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CONVERSION OF GUANINE TO HYPOXANTHINE IN MAMMALIAN RED BLOOD CELLS

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SUMMARY

1. Experimental data are reported which indicate that human and rabbit erythrocytes are capable of converting exogenously supplied guanine to hypoxanthine.

2. Evidence is adduced to show that the overall system involves transformation of guanine into guanosine 5'-phosphate prior to its reductive deamination to inosine 5'-phosphate.

3. The enzyme catalyzing the reductive deamination utilizes reduced nicotinamide adenine dinucleotide phosphate as a specific hydrogen donor; it also requires the presence of a sulfhydryl compound.

4. Evidence to support the above conclusions as well as the role of the present system within the general framework of purine nucleotide metabolism of the red cell, are discussed.

INTRODUCTION

The utilization of exogenously supplied guanine for the synthesis of nucleotide-bound adenine has been demonstrated in a number of different microorganisms by means of isotopic and nutritional procedures[1-3]. In *Escherichia coli*, and related species, the transformation of guanine into hypoxanthine has been shown to take place at the nucleotide level and to be accomplished by a NADPH-linked enzyme, catalyzing the reductive deamination of GMP to IMP[4]; the latter compound may be then channelled to the known biosynthetic route leading to AMP[5].

Relevant information with regard to higher organisms is both scanty and controversial. ABRAMS AND GOLDINGER[6] noted a substantial production of hypoxanthine following incubation of guanine with rabbit-bone-marrow slices. ABRAMS[7] also observed that [^{14}C]guanine injected into rats undergoes a relatively slight but significant incorporation into the adenine of nucleic acid. GUARINO AND JÜREGIR[8] described the conversion of guanine to hypoxanthine, brought about by rat-liver extracts, and postulated, that this reaction is catalyzed by a system essentially analogous to the above mentioned bacterial GMP reductase (NADPH:GMP oxidoreductase (deaminating)) (see ref. 4). The latter view, however, has been challenged by BISWAS AND

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ABRAMS[9], who confirmed and amplified the experimental data of the former authors but suggested an entirely different enzymic mechanism. According to BISWAS AND ABRAMS[9], the conversion of guanine to hypoxanthine in rat-liver extracts occurs at the free purine level in two successive steps, *viz.*: the hydrolytic deamination of guanine to xanthine through the action of guanine deaminase (EC 3.5.4.3) and the subsequent reduction of xanthine to hypoxanthine by the xanthine oxidase–NADH system.

The results reported in the present communication establish the occurrence in erythrocytes of a NADPH-linked pathway mediating the conversion of GMP to IMP. A brief account of this study has been published elsewhere[10].

MATERIALS AND METHODS

Chemicals

All chemicals used were commercial products of tested purity or analytical grade. The crystalline enzymes: glutamate dehydrogenase (EC 1.4.1.3) and lactate dehydrogenase (EC 1.1.1.27), pyruvate kinase (EC 2.7.1.40) and creatine kinase (EC 2.7.3.2) were supplied by the Boehringer Co.; a purified preparation of yeast hexokinase (EC 2.7.1.1) (type III) was purchased from the Sigma Chemical Co. [8-^{14}C]Guanine, [8-^{14}C]adenine and [8-^{14}C]hypoxanthine were acquired from the Amersham Radiochemical Centre. [8-^{14}C]Xanthine was prepared from [8-^{14}C]guanine by deamination with nitrous acid and was found to be chromatographically pure. [8-^{14}C]Guanosine which was purchased from Schwarz Laboratories Inc. contained about 5 % of free guanine; this contamination, however, did not interfere with the results. Labelled guanosine 5'-phosphate was prepared enzymically by incubating a haemolyzate of human red cells with [8-^{14}C]guanine, ribose 5-phosphate and an ATP-regenerating system (ATP, phosphoenolpyruvate and pyruvate kinase) and the other components of the "standard reaction mixture" (see legend to Table III). The labelled nucleotide thus produced was adsorbed on to charcoal ("Darco") and subsequently eluted with 50 %, (v/v) aqueous ethanol containing 2.5 % (v/v) of conc. NH_4OH. The material was further purified by passing through a Dowex-1 formate column (1 × 10 cm) using a gradient-elution system consisting of 4 N formic acid in the reservoir and 250 ml of water in the mixing chamber. The fraction containing the eluted GMP fraction was evaporated to dryness *in vacuo*. The isolated compound migrated as a single, radioactive spot corresponding to GMP on paper-electrophoresis.

