Magnesium Requirement for the Incorporation of Cytidylic Acid into Deoxyribonucleic Acid in Chick Embryo Extracts*

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ABSTRACT: The incorporation of ^{14}C-labeled cytidine 5′-monophosphate into deoxyribonucleic acid by soluble enzyme extracts from 5-day chick embryos with the Reichard system [Reichard, P., Canellakis, Z. N., and Canellakis, F. S. (1961), *J. Biol. Chem. 236*, 2514] was studied, and a marked effect of magnesium ion concentration on the activity of this enzyme system was noted. Maximal activity could only be obtained in a very narrow range of relatively low magnesium concentrations. Increased magnesium concentration favored the rapid destruction of cytidylic acid by dephosphorylation. Some evidence was obtained that the cytidylic acid deaminase reaction, another competing reaction, is metal dependent and that the metal required is probably not magnesium. The possibility that variations in magnesium concentration, *in vivo*, can exert a control on deoxyribonucleic acid synthesis is suggested.

Preparations derived from gel filtration of these enzyme extracts incorporated ^{14}C-labeled cytidine 5′-monophosphate into deoxyribonucleic acid as rapidly as the untreated protein fractions in spite of the fact that cytidylic acid and adenosine triphosphate were the only nucleic acid substrates added to the final incubation mixture. The other required substrates were apparently carried through the gel filtration procedure tightly bound to the appropriate enzymes.

During the past few years much interest has centered on the mechanism of control of DNA synthesis *in vivo* (Brown and Roll, 1955). This control mechanism is, quite obviously, of central importance in the reproduction, growth, and maintenance of cells both normal and malignant. Reichard *et al.* (1961) have obtained cell-free extracts from chick embryos which have the capability of synthesizing DNA from ribonucleotide precursors. It was felt that further study of this system of enzymes *in vitro* should give new insight into possible control mechanisms and also permit studies of the specific effect of a variety of inhibitors of DNA synthesis. Some observations on the requirements of this enzyme system are presented here.

Experimental Procedure

Materials. ATP[1] and NADPH were obtained from Sigma Chemical Co., the unlabeled nucleotides and the [2-^{14}C]cytidine 5′-monosphosphate ([2-^{14}C]CMP) from Schwarz Bioresearch. Uniformly labeled [U-^{14}C]CMP was obtained from the New England Nuclear Corp. The DNA used was the highly polymerized sodium nucleate of salmon sperm from the California Corp. for Biochemical Research. DNA was assayed by the diphenylamine reaction (Zamenhof, 1957), protein by the method of Lowry (Lowry *et al.*, 1951). Determinations of ^{14}C were usually made in a Tracerlab Omni-Guard low-background Geiger counter.

Preparation of Enzyme Extracts from Chick Embryos. Five-day-old chick embryos were removed from eggs and washed twice with cold isotonic saline and three times with isotonic saline–0.10 M Tris buffer, *p*H 7.5 (2:1).[2] The embryos were then homogenized with one-half volume of 0.10 M Tris buffer, *p*H 7.5, in 0.001 M mercaptoethanol in a Potter-Elvehjem homogenizer equipped with a Teflon pestle. The homogenate was centrifuged at 42,000 rpm for 20 minutes in a Spinco centrifuge using a No. 50 head (140,000*g* at bottom of tube) The *p*H of the supernatant was checked and adjusted to the desired value, when necessary.

Gel Filtration of Enzyme Extracts. The extract from the above procedure was placed on a column of Sephadex G-25 (medium) which had previously been equilibrated with 0.05 M Tris buffer, *p*H 7.5, in 0.001 M mercaptoethanol, and the protein fraction was eluted in as small a volume as possible with the same buffer (e.g., 2.0 ml of extract was placed in a 1.0- × 21-cm column and 4.0–5.0 ml of protein eluate obtained.

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[1] Abbreviations used in this work: ATP, adenosine triphosphate; NADPH, reduced triphosphopyridine nucleotide; CMP, cytidine 5′-monophosphate; GMP, guanosine 5′-monophosphate; dATP, dCMP, and dGMP refer to the 2′-deoxy compound. All nucleotide phosphates used were 5′-phosphates. In our experiments there was essentially no difference when either the uniformly or the specifically labeled nucleotides were used.

