Studies on the Biosynthesis of Deoxyribonucleic Acid by Soluble Mammalian Enzymes*

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A variety of reactions associated with the anabolism of ribonucleotides and deoxyribonucleotides as well as with their incorporation into ribonucleic acid and deoxyribonucleic acid, respectively, has been shown to occur in the high speed, particle free, supernatant fraction of rat liver. Such a cellular fraction obtained from normal rat liver has been shown to convert UMP (1), dAMP, dCMP, and dGMP (2) to the respective di- and triphosphates, and to catalyze the incorporation of UMP (3) and AMP (4) into RNA. The comparable fraction obtained from regenerating rat liver differs from the fraction of the normal rat liver in that it is capable of converting not only dAMP, dCMP, and dGMP, but also dTMP (5) to the respective di- and triphosphate forms. In addition, this latter fraction has been shown to catalyze the incorporation of C¹⁴-labeled dAMP, dCMP, dGMP, and dTMP (6), as well as tritiated thymidine (7), and tritiated thymidine from tritiated thymidine triphosphate (8) into DNA. The incorporation of tritiated thymidine into DNA by cell-free extracts of Ehrlich mouse ascites tumor cells has also been demonstrated (9).

The mechanism of DNA biosynthesis by highly purified bacterial preparations has been elegantly elucidated by the studies of Lehman et al. (10) and Bessman et al. (11). Because of our basic interests in the interdependence of enzymatic reactions associated with the process of nucleic acid biosynthesis in mammalian tissues we have undertaken a study of the reactions associated with DNA biosynthesis in these tissues. We have thus far confined our investigations to a study of crude cellular extracts in order to establish the associated enzymatic pathways which exist as well as the conditions for their interaction. To this end we have developed an enzyme system which, under the described experimental conditions, will respond maximally to environmental changes. This enzyme system may be used as an assay for compounds which may be involved in, or may interfere with, the over-all process of DNA synthesis by affecting any of a number of biosynthetic reactions, i.e. synthesis of DNA precursors, incorporation and polymerization reactions, interactions of DNA with other cellular components, and others. It has been found that a greater than 30-fold stimulation of deoxyribonucleotide incorporation into DNA can be attained by the judicious use of optimal substrate and cation concentrations.

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EXPERIMENTAL

Materials and Methods

The creatine phosphate and nonradioactive deoxyribonucleoside 5'-monophosphates were purchased from the California Foundation for Biochemical Research. ATP and Tris¹ were commercial products of the Sigma Chemical Company. Creatine kinase was prepared according to the method of Kuby et al. (12). RNase and DNase were products of the Worthington Biochemical Corporation. The deoxyribonucleoside di- and triphosphates of adenine, thymine, guanine, and cytosine were chemically synthesized from the respective deoxyribonucleoside 5'-monophosphates by the methods of Khorana (13), Chambers and Khorana (14), and Potter et al. (15). The corresponding di- and triphosphates of 5'M-dCMP were obtained by incubating the "enzyme" (described below) with the monophosphate under conditions already described (5). All compounds used during the assay were added as the neutral sodium salts.

Randomly labeled C¹⁴-deoxyribonucleoside 5'-monophosphates were obtained by enzymatic hydrolysis of the DNA of Escherichia coli grown on a minimal medium containing 10 mc. of C¹⁴O₂, according to the method of Downing and Schweigert (16). Separation of the deoxyribonucleotides was accomplished by using a modification of the ion-exchange chromatography technique of Hurlbert et al. (17) which gave excellent resolution and good yields. The details and advantages of this modified fractionation procedure have been reported in an earlier communication (5). The specific activities of the C¹⁴-labeled deoxyribonucleotides were 4.5 × 10⁶ to 8.5 × 10⁶ c.p.m. per amole, as measured in a gas flow counter. DNA was isolated from normal rat liver and freed of RNA contamination by the methods described by Zamenhof (18) and was assayed with diphenylamine by the Dacie colorimetric reaction (19).