Red blood cells

Human blood from healthy donors or polycythaemic patients was collected by venipuncture in heparinized containers. Rabbit-blood was withdrawn by cardiac puncture. The red cells were separated from the plasma by centrifugation at 2000 × *g* for 15 min and washed twice with 3–4 volumes of physiological saline buffered with 0.01 M phosphate buffer (pH 7.4); while aspirating the supernatant fluid, care was taken to remove as thoroughly as possible the buffy coat.

Hemolyzates were prepared by freezing and thawing the washed, packed, red blood cells followed by the addition of an equal volume of water. Ghost cells and erythrocytes which had not lysed were sedimented by centrifugation at 30 000 × *g* for 20 min. The sediment was discarded and the supernatant fluid was used as a source of enzymes. This preparation could be stored at — 12° for at least 2 months without any appreciable loss of activity.

Assay procedures

The conversion of guanine to hypoxanthine was assayed by the following routine procedure:

The reaction was stopped by adding to the incubation mixture an equal volume of 2 N perchloric acid. The resulting precipitate was separated by centrifugation and washed twice with 5-ml portions of 1 N perchloric acid. The combined supernatant fluids were heated in a boiling-water bath for 1 h in order to hydrolyze the purine nucleotides. Following the addition of 5 μmoles of adenine and 0.5 μmoles of hypoxanthine as carriers, the purines were precipitated as silver salts with 2 ml of concentrated ammonia (25 %) and 3 ml of 10 % silver nitrate and left overnight in the cold. The precipitate was washed with 5 ml of distilled water and then treated twice with 3-ml portions of 0.1 N HCl to bring the purines into solution. The supernatant fluids were combined and evaporated to dryness. The residue was redissolved in a small volume of 0.1 N HCl. The solution of purines was quantitatively transferred to Whatman No. 1 filter paper and subjected to descending chromatography for about 12 h using isoamyl alcohol–5 % Na_2HPO_4 as the solvent system[11]. This system enabled a good separation of both xanthine and hypoxanthine from the other purines. To achieve a separation of adenine, guanine and hypoxanthine by descending paper chromatography, the *n*-butanol–water–formic acid mixture (77:13:10, v/v) was used[11]. The purines were located as dark spots on the paper when illuminated by ultraviolet light and the radioactivity was scanned with the aid of the Vanguard automatic, gas-flow radioscanner. The radioactive spots were cut out and the purines were eluted from the paper by heating with 5 ml of 0.1 N HCl at 80° for 15 min. A measured sample of the eluate was transferred to a planchet, dried and the radioactivity was estimated with the aid of a Nuclear Chicago low background gas-flow counter equipped with an automatic sample changer and scaler. The figures (counts/min) for hypoxanthine were corrected for the radioactivity obtained in the zero-time blanks run concurrently, which was usually within 1–2 % of the values obtained in the positive incubation experiments. Another aliquot was used for determining the spectral characteristics of the isolated purine.

In some instances hypoxanthine and xanthine were isolated by adsorption on a Dowex-50 (H^+) resin (200–400 mesh) column (0.6 × 10 cm) followed by gradient-elution with 1 N HCl in the reservoir and 500 ml of water in the mixing chamber.

In the experiment designed to isolate and identify the purine nucleotides, the supernatant fluids resulting from the perchloric acid precipitation were treated with 50 mg of acid-washed charcoal (Darco). The charcoal was washed with 10 ml of water and the adsorbed nucleotides were then eluted with a 2.5 % solution of conc. NH_4OH in 50 % (v/v) aqueous ethanol. The eluates were evaporated to dryness in a flash-evaporator. The residue was taken up in a small amount of water and then subjected to electrophoresis at 70 V/cm on Whatman No. 1 filter paper in 0.025 M citrate buffer (pH 3.5) for 90 min. With this method a satisfactory separation of IMP from the guanosine 5'-phosphates was obtained.