[2] These and all subsequent operations on these enzyme extracts were carried out at or near 4°.

Since these extracts contained small amounts of heme-containing proteins, the progress of the protein fraction through the column could be easily followed).

Assay of the Incorporation of CMP into DNA. The reaction mixture in the standard assay contained in a final volume of 0.825 ml: ATP, 3 μmoles; $MgCl_2$, 15 μmoles; heat-treated DNA, 0.2 mg; [2-^{14}C]CMP, 1.5 μmoles (6×10^5 dpm, 1.2×10^5 cpm); enzyme extract, 0.6 ml (5–7 mg of protein). With fractions from Sephadex columns, 0.12 μmole of NADPH was added. The ATP, DNA, and $MgCl_2$ solutions were made up in 0.05 M Tris buffer. All were adjusted to *p*H 7.5. The mixtures were incubated for 1 hour at 37° in a Dubnoff shaker and the reaction was terminated by quick freezing.

To each sample 2.0 mg of DNA was added as carrier and the protein and nucleic acids were precipitated with 3.0 ml of cold 10% trichloroacetic acid. The residues were washed two times with cold ethanol. (The trichloroacetic acid solutions were combined and, at times, analyzed for acid-soluble materials.) Lipids were extracted with two washes of alcohol-ether (3:1) followed by two extractions with ether. The ether-wet residues were dried and sodium nucleates prepared by extraction with 2.0 ml of neutral 2 M NaCl on a steam bath for 4 hours. The protein residues were then re-extracted for 2 hours with an additional 1.0 ml of 2 M NaCl. The NaCl extracts were precipitated with 2.5 volumes of cold 100% ethanol, washed with cold 80% ethanol, redissolved in 1.0 ml of 1 M NaCl, reprecipitated with 2.5 volumes of cold ethanol, washed with 80% ethanol, and air-dried. The sodium nucleate residue was incubated at 37° in 0.2 ml of 1 N NaOH for 16 hours, chilled, and neutralized with 1 N HCl, and enough 20% cold trichloroacetic acid was added to make a final trichloroacetic acid concentration of 4%.

The DNA precipitate was collected by centrifugation and washed with a small volume of cold 4% trichloroacetic acid and two times with ethanol. It was dissolved in 1.5 ml of water, a suitable aliquot (usually 1 ml) was dried in a 5.08 cm (2 in.) aluminum planchet and the radioactivity was measured. Aliquots of this solution were assayed for DNA and the activities were corrected for percentage of carrier recovered.

Analysis of Acid-soluble Fractions. The acid-soluble fractions were taken to dryness *in vacuo* and dissolved in several ml of H_2O, and the residual trichloroacetic acid was removed by three extractions with ether. The aqueous residues were again taken to dryness, dissolved in 10 ml of 1.0 N HCl, and heated at 100° for 10 minutes to hydrolyze the di- and triphosphates. The solutions were taken to dryness *in vacuo* several times to eliminate all the HCl. (At times it was necessary to redissolve the residue at this point and re-extract with ether to remove the last traces of trichloroacetic acid.) The residues were dissolved in small volumes of dilute NH_3 to neutralize all acidic components, taken to dryness *in vacuo*, and redissolved in 2.0 ml of a solution containing 2.0 mg each of cold dCMP, CMP, and cytidine to act as chromatographic markers. Aliquots of 50 and 100 μl of the solutions were streaked on two sets of strips of Whatman No. 1 paper. One set was run as ascending chromatograms with the NH_3–isobutyric acid system (Krebs and Hems, 1952) and the other with the 1-butanol-pyridine-water system (Morrison, 1953). The paper strips were scanned for radioactivity in a 4π strip counter (Baird-Atomic 4π strip counter run at 12 in./hour using a 30 second time constant and 0.45-cm windows.) The two different systems were necessary since CMP and uridine run together in the first, and CMP and UMP are too close for adequate resolution in the second. The radioactive peaks on the recorder tracings were integrated and the size of each peak was recorded as the percentage of the total radioactivity on the strip.

