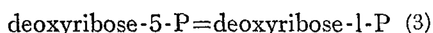
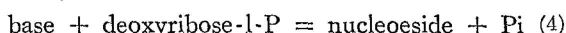
FORMATION OF DEOXYRIBONUCLEOTIDES IN *ASPERGILLUS FLAVUS**

Jason Chia-Hsing Shih

In 1952, Racker (1) described an enzyme from mouse liver and *E. coli* which catalyzes the aldol condensation of glyceraldehyde-3-phosphate and acetaldehyde to form deoxyribose-5-phosphate. A similar enzyme has recently been purified by Pricer and Horecker (2) from extracts of *Lactobacillus plantarum*. Enzymes which are known will synthesize deoxyribonucleotides from deoxyribose-5-phosphate by the following pathway:


deoxyribose-5-P=deoxyribose-1-P (3)


base + deoxyribose-1-P = nucleoeside + Pi (4)

But evidence accumulated over the last ten years makes it unlikely that the reactions are of importance for the synthesis of deoxyribonucleotides, and their function appears to be primarily catabolic (5, 6).

The other alternative pathway leading to deoxyribonucleotides is the direct reduction of ribonucleotides to the corresponding deoxyribonucleotides. There is by now good evidence that such a conversion actually takes place.

The first indication came from the work with N^{15}-labeled cytidine and uridine by Hammarsten et al in 1950 (7). It was found that these compounds were used for the synthesis of both RNA- and DNA-pyrimidines by the rat. Subsequent experiments by Rose and Schweigert (8) directly demonstrated such a conversion. Using cytidine-C^{14}, labeleled in both the pyrimidine and ribose moieties, it was found that the ratio between the specific activities of cytosine and ribose in the injected cytidine was the same as that between the specific activities of the pyrimidine and deoxyribose in the DNA of the rat. These have been confirmed and extended to cytidylic acid (9), uridylic acid (9), and uridine (10).

Such results almost definitely establish that the ribosides are incorporated intact into DNA and that the ribose-deoxyribose transformation takes place without breakage of the base-sugar bond.

The same results have also been observed in Neurospora (11, 12) and bacteria (13).

But the question of the conversion could not be definitely decided until the study of an appropriate enzyme involved in the reaction was carried out.

In 1957, Grossman (14, 15) in America began to find that extracts of *Salmonella typhimurium* had the capacity to transform both ribosides and ribotides to the corrresponding deoxyribosides and deoxyribotides. 1958, Reichard (16) in Stockholm found that a soluble fraction from a chick embryo homogenate could transform UMP to dUMP, and he also pointed out that such transformation was at the ribotide level.

Subsequently, crude extracts of *E. coli* (17, 18), Novikoff ascites tumor (19, 20), thymus, and

*This work was submitted by the author to the Graduate Institute of Biochemistry of National Taiwan University in partial fufillment of the reguirements for the degree of Master of Science.

Ehrlich ascites (21) were described to have the same capacity to convert CMP to dCMP.

In 1962, Reichard (22) started to purify the enzyme system from *E. coli* extract. A partially purified system, so called ribonucleotide reductase system, showed absolute requirements for ATP, Mg^{++}, and reduced lipoic acid. The reduction of cytidine phosphates was at diphosphate level.

Later, it was found that the same system could catalyze the conversions of both CDP to dCDP and UDP to dUDP (23). In 1964 Reichard et al succeeded in isolation of a protein, thioredoxin, which could replace reduced lipoic acid as the hydrogen donor (24, 25), and in purificatication of thioredoxin reductase which could reduce the one cystine of thioredoxin in the presence of TPNH (26). However, the mechanism of such conversion is still unclear, although

