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THE CONVERSION OF GUANINE TO HYPOXANTHINE IN RAT-LIVER EXTRACTS

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SUMMARY

Crude dialyzed homogenates of rat liver convert [^{14}C]guanine to hypoxanthine. This conversion is stimulated by the addition of ATP, ribose-5-phosphate, and DPN.H. The data presented are consistent with the idea that the reductive deamination takes place at the nucleotide level and by a pathway not involving opening of the purine ring.

INTRODUCTION

In a series of studies involving the utilization of [8-^{14}C]guanine by extracts of rat liver, we noted the appearance of isotope in free hypoxanthine. ABRAMS AND GOLDINGER[1] also noted the formation of labeled hypoxanthine in experiments designed to study the incorporation of labeled guanine and adenine into the nucleic acid of rabbit bone marrow. Hydrolytic deamination of the free base could easily account for the results obtained using [8-^{14}C]adenine as substrate, but with guanine this would result in the formation of xanthine a compound requiring further reduction.

From the work of CARTER AND COHEN[2,3], LIEBERMAN[4], ABRAMS AND BENTLY[5,6], MAGASANIK *et al.*[7-9], and LAGERKVIST[10,11], guanine and adenine formation from *de novo* synthesized purine may be expected to take place at the nucleotide level. Since

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other purine interconversions are known to occur at the free base and nucleoside level, we became interested in determining at what level this reductive deamination was taking place. This report presents evidence that the conversion of [8-^{14}C]guanine to [^{14}C]hypoxanthine in rat-liver extracts probably occurs at the nucleotide level, and by a pathway in which the purine skeleton remains intact.

While this work was in progress, the paper of MAGER AND MAGASANIK[12] appeared in which they reported the reductive deamination of guanosine-5'-phosphate to inosine-5'-phosphate in purine auxotrophs. The results of our work indicate that a similar pathway may exist in rat liver.

MATERIALS AND METHODS

[8-^{14}C]Guanine and [2-^{14}C]guanine were purchased from the California Foundation for Biochemical Research. The [8-^{14}C]guanine was co-chromatographed on Dowex-50-H$^+$ (200–400 mesh 10 ×) with carrier uric acid, xanthine, hypoxanthine, guanine, and adenine. The system used led to a complete separation of these five purines (Fig. 1). No radioactivity was observed in any of the purines eluted except guanine itself. Guanine, hypoxanthine, xanthine, and adenine were purchased from Schwarz Laboratories and were used without further purification. Adenosine triphosphate (ATP), 95 % pure, was purchased from Pabst Laboratories. Xanthosine-5'-phosphate was prepared by nitrous acid treatment of guanosine-5'-phosphate. Guanosine-5'-phosphate was purchased from Pabst Laboratories. Ribose-5-phosphate was prepared by acid hydrolysis of adenosine-5'-phosphate. Adenosine-5'-phosphate was purchased from Pabst Laboratories. Xanthine oxidase was prepared from milk[13]. Reduced diphosphopyridine nucleotide (DPN.H) was purchased from Sigma Chemical Company. Ion exchange chromatography utilized Dowex-50-H$^+$ (200–400 mesh 10 X)