Rat liver nuclei were obtained by the method described by Hogeboom (20). The soluble extract of cell nuclei used in these experiments was prepared by repeated thawing and freezing of the cell nuclei of four adult rat livers in 5.0 ml. of 0.06 M Tris buffer, pH 7.4. The supernatant fraction obtained after centrifugation at 20,000 × g in an International centrifuge was used.

Preparation of Soluble Extract of Regeneration Rat Liver—Male albino rats (Sprague-Dawley) weighing 160 to 220 gm. were partially hepatectomized under ether anesthesia, between 8 and

¹ The abbreviations used are: d, in combination with accepted abbreviations, deoxy; Tris, tris(hydroxymethyl)aminomethane; 5'M-dCMP, 5-methyldeoxycytidine monophosphate.
0 a.m., according to the method described by Higgins and Anderson (21). The animals were sacrificed by decapitation 36 hours postoperatively and the livers rapidly removed, chilled on ice, and homogenized in 0.4 volume of ice-cold 0.1 m sodium phosphate buffer, pH 7.4. The homogenate was then centrifuged at 106,000 \times g in a Spinco ultracentrifuge (Model L, Rotor No. 40). After centrifugation the supernatant fraction was pipetted off with a pro-pipette, care being exercised to avoid any contamination by cellular particles. This fraction was then dialyzed in the cold room at 3-5° for 24 hours against two 41. changes of 0.05 m Tris, pH 7.4, centrifuged once again at 20,000 \times g at 0°, and then stored in aliquot portions at -20° until used. The protein content of this preparation was measured spectrophotometrically by the method of Lowry as reported by Kalesar (22). All experiments reported in this work were performed using this crude preparation, henceforth referred to as "enzyme," without any further attempt at purification. Different "enzyme" preparations were used for most experiments.

Preparation of normal rat liver "enzyme" has been described elsewhere (23).

Assay of Reaction Mixtures—The following procedure, which is essentially a combination of the Schmidt-Thannhauser (24) and Schneider (25) methods, was used routinely to assay for the extent of incorporation of radioactive deoxyribonucleotides into DNA. The following steps, up to the alkaline digestion of the residue, were performed with ice-cold reagents and at 0 to 4°. After incubation the reaction vessel was chilled on ice and 8.0 ml of 0.4 N HClO4 was added to the mixture. Whenever necessary, a suitable amount of carrier protein and DNA, in the form of a concentrated rat liver homogenate, was added to the denatured reaction mixture in order to obtain a workable precipitate. The precipitated mixture was centrifuged and the residue was washed three more times with 6.0-ml portions of 95 per cent HClO4, followed by washings with 6.0-ml portions of 95 per cent ethanol, ethanol-ether (3:1), and finally with ether. The residue was then dried at room temperature and digested overnight with 1.0 ml of 0.2 N NaOH at 37° in order to hydrolyze the RNA. After alkaline hydrolysis the reaction vessel was chilled on ice, and 0.2 ml of ice-cold 1.0 N HCl was added to the digest followed by 8.0 ml of ice-cold 7 per cent trichloroacetic acid. The mixture was centrifuged at 0° and, after washing the residue twice with 5.0-ml portions of the above ice-cold trichloroacetic acid, it was extracted with 5.0 ml of 5 per cent trichloroacetic acid at 90° for 30 minutes and centrifuged at room temperature. The resulting supernatant fraction was concentrated to a volume of about 0.5 ml by heating in test tubes immersed in a boiling water bath. The concentrate was then plated on stainless steel planchets and dried, first in a desiccator over phosphorus pentoxide and under reduced pressure, and then overnight in an oven at 80°. The radioactivity of the sample was finally recorded with a gas flow counter. Under these conditions no visible corrosion of the planchets occurs and the counts obtained are comparable to those obtained at infinite thinness.