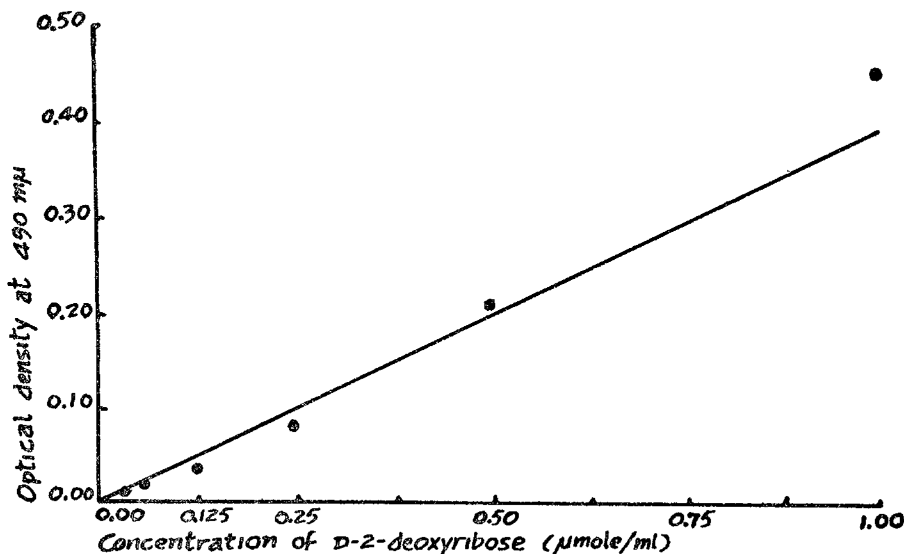


Fig.1 Standard Curve for the determination of D-2-deoxyribose by the reaction with cysteine and sulfuric acid.

Table 1 Determination of molar extinction coefficient of D-2- deoxyribose in the reaction with cysteine and H_2SO_4

Concentration (μmole/ml)	OD_{490}	E=OD/C
1.00	0.478	0.478
0.50	0.210	0.420
0.25	0.083	0.332
0.125	0.043	0.344
0.0625	0.019	0.304
0.03125	0.014	0.448

Procedure: 0.05 ml of 5% cysteine and 5 ml of 75 vol % H_2SO_4 were added to 0.5 ml of D-2-deoxyribose solution. After stirring and standing 10 minutes, OD at 490 mμ was measured with Beckman DU spectrophotometer.

Molar extinction coefficient, $E^{M}_{cm}=0.388\times10^{3}$ liters/mole-cm

some hypotheses have been proposed (22, 27).

A simlar enzymatic conversion of ribonucleotides to deoxyribonucleotides in *Lactobacillus leichmannii* has recently been observed by Downing et al (28).

In addition to these, this paper presents evidence of such a direct reduction of ribonucleotides to deoxyribonucleotides in another organism. The crude extract prepared from the mold *Aspersgillus flavus* was proved to have the capacity to transform adenine ribonucleotides to deoxyribonucleotides. A preliminary account of this work is described.

MATERIALS AND METHODS

Materials: ATP, ADP, and AMP were purchased from Sigma Chemical Company. D-2-deoxyribose was the product of Nutritional Biochemicals Corporation. Cysteine hydrochloride was purchased from Eastman Kodak Company. The 75 vol% H_2SO_4 was prepared by mixing 70 volumes of conc. H_2SO_4 and 30 volumes of distilled water. Biuret reagent was prepared by the method described by Gornall et al (32). Aspergillus flavus 3734/10 was donated by Tropical Product Institute, London.

Preparation of Extracts from *Aspergillus flavus*: *Aspergillus flavus* was grown in the Czepeck's solution enriched with rice powder (29) at the temperature of 25°-30°C. After three days the cells were harvested by skimming the floating mycelium from the growth medium. The mycelium was frozen and stored at -50°C.

When undertaking extraction, 4 gm of frozen mycelium was weighed and ground with 4 gm of precooled sea sand in a precooled mortar. Grinding was carried out until the cells were completely crushed and melted.

Twenty ml of cold distilled water was added to it, stirring for two minutes. The suspension was then centrifuged at 25,000×g for 30 minutes. The clear extract was used for experiment the same day.