As a check on the method, the radioactive spots on one paper were eluted and aliquots were plated on aluminum planchets. The planchets were assayed in the Tracerlab low-background counter, and it was found that all of the radioactivity within the limits of accuracy of the counting system, 100 ± 2%, was recovered.

Results and Discussion

Effect of Variation of Enzyme and Substrate Concentrations and Incubation Time. These studies on chick embryo extracts confirmed much of the detail reported by Reichard *et al.* (1961). At the optimal *p*H of 7.5 for the over-all incorporation of CMP into the dCMP of DNA, however, incorporation was variable and seldom exceeded 0.05 mμmole CMP/mg of protein at a substrate level of 0.3 mM CMP.

In agreement with Reichard *et al.* (1961), we have also noted the incorporation of GMP into DNA by this enzyme system and the appearance of label in the dCMP with the CMP substrate. Reichard showed by means of [^{32}P]CMP that the label in the DNA was in the dCMP residues and that the bases adjacent to the incorporated dCMP were random, arguing against the possibility of chains of dCMP residues at the ends of the primer molecules. In some of our experiments about 1 μg of CMP was incorporated with 200 μg of primer. The primer used was highly polymerized and 2×10^6 probably represents a minimal molecular weight for this material. This incorporation represents *ca.* 30 dCMP residues per DNA molecule, a fact that would be difficult to reconcile with the incorporation of our CMP into anything but new DNA molecules considering the aforementioned ^{32}P study and the parallel incorporation of GMP. Using the "standard" enzyme assay procedure (see Experimental), we studied the effect of increasing CMP concentration in the range 0.36–3.6 mM (final volume = 0.825 ml). Incorporation was found to be proportional to the substrate concentration in this tenfold range, with no hint of enzyme saturation.

A time study indicated that essentially all incorporation occurred during the first 30 minutes of incubation with about 70% of the incorporation occurring during the first 15 minutes.

In another set of experiments varying amounts of enzyme were incubated under "standard" conditions. Figure 1 is a semilogarithmic plot of the variation of

the specific activity of the enzyme with dilution. Points for 30-, 45-, and 60-minute incubation periods are included; maximum incorporation appears to be reached after 30 minutes under these conditions. The specific activity of the enzyme varies inversely with the enzyme concentration in the range of concentrations examined. Extrapolation of the curve to zero concentration indicates a maximum specific activity in the vicinity of 0.25 mμmole of CMP incorporated into DNA per mg of protein at the 1.8 mM CMP level.

Thus, three anomalies appeared: First, the enzyme system could not be saturated with CMP even at ten times the level of CMP recommended; incorporation appeared to be proportional to the CMP concentration at all levels of CMP. Second, the reaction continued for only a relatively short time; the rate approached zero after 30 minutes. Third, dilution of the enzyme extract gave rise to a large increase in the specific activity of the enzyme.

In order to explain these anomalies, it was proposed that the substrate (CMP in this case) was being utilized in several different reactions and that reduction of the CMP to dCMP and subsequent incorporation into DNA was relatively slow compared to its utilization in other pathways. If the relatively large amounts of CMP (up to 3.6 mM) employed in these experiments were very rapidly consumed in the competing reactions, then it should be virtually impossible to saturate the enzyme system, and the incorporation should stop after a relatively short time. The large increase in specific activity with dilution of enzyme could then be attributed to the increased period during which the CMP concentration remained high due to the concomitant dilution of the enzymes of the more rapid competing reactions. In line with this reasoning, an attempt was made to find conditions under which the undesired reactions would be inhibited. Phosphatases were considered as the most likely competing enzymes and attempts were made to minimize their activity.

Effect of Gel Filtration of Enzyme Extracts. The incorporation of Sephadex-treated preparations was compared with that of the untreated preparations. The average values for a number of experiments are shown in Table I. In ten completely independent experiments the specific activities of the enzyme preparations, using standard conditions, showed an average fourfold increase after fractionation on Sephadex G-25 columns. Although these figures show a large amount of scatter, the probability that the increased activities of the Sephadex fractions is real is greater than 99.5%, and the probability that this increase is threefold or more is greater than 95%.

