THE ENZYMIC CONVERSION OF ADENINE TO ADENOSINE PHOSPHATES*

by

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The mechanism of formation of adenosine-5'-phosphoric acid (AMP) by the phosphorylation of adenosine has been well established through the work of OSTERN AND TERSZAKOWEC[2], CAPUTTO[3] and KORNBERG AND PRICER[4], but the participation of the free purine, adenine, in nucleotide formation has received little attention. The formation of inosinic acid, however, from hypoxanthine[5] or from purine precursors such as glycine and formate[6] is currently the subject of intensive investigation. During a study of the incorporation of labelled adenine into the ribonucleic acid of pigeon liver homogenates[7] it was found that these preparations could readily convert the purine to acid-soluble nucleotides. The formation of acid-soluble nucleotides from labelled adenine has also been demonstrated in perfused rabbit liver*** and in mice[8]. The work described in this communication is the start of a study of the pathway of the formation of AMP from adenine.

EXPERIMENTAL

Adenine-8-^{14}C hydrochloride was prepared by CLARK AND KALCKAR[9] and contained 0.8 μC/μM corresponding to $2.1 \cdot 10^5$ c.p.m./μM using an end window counter of about 12 % efficiency. Samples with a total weight of less than 0.1 mg were plated directly as 1 cm^2 circles on aluminum planchets and enough counts registered to ensure 3–4 % accuracy.

The homogenates were prepared and incubated as previously described[7]. After the incubation period the reaction was stopped by chilling and addition of cold perchloric acid to a final concentration of 4%. The acid-insoluble material was removed by centrifugation. In some experiments the soluble nucleotides were precipitated as the barium salts according to the method of LE PAGE[10].

Column chromatography of the nucleotides was carried out according to the method of COHN[11] with a slight modification which is discussed later. Paper chromatography was done on Whatman No. 1 filter paper in the indicated solvents and the spots were detected under a mineralight.

Analyses for adenosine, the adenosine-5-phosphates, and hypoxanthine were done by differential enzymic spectrophotometry according to KALCKAR[12] and for adenine by the method of KLENOW[13] using a Beckman model DU spectrophotometer.

RESULTS AND DISCUSSION

Conversion of adenine to hypoxanthine

After incubation of 0.15 μM of adenine-8-^{14}C hydrochloride for 90 minutes with a homogenate containing about 0.5 g of tissue, the acid-soluble fraction was extracted

* Aided by grants from The Rockefeller Foundation, Carlsberg Fondet, The Williams-Waterman Foundation and the Lederle Laboratories Division of The American Cyanamid Company. This work was done while the author was a Fellow of the American Cancer Society as recommended by the Committee on Growth of the National Research Council. A preliminary report has been published[1].

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*** KALCKAR, H. M. AND BENNETT, E. L., Unpub.

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with *n*-butanol saturated with water until no counts were found in the non-aqueous layer. As a result of this extraction, the nucleotides and inosine which remained in the water phase were separated from the purines and the other nucleosides. The butanol extract was then concentrated and examined by paper chromatography in the butanol, water system described by HOTCHKISS[14]. There was no detectable adenine found and all of the isotope present was recovered as hypoxanthine. The hypoxanthine isolated accounted for about 60% of the total isotope in the acid-soluble fraction, and had a specific activity (c.p.m./μM) one-tenth that of the original adenine.

Since there was a report of a possible direct conversion of adenine to hypoxanthine by hyperplastic rabbit marrow[15], the pigeon liver preparations were examined for the presence of an adenase. After incubation of the homogenate in the presence of 0.5 μM of adenine for 130 minutes, 1.58 μM of hypoxanthine were found as compared with 1.65 μM recovered after incubation without added adenine. These results indicated that there was no direct formation of hypoxanthine from adenine. Since pigeon liver preparations were found to contain no xanthine oxidase, in agreement with the finding of MORGAN[16], there can be no question of the oxidation of any hypoxanthine which might have been derived directly from adenine.