Conditions of Incubation: Two different systems were used during this work: (a) in testing the effect of temperature and pH, the conditions were after Reichard et al (18), i.e. 0.8 ml of the cell extract was incubated 10 minutes with 0.6 μmole of AMP, 4 μmoles of ATP, 20 μmoles of $MgCl_2$, and 0.08 ml of M Tris-HCl buffer to make a final volume of 1.0 ml; (b) standard conditions of incubation was as follow: 0.8 ml of the cell extract was inubcated 10 minutes at 45°C with 4 μmoles of ATP, ADP, or AMP, 20 μmoles of $MgCl_2$, and 0.08 ml of M Tris-HCl buffer (pH7.2) to make a final volume volume of 1.0 ml. The reaction was stopped by addion of 0.5 ml of 70% $HClO_4$ and the aliquot was used for the assay of deoxyribonucleotides.

Assay of Deoxyribonucleotide Formation: Stumpf's modification of the cysteine reaction of D-2-deoxyribose with 75 vol% H_2SO_4 (30) was used here to determine the amount of deoxyribonucleotides. Since the molar extinction coefficients of D-2-deoxyribose and adenine deoxyribonucleotides do not differ significantly in this method (31), the standard curve (Fig. 1) plotted OD vs. standard concentration of D-2-deoxyribose was used to determine the amount of deoxyribonucleotides. The molar extinction coefficient was found to be 0.338×10^3 liters/mole-cm (Table 1).

During the experiment, 0.05 ml of 5% cysteine-HCl and 5 ml of 75 vol% H_2SO_4 were added to 0.5 ml of the aliquot which was taken from the reaction mixture after being stopped by adding $HClO_4$. After completely stirring and standing for 10 minutes, the OD at 490 mμ of the sample was measured with Beckman DU spectrophotometer. Since the OD will rapidly increase along with the time of standing, standing time must be exactly 10 minutes.

The formation of deoxyribonucleotides was calculated from the difference of OD_{490} between the sample and the control which was with zero time of incubation. The calculation was worked out by the following equation:

$$\text{deoxyribonucleotide formed } (\mu\text{moles}) = \frac{\triangle OD \times 1.5}{0.338 \times 1.0}$$

Determination of Protein: The amount of protein in the cell extract was measured by biuret method (32)

RESULTS

Temperature Optimum: The highest activity of incubation was carried out at 45°C, with sharp decrease at higher temperature (Fig. 2).

pH Optmum: Only four different pH values of Tris-HCl buffer were tried, a rather broad pH optimum between 7.2 and 7.6 (Table 2). The actual pH value of the incubation mixture was slightly lower (0.13 unit) than that of buffer used. During incubation, the shift of pH did not exceed 0.1 unit (Table 3).

Table 2 Test for pH optimum

	pH of buffer added			
	7.2	7.6	8.0	8.8
Formation of deoxyribsyl compounds (μmole)	0.23	0.23	0.19	0.11

Conditions of incubation: 0.8 ml of cell extract was incubated with 0.6 μmole of AMP, 4 μmoles of ATP, 20 μmoles of Mg^{++}, and 0.08 ml of M Tris-HC1 buffer to make a final volume of 1.0 ml at 45°C for 10 minutes.

Table 3 pH change during incubation

	before incubation	after 45°C-10'
pH	7.07	7.13

The pH of M Tris-HCl buffer added is 7.2

Requirements for Deoxribonucleotide Formation: ATP was absolutely required for the reaction. AMP had little effect in this reaction. Mg^{++} was also required. (Table 4)

Specific Activity Performed by Individual Adenine Ribonucleotides: ATP alone can give good production of deoxyribosyl compounds, but ADP or AMP alone do poorly (Table 5). Changing the amount of addition of each ribonucleotides showed more obviously the activity performed by ATP and the much less actity given by ADP or AMP (Fig. 3).

Table 4 Test for the components of the incubation system

	complete system*	without AMP	without ATP	without Mg^{++}
Formation of deoxyribosyl compounds (μmole)	0.25	0.30	0.04	0.20

*The complete system was after Reichard (18): 0.8 ml of cell extract was incubated with 0.6 μmole of AMP, 4 μmoles of ATP, 20 μmoles of Mg^{++} and 0.08 ml of M Tris-HCl buffer (pH 7.2) at 45°C for 10 minutes.