Unless otherwise specified all reaction mixtures were incubated at 37° for a period of 1 hour. The composition of the three basic reaction mixtures (A, B, and C) used in this work was as follows: (A) 1.0 mmoles each of chemically synthesized dATP, dCTP, and dGTP, 1.0 mmoles of dTMP having 3.0 \times 10^4 c.p.m., 3.3 mmoles of ATP, 15.3 mmoles creatine phosphate, 200 \mu g. of creatine kinase, 62 mmoles of Tris buffer; pH 7.4; 75 \mu g. of rat liver DNA; "enzyme" (10 mg. of protein), MgCl2 in the amounts given for each experiment, and distilled water to a final volume of 1.6 ml., (B) 1.0 mmoles each of dAMP, dCMP, dGMP, and 5M-dCMP, 1.0 mmoles of dTMP having 3.0 \times 10^4 c.p.m., 3.3 mmoles of ATP, 7.7 mmoles of creatine phosphate, 200 \mu g. of creatine kinase, 62 mmoles of Tris buffer, pH 7.4, 75 \mu g. of rat liver DNA, 11 mmoles of MgCl2, 4.0 mmoles of CaCl2, "enzyme" (3.4 mg. of protein), and distilled water to a final volume of 1.6 ml., (C) 10 mmoles of dAMP, 10 mmoles of dCMP, 20 mmoles of dGMP, 5.0 mmoles of 5M-dCMP, 20 mmoles of dTMP, 3.3 mmoles of ATP, 7.7 mmoles of creatine phosphate, 200 \mu g. of creatine kinase, 62 mmoles of Tris buffer, pH 7.4, 100 \mu g. of rat liver DNA, MgCl2, CaCl2, and MnCl2 in concentrations indicated for each experiment, "enzyme" (3.4 mg. of protein), and distilled water to a final volume of 1.6 ml.

In this type of reaction mixture 3.0 mmoles of the total amount of either dTMP or dGMP was present as the radioactive deoxyribonucleotide having 9.0 \times 10^4 c.p.m. The concentrations of the deoxyribonucleotides listed above represent optimal substrate concentrations for the incorporation of C14-dTMP into DNA as determined by the experimental procedure described further in the text.

RESULTS AND DISCUSSION

Phosphorylation of Deoxyribonucleoside 5'-Monophosphates

As has been previously reported (5) the "enzyme" from regenerating rat liver can phosphorylate the deoxyribonucleoside 5'-monophosphates of adenine, guanine, cytosine, and thymine to the di- and triphosphate forms, as well as the 5'-monophosphate of 5-methyldeoxycytidine (see "Materials and Methods"). Interestingly enough, although the soluble cytoplasmic fraction of normal rat liver can phosphorylate the deoxyribonucleoside 5'-monophosphates of adenine, guanine, and cytosine to the di- and triphosphate stage it cannot phosphorylate dTMP to the di- and triphosphates (2). Similar differences in the capacity of normal and regenerating rat liver to phosphorylate IP-thymidine have been reported by Bollum (8). This may represent a critical point of difference between normal and regenerating rat liver and it may afford a mechanism for the control of DNA synthesis, especially when this difference is considered in conjunction with the high rate of degradation of uracil by normal rat liver as compared to the low rate of degradation of this compound by regenerating rat liver (26).

Several investigators (10, 27-32) have previously reported the presence of kinases, in bacterial and animal tissues, capable of phosphorylating deoxyribonucleoside monophosphates to the di- and triphosphate stages. From the results of our previous experiments on the phosphorylation of deoxyribonucleoside monophosphates (5), it is tempting to designate the triphosphates as the precursors for DNA synthesis in the system under investigation, as has been shown to be the case for the bacterial system (10). However, such an assumption cannot be made on the basis of a limited number of experiments with a crude enzyme preparation. More conclusive evidence as to the nature of DNA precursors must await experiments with the purified polymerase fraction of the "enzyme" preparation and establishment of the optimal conditions necessary for its interaction with likely precursor substrates.
Comparison of phosphorylation of C14-dGMP in presence of various energy sources by normal and regenerating rat liver enzymes

The reaction mixture contained approximately 2 mmoles of C14-dGMP (6 × 10⁶ e.p.m.), 3.3 mmoles of ATP, 15.3 mmoles of creatine phosphate, 90 μg of creatine kinase, 35 mmoles MgCl₂, 62 mmoles of Tris, pH 7.4, "enzyme" (10 mg protein), and distilled water to a total volume of 1.3 ml. When used alone, 35 mmoles of either ATP, UTP, ADP, or UDP were added. Incubation time was 20 minutes at 37°. After incubation the reaction mixture was analyzed as previously described (5).