The disappearance of labelled adenine and the appearance of labelled products were studied as a function of time in order to clarify the mechanism of formation of the labelled hypoxanthine. Seven ml of a 15% homogenate of pigeon liver were incubated with 1 μM of labelled adenine, aliquots were removed at the indicated times and pipetted into cold 10% perchloric acid. The acid-soluble fraction was separated, neutralized and extracted eight times with 2 volumes of water-saturated butanol. This extraction procedure was found to be sufficient to remove all the butanol-soluble isotope. The dried butanol-soluble fraction was dissolved in a small volume of water and examined by paper chromatography in the butanol-water system, and the butanol-insoluble fraction which contained the soluble nucleotides and inosine was examined by chromatography in the propanol, water, ammonia system of HANES AND ISHERWOOD[17], with 20 μg each of AMP, ADP, ATP and inosine added to the paper as carriers. The spots were eluted and the eluates dried and counted. The results of this experiment are shown graphically in Fig. 1. The curve labelled "nucleotides" represents the sum of the ^{14}C content of the spots representing AMP, ADP, ATP and another substance which had an R_F between that of AMP and ADP. This material, as yet unidentified, appears to be an adenine nucleotide but is not identical with those already mentioned or with adenylic acids "a" and "b", or with any of the common adenine or hypoxanthine compounds. The four substances accounted for all of the isotope contained in the butanol-insoluble fraction. The sum of the ^{14}C contents of the compounds isolated from both

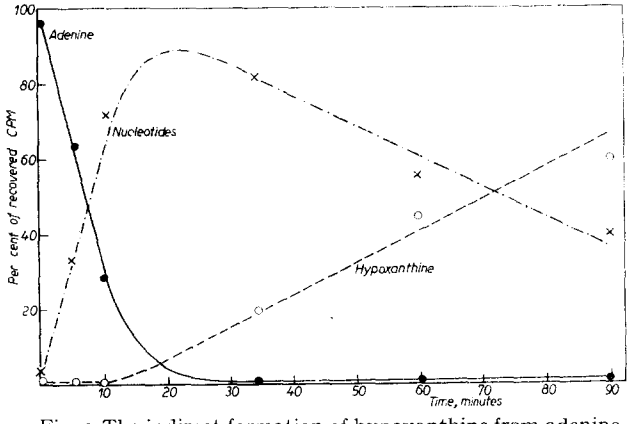

Fig. 1. The indirect formation of hypoxanthine from adenine by pigeon liver homogenate. For experimental details see text.

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the butanol-soluble and butanol-insoluble fractions accounted for about 80% of that in the total acid-soluble fraction at each time interval.

It is obvious from the figure that added adenine is rapidly converted to its nucleotides and that hypoxanthine, in this tissue, is not formed directly from adenine. It is possibly derived from inosine formed by dephosphorylation and deamination of the adenine nucleotides, although no inosine could be detected in any acid-soluble fraction.

The formation of nucleotides from adenine

The results of a typical experiment with a short time of incubation, for the purpose of studying the formation of acid-soluble nucleotides from adenine, are shown in Fig. 2. In this experiment 2.5 μM of adenine-8-^{14}C were incubated at 37° for 20 minutes with 15 ml of a pigeon liver homogenate containing about 2 g of tissue. After the reaction was stopped and the acid-insoluble material removed, the barium nucleotides were precipitated. The precipitate was washed thoroughly, dissolved in cold 1 M hydrochloric acid and the barium removed as the sulfate. The pH was then brought to 5.6 and the nucleotide fraction separated into its constituents on a column (1 cm × 1 cm^2) of Dowex-1 in the chloride form.

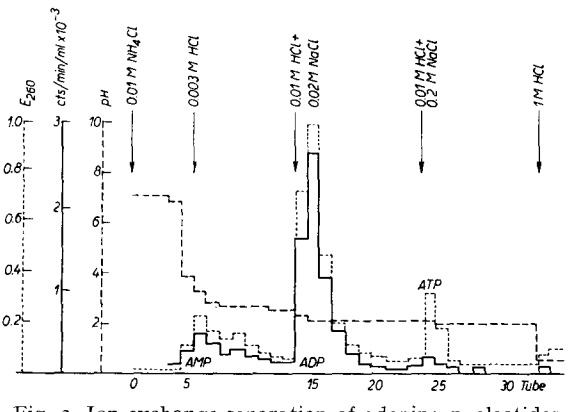

Fig. 2. Ion-exchange separation of adenine nucleotides after incubation of pigeon liver homogenate with adenine-8-^{14}C. For experimental details see text.

