to unequivocal conclusions. A simple way to explain the experimental data is to assume that, during the lag phase, hypoxanthine is formed within the erythrocytes but that the release of the purine base does not appear owing to its reutilization through the hypoxanthine–guanine phosphoribosyltransferase-catalyzed reaction (Giacomello & Salerno, 1978). In line with this assumption are the observations that the lag phase disappears in the absence of intracellular 5-phosphoribosyl 1-pyrophosphate and that the lag time is a direct function of the amount of 5-phosphoribosyl 1-pyrophosphate remaining within the cells after the uptake of hypoxanthine added to the suspending medium. According to this hypothesis, the observation that formycin B in the incubation medium leads to a small increase in the uptake of hypoxanthine could be attributed to a sparing effect on intracellular 5-phosphoribosyl 1-pyrophosphate, whose rate of consumption in the salvage of hypoxanthine formed from intracellular IMP is decreased owing to the inhibition of the catabolic path of this nucleotide.

The results obtained in the presence of guanine in the suspending medium cannot be explained by assuming that the catabolic path for IMP degradation involves only nucleotidase and purine nucleoside phosphorylase. In fact, the addition of guanine to the suspending medium leads to an increase in the rate of hypoxanthine release, which is unaffected by the presence of formycin B.

Although a note of caution is necessary in interpretation of metabolic events occurring within the cells, the possibility that the catabolic path of IMP in the presence of guanine is different from that occurring in the absence of guanine must be considered. It has been established (Hershko et al., 1967) that guanine is taken up by the cells and converted to GMP through the hypoxanthine–guanine phosphoribosyltransferase-catalyzed reaction with consumption of 5-phosphoribosyl 1-pyrophosphate. Guanine is taken up by IMP-enriched erythrocytes as GMP. However, this can occur even at an intracellular concentration of 5-phosphoribosyl 1-pyrophosphate unassayable and insufficient to allow a further uptake of hypoxanthine as IMP. Although more complex pathways cannot be excluded, the simplest mechanism that can explain the guanine-induced formycin B independent hypoxanthine release associated with GMP synthesis is the direct transfer of the phosphoribosyl moiety of IMP to guanine through the hypoxanthine–guanine phosphoribosyltransferase-catalyzed IMP–GMP exchange (Salerno & Giacomello, 1979). The equilibrium of this reaction favors GMP and hypoxanthine formation (K_{eq} = 0.4) (Salerno & Giacomello, 1982). According to this hypothesis, hypoxanthine–guanine phosphoribosyltransferase could be, at least in some conditions, the chief enzyme involved in IMP degradation.

Registry No. IMP, 131-99-7; GMP, 85-32-5; phosphate, 14265-44-2; 5-phosphoribosyl 1-pyrophosphate, 7540-64-9; inosine, 58-63-9; hypoxanthine, 68-94-0; guanine, 73-40-5.

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Extracellular cGMP Phosphodiesterase Related to the Rod Outer Segment Phosphodiesterase Isolated from Bovine and Monkey Retinas

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ABSTRACT: A phosphodiesterase (PDE) has been characterized in the interphotoreceptor matrix (IPM) of light-adapted fresh bovine retinas. It is obtained through a gentle rinsing of the retinal surface under conditions where the light-activated rod outer segment (ROS) enzyme remains attached. The enzyme has an apparent native molecular weight of 350000 by gel filtration and appears as a doublet at M_r 47000 and 45000 on sodium dodecyl sulfate–polyacrylamide gels. It has an apparent K_m value for cGMP of 33 μM and an apparent K_m value for cAMP of 2200 μM. It is activated 3–6-fold by protamine and over 40-fold by trypsin. Protamine has no effect on the K_m for cGMP while trypsin decreases the K_m for cGMP by a factor of 2. The enzyme occurs in at least two forms as evidenced by two distinct peaks of activity after gel electrophoresis under nondenaturing conditions. A heat-stable inhibitor is tightly bound to the enzyme. The inhibitor obtained from the IPM PDE inhibits 98% of the activity of the trypsin-activated ROS PDE: conversely, the inhibitor obtained by boiling the ROS PDE completely inhibits the trypsin-activated IPM enzyme. A high-affinity monoclonal antibody to the active site of the ROS PDE, ROS 1 [Hurwitz, R., Bunt-Milan, A. H., & Beavo, J. (1984) *J. Biol. Chem. 259*, 8612–8618], quantitatively absorbs the IPM PDE. These observations indicate a clear relationship between these two PDEs even though their location, sizes, and specific functions in the retina appear to be distinct.

The major cGMP phosphodiesterase (PDE)[1] activity in retina is present in the rod outer segment organelle of the photoreceptor cell (Miki et al., 1975; Baehr et al., 1979). The ROS PDE is activated by light (Miki et al., 1973; Chader et al., 1974) and is thought to play a role in phototransduction through regulation of cGMP levels (Bitensky et al., 1978; Miller, 1983). The enzyme is a peripheral protein tightly bound to the photoreceptor disk membranes when exposed to isotonic salt and Mg^{2+}, but it can be released by vigorous washing with low ionic strength buffer (Kuhn, 1982; Miki et al., 1975; Baehr et al., 1979). The purified ROS PDE has a molecular weight of 185000 and is composed of three subunits: M_r 88000 (α), M_r 84000 (β), and an inhibitory subunit, M_r 13000 (γ) (Baehr et al., 1979; Hurley & Stryer, 1982).

The photoreceptor cell outer segments and the processes of the pigment epithelial cells interdigitate. The extracellular material that surrounds these opposing surfaces is known as the interphotoreceptor matrix (IPM). There has been a recent upsurge of interest in the IPM as a separate, defined extracellular compartment important to visual function, specifically in connection with its role in retinol (vitamin A) transport and as a potentially excellent model for studying the components and functions of the extracellular matrix (Adler & Martin, 1982; Lai et al., 1982; Liou et al., 1982; Pfeffer et al., 1984).

When the IPM material is run on SDS–PAGE, about a dozen Coomassie blue staining protein bands can be visualized (Adler & Severin, 1981). However, prior to the present study, only one of these proteins had been purified and partially characterized. This protein is the interphotoreceptor retinoid binding protein (IRBP), a glycoprotein that could be involved in retinol transport between the pigment epithelium and retina (Lai et al., 1982; Liou et al., 1982; Adler & Martin, 1982; Pfeffer et al., 1984).

We have now identified a second protein in the IPM, a cGMP PDE (designated IPM PDE). It can be easily isolated through an extremely gentle, isotonic wash of whole, light-adapted retinas. This yields the constituents of the IPM and, at the same time, leaves the ROS PDE membrane bound. The

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[1] Abbreviations: PDE, phosphodiesterase; IPM, interphotoreceptor matrix; ROS, rod outer segments; IRBP, interphotoreceptor retinol binding protein; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; Con A, concanavalin A; HPLC, high-performance liquid chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; BSA, bovine serum albumin; Cl_3CCOOH, trichloroacetic acid.