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Commentary by

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on 'Formation of deoxycytidine 5′-phosphate from cytidine 5′-phosphate with enzymes from *Escherichia coli*'
by P. Reichard and L. Rutberg
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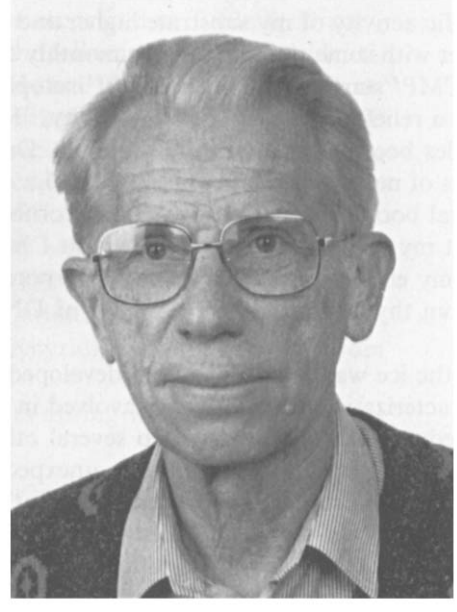
The period between 1950 and 1960 was the golden age of intermediary metabolism. Then, most of the enzymes involved in the major pathways leading to the formation of carbohydrates, lipids, amino acids and nucleotides were discovered and characterized. A milestone for our understanding of nucleotide synthesis was the discovery of PRPP by Arthur Kornberg in 1952. Starting from this compound, the pathways for the synthesis of both purine and pyrimidine ribonucleotides, and ultimately RNA, were unraveled. At the end of the decennium one of the few unresolved questions concerned the synthesis of deoxyribose, required for DNA replication. One candidate enzyme in this connection was a ubiquitous aldolase discovered by Ephraim Racker in 1951 which catalyzes the interconversion of acetaldehyde + glyceraldehyde 3-phosphate and deoxyribose 5-phosphate. However, for several reasons it seemed likely that the enzyme had a catabolic rather than anabolic function, in particular since cells hardly contain the amounts of acetaldehyde required to drive the reaction towards synthesis. Starting already in 1950 my own work had instead suggested that deoxyribose synthesis occurred by a direct reduction of ribonucleotides to the corresponding deoxyribosyl compounds. The experiments involved the administration of labeled ribonucleosides to animals or intact cells followed by the demonstration that large amounts of isotope could be recovered in DNA, apparently without cleavage of the bond between the ribose and the base. However, my attempts to demonstrate the conversion of a ribonucleotide to a deoxyribonucleotide in a soluble enzyme system met with no success. I got little encouragement for my ideas from discussions with colleagues who obviously knew more organic chemistry than I did. Organic chemistry did not know a direct replacement of a carbon-bound OH group by a hydrogen atom and such a reaction could consequently hardly occur in biochemistry. For most of the time my faith in the evidence remained strong, however. Between 1950 and 1960 I must have prepared thousands of extracts, from *E. coli* or other rapidly growing cells, to look for the formation of a labeled deoxyribonucleotide from an isotopic ribonucleotide. On some occasions I had a few counts, usually there was nothing. Certainly not enough to provide me with the required support from the Swedish Medical Research Council. Therefore, looking for labeled deoxyribonucleotides became an extracurricular activity that I could enjoy whenever I had accumulated sufficient results from my bread and butter research on enzymes involved in the biosynthesis of the pyrimidine ring.



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But finally it worked, with the results reported in the BBA paper. Why did it take such a long time? One important reason was that ribonucleotide reduction in *E. coli* occurs at the diphosphate level and that finding

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the activity required the addition of just the right amounts of ATP and Mg^{2+} to maintain enough of the substrate as CDP. A second reason was that ATP in addition acts as an allosteric effector. And, last but not least: I had expected too much. From the in vivo rate of bacterial DNA synthesis one can calculate the enzyme activity required for a sufficient supply of deoxyribonucleotides. The amount found in the BBA paper actually corresponds to only a few percent. To this date it remains an unexplained and embarassing fact that, even when assayed under optimal conditions, ribonucleotide reductase activity in *E. coli* extracts is not sufficient for the rate of DNA replication. Not until 12 years later, when the first ts mutant of ribonucleotide reductase had been identified, did we feel safe that ribonucleotide reduction indeed provides all the deoxyribose required for DNA replication. At the time, the small amount of activity meant that I had to push the specific activity of my substrate higher and higher. I remember with some trepidations the monthly syntheses of [^{32}P]CMP, starting from 30 mCi of isotope. It was certainly a relief when high specific activity, ^{3}H-labeled nucleotides became commercially available. During the final days of negative experiments I received a considerable moral boost from a visit of Arthur Kornberg, who looked at my few counts and told me that I had found more in my end-product than he had incorporated into DNA from thymidine in the early days of DNA replication.