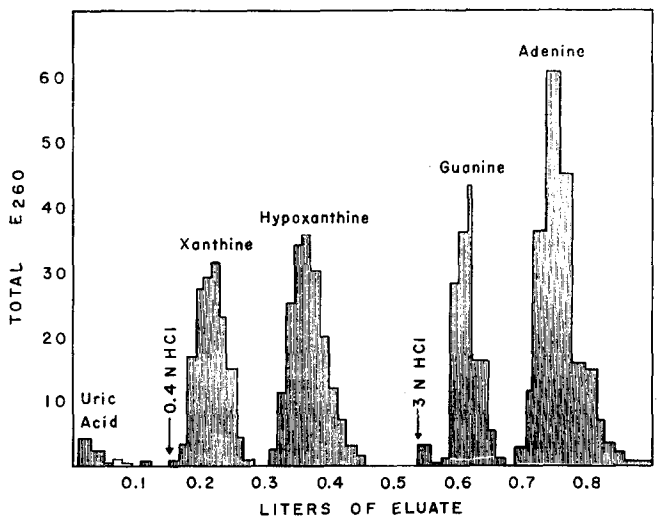

Fig. 1. Chromatography of purines on Dowex-50-H$^+$. Approximately 5 μmoles each of carrier xanthine, hypoxanthine, guanine, and adenine were added to the acid-hydrolyzed acid-soluble extract of rat liver. The purines were adsorbed onto a Dowex-50-H$^+$ column (2 cm in diameter by 10 cm long) at a flow rate of 1.5 ml/min. The column was washed with an equal volume of water and the purines eluted with HCl as indicated above.

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for cation exchange, and Dowex-1-Cl (200–400 mesh 10 X) for anion exchange. Compounds eluted from ion exchange columns were collected on a Gilson Medical Electronics Fraction collector. All aliquot portions of pooled fractions from ion-exchange columns were plated directly on stainless steel planchets and counted in a nuclear gasflow Geiger counter with a micro-mill window using an automatic sample changer. Pentose was determined by the method of MEJBAUM[14]. Phosphate was determined by KING's modification[15] of the FISKE-SUBBAROW[16] procedure.

EXPERIMENTAL

Rats were stunned by a blow on the head, exsanguinated, and the liver quickly excised and placed on ice. The liver was minced with scissors and homogenized in a Potter-Elvehjem homogenizer in 1.5 volumes of buffer containing 0.013 *M* sodium phosphate pH 7.4, 0.04 *M* KCl, and 0.004 *M* $MgCl_2$. The crude homogenate was centrifuged in the number 30 rotor of a Spinco Model L Ultracentrifuge at 28,000 rev./min for 1 h. The supernatant obtained by this procedure was then dialyzed 24 h against a phosphate buffer of the same pH and molarity as that used in preparing the homogenate. The precipitate which formed was removed by centrifugation. The clear supernatant from this centrifugation was used directly as the source of enzyme.

A typical experiment was conducted as follows: 20 ml of the enzyme prepared as described above was incubated at 37° for 0.5 h with 20 μmoles of [8-^{14}C]guanine (specific activity of 75,000 counts/min/μmole), 40 μmoles of sodium glutamate, and appropriate co-factors to be tested, in a final volume of 35 ml. At the end of this incubation period the reaction was stopped by adding an equal volume of 1 *M* $HClO_4$. The precipitated protein was washed twice with small volumes of 0.5 *M* $HClO_4$, the washings being combined with the original supernatant. The solution was adjusted to a final concentration of 1 *M* $HClO_4$ and heated in a boiling water bath for 1 h to liberate the purines. The hydrolysate was adjusted to pH 8 by the addition of 5 *N* KOH. The solution was chilled overnight at 2° to produce more extensive precipitation of $KClO_4$, which was removed by centrifugation. The precipitated salt was not washed. 5 μmoles each of carrier hypoxanthine, xanthine, guanine, and adenine were then added, the pH adjusted to 2 by the addition of HCl, and the free purines adsorbed onto a Dowex-50-H^+ column (2 cm in diameter by 10 cm long) at a flow rate of 1.5 ml/min. The column was washed with an equal volume of water and the purines eluted with HCl as shown in Fig. 1. The hypoxanthine peak was combined and an aliquot taken for counting.

In experiments in which the action of various cofactors was tested, the same liver homogenate was taken, one-half being used with the desired cofactor, the other half in the absence of the cofactor, as a control. Both separations were carried out simultaneously using a tandem operation of the fraction collector.