<table>
<thead>
<tr>
<th>Enzyme source (rat liver)</th>
<th>Energy source</th>
<th>Distribution of deoxyribonucleotides*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mono-P</td>
<td>Di-P</td>
</tr>
<tr>
<td>Normal</td>
<td>CP, CK, ATP</td>
<td>10</td>
</tr>
<tr>
<td>Regenerating</td>
<td>CP, CK, ATP</td>
<td>5</td>
</tr>
<tr>
<td>Normal</td>
<td>ATP</td>
<td>30</td>
</tr>
<tr>
<td>Regenerating</td>
<td>UTP</td>
<td>55</td>
</tr>
<tr>
<td>Normal</td>
<td>UTP</td>
<td>30</td>
</tr>
<tr>
<td>Regenerating</td>
<td>ADP</td>
<td>60</td>
</tr>
<tr>
<td>Normal</td>
<td>ADP</td>
<td>90</td>
</tr>
<tr>
<td>Regenerating</td>
<td>UDP</td>
<td>100</td>
</tr>
</tbody>
</table>

* The radioactivity present in the three forms is expressed as per cent of the total in the three forms recovered in the acid soluble fraction.
† The di- and triphosphates were identified by comparison with synthetic di- and triphosphates (12-14).
‡ CP, creatine phosphate; CK, creatine kinase.

Reaction—Table I presents experimental results comparing the relative abilities of the normal and regenerating rat liver "enzymes" to catalyze the conversion of C14-dGMP to higher phosphates while utilizing various high energy phosphate donors as energy sources. It can be seen that even though the creatine kinase-creatine phosphate-ATP system is optimal, ATP or UTP alone will also serve in a similar capacity, although less efficiently; however, ADP and UDP are essentially inactive.

Incorporation of C14-labeled deoxyribonucleotides into DNA

General Properties and Requirements—In addition to the requirement for a high energy phosphate source the enzymatic reactions being investigated were found to be dependent upon the addition of rat liver DNA and the presence of certain cations, namely Mg++ or Mn++. It was also observed that although equimolar concentrations of dAMP, dCMP, dGMP, and dTMP were incorporated into DNA to the same extent separately, a stimulation of incorporation was obtained when a full complement of all four deoxyribonucleotides was incubated concurrently (6). It has been found that this stimulatory effect can be further increased by the addition of 5M-dCMP to the remaining four deoxyribonucleotides. Experimental evidence indicating that the incorporated radioactivity was associated with DNA has already been presented in an earlier communication (6).

The optimal concentrations of deoxyribonucleotides and of metal cations required for maximal incorporation of deoxyribonucleotides into DNA by this "enzyme" system have also been established. It has been found that the extent of incorporation is not only dependent on the type of metal cation and deoxyribonucleotide present in the reaction mixture but also on the concentration of these reactants. A concentration of 7.0 × 10⁻³ M MgCl₂ was found to be optimal in reaction mixtures whose basic composition is similar to those used here (see "Materials and Methods"). No appreciable incorporation of dTMP into DNA was observed when Mg++ was omitted from the reaction mixtures. The addition of CaCl₂ at an optimal concentration of 2.5 × 10⁻² M to reaction mixture type A containing an optimal Mg++ concentration resulted in an enhancement of the incorporation of dTMP by approximately a factor of 5. Although a stimulatory effect is observed with combinations of Ca++ and Mg++ no incorporation can be detected in reaction mixtures containing Ca++ alone. It has also been found that the presence of Mn++ at an optimal concentration of 3.0 × 10⁻² M could replace the requirement of this system for Mg++ and that the incorporation observed in the presence of Mn++ alone is equivalent to that obtained in the presence of optimal concentrations of Ca++ and Mg++. All of the metal cation concentration curves were bell-shaped in appearance with well-defined maxima and in each case superoptimal concentrations were found to be inhibitory.