The synthesis of ribonucleotides from [^{14}C]purines was determined in the following manner[12]: The incubation mixture was deproteinized with an equal volume of 2 N $HClO_4$. 1 ml of the clear supernatant fluid, supplemented with 10 μmoles of sodium phosphoglycerate as carrier, was neutralized with NH_4OH (10 %, v/v) using phenol red as an indicator. Subsequently, 0.3 ml of a 25 % solution (w/v) of barium acetate

and 4 volumes of neutralized 95 % ethanol were added. The mixture was chilled in ice for a period of from 7 to 10 min and the precipitate was collected by centrifugation. Following 4 washings with chilled 80 % ethanol, the final precipitate was taken up in a small volume of an ethanol–ether mixture (3:1, v/v) and transferred to planchets. The radioactivity of the dried sample was counted at infinite thickness, as described above.

Miscellaneous techniques

The purity of the NAD^+ and $NADP^+$ preparations supplied by the Sigma Co. was checked by enzymic reduction with crystalline yeast alcohol dehydrogenase[13] (EC 1.1.1.1) or pig-heart isocitrate dehydrogenase[14] (EC 1.1.1.42), respectively, and was found to be close to 100 %.

NADH in the presence of red-cell haemolyzates was estimated by its oxidation in the presence of an excess of sodium pyruvate and crystalline beef-heart lactate dehydrogenase. The equivalent amount of the pyruvate which disappeared was determined by the method of FRIEDEMANN AND HAUGEN[15].

RESULTS

Experiment with intact red blood cells

Quantitative significance of guanine to hypoxanthine conversion

Appreciable amounts of labelled hypoxanthine were formed when human, or rabbit, erythrocytes were incubated in Krebs–Ringer phosphate medium supplemented with [8-^{14}C]guanine. A quantitative examination revealed that, under comparable conditions, the conversion of guanine to hypoxanthine constituted the most prominent reaction among the various purine interconversions catalyzed by human red blood cells (Table I). The data recorded in Table I are only approximate conversion rates. Substantial corrections would have to be introduced to account for the extent of isotope dilution in each of the different purines tested, which may vary considerably, with the size of the internal pool of each corresponding nucleotide. Thus, the average purine values obtained in analyses performed on washed human erythrocytes were as follows[16] (in μmoles/ml of packed erythrocytes): adenine,

TABLE I

INTERCONVERSION OF EXOGENOUSLY SUPPLIED PURINE BASES IN HUMAN ERYTHROCYTES

1 ml of packed, washed, red blood cells was suspended in 1.5 ml of Krebs–Ringer phosphate solution supplemented with 22 μmoles of glucose and 0.5 μmole of the [8-^{14}C]purine tested (about $5 \cdot 10^4$ counts/min, each). The mixture was incubated in 10-ml flasks at 37° for 60 min, with continuous shaking. The incubation was terminated by adding 2.5 ml of 2 N $HClO_4$ and the incorporation into the respective intracellular purines was assayed as described under MATERIALS AND METHODS.

[8-^{14}C]Purine added	Radioactivity incorporated (total counts/min)		
	Hypoxanthine	Adenine	Guanine
Hypoxanthine		193	79
Adenine	75		0
Guanine	2580	82	

0.98 ± 0.14; guanine 0.08 ± 0.012; hypoxanthine, 0.04 ± 0.017; these figures come rather close to those computed from the data of BISHOP *et al.*[17] relating to human whole blood. However, even if it be assumed, for the sake of simplicity, that a rapid and complete equilibration occurs between the exogenous purines and their intracellular nucleotide congeners, the preponderance of the guanine to hypoxanthine conversion over the other interconversion systems still prevails.