The fact that the Sephadex fractions were active was somewhat surprising. Only ATP, CMP, Mg^{2+}, NADPH, and primer DNA were added to these fractions. Since it has been shown that all four trinucleotides are needed for the activity of DNA polymerase (Bessman *et al.*, 1958; Reichard *et al.*, 1961), one must assume that this system can either synthesize the missing bases from those that are available or that sufficient concentrations of these bases are bound to the protein during Sephadex chromatography. There seems to be little reason to doubt that this is the same reaction that occurs with the untreated enzyme preparations. We have performed experiments showing that GMP was easily incorporated by these preparations and that the incorporated label (from CMP experiments) was rendered entirely acid soluble by DNAase treatment. In experiments described later in this paper, it will be seen that the Mg^{2+} requirements, the effect of EDTA, and the activities of the crude and Sephadex-treated enzyme systems were found to be approximately the same. While there is a great deal of evidence that thymidylic acid is readily synthesized from CMP (Maley and Maley, 1962), the larger number of steps required to convert ATP to dGMP makes it unlikely that enough

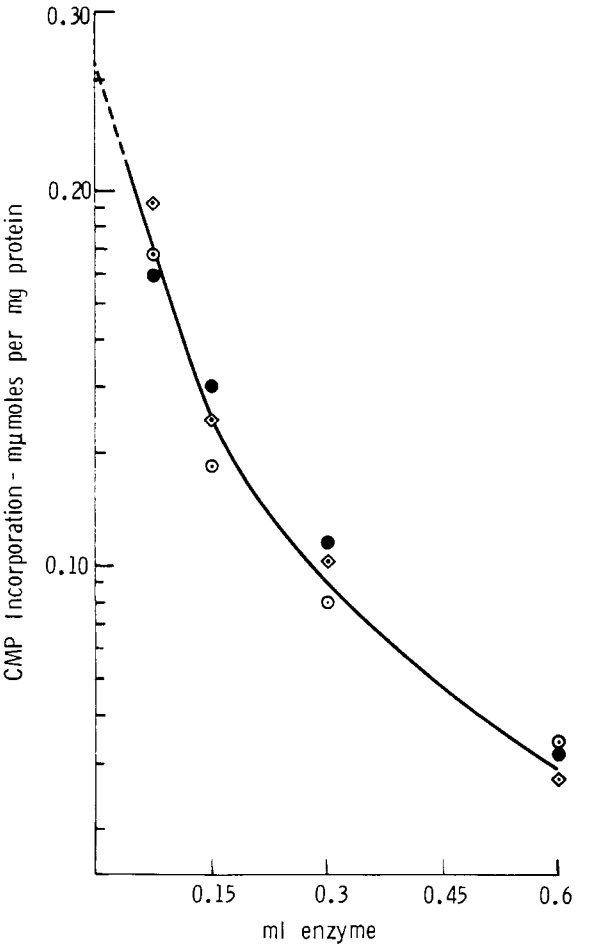


FIGURE 1: Variation of incorporation of [2-^{14}C]CMP into DNA with enzyme dilution. The enzyme dilution was carried out in 0.033 M Tris–0.001 M mercaptoethanol buffer at *p*H 7.5. Each tube contained in a final volume of 0.825 ml: ATP, 3 μmoles; $MgCl_2$, 15 μmoles; heat-treated DNA, 0.2 mg; [2-^{14}C]CMP, 1.5 μmoles (1.2×10^5 cpm); enzyme extract, 0.6 ml. Protein concentration of the undiluted enzyme preparation = 12.8 mg/ml. ⊙ 30 minutes incubation; ◈ 45 minutes incubation; ● 60 minutes incubation.