Optimal substrate concentrations for dAMP, dCMP, dGMP, dTMP, and 5M-dCMP were also established and were found to lie within a range of approximately 3.0 to 13.0 mmoles per ml. when determined in reaction mixtures whose basic composition was similar to that of reaction mixture C.

Time Curve—Fig. 1 presents experimental data obtained from a time study of the enzymatic reaction investigated with the use of two differently aged "enzyme" preparations. It can be seen that both time curves display a lag period which precedes the

![Fig. 1. The incorporation of C14-dTMP into DNA at low "enzyme" concentrations as a function of time.](http://www.jbc.org/)

Downloaded from http://www.jbc.org/ by guest on July 1, 2017
The incorporation of C14-dTMP into DNA as a function of "enzyme" concentration. The concentration of "enzyme" is expressed in terms of protein per ml. The composition of the incubation mixture is similar to reaction mixture type "B" with the exception of the amount of "enzyme" which was varied as shown. Incubation time was 1 hour at 37°.

Ascending limb of the curve. This lag period is much greater for the "enzyme" which was stored at -20° for 1 month than for the 3 day old "enzyme." The longer lag period of the 1-month old "enzyme" may be a reflection of the decreased capacity of the "enzyme" to phosphorylate the deoxyribonucleotide monophosphates to higher phosphates. Due to the instability of the "enzyme" most experiments were performed within 10 days after preparation of the "enzyme."

"Enzyme" Concentration Curve—As can be seen from Fig. 2 the "enzyme" concentration curve is S-shaped and the upper plateau of the curve appears at a level of approximately 9.0 mg. of protein per ml. The plateau is probably the result of a combination of several factors such as the exhaustion of the energy source due to side reactions and subsequent degradation of substrates, the degradation of added and synthesized DNA, as well as the inactivation of enzymes involved in the reactions under study.

Effect of Added DNA on C14-dTMP Incorporation—Fig. 3A illustrates the variation in the incorporation of C14-dTMP obtained in reaction mixtures containing varying amounts of rat liver DNA and having different cationic compositions. In the absence of added DNA no appreciable incorporation of dTMP was observed. The extent of incorporation in reaction mixtures which contained both MgCl₂ (11 μmoles per 1.6 ml. of sample), and CaCl₂ (4.0 μmoles per 1.6 ml. of sample), showed consistently higher incorporation and did not reach a plateau value even with DNA concentrations as high as 200 μg. per sample. The effects produced by Ca++ and other divalent cations is discussed in a later section of this paper.

The effect of adding rat liver DNA to reaction mixtures containing Ca++ and at low "enzyme" concentration (3.4 mg. of protein) is shown in Fig. 3B. Under these conditions it can be seen that the stimulation achieved by the addition of increasing amounts of rat liver DNA reached a plateau at approximately 75 μg. of DNA per 1.6 ml. of sample. At DNA concentrations above 150 μg. per sample an inhibition of the incorporation of thymidyllic acid was observed. This curve supplements the upper curve of Fig. 3A which shows the results obtained with 10 mg. of "enzyme" protein and which could not be extended to higher DNA concentrations because of technical difficulties. In subsequent experiments a nonlimiting concentration of 100 μg.
zyme" protein.

Effect of RNase and DNase on Incorporation—Fig. 4 shows that the addition of DNase in concentrations of approximately 6 µg. per ml. to reaction mixtures results in an inhibition of deoxyribonucleotide incorporation of about 33 per cent, whereas the addition of DNase in concentrations of 1.0 µg. per ml. results in an inhibition of incorporation by approximately 33 per cent. It can also be seen that RNase under similar experimental conditions does not influence the extent of incorporation to any appreciable degree.