Role of glucose metabolism

Table II shows that the rate of transformation of guanine into hypoxanthine observed with washed erythrocytes was critically dependent upon the concomitant supply of both glucose and phosphate; the reaction was almost completely inhibited by approx. $5 \cdot 10^{-3}$ M monoiodoacetate. The crucial role of glucose metabolism in the system was also reflected in the characteristic time course of the conversion process.

TABLE II

FACTORS AFFECTING CONVERSION OF GUANINE TO HYPOXANTHINE BY HUMAN ERYTHROCYTES

1 ml of washed red blood cells was suspended in 2 ml of Krebs–Ringer phosphate medium supplemented with 200 μmoles Tris–chloride buffer (pH 8.5), 50 μmoles glucose and 0.5 μmole 8-^{14}C-guanine (approx. $5 \cdot 10^4$ counts/min). Where indicated, 20 μmoles sodium monoiodoacetate were added. Conditions otherwise as in Table I.

Reaction mixture	*Radioactivity in the isolated hypoxanthine (total counts/min)*
Complete	3025
Complete — glucose	295
Complete — phosphate	250
Complete + monoiodoacetate	60

The prolonged lag period (45–60 min) which manifested itself under the usual test conditions, could be completely eliminated by preincubating the washed red blood cells for 60 min in the presence of glucose prior to the addition of the labeled guanine. The formation of some essential metabolites, arising in the course of glucose breakdown, appears to be a necessary prerequisite for the operation of the conversion system.

Experiments with haemolyzed red blood cells

Specific requirement for NADPH

Conversion of guanine to hypoxanthine could also be demonstrated with cell-free haemolyzates of both human, and rabbit erythrocytes, provided that the standard reaction mixture was supplemented with an appropriate NADPH-regenerating system. As illustrated in Table III, NADPH could be generated *in situ* by adding $NADP^+$ and either glucose 6-phosphate, or isocitrate, as substrate for the respective NADP-specific enzymes contained in the haemolyzate. Alternatively, glutamate and a crystalline preparation of beef-liver glutamate dehydrogenase were employed as a couple suitable for generating both NADH and NADPH, owing to the ability of the latter enzyme to link up with either NAD^+, or $NADP^+$, as the hydrogen acceptor[18].

The requirement for NADPH could not be fulfilled by NADH (Table III). It could be argued that the inadequacy of NADH as a hydrogen-donor in the present

system might be due to its rapid destruction, or oxidation, by competing enzymes present in the crude red-cell haemolyzate. The latter argument, however, was refuted by the finding that out of 10 μmoles of NADH added to the reaction mixture about 6 μmoles could be recovered at the end of incubation period of 2 h.

TABLE III

FORMATION OF HYPOXANTHINE FROM GUANINE IN RED CELL HAEMOLYZATES: SPECIFIC REQUIREMENT FOR NADPH

Each tube contained, in a total volume of 3 ml, the "standard reaction mixture" of the following composition: 200 μmoles of Tris–chloride buffer (pH 8.5), 20 μmoles of $MgSO_4$, 100 μmoles of KCl, 10 μmoles of GSH, 0.5 μmoles of [8-^{14}C]guanine (approx. $5 \cdot 10^4$ counts/min), 2 ml of red-cell haemolyzate. The supplements were added in the following amounts: sodium glucose 6-phosphate, 10 μmoles; DL-sodium isocitrate, 20 μmoles; sodium-L-glutamate, 50 μmoles; NADP, 1 μmole; NADH, 10 μmoles; crystalline beef-liver glutamate dehydrogenase, 100 μg. The final mixtures were incubated at 37° for 120 min.