It was found that the unidentified nucleotide-like material which was mentioned earlier was not adsorbed to the Dowex column when the input solution was at pH 5.6 while AMP was. At pH 11 which is usually used for the separation of the adenine nucleotides[10] both AMP and the unknown nucleotide are adsorbed completely. By use of this difference in behaviour on the ion-exchange resin it was possible to separate a small amount of the unknown nucleotide. The unadsorbed material was shown to contain adenine, ribose and phosphorus but not enough has been obtained to characterize it further. In some experiments this material contained almost one-half the added isotope and would appear to be potentially important in adenine metabolism in this tissue.

For each fraction which was eluted from the column the total ^{14}C content, pH and extinction at 260 mμ were determined. In addition, an aliquot from the maximum of each of the three peaks was analyzed for AMP, ADP and ATP by the use of adenylic acid deaminase and a preparation of potato apyrase[11]. In the case of the peaks called AMP and ADP all of the material absorbing light at 260 mμ was recoverable as the indicated compound. However, the content of ATP determined enzymically was about one-half that indicated by the ultraviolet absorption, showing contamination in that fraction. This discrepancy probably can account for the lower relative count observed for ATP. The ^{14}C content of the AMP and ADP indicated a four-fold dilution of the original adenine in the formation of these compounds.

Analysis of the barium-alcohol soluble fraction by paper chromatography showed

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that there was no adenine left and that only hypoxanthine was labelled. The ^{14}C content of the isolated hypoxanthine indicated a six-fold dilution of the original adenine.

The role of adenosine in AMP formation

The possibility that adenosine is an intermediate between adenine and AMP was examined by use of a "bank" of unlabelled adenosine. A comparison of the specific activities of AMP isolated after parallel incubations of homogenate with labelled adenine and both with and without a large amount of unlabelled adenosine was made. The time of incubation was chosen so that enough adenine would remain for isolation and the determination of its specific activity, and for isolation of adenosine. It was expected that, if adenosine were on the pathway between adenine and AMP, the presence of large amounts of the nucleoside ought to dilute the final AMP obtained. The AMP which was counted was obtained from the total nucleotide fraction separated after chromatography in the butanol, water system. This fraction was then separated into its constituent nucleotides by chromatography in the propanol, water, ammonia system. The results of such an experiment are indicated in Table I. While the AMP obtained after incubation

TABLE I

SPECIFIC ACTIVITIES OF COMPOUNDS ISOLATED AFTER INCUBATION WITH LABELLED ADENINE IN PRESENCE OF UNLABELLED ADENOSINE

2 ml of 20% pigeon liver homogenate incubated 10 minutes at 37° with 0.18 μM of adenine-8-^{14}C

Compound	*With 8.2 μM unlabelled adenosine*	*With no added adenosine*
AMP	2,200	6,900
Adenosine	550	—
Adenine	195,000	218,000

in the presence of adenosine did have a specific activity appreciably lower than AMP obtained from preparations containing no adenosine, the fact that the adenosine isolated had a lower specific activity than the AMP demonstrates that adenosine was not the sole precursor of AMP. The nucleotide was formed by at least two distinct pathways: not only by phosphorylation of adenosine, but also from adenine without the necessary formation of adenosine as an intermediate. This second pathway might be something like the direct condensation of ribose-1,5-diphosphate with the purine to form AMP and inorganic phosphate[18]. This may also be the case in the formation of inosinic acid from hypoxanthine which KORN AND BUCHANAN[19] have demonstrated does not proceed through the intermediate formation of inosine. The very small fall in specific activity of the isolated adenine indicates that there was no appreciable hydrolysis of adenosine to adenine which might have accounted for the dilution of isotope in AMP; it also indicates that there is no detectable pool of adenine in this tissue.