Effect of Concentration and of Various Combinations of Deoxyribonucleotides—In order to establish the optimal concentrations of deoxyribonucleotides required for maximal incorporation the following experimental approach was used. The amount of incorporation of C14-dTMP was first measured in the presence of increasing concentrations of nonradioactive dAMP. After the optimal concentration of dAMP was established the effect of increasing concentrations of dCMP in reaction mixtures containing C14-dTMP and optimal concentrations of dAMP was determined. Similarly, optimal concentrations of dGMP, 5M-dCMP, and C14-dTMP were determined in that respective order. The results of these experiments are presented in Fig. 5. Optimal concentrations for the substrates studied lie within a range of approximately 3.0 to 13 µmoles per ml., and with the use of optimal concentrations an approximately 5-fold increase in the incorporation of C14-dTMP could be obtained. It is recognized that the above order of determining the optimal concentrations for the deoxyribonucleotide substrates is certainly not exhaustive and that a more efficient order may exist. However, it did not appear practical to screen other possible combinations at this stage of the experimental work. When optimal deoxyribonucleotide concentrations were determined in the previously mentioned order it was found that superoptimal concentrations of both dAMP and 5M-dCMP nullified the stimulatory effect which these compounds produced when added in optimal concentrations as is shown in Fig. 5. This effect contrasts to that obtained for the other substrates tested; at superoptimal levels these did not inhibit the incorporation of C14-dTMP into DNA. It is also noteworthy that on reevaluating the optimal concentration of Ca++ at optimal concentrations of deoxyribonucleotides it was found to increase slightly, shifting from 2.5 × 10^{-3} M to 3.0 × 10^{-3} M.

The experimental data presented in Table II indicate that the deoxyribonucleotides whose bases are thought of as normally being hydrogen bonded to each other in the DNA structure as proposed by Watson and Crick (33) stimulate the incorporation of each other. For example, the data in part 1 of Table II indicate that neither the addition of dCMP or dGMP nor a combination of dCMP and 5M-dCMP, increases, to any appreciable extent, the incorporation of C14-dTMP, which is regarded as being hydrogen bonded to dAMP in the DNA helix. However, the addition of dAMP or dGMP to similar mixtures containing dCMP and 5M-dCMP, results in an enhancement in the incorporation of C14-dTMP. This enhancement is further increased by the addition of the last remaining deoxyribonucleotide of the series (dGMP or dAMP, respectively). Part 2 of Table II shows that the incorporation of C14-dTMP can be stimulated by the addition of dAMP alone, to a greater extent than when dCMP or dGMP are added alone (compare part 1). Upon addition of dCMP to a reaction mixture containing both dTMP and dAMP another increase of incorporation occurs and further increases can be obtained by the addition of dGMP and 5M-dCMP.

Effect of Various Cations on Incorporation of Deoxyribonucleotides into DNA—In an earlier communication (6) from this laboratory we reported that the incorporation of deoxyribonucleotides into DNA could be stimulated by the presence of an extract prepared from regenerating rat liver cell nuclei isolated by the method of Hogeboom (20). This isolation procedure involves the use of media containing small amounts of CaCl2 to aid in maintaining the morphological characteristics of the cell nuclei. Table III presents the results of a series of experiments which established the presence of contaminating Ca++ in the nuclear extract as being the factor largely responsible for the stimulating effect observed with the nuclear extract. Experiment I shows that the stimulation of incorporation observed by the addition of nuclear extract prepared by the calcium method, although not lost by heating, is destroyed by dialysis of the nuclear extract. In addition, this experiment shows that nuclear extracts prepared from rat liver nuclei, which were isolated by the same method, but without the addition of calcium did not stimulate the reaction under study. As shown in experiment 2, addition of calcium in varying amounts to incubation mixtures resulted in a stimulation of incorporation, thus offering fairly conclusive evidence that Ca++ was responsible for the activating effect previously reported.