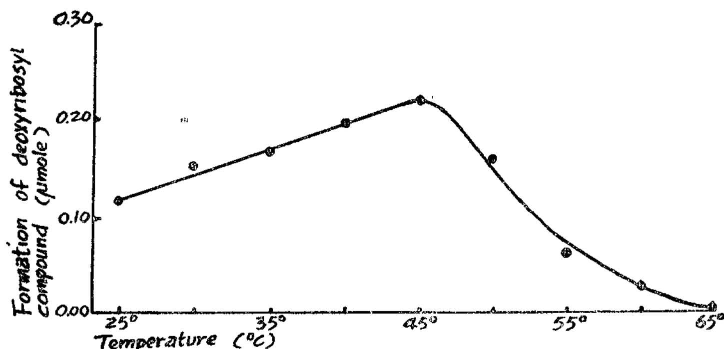


Fig.2 Test for temperature optimum

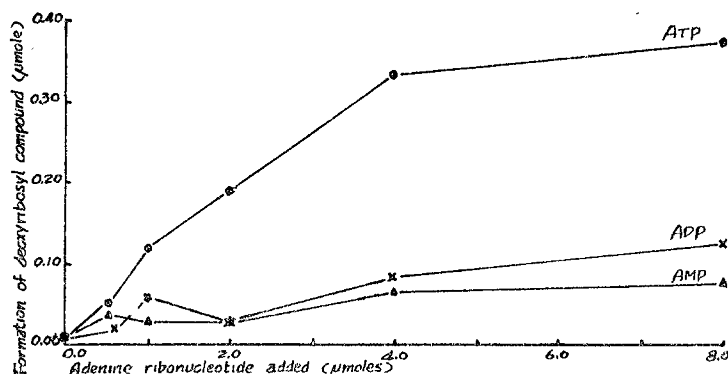


Fig.3 Comparison of the individual activity of each adenine ribonucleotide in changing amount of addition. Incubation was at 45°C for 10 minutes.

Time Course of the Reaction, Taking ATP as the Substrate: The reaction reached maximum and began to level off after 20 minutes of incubation. The formation of deoxyribosyl compounds during the initial 10 minutes was in linear relation with incubation time (Fig. 4).

The Reactivtty of ADP in the Presence of ATP: The total activity performed by ATP and ADP was equal to the sum of their individual activties, But, in the presence of higher concentration of ATP and after a longer period of incubation (30 minutes), ADP can perform with considerably high activity (Fig. 5).

The Reactivity of AMP in the Presence of ATP:No stimulating effect given by ATP on the reactivity of AMP was observed. The function of ATP in the formation of deoxyribosyl compound was reduced (Fig. 6).

Table 5 Specific activity of individual adenine ribonucleotide

	ATP	ADP	AMP
Formation of deoxyribosyl compounds (μmole)	0.30	0.08	0.04
Specific activity (μmole/mg of protein)	0.19	0.05	0.03

Conditions of incubation: 0.8 ml of cell extract was incubated with 4 μmoles of ATP, ADP or AMP,20 μmoles of Mg^{++}, and 0.08 ml of M Tris-HCl buffer (pH 7.2) to make a final volume of 1.0 ml at 45°C for 10 minutes.