Exogenous components of the hydrogen-donating system			*Radioactive hypoxanthine produced by the haemolyzate (total counts/min)*	
Substrate	*Enzyme*	*Coenzyme*	*Human*	*Rabbit*
Glucose 6-phosphate	—	$NADP^+$	3170	2325
Glucose 6-phosphate	—	—	60	
—	—	$NADP^+$	35	
Isocitrate	—	$NADP^+$	4625	
Isocitrate	—	—	125	
Glutamate	Glutamate dehydrogenase	$NADP^+$	2645	2020
Glutamate	—	$NADP^+$	230	
—	Glutamate dehydrogenase	$NADP^+$	120	
Glutamate	Glutamate dehydrogenase	NAD^+	130	85
—	—	NADH	90	

Inertness of xanthine

A reaction sequence involving xanthine as an intermediate[9] appears to be ruled out by the negative outcome of the following experiments:

Addition of an excess of non-radioactive xanthine to the standard test system did not result in a dilution of the isotope content of the hypoxanthine produced from [8-^{14}C]guanine. Lack of guanine deaminase activity in the haemolyzate is inferred from the failure of radioactive xanthine to accumulate when the reduction of guanine to hypoxanthine was prevented by the omission of NADPH from the otherwise complete standard reaction mixture (see legend to Table III). Moreover, [8-^{14}C]-xanthine did not give rise to any detectable amount of hypoxanthine when tested under the optimal conditions furthering the conversion of guanine to hypoxanthine. In addition, no formation of radioactive uric acid was noted on incubating the haemolyzate with labelled xanthine or hypoxanthine, thus corroborating the earlier reports in the literature[19,20] concerning the absence of xanthine oxidase in human whole blood.

Requirement for ribose 5-phosphate, Mg^{2+}, ATP and thiol compounds

Red blood cells are able to utilize preformed guanine for the synthesis of GMP by either of the following two pathways A and B (see ref. 21):

A. Ribose 5-phosphate + ATP $\xrightarrow{Mg^{2+}}$ 5-phosphoribosyl pyrophosphate + AMP (1)

Guanine + 5-phosphoribonyl pyrophosphate $\xrightarrow{Mg^{2+}}$ GMP + PP_i (2)

B. Ribose 5-phosphate $\rightleftharpoons$ Ribose 1-phosphate (3)

Guanine + Ribose 1-phosphate $\rightleftharpoons$ Guanosine + P_i (4)

Guanosine + ATP $\xrightarrow{Mg^{2+}}$ GMP + ADP (5)

A scrutiny of the above reaction schemes shows that both mechanisms involve ribose phosphate, ATP and Mg^{2+} as essential components. Consequently, if the conversion of guanine to hypoxanthine takes place at the nucleotide level, the overall system concerned should show a dependence on the above substances.

As mentioned before, our system when examined in crude haemolyzates of red cells exhibited maximum activity with no supplements other than NADPH. It appeared likely, however, that additional requirements might become manifest following the removal from the haemolyzate, of its high content of endogenous metabolites (see ref. 22).

In fact, dialysis of the haemolyzate was found to result in complete inactivation of the system studied. The activity could be fully restored, however, by adding GSH and ribose 5-phosphate. Furthermore, the essential role of Mg^{2+} was revealed by the loss of activity following its omission from the reaction mixture (Table IV).

The latter system showed no overt demand for added ATP, even when the dialyzed haemolyzate was treated with activated charcoal in order to remove the remaining traces of nucleotides. Indirect evidence, however, for the participation of ATP in the conversion process of guanine to hypoxanthine was provided by the drastic inhibition induced by the addition of glucose and an excess of yeast hexokinase as an ATP-

TABLE IV

REQUIREMENTS FOR CONVERSION OF GUANINE TO HYPOXANTHINE AND RIBONUCLEOTIDE FORMATION WITH A DIALYZED HUMAN HAEMOLYZATE

The complete system contained, in addition to the "standard reaction mixture" (see legend to Table III), the following ingredients: 10 μmoles of GSH, 2.5 μmoles of ribose 5-phosphate, and the NADPH-regenerating system consisting of 1 μmole of $NADP^+$, 50 μmoles of sodium glutamate and 100 μg of glutamate dehydrogenase. Where indicated, 5 mg of a hexokinase preparation (approx. 8000 Kunitz units) and 20 μmoles of glucose were added. The ATP-regenerating system consisted of 0.2 μmole of ATP, 20 μmoles of creatine phosphate (sodium salt) and 20 μg of crystalline creatine kinase. The haemolyzate was dialyzed in the cold room (6–8°) against 2 changes of 0.01 M Tris buffer (pH 7.4) for about 15 h with continuous, magnetic stirring. Conditions otherwise were as in Table III