A relative comparison of the activity of various metal cations on the incorporation of C14-dGMP into DNA is presented in Table IV. In all experiments the chloride salts of the respective cations were added to incubation mixtures at optimal concentrations which were previously determined by concentration curves. The optimal concentrations were 7.0 × 10^{-2} M for Mg++, and 3.0 × 10^{-3} M for Mn++ and Ca++ as determined in reaction mixtures of type C. The relative activity of the cations was compared on the basis of the maximal stimulation attained by Mg++ which was assigned an arbitrary value of 1.0. The results obtained in various experiments showed good agreement except for the variability noted when combinations of Mg++ plus Mn++ plus Ca++ were used. In this case the values obtained ranged from no appreciable stimulation by Ca++ to a 50 per cent stimulation over that obtained in the presence of optimal con-
centrations of Mn++ and Mg++. The cause of this variation is not yet clear, although it appears to be related to the age of the "enzyme," older preparations appearing to be activated to a greater extent. Such a variation was not observed with combinations of Mg++ plus Mn++. Of the ion combinations listed above, that of Mg++ plus Mn++ appears to be most effective, with the exception of the at times higher but variable result obtained by a combination of Mg++ plus Mn++ plus Ca++.

Stansly (34) has recently described two DNA-degrading enzymes in Ehrlich ascites tumors, one of which (Enzyme II) is specific in its requirements for cations, being activated by Mg++ but not by Na+, Ca++, or Mn++, each of which inhibits its activity. The inhibitory effect observed by cations similar to those studied in this investigation suggested the possibility that Ca++ and Mn++ may be acting to inhibit such enzymes in our preparation, thereby eliminating extensive degradation of DNA during the incubation period. However, Enzyme II is inactivated by dialysis (dialysis is included in our isolation procedure) and
The reaction mixtures were identical with reaction mixture C containing 11 pmoles of MgCl₂ and 4.8 pmoles of CaCl₂, but no MnCl₂ in a final volume of 1.6 ml. Incubation time was 1 hour at 37°C.

**Table II**

| Experiment | Additions | Relative incorporation
<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>Control</td>
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</tr>
<tr>
<td></td>
<td>Nuclear extract prepared with Ca++</td>
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<tr>
<td></td>
<td>(boiled)</td>
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<tr>
<td></td>
<td>Nuclear extract prepared with Ca++</td>
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</tr>
<tr>
<td></td>
<td>(dialyzed)</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>Nuclear extract prepared without Ca</td>
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<td>1.</td>
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<td></td>
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<tr>
<td></td>
<td>Calcium chloride 0.0030 M</td>
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</tr>
</tbody>
</table>

**Table III**

Effect of order of addition of nonradioactive deoxynucleotides on incorporation of C¹⁴-thymidylic acid into DNA

The composition of the reaction mixtures is similar to that given for reaction mixture A. All reaction mixtures contained 11.0 pmoles of MgCl₂ per 1.6 ml. Incubation time was 1 hour at 37°C.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Additions</th>
<th>Addition to DNA</th>
<th>Total c.p.m. incorporated into DNA expressed as % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>None</td>
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<td>100</td>
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<tr>
<td>1.</td>
<td>Nuclear extract prepared with Ca++</td>
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<tr>
<td></td>
<td>(boiled)</td>
<td></td>
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<tr>
<td></td>
<td>(dialyzed)</td>
<td></td>
<td>90</td>
</tr>
<tr>
<td>2.</td>
<td>Calcium chloride 0.002 M</td>
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<td>450</td>
</tr>
<tr>
<td></td>
<td>Calcium chloride 0.0025 M</td>
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<td>500</td>
</tr>
<tr>
<td></td>
<td>Calcium chloride 0.0030 M</td>
<td></td>
<td>420</td>
</tr>
</tbody>
</table>

The enzymatic reactions involved in the over-all process of incorporation of deoxribonucleotides into DNA are dependent upon a source of high energy phosphate, upon the addition of rat liver DNA, and of either Mg++ or Mn++. The degree of incorporation obtained is also affected by the addition of Ca++. Optimal concentrations of these reactants and experimental conditions required for a given metal cation most probably represent an average optimum for the various reactions occurring in the system under the prescribed experimental conditions. The optimal concentrations required by the purified fraction of this enzyme preparation may very well prove to be different. Nevertheless, our results suggest that a delicate balance and interplay of certain metal cations may be involved in controlling this critical biosynthetic process by regulating certain key reactions either as activators or inhibitors.