Identification of hypoxanthine

In a typical run approximately 7 % of the recovered radioactivity was found in the peak identified as hypoxanthine. This material was characterized by its absorption spectrum both in acid and alkali in an experiment in which no carrier hypoxanthine was added. In another experiment the hypoxanthine-rich fractions as obtained from the Dowex-50-H^+ column were pooled, neutralized, and incubated with purified

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cream xanthine oxidase in glycylglycine buffer pH 5.7. Carrier uric acid, xanthine, and hypoxanthine were added to the enzymically treated material, and rechromatographed on Dowex-50-H^+. All radioactivity was now associated with the uric acid peak and none with the hypoxanthine.

If the conversion of guanine to hypoxanthine took place at the nucleotide level in these dialyzed extracts, then the addition of ribose-5-phosphate and ATP to the dialyzed preparation might be expected to result in a stimulation of isotope incorporation into hypoxanthine. As can be seen in Table I, adding ribose-5-phosphate and ATP to the dialyzed preparation resulted in a three-fold stimulation of incorporation of label into hypoxanthine. That the stimulatory effect of these substances is not due entirely to the ATP alone is shown in the same table by an experiment in which ATP was added to both the control and experimental vessel, the latter containing in addition ribose-5-phosphate. When DPN.H was added to a dialyzed preparation containing ATP and ribose-5-phosphate a further stimulation of isotope incorporation into hypoxanthine was noted (Table I).

TABLE I

THE EFFECT OF VARIOUS SUBSTANCES ON THE TRANSFER OF ISOTOPE FROM [8-^{14}C]GUANINE INTO HYPOXANTHINE IN CRUDE RAT-LIVER PREPARATIONS

All vessels contained 20 ml of the dialyzed extract and 20 μmoles of [8-^{14}C]guanine (specific activity of 75,000 counts/min/μmole) in addition to the substances listed below.

Expt.	*Additions*	*Total counts recovered in the isolated hypoxanthine*
1	None	7,000
2	20 μmoles of ATP and 40 μmoles of ribose-5-phosphate	22,000
3	20 μmoles of ATP	16,000
4	20 μmoles of ATP, 40 μmoles of ribose-5-phosphate, and 50 μmoles of DPN.H	45,000

Although we routinely liberate all purines by acid-hydrolysis at the end of the experiment, we decided to omit the hydrolysis in one experiment and to isolate inosinic acid from the reaction vessel. 20 μmoles of ATP, 40 μmoles of ribose-5-phosphate, 20 μmoles of [8-^{14}C]guanine (specific activity of 75,000 counts/min/μmole), 20 ml of dialyzed extract, and 100 μmoles of sodium inosinate were incubated in a final volume of 35 ml for 15 min. At the end of this period of time the reaction was stopped by adding an equal volume of ice-cold 1 *M* $HClO_4$, and the acid-soluble extract was made as described above. The solution was adjusted to pH 8 and the acid-soluble nucleotides adsorbed onto a Dowex-1-formate column (1 cm diameter by 10 cm high). 10-ml fractions were collected at a flow rate of approx. 0.5 ml/min. Gradient elution was used, forming the first gradient by placing 2 *M* formic acid in the reservoir and water in the mixing vessel. Reservoirs were then substituted containing in turn: 4 *M* formic acid; 0.2 *M* sodium formate and 4 *M* formic acid; and finally, 0.4 *M* sodium formate and 4 *M* formic acid. Pooled fractions 75 through 93, eluted at the time 4 *M* formic acid was in the reservoir, were found to contain 6,380 total counts and an absorption spectrum characteristic of inosinic acid. Pentose, phosphate, and base (calculated on the basis of the extinction coefficient for inosine as $1.2 \cdot 10^4$) ratios were found to be 0.93:1.2:1.