Reaction mixture	*Radioactivity incorporated (total counts/min)*	
	Hypoxanthine	*Ribonucleotide fraction*
Complete	2500	28 600
Complete − ribose 5-phosphate	110	178
Complete − GSH	80	30 000
Complete − $MgSO_4$	350	1 200
Complete + ATP-regenerating system	2850	28 600
Complete + hexokinase + glucose	350	2 500
Complete + hexokinase + glucose + ATP-regenerating system	2080	28 000

trapping device; the inhibition could be reversed by including in the reaction mixture a suitable ATP generator, such as creatine phosphate and the corresponding kinase (Table IV).

Moreover, a supply of continuously generated ATP became essential when GSH was replaced by cysteine as an alternative thiol compound (Table V).

TABLE V

INTERRELATIONSHIP BETWEEN THE EFFECTS OF THIOL COMPOUNDS AND OF ATP ON THE CONVERSION OF LABELLED GUANINE TO HYPOXANTHINE

The reaction mixture was identical with the complete system described in Table IV. The ATP-regenerating system added was as specified in Table IV. Where indicated, GSH was replaced by 10 μmoles of L-cysteine hydrochloride (neutralized). The dialyzed haemolyzate (see Table IV) was briefly stirred with acid-washed charcoal (Darco); the charcoal was subsequently removed by centrifugation at 30000 × g for 20 min and at about 6°. Other conditions were as in Table III.

Thiol compound added	*ATP-regenerating system added*	*Radioactivity incorporated (total counts/min)*	
		Hypoxanthine	*Ribonucleotide fraction*
None	−	410	30000
None	+	375	29000
GSH	−	2250	27600
GSH	+	2000	31000
Cysteine	−	155	8500
Cysteine	+	1675	27000

The above results suggest that the system employed in the present study possesses an efficient device for ATP generation which obviates the need for added ATP. The precise nature of the underlying mechanism, as well as the rather intricate interplay between it and the thiol compounds, has not yet been clarified. These aspects are being currently investigated and the results will be dealt with in detail in a separate communication. It may be stated briefly at present, however, that the available data are consistent with the interpretation that cysteine shares with GSH the capacity to activate some essential component of the system which catalyzes the conversion of guanine to hypoxanthine. As mentioned above, however, cysteine was found to differ from GSH in being unable to perform its activating function without ATP added. The peculiar association between the action of cysteine and the requirement for ATP appears to be attributable to the inhibitory effect exerted by cysteine on the endogenous generation of ATP. This conclusion is based on the finding that the pronounced cysteine-induced inhibition of the incorporation of guanine into the ribonucleotide fraction was completely relieved by exogenously supplied ATP (Table V). The latter interpretation seems to afford at least a partial explanation for the seemingly specific ability of GSH to promote the conversion of guanine to hypoxanthine in the absence of an exogenous source of ATP.

As illustrated in Tables IV and V, the synthesis of GMP from guanine and the conversion of guanine to hypoxanthine displayed a parallel response to ATP, ribose 5-phosphate and Mg^{2+}. This correlation favours the conclusion that in the overall process mediating the transformation of guanine into hypoxanthine the synthesis of GMP constitutes a preparatory step for the eventual reductive deamination of

GMP to IMP. Evidently, it is the conversion of GMP to IMP which requires activation by thiol compounds, since the extent of ribonucleotide formation from guanine remains unimpaired in the absence of GSH (see Tables V, VI and VII). The notion of a two-stage mechanism appears to be borne out also by the shapes and by the parallel course of the corresponding time–activity curves (see Fig. 1).