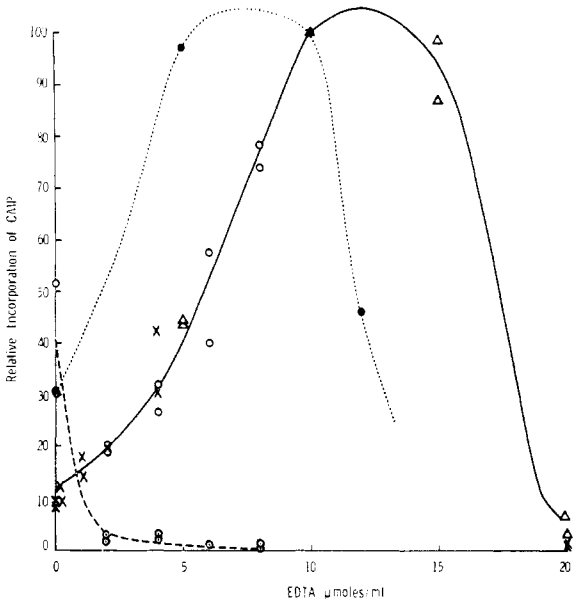


FIGURE 2: Effect of EDTA on the incorporation of [2-^{14}C]CMP into DNA with untreated and Sephadex-treated enzyme preparations. Incorporation of 100% represents *ca.* 0.2 mμmole CMP per mg protein. Different symbols indicate independent experiments. ——— Standard enzyme assay; ····· Sephadex-treated enzyme assay; – – – – standard enzyme assay without Mg^{2+}.

TABLE I: Incorporation of CMP into DNA. A Comparison of "Standard" Enzyme Preparations with Sephadex-treated Preparations.

	No. of Samples	Incorporation (mμmoles/mg protein)
"Standard"	19	0.022 ± 0.005[a]
Treated[c]	10	0.125 ± 0.026[a]
Ratio (T/S)		4.0[b] ± 1.3

[a] Mean ± standard error of the mean. [b] Geometric mean obtained through log conversion. [c] Treated enzyme preparations. Protein eluates from Sephadex G-25 columns (see Methods) incubated under "Standard" assay conditions with the addition of 100 μg of NADPH per tube.

dGMP could be synthesized during the short incubation period (Buchanan, 1960). In analogy to some recent work by Stonehill and Balis (1965) in which they showed that pyrophosphorylase preparations from bacteria and from leucocytes carried 5-phospho-α-ribosyl pyrophosphate and purines along during gel filtration, it appears more probable that the bases needed for DNA synthesis are bound to the appropriate enzyme proteins.

Effect of Inorganic Ions, EDTA, and Variation in Extraction Procedure. Table II (first column) summarizes the effect of the addition of a variety of inorganic ions and of ethylenediaminetetraacetic acid (EDTA) on the incorporation of CMP into DNA by untreated and Sephadex-treated enzyme preparations. Of those substances tested, only fluoride and EDTA increased activity appreciably. Fe^{3+} and Co^{2+} had almost no effect. The others inhibited incorporation markedly. It should be noted, however, that Ca^{2+} and Mn^{2+} were tested at relatively high concentrations and that all these materials were added in the presence of 17 mM Mg^{2+}.

TABLE II: Effect of Inorganic Ions and EDTA on the Incorporation of Cytidine 5'-Monophosphate into DNA and Comparison of Different Methods of Enzyme Extraction.

	Incorporation (mμmoles/mg protein)		
Addend to Standard System (μM)	Normal Tris Extract	Tris–0.25 M Sucrose Extract	Tris–Mg^{2+}–0.25 M Sucrose Extract
None	0.038	0.052	0.031
2 EDTA	0.067	0.082	0.065
1 F^-	0.076	0.071	0.053
15 Ca^{2+}	0.003	0.004	
15 Mn^{2+}	0.006	0.005	
1 Co^{2+}	0.033	0.034	
1 Cu^{2+}, Zn^{2+}	0.005	0.004	
1 Fe^{3+}	0.047	0.041	
	Sephadex-treated Enzymes		
None	0.171	0.132	
2 EDTA	0.319	0.294	
1 F^-	0.232	0.176	

Figure 2 shows the variation of enzyme activity with EDTA concentration. With our standard incubation mixture which contained 17 μmoles Mg^{2+}/ml in addition to the endogenous Mg^{2+}, about 12 μmoles EDTA/ml are required for maximal activity. With the Sephadex-treated preparations, which have less endogenous Mg^{2+}, about 8 μmoles EDTA/ml are needed. EDTA decreases the enzyme activity in the absence of added Mg^{2+} or when it is added in excess of the optimal concentration. Since both F^- and EDTA bind magnesium, which many phosphatases require (Heppel, 1961), a more precise evaluation of the Mg^{2+} requirement of this system was undertaken.