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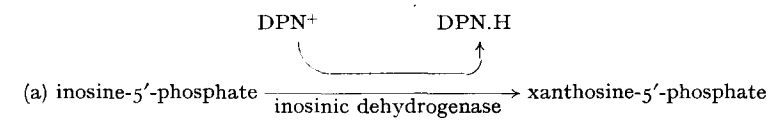
Accordingly the labeled product was considered to be inosinic acid which would be consistent with the idea that the conversion took place at the nucleotide level. As to the pathway of conversion an important possibility is a reversal of the inosinic dehydrogenase reaction (a)



especially since DPN.H stimulates the overall reaction. MAGASANIK[8] reported that he could not show a reversal of this reaction with a bacterial enzyme preparation. We tried to show a reoxidation of DPN.H dependent upon the presence of xanthosine-5'-phosphate with the liver preparation. Using the crude preparation as described above as well as various $(NH_4)_2SO_4$ fractions from this preparation, we have so far been unable to show the reversal of this reaction.

In view of the known lability of the C-2 position of the purine ring the possibility had to be considered that an opening of the ring occurred with a loss of carbon 2 and subsequent replacement with a carbon atom at a lower level of oxidation. [2-^{14}C]-guanine was tried in place of the [8-^{14}C]guanine in an attempt to show a dilution of isotope in the hypoxanthine isolated from the C-2 labeled precursor. The incubation mixtures contained 20 μmoles of ATP, 40 μmoles of ribose-5-phosphate, 50 μmoles of DPN.H, 20 ml of the enzyme extract, and in one case 20 μmoles of [2-^{14}C]guanine (specific activity of 75,000 counts/min/μmole) and in the other 20 μmoles of [8-^{14}C]-guanine of the same specific activity. The two experiments were run in tandem and as can be seen in Table II, no dilution of isotope occurred in the case in which the C-2 labeled compound was used as a substrate. A second set of experiments were made in the same way except that [2-^{14}C]guanine was used in both cases, with the added difference that the experimental vessel contained in addition 100 μmoles of sodium formate. As can be seen in Table II, the presence of formate in the reaction mixture did not dilute the isotope content of the isolated hypoxanthine. Of course this does not exclude the possibility that some substance other than formate may be the one entering an exchange. MAGASANIK[17] has been able to show an incorporation of isotope from C-2 labeled guanine to histidine in a bacterial system. We tried a similar experiment in the rat liver system. 40 μmoles of histidine were incubated with [2-^{14}C]-guanine in an experiment as described in experiment three of Table II. In two trials we found no dilution of label in the isolated hypoxanthine. In one experiment the

TABLE II

COMPARATIVE ISOTOPE TRANSFER FROM [^{14}C]GUANINE IN THE CRUDE LIVER PREPARATION

All vessels contained 20 μmoles of ATP, 40 μmoles of ribose-5-phosphate, 50 μmoles of DPN.H, and 20 ml of extract, in addition to the substances listed below.

Expt.	*Additions*	*Total counts*
1	20 μmoles of [2-^{14}]guanine (specific activity of 75,00 counts/min/μmole)	43,000
2	20 μmoles of [8-^{14}C]guanine (specific activity of 75,000 counts/min/μmole)	45,000
3	20 μmoles of [2-^{14}C]guanine (specific activity of 75,000 counts/min/μmole) and 100 μmoles of sodium formate	43,000

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histidine was reisolated at the end of the experiment by ion exchange chromatography and found still entirely unlabeled. These experiments make unlikely a reduction involving a ring opening.

MAGASANIK[12] has recently reported that a reductive deamination of guanosine-5'-phosphate to inosine-5'-phosphate in bacteria requires TPN.H. We did not add TPN.H to our experimental vessels, but since we used a crude enzyme preparation the presence of transhydrogenating activity could easily explain the stimulation by DPN.H in the liver enzyme system.

The results of this work eliminate several alternative possibilities for the conversion of guanine to hypoxanthine in rat-liver homogenates. The results are in agreement that a pathway for reductive deamination may exist in mammalian systems similar to that found in bacteria. The implications of such a pathway have been presented by MAGASANIK[12].

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