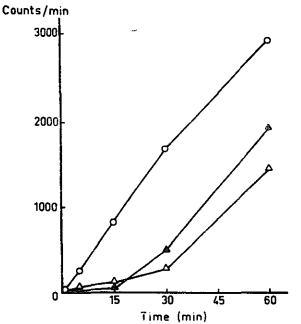

Fig. 1. Time course of the conversion reactions. O—O, GMP to IMP; △—△, guanine to IMP; ▲—▲, guanine to guanine nucleotides (the actual values on the latter curve were: counts/min × 10). Conditions as in Tables VII and V, respectively.

Consonant with the latter interpretation was the additional finding that replacement of guanine by guanosine as a substrate in the otherwise unaltered assay system eliminated the need for the addition of ribose 5-phosphate. On the other hand, the pattern of requirements for ATP, thiol compounds and Mg^{2+}, remained essentially the same as that observed with guanine (Table VI). Finally, when the reaction products were examined by paper electrophoresis, without prior hydrolysis (which was otherwise applied as a routine procedure in the standard quantitative assay), the only radioactive spots detected were those corresponding to the guanosine 5′-phosphates (GMP, GDP and GTP) and IMP.

The results were practically identical regardless of whether guanine, or guanosine, was used as the ^{14}C substrate. (The identity of the eluted spots was confirmed by paper chromatographic separation of the purine bases liberated by acid hydrolysis.)

Conversion of GMP to IMP

The conclusion arrived at in the preceding chapter that the conversion of guanine to hypoxanthine takes place at the nucleotide level was fully borne out by the results of the experiments in which [8-^{14}]GMP was used as a substrate in place of guanine.

In accordance with the previous data obtained with guanine and guanosine, the formation of labelled hypoxanthine from [^{14}C]GMP was found to depend on the supply of NADPH which could not be substituted for by NADH. In addition, the dialysed and charcoal-treated haemolyzate exhibited an essentially non-specific requirement for thiol compounds (Table VII).

TABLE VI

INCORPORATION OF LABELLED GUANOSINE INTO GUANINE RIBONUCLEOTIDES AND INOSINE 5'-PHOSPHATE CATALYZED BY A HUMAN, RED CELL HAEMOLYZATE

The complete system consisted of the standard reaction mixture with [8-^{14}C]guanine replaced by an equivalent amount of [8-^{14}C]guanosine (see Table III), and fortified with 10 μmoles of GSH together with the regenerating systems, for NADPH and ATP, described in Table IV. Where indicated, GSH was replaced by 10 μmoles of L-cysteine. Conditions otherwise were as in Table V. The nucleotides produced in the course of incubation were identified by paper-electrophoresis and, following hydrolysis, were assayed as free purines, using the standard procedures described under METHODS.

Reaction mixture	*Radioactivity incorporated (total counts/min)*	
	Guanosine 5'-phosphate	*Inosine 5'-phosphate*
Complete	18 500	1250
Complete — ATP-regenerating system	600	32
Complete — NADPH-regenerating system	18 500	0
Complete — Mg^{2+}	1 500	128
Complete — GSH	17 000	320
Complete — GSH + cysteine	16 500	1250

TABLE VII

CONVERSION OF LABELLED GUANOSINE 5'-PHOSPHATE TO INOSINE 5'-PHOSPHATE CATALYZED BY A HUMAN RED CELL HAEMOLYZATE

The complete system contained the "standard reaction mixture" described in Table III (with [8-^{14}C]guanine replaced by an equivalent amount of [8-^{14}C]GMP) supplemented with 10 μmoles of GSH and 1 μmole of NADPH. Where indicated, GSH was replaced by 10 μmoles of L-cysteine. The treatment of the haemolyzate and other conditions were as detailed in Tables V and VI.

Reaction mixture	*Radioactivity incorporated (total counts/min) IMP*
Complete	1200
Complete — NADPH	22
Complete — Mg^{2+}	1150
Complete — GSH	280
Complete — GSH + cysteine	1087

Predictably, the conversion of GMP into IMP took place without addition of ribose 5-phosphate and ATP; omission of Mg^{2+} did not affect the conversion (see Table VII). Furthermore, GMP and IMP proved to be the only purine derivatives detectable when examined directly (without prior hydrolysis) by paper electrophoresis.