The variation in enzyme activity with Mg^{2+} concentration is represented in Figure 3. The optimal concentration of added Mg^{2+} for the untreated enzyme extract is about 1.7 mM and that for the Sephadex-treated preparation about 6 mM.

Analysis of a standard enzyme preparation showed

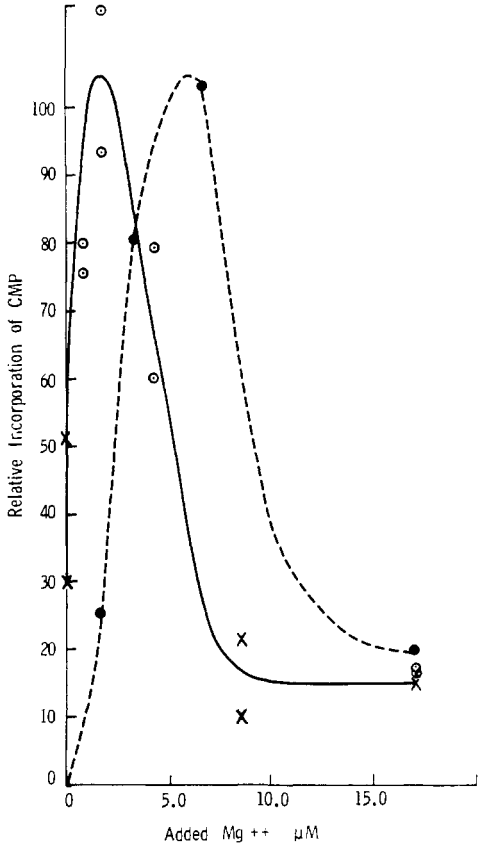


FIGURE 3: Effect of Mg^{2+} concentration on the incorporation of [2-^{14}C]CMP into DNA with untreated and Sephadex-treated enzyme preparations. Incorporation of 100% represents *ca.* 0.2 mμmole CMP per mg of protein. Different symbols indicate independent experiments. ——— Standard enzyme preparation in standard assay system except that Mg^{2+} concentration is as indicated; ·· – – – Sephadex-treated enzyme.

an endogenous Mg^{2+} concentration of 2.0 mM. Sephadex-treated preparations were less than 0.2 mM.[3]

The optimum of 1.7 mM exogenous Mg^{2+} [only one-tenth the amount recommended by Reichard *et al.* (1961)], was very sharp. The same activity could be obtained at the 17 mM level if 12 μmoles/ml of EDTA was added.

The last two columns of Table II show the results of experiments in which the enzyme extracts were made in 0.25 M sucrose–0.10 M Tris–0.001 M mercaptoethanol at *p*H 7.5 or 0.25 M sucrose–0.10 M Tris–0.01 M $MgCl_2$–0.001 M mercaptoethanol at *p*H 7.5. The incorporation by these extracts was not appreciably different from that of the standard extracts.

Upon repetition of the time study using either optimal amounts of Mg^{2+} (1.7 mM) or the standard amount of Mg^{2+} (17 mM) with the addition of 12 μmoles EDTA/

[3] Schwarzkopf Microanalytical Laboratory, Woodside 77, N.Y.

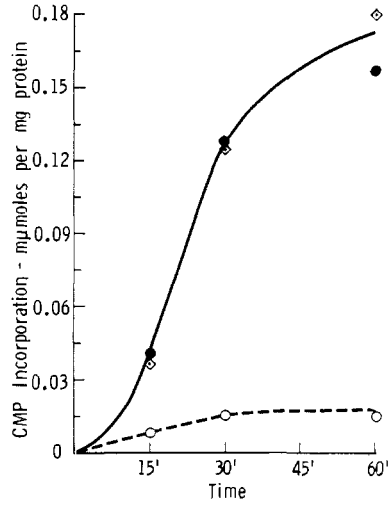


FIGURE 4: Effect of variation of incubation time on the incorporation of [2-^{14}C]CMP into DNA. ○ Standard assay system; ◈ assay with 1.5 μmoles Mg^{2+} per tube; ● standard assay with 12 μmoles EDTA/ml.