**Table IV**

Comparison of effect of various cations on incorporation of C¹⁴-dGMP into DNA

<table>
<thead>
<tr>
<th>Cations†</th>
<th>Optimal concentration of chloride salt</th>
<th>Relative incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg++</td>
<td>7.0 × 10⁻⁶</td>
<td>1.0</td>
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<tr>
<td>Mg++</td>
<td>7.0</td>
<td>3.5</td>
</tr>
<tr>
<td>Mn+++</td>
<td>3.0</td>
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<tr>
<td>Ca++</td>
<td>7.0</td>
<td></td>
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</tbody>
</table>

* Reaction mixture C was used to which increasing concentrations of the cations were added.
† No incorporation occurred in the presence of Ca++ alone.
‡ Values are expressed as multiples of the incorporation obtained in the presence of Mg++ alone.

which would permit only a stimulating effect of Mg++ to be disclosed at optimal concentrations of Mn++. Other possible sites of action for these divalent metal cations may be the conversion of deoxyribonucleoside monophosphates to their corresponding precursors for the synthesis of DNA, the inhibition of phosphatases, and the mediation of reactions between DNA and other cellular components. Thus, at the present time it is difficult to assign an exact function to each of the above metal cations in the over-all process of the biosynthesis of DNA in crude cell extracts. Further experimentation is required to localize the sites of action of these cations. The optimal concentrations found for a given metal cation most probably represent an average optimum for the various reactions occurring in the system under the prescribed experimental conditions. The optimal concentrations required by the purified fraction of this enzyme preparation may very well prove to be different. Nevertheless, our results suggest that a delicate balance and interplay of certain metal cations may be involved in controlling this critical biosynthetic process by regulating certain key reactions either as activators or inhibitors.

**SUMMARY**

A study has been made of enzymes found in the high speed supernatant fraction of regenerating rat liver which are involved in the biosynthesis of deoxyribonucleic acid (DNA). This crude cellular fraction contains enzymes which catalyze the phosphorylation of the monophosphates of deoxyadenosine, deoxythymidine, deoxyguanosine, 5-methyldeoxycytidine, and thymidine to the corresponding di- and triphosphates, as well as enzymes which catalyze the incorporation of deoxyribonucleotides into DNA.

The enzymatic reactions involved in the over-all process of incorporation of deoxribonucleotides into DNA are dependent upon a source of high energy phosphate, upon the addition of rat liver DNA, and of either Mg++ or Mn++. The degree of incorporation obtained is also affected by the addition of Ca++. Optimal concentrations of these reactants and experimental conditions required for a given metal cation most probably represent an average optimum for the various reactions occurring in the system under the prescribed experimental conditions. The optimal concentrations required by the purified fraction of this enzyme preparation may very well prove to be different. Nevertheless, our results suggest that a delicate balance and interplay of certain metal cations may be involved in controlling this critical biosynthetic process by regulating certain key reactions either as activators or inhibitors.
conditions favoring a maximal rate of incorporation have been determined.

The incorporation of a given deoxyribonucleotide can be stimulated by the addition of other deoxyribonucleotides. The incorporation of radioactive thymidylic acid into DNA is enhanced to a greater extent by the addition of deoxyadenylic acid at optimal concentrations than by any of the other deoxyribonucleotides tested. However, superoptimal concentrations of either deoxyadenylic acid or 5-methyldeoxycytidylic acid, nullified the stimulatory effect which these compounds produced when added in optimal concentrations.

Ca++ acts synergistically with Mg++ to increase the incorporation of deoxyribonucleotides into DNA, but has little effect on the action of Mn++ in this respect. Mn++ alone permits incorporation to occur to about the same degree as Mg++ plus Ca++. Mg++ added to Mn++ provides additional stimulation.

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