Under the conditions employed, the conversion of GMP to IMP appeared to follow zero order kinetics, as reflected in the practically linear time course of the reaction. This behaviour contrasted with the appreciable initial lag observed when studying the rate of conversion of guanine to hypoxanthine (see Fig. 1). The pH-activity curve of the reaction showed a rather broad optimum extending from pH 7.6 to 8.5.

DISCUSSION

The elucidation of the pathway for the conversion of guanine to hypoxanthine in erythrocytes was greatly facilitated by the absence, in these cells, of guanine deaminase

and xanthine oxidase, enzymes which would tend to obscure the picture by side-tracking the substrate towards xanthine and uric acid. From the experiments carried out on cell-free haemolyzates the conclusion is drawn that the formation of hypoxanthine from guanine involves the transformation of guanine into GMP prior to the reductive deamination of GMP to IMP. The main evidence is based on the finding that the overall system exhibited a specific requirement for NADPH, regardless of whether guanine, guanosine, or GMP, was used as substrate; on the other hand, the additional demand for a joint supplement of ribose 5-phosphate, ATP and Mg^{2+}, or for ATP and Mg^{2+} only, was shown to be associated with the particular enzymic reactions catalyzing the conversion of guanine or guanosine, respectively, to GMP. This interpretation is further substantiated by the identification of guanine nucleotides and IMP as the sole reaction products.

Thus, the enzymic system which governs the conversion of guanine to hypoxanthine in erythrocytes is entirely different from the system postulated by BISWAS AND ABRAMS[9] in the case of rat-liver extracts; it seems, however, to be identical essentially with the corresponding system described in *E. coli*[4]. The GMP-reducing enzyme discovered in the red blood cells and the bacterial GMP-reductase closely resemble each other in their general characteristics, such as substrate-, and coenzyme-specificity, optimum pH and requirement for a thiol compound. It appears likely that this analogy extends also to the presumed physiological function performed by these enzymes[4].

GMP-reductase confers upon the cell the ability to utilize guanine for the synthesis of inosinic acid, thus parallelling the metabolically analogous function of adenosine deaminase (EC 3.5.4.4) which is known to be present in red blood cells[23]. Since IMP serves as a common biosynthetic precursor of both AMP and GMP[5], the continuous replenishment of the IMP pool through the concerted action of these enzymes provides a flexible mechanism for adjusting the intracellular level of the individual purine nucleotides to both the varying supply of exogenous purines and to the momentary metabolic needs of the cell.

Such a mechanism may be of vital importance in the bone marrow which according to the data of LAJTHA AND VANE[24] possesses a limited capacity for synthesizing its own purine nucleotides and depends to a large extent on the supply of preformed purines manufactured by the liver. The same consideration applies with even greater cogency to the mature red blood cell which has lost completely the ability to produce purine nucleotides *de novo*[25]. Furthermore, the task of maintaining a constant and a properly balanced pool of free purine nucleotides poses particular problems in the red cell which is unique in its inability to synthesize nucleic acids. It may be relevant to mention in this connection the work of HENDERSON AND LE PAGE[26] which indicates that the uptake of preformed purines *in vivo* constitutes a major function of the red blood cells of the mouse. The hypothesis advanced by the latter authors that erythrocytes may serve as a vehicle for transporting the purines produced in the liver to the other tissues is consistent with the fact that these cells lack the catabolic enzymes involved in uric acid formation.

Some further insight into the physiological role of GMP-reductase might be gained perhaps by exploring the metabolic status of the guanine nucleotides in red blood cells afflicted with a congenital deficiency of glucose-6-phosphate dehydrogenase[27] (EC 1.1.1.49). It appears safe to antiticipate that this condition should create a

functional derangement of the GMP-reductase owing to the inadequate supply of NADPH and the diminished content of GSH[27].

It is hoped that progress in the isolation and purification of GMP-reductase from red blood cells will enable further characterization of this enzyme and permit the elaboration of the stoichiometry of the reaction as well as a study of the regulatory mechanism involved.

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