Fractionation of the enzymes

In an attempt to concentrate the enzyme or enzymes involved in AMP formation the crude homogenate was subjected to high speed centrifugation. The separation was carried out in a Spinco model L preparative ultracentrifuge at 81,000 g for 60 minutes yielding a clear supernatant. A small amount of fat floating on the surface was discarded. Each fraction of the homogenate, including the reconstituted whole homogenate, was

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then incubated with 0.15 μM of labelled adenine for 30 minutes at 37°. After removal of the acid-insoluble fraction, the nucleotides were precipitated as the barium salts, the barium removed, the total amount of nucleotide determined by absorption at 260 mμ, and the ^{14}C content was determined. The effect of storage of the homogenate in the frozen state was also determined. The results of this experiment are shown in Table II in which the enzymic activity of the fractions are expressed as per cent of the specific activity of the acid-soluble nucleotides found in the whole homogenate.

TABLE II

ABILITY OF FRACTIONS OF PIGEON LIVER HOMOGENATE IN FORMING ADENINE NUCLEOTIDES FROM ADENINE-8-^{14}C

Fraction	*Relative activity*
Whole homogenate	100
Sedimentable fraction	8
Supernatant fraction	120
Supernatant + sedimentable fraction	140
Whole homogenate stored at −20° for 2 days	2

The author is greatly indebted to Professor H. M. KALCKAR for generously supplying soluble constituents of the cell and that there is little, if any, effect of the particulate fraction. It can also be seen that the enzymic activity is lost upon storage of the whole homogenate at low temperature. Subsequent experiments, however, have shown that the high speed supernate can be stored in the frozen state without appreciable loss of activity. Further attempts at purification of this extract of pigeon liver are in progress.

ACKNOWLEDGEMENT

The author is greatly indebted to Professor H. M. KALCKAR for generously supplying the labelled adenine and for his constant stimulating discussions, guidance and criticism during the course of this work.

SUMMARY

A cell-free preparation of pigeon liver has been shown to form 5-adenylic acid and its phosphorylated derivatives from adenine. The formation of adenylic acid has been shown to occur by at least two pathways; one directly from adenosine and the other from adenine without the intermediate formation of the nucleoside. The enzymes responsible for the formation of adenine nucleotides from adenine are found in the soluble fraction of the homogenate after high speed centrifugation.

It has been demonstrated that hypoxanthine is not derived directly from adenine in pigeon liver homogenates. The presence of an unidentified adenine compound, probably a nucleotide, which is potentially important in adenine metabolism has been reported.

RÉSUMÉ

Les auteurs ont montré qu'un extrait acellulaire de foie de pigeon transforme l'adénine en acide 5-adénylique et en ses dérivés phosphorylés. La formation d'acide adénylique a lieu selon deux voies; l'une part directement de l'adénosine et l'autre de l'adénine sans apparition intermédiaire de nucléoside. Les enzymes responsables de la formation des adénine nucléotides à partir de l'adénine se trouvent dans la fraction soluble d'un homogénat après centrifugation à grande vitesse.

L'hypoxanthine ne provient pas directement de l'adénine dans les homogénats de foie de pigeon. Les auteurs signalent l'existence d'un dérivé de l'adénine, non identifié, probablement un nucléotide, qui joue un rôle important dans le métabolisme de l'adénine.

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ZUSAMMENFASSUNG

Es wurde gezeigt, dass ein zellfreies Taubenleber-Präparat aus Adenin 5-Adenylsäure und seine phosphorylierten Derivate bildet. Es wurde gezeigt, dass die Bildung der Adenylsäure zumindest auf zwei Wegen erfolgt. Einer geht direkt vom Adenosin aus und der andere vom Adenin ohne die Zwischenbildung des Nucleosids. Die für die Bildung der Adeninnucleotide aus Adenin verantwortlichen Enzyme finden sich nach Zentrifugation mit grosser Geschwindigkeit in der löslichen Fraktion des Homogenates.

Es wurde gezeigt, dass Hypoxanthin in Taubenleberhomogenaten nicht direkt vom Adenin stammt. Es wurde die Gegenwart einer nicht identifizierten Adeninverbindung, wahrscheinlich eines Nucleotids, das beim Adeninstoffwechsel potentiell wichtig ist, berichtet.

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Received October 17th, 1953

Note added in proof

Since this paper was submitted for publication, results have been published of experiments which strongly indicate that one mechanism of AMP formation by pigeon liver enzymes does involve the condensation of adenine with ribose-1,5-diphosphate [M. SAFFRAN AND E. SCARANO, *Nature*, 172 (1953) 949].