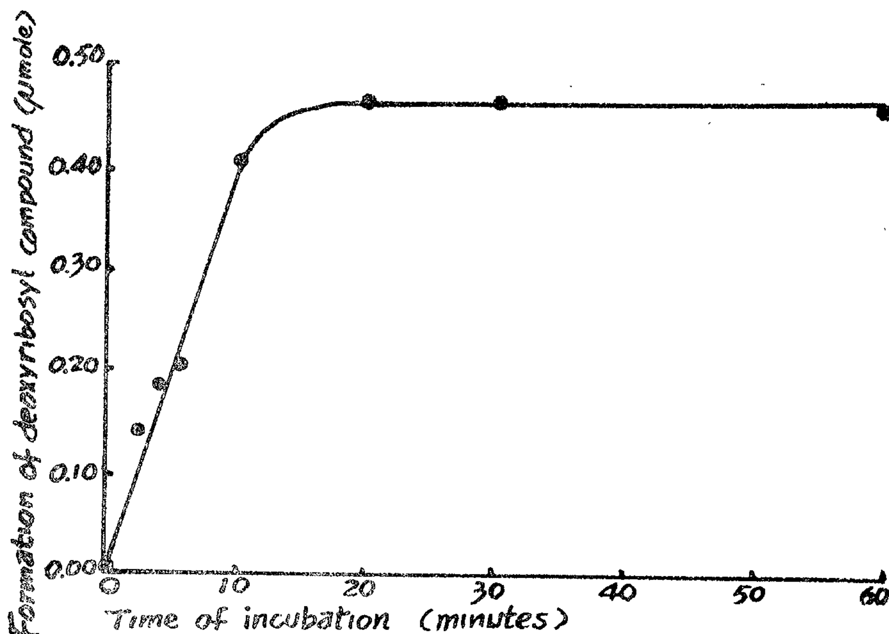


Fig. 4 Time course of the reaction, taking ATP as the substrate.
Conditions of incubation: 0.8 ml of cell extract was incubated with 4 μmoles of ATP, 20 μmoles of Mg^{++}, and 0.08 ml of Tris-HCl buffer (pH7.2) to make a final volume of 1.0 ml at 45°C.

DISCUSSIONS

This paper describes a probable new source of the ribonucleotide reductase system. Addition of adenine ribonucleotides to the crude extract of *Aspergillus flavus* will certainly cause an increase of deoxyribosyl compouds, which are detectable by the color reaction with cysteine and H_2SO_4. Although the products have not been isolated and identified, they are believed to be the adenine deoxyribonucleotides. There is no other possibility that can be imagined to give such a high activity with this assay method, a method which is highly specific for D-2-deoxyribose and its derivatives of nucleosides and nucleotides.

Since AMP has little effect in deoxyribosyl compound formation, the transformati on of ribose to deoxyribose can not take place at nucleoside level after dephosphorylation of AMP.

Individually, ATP is the best substrate among the three adenine ribonucleotides. ADP can also perform some activity in the presence of ATP (Fig. 5).

On the other hand, AMP not only shows poor activity while alone but also seems to have inhibitory effect on the reactivity of ATP (Fig. 6). The mechanism of inhibition is unknown, but one possibility could be predicted. In the presence of AMP, ATP could be consumed to become the less active substrate, ADP. It seems, however, that the conversion of ribotides to deoxyribotides takes place at triphosphate level in *Aspergillus flavus* rather than at diphosphate level as in the bacteria(22).

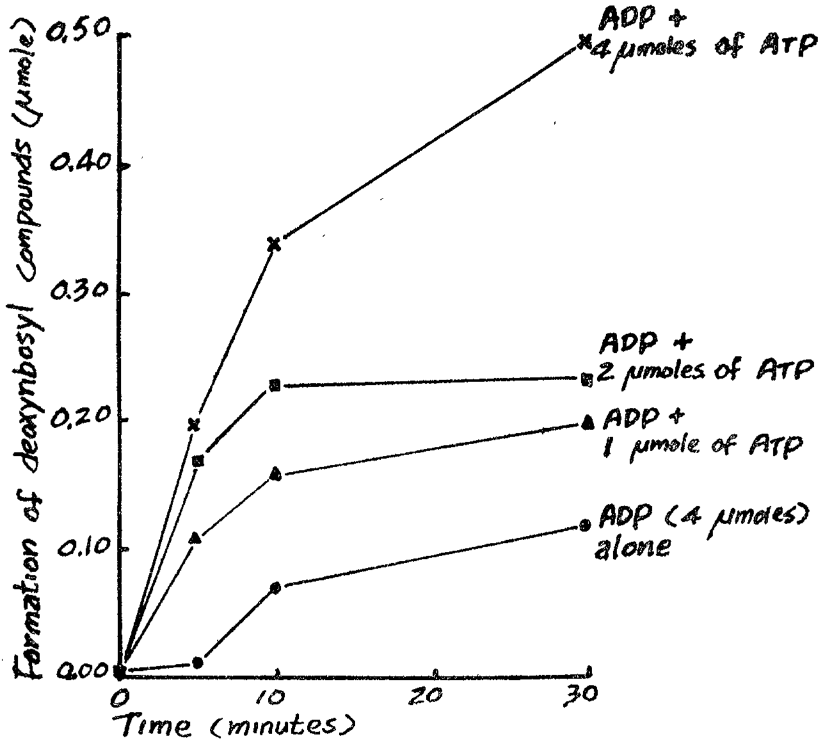


Fig.5 The reactivity of ADP in the presence of ATP.