ml, the results depicted in Figure 4 were obtained. Not only was incorporation much greater under these conditions, about seven times as great as with the standard level of Mg^{2+}, but it had not yet leveled off after 60-minutes' incubation. The optimal rate of incorporation in these experiments is about 6×10^{-3}% of the initial CMP per mg of soluble protein in 15 minutes. Since these experiments started with 1.5 μmoles of CMP, this would represent the incorporation of 0.09 mμmole of CMP per 15 minutes. This is very close to the value obtained by extrapolating the curve in Figure 3 to infinite dilution.

The results of a repetition of the experiments on the effect of substrate concentration on CMP incorporation, this time with optimal Mg^{2+} concentration, are summarized in Table III. Under these conditions it was found that the enzyme was more than saturated at the levels of CMP tested, 0.19–1.5 mM. In fact, there was greater than 50% inhibition of incorporation at the

TABLE III: Effect of Variation of Substrate Level on the Incorporation of CMP into DNA using Optimal Mg^{2+} Concentration.[a]

CMP Level (mM)	Incorporation (mμmoles CMP/mg protein) Experiment I	Experiment II
0.19	0.37	...
0.38	0.35	0.51
0.75	0.32	0.43
1.50	0.16	0.28

[a] Added Mg^{2+}, 1.7 mM.

TABLE IV: Distribution of CMP Label into Acid-soluble Components. Comparison of Standard Assay with Assays with Optimal Mg^{2+} or EDTA.

Assay System[a]	Incubation Time (min)	Acid-soluble Label (% of total)[b]			
		CMP	Cytidine	UMP	Uridine
I. Standard Assay	15	16.5	66.3	3.2	16.6
	30	13.8	66.4	3.1	15.5
	60	6.0	72.8	1.6	17.9
II. Optimal Mg^{2+}	15	48.2	24.9	4.7	21.2
	30	38.4	35.8	3.6	18.6
	60	31.1	42.6	3.6	22.0
III. EDTA	15	44.6	48.9	2.0	3.7
	30	...	...	1.2	9.8
	60	23.0	69.4	1.3	5.6

[a] Standard assay: 17 mM added Mg^{2+}; optimal Mg^{2+}: 1.7 mM added Mg^{2+}; EDTA assay: 17 mM added Mg^{2+} + 12 μmoles/ml EDTA. [b] Acid-soluble fractions analyzed as in methods section. Each value represents the average of at least two separate determinations on each of two duplicate experiments.

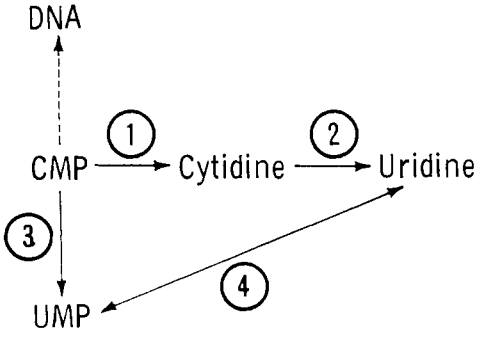
upper level. The maximum incorporation recorded in this set of experiments was 0.16 μg (0.5 mμmole) CMP/mg of soluble protein. Since the time study (Figure 4) shows that under optimal conditions of Mg^{2+} concentration better than one-half of the incorporation occurs in 15 minutes, one can expect the incorporation of 0.08 μg CMP/mg of protein per 15 minutes, or, assuming equal concentrations of all four bases in DNA, about 0.32 μg DNA/mg of protein per 15 minutes or about 32 μg of new DNA per day per mg of soluble protein.