Once the ice was broken the field developed rapidly. The characterization of the system involved in ribonucleotide reduction branched out into several other areas of biochemistry, sometimes with quite unexpected consequences. I was promoted to professor, first at Uppsala University, later at the Karolinska Institute in Stockholm, and was now in a good position to attract excellent enthusiastic graduate students whose input had a decisive influence on the forward movement. Together with first Agne Larsson, later Lars Thelander and Arne Holmgren and finally Britt-Marie Sjöberg, I could tackle questions concerning the protein structure of the system, the cofactor requirements of the reaction, its mechanism and control. Enzyme purification was the major tool at first, later joined by genetic and biophysical techniques and finally DNA technology. By that time the students had become Ph.D.s, had begun to dig in their own gardens and eventually became professors and heads of their own departments. In retrospect, it is a marvel how this little paper in BBA has branched out, both into related new areas of research and into the careers of so many able young scientists. At the time of its publication we still had to discover the thioredoxin system involved as a hydrogen carrier in the reaction. But which also, as demonstrated by Holmgren later, functions as a general disulfide oxide reductase in many other systems, including photosynthesis.

The allosteric properties were first tackled by Agne Larsson and were found to regulate the substrate specificity of the bacterial reductase in a highly complex manner. Fifteen years later, Lars Thelander had obtained the mammalian enzyme in pure form and found the same intricate regulatory pattern, responsible for the enzyme being able to provide a balanced supply of the four building blocks for DNA replication and repair. Disturbances of the balance lead to genetic abnormalities and disease. Thelander also carried out much of the work that led to an understanding of the subunit structure of the *E. coli* enzyme and to the discovery that the enzyme contains non-heme iron. When Anders Ehrenberg of the Department of Biophysics subsequently applied EPR spectroscopy to characterize the iron center we were in for a surprise: the enzyme contains a stable organic free radical as part of its structure. This was certainly not expected from a protein that had spent 2 weeks in aqueous solution during its purification. Britt-Marie Sjöberg then became heavily involved in the characterization of this radical and finally localized it to one specific tyrosine in one of the four polypeptide chains. Her work, and that of Joann Stubbe in the U.S.A., led to an understanding of the function of the radical during catalysis. I hope that some of my friends in organic chemistry from the fifties have read their papers. For some time the reductase was the only known 'radical' enzyme, but recently the presence of tyrosyl radicals as part of the protein structure has been found also in other enzyme systems.

What next? These days, work on ribonucleotide reductases proceeds in many laboratories. While the *E. coli* enzyme remains the prototype for the enzyme of multicellular organisms, including plants, other kinds of reductase are often found in bacteria. Bacteriophages and animal viruses sometimes carry information for their own reductase and such enzymes, as well as enzymes in rapidly growing cancer cells, may be candidate targets for chemotherapy. My own recent work aims at an understanding of the interplay between mammalian ribonucleotide reductase with other enzymes of deoxyribonucleotide synthesis and degradation, and the connection with DNA synthesis. To this purpose I have returned to labeled nucleosides and intact cells, which can now be used in much more sophisticated experiments than in the fifties. I am also still intrigued by the presence of the tyrosyl radical. How does it get there? Does a balance between radical-containing and radical-free enzyme reflect an additional regulatory mechanism? Radical formation requires the presence of oxygen. How does *E. coli* manage during anaerobic conditions? A new anaerobic reductase is waiting around the corner.

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Preliminary Notes

Formation of deoxycytidine 5′-phosphate from cytidine 5′-phosphate with enzymes from *Escherichia coli*

Deoxyribonucleotides can directly be formed through a reduction of ribonucleotides in different types of cells (see *e.g.* ref. 1). Most of the evidence for such a type of reaction comes from experiments with intact cells. We have now found that a "particulate-free" extract from *E. coli* B carries out the transformation of CMP to deoxy-CMP, and that this reaction depends on the presence of ATP, Mg^{++} and TPNH.

The requirements for the overall reaction are demonstrated in Table I. Deoxy-CMP was identified as the product of the reaction by the following criteria: (1) identical amounts of product were obtained with either ^3H- or ^{32}P-labelled CMP; (2) on paper chromatography[1] the product obtained from either ^3H- or ^{32}P-labelled CMP moved as deoxy-CMP; (3) after dephosphorylation with prostatic phosphatase the product obtained from [^3H]CMP behaved as deoxycytidine on Dowex-50[1] or on paper chromatography.

A strong stimulation of deoxy-CMP formation with ATP + Mg^{++} appears directly with the crude extract. The dependence on TPNH, however, emerges first after treatment of the enzyme with Dowex-2. DPNH stimulated much less.