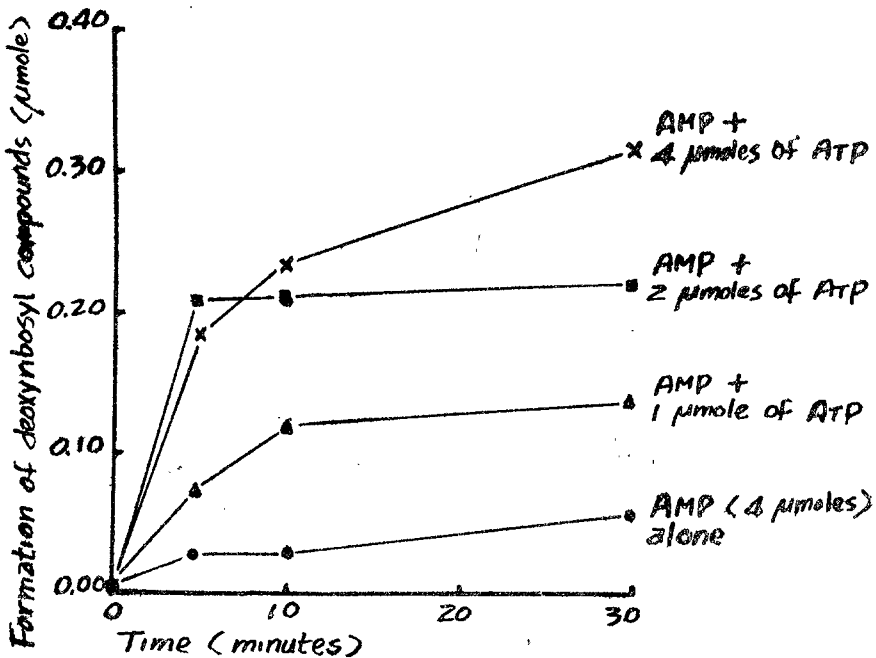


Fig.6 The reactivity of AMP in the presence of ATP.

During extraction, an alternative method was used but failed. The mycelium was first crushed in a Waring Blendor, then underwent supersonic vibration at 20 kc for 30 minutes. The supernatant from the centrifugation at 25,000xg for 30 minutes gave poor extraction of protein and enzyme activity. The treatment was too mild to overcome the resistance of cell wall of a mold.

Older cultures (8 days) have also been tried for extraction of the same enzyme activity, but the extracts appear poor in both enzyme activity and protein. Two explanations are suggested here: first, the relative amount of protein is very low in the old cells where a large amount of cell wall substances exist; second, this enzyme system is very important for DNA synthesis and must be highly active in the young culture for rapid growth and cell division but less active in an old culture.

A little experimentation to test the stability of this enzyme system has been done. Incomplete data showed that the enzyme activity can last 2 days under the storage at −15°C, but it almost completely disappeared on the fourth day.

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黃麴菌內 deoxyribonucleotide 之合成

石 家 興

將培養三天之黃麴黴菌磨碎，在高速離心後，取其水溶液做種種之實驗。結果發現此抽出液與 adenine ribonucleotides 在 45°C 作用之後，有 deoxyribosyl compounds 的生成，其生成物可由 Cysteine 和硫酸作用呈色而測定之。在三種 Adenine ribonucleotides 中，以 ATP 的作用最爲顯著。由此試驗可以推斷一種可能性，就是黃麴菌體內有一種還原酵素，它能够把 ribonucleotides 變爲 deoxyribonucleotides。

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The crude extract prepared from Aspergillus flavus was incubated with adenine ribonucleotides. After incubation, an increase of deoxyribosyl compounds was detected by the reaction with cysteine and H_2SO_4. It was believed that this experiment indicated a pathway of direct reduction of ribonucleotides to dexyribonucleotides in the mold. Experiments showed that ATP was the favorable substrate.