Since one 5-day embryo yielded about 1.5 mg of soluble protein, these enzyme extracts would produce about 48 μg of DNA per embryo per day. From the data of Novikoff and Potter (1948), one can ascertain that 4- to 5-day chick embryos contain about 1.5 mg DNA/g of wet weight and that they double their weight in about 24 hours. These embryos weighed about 100 mg each and would thus have to produce about 150 μg of DNA per day. This enzyme extract has the capability of producing about one-third of the required DNA. The observed activity is close to that exhibited *in vivo* in view of the relatively crude extraction technique and the reported instability of this system (Reichard *et al.*, 1961).

Analysis of Acid-soluble Fractions. Table IV summarizes the analysis of the acid-soluble fractions of a time study under three sets of assay conditions: I, standard; II, 1.7 mM Mg^{2+}; III, standard with the addition of 12 μmoles EDTA/ml. Under standard conditions (17 mM Mg^{2+}) the CMP concentrations dropped very rapidly; about 83% was consumed in 15 minutes, confirming our initial suppositions about the puzzling kinetics of this enzyme system at this Mg^{2+} level. With 1.7 mM added Mg^{2+} only about 52% was consumed after 15 minutes and, similarly, with the Mg^{2+} + EDTA system only 55% was consumed.

Further confirmation was available from the studies on the Sephadex-treated preparations. These preparations had less than 0.2 μmole endogenous Mg^{2+}/ml and were also diluted *ca.* 2.5 times on the Sephadex columns. With 17 mM added Mg^{2+} they consistently incorporated more CMP per mg of protein than the untreated preparations. In this case, the increased activity can, in part, be attributed to the decrease in the over-all Mg^{2+} concentration and, in part, to the dilution of the undesired enzymes. Since the endogenous Mg^{2+} concentration is lower in these Sephadex-treated preparations, less additional Mg^{2+} is required for optimal activity and less EDTA is needed to bind the excess Mg^{2+} when the standard amount of Mg^{2+} (17 mM) was added.

The analyses of the acid-soluble fractions also indicated some deamination of CMP to UMP and uridine, possibly via one of the interconversions indicated in Scheme 1. The data in Table IV indicate that most of the uridine is produced during the first 15 minutes, when the CMP concentration is relatively high and the cytidine concentration low. Furthermore,



Scheme 1. Possible Pathways of CMP Metabolism

in the standard series where the cytidine concentration is high, uridine concentration is less than in the optimal Mg^{2+} series where the cytidine concentration is considerably lower. From these considerations, the deamination of cytidine to uridine, reaction 2, can be eliminated as an important pathway in this system. Since UMP concentration decreases with time and appears to be related to CMP concentration rather than to uridine concentration, it appears that the major pathway to UMP in this system must be via the direct deamination of CMP rather than through uridine (via reaction 3 rather than reaction 4). Furthermore, in the presence of EDTA both UMP and uridine formation are depressed although reaction 1, the dephosphorylation of CMP to cytidine, is not affected. It would be difficult to contend that the UMP phosphatase has a much greater requirement for Mg^{2+} than the CMP phosphatase since uridine formation is almost the same on both the high and low Mg^{2+} systems. The effect of EDTA in depressing uridine formation must lie then in reaction 3, the CMP deaminase reaction. Further, the probability that this reaction has a requirement for a metal other than Mg^{2+} is suggested since the deamination is inhibited by EDTA in the presence of excess Mg^{2+}.

Although all these experiments were performed *in vitro*, it is tempting to speculate on the possible importance of Mg^{2+} and other metal ion concentrations in the control of DNA synthesis *in vivo*. Reichard *et al.* (1961) have indicated that the Mg^{2+} requirement for nucleotide reduction is quite different than that required for the DNA polymerase. From our data it becomes apparent that the optimal concentration of Mg^{2+} for the over-all series of reactions from nucleotide to DNA is very sharp and, further, that the relative rates of the CMP phosphatase and the CMP deaminase reactions are dependent on Mg^{2+} concentration. The balance between the relatively slow incorporation of CMP into DNA and its destruction by dephosphorylation can readily be controlled *in vitro* by manipulation of the Mg^{2+} concentration; similar mechanisms might conceivably be operative *in vivo*.

Acknowledgment

The authors wish to thank Dr. George B. Brown for his interest in this work and his many helpful suggestions, and Steven Schimmel for his technical assistance.

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