Negligable amounts of deoxy-CMP (and deoxycytidine) were formed from cytidine 2′(3′)-phosphate or cytidine.

Table II demonstrates results of three experiments during which incubation was

TABLE I

REQUIREMENTS FOR THE FORMATION OF DEOXY-CMP

The complete system contained: 0.05 μmole of ^3H- or ^{32}P-labelled substrate, 1 μmole ATP, 5 μmoles $MgCl_2$, 10 μmoles Tris buffer, pH 7.4. Incubation for 10 min at 37° with 3.4 mg enzyme, final vol. 0.25 ml. The reaction was stopped with 1 ml 1 *M* $HClO_4$. After centrifugation the supernatant was boiled for 10 min, and CMP and deoxy-CMP were separated on Dowex 50[1]. The amounts of deoxy compounds formed were calculated from the radioactivity.

	Radioactive substrate	*mμmole deoxy-CMP formed*
Complete system	[^3H]CMP	0.55
Complete system	[^{32}P]CMP	0.53
Minus ATP and Mg^{++}	[^{32}P]CMP	0.03
Complete system	[^{32}P]cytidine 2′(3′)-phosphate	0.01
Complete system	[^3H]cytidine	0.04
Complete system*	[^{32}P]CMP	0.04
Complete system* + TPNH (30 mμmoles)	[^{32}P]CMP	0.35
Complete system* + DPNH (30 mμmoles)	[^{32}P]CMP	0.10

* Dowex-2-treated enzyme.

Abbreviations: CMP, CDP and CTP, cytidine 5′-mono-, di- and triphosphate, respectively; ATP, adenosine 5′-triphosphate; DPNH and TPNH, reduced di- and triphosphopyridine nucleotide, respectively; Tris, tris(hydroxymethyl)aminomethane.

carried out in the presence of large amounts of non-labelled deoxy-CMP, deoxy-CDP and deoxy-CTP, respectively. In each case the specific activities of the three deoxynucleotides were determined at different time intervals. Only one early-time point is given for each experiment in Table II. It is evident that in all three experiments the highest specific activity was found in deoxy-CDP, demonstrating that this nucleotide was the first product of the reaction.

TABLE II

DEOXY-CDP AS THE FIRST PRODUCT OF THE REACTION

[^{32}P]CMP (1.7 μmoles, $23 \cdot 10^6$ counts/min/μmole), 10 μmoles ATP, 50 μmoles $MgCl_2$, 3 μmoles deoxy-nucleotide and 31 mg enzyme, pH 7.4, were incubated for 4 min at 37° (vol., 2.5 ml). Mono-, di- and triphosphates were separated on Dowex-2, the ribo- and deoxyribonucleotides within each group were then separated on Dowex-50 and their specific activities were determined.

Deoxynucleotide added at start of incubation	*Counts/min/μmole after 4 min*		
	deoxy-CMP	*deoxy-CDP*	*deoxy-CTP*
deoxy-CMP	13,000	77,000	75,000
deoxy-CDP	9,000	27,000	14,000
deoxy-CTP	5,000	20,000	11,000

These experiments thus lead us to the following formulation of the reaction sequence during the reduction of CMP to deoxy-CMP:

$$\text{CMP} \xrightarrow{\text{ATP, Mg}^{++}} \text{CDP} \xrightarrow{\text{TPNH}} \text{deoxy-CDP} \longrightarrow \text{deoxy-CMP.}$$

With CDP as substrate the formation of deoxy compounds was still strongly stimulated by ATP and Mg^{++}. This might indicate that the actual reduction step involves a more complicated course than that shown in the above scheme and that it might involve the synthesis of an intermediate which requires ATP and Mg^{++}.

Our results are not in agreement with those reported earlier by GROSSMAN AND HAWKINS[2]. It was reported that deoxyribosyl compounds could be formed in a rather unspecific way from ribonucleotides or ribonucleosides with an extract of *Salmonella typhimurium*. The reaction did not require ATP or TPNH, but was strongly stimulated by dithiopropanol. Besides, considerably larger amounts of deoxyribosyl compounds were formed than in our experiments.

Using the techniques reported by GROSSMAN AND HAWKINS we have to a large extent been able to confirm the results of these authors with *S. typhimurium*. However, when comparing the amounts of deoxyribosides formed as measured by deoxyribose analysis with the results of isotopic assays, it became evident that only a very small fraction of the deoxyribosyl compounds conceivably could have arisen through a direct reduction of ribosyl compounds. Instead, it seems likely that the results with *S. typhimurium* can be best explained on the basis of the action of deoxyribosyl-transferring enzymes.

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