Mammalian DNA Ligases

SEROLOGICAL EVIDENCE FOR TWO SEPARATE ENZYMES

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Mammalian cells contain two DNA ligase activities with different chromatographic properties, referred to as DNA ligase I and II. The major ligase activity present in calf thymus cell extracts, DNA ligase I, has been purified 1000-fold. After repeated injections of this enzyme with complete Freund's adjuvant into a rabbit, antibodies were induced that inhibit DNA ligase I from calf, human, mouse, and rabbit tissues. This antisera did not affect DNA ligase II from the same sources to a detectable extent, even at a concentration 10-fold higher than that required for 98% inhibition of DNA ligase I. These data strongly indicate that the two mammalian DNA ligase activities are due to two separate enzymes, and not to two forms of the same enzyme. Both enzymes are present in the nuclear fraction, but are also found in the cytoplasmic fraction. Rapidly dividing cells (mouse ascites tumor cells and calf thymus) contain higher amounts of DNA ligase I than other cells (calf liver and spleen, human placenta, and rabbit spleen), while no such correlation was observed for DNA ligase II.

The major DNA ligase activity present in cell extracts from mouse embryo fibroblasts (1), calf thymus (2), and the human heteroploid cell line EUE (3) is an ATP-requiring enzyme with a molecular weight of 175,000 to 220,000. This DNA ligase, which we call mammalian DNA ligase I, apparently acts by the same mechanism as the phage T4-induced DNA ligase in Escherichia coli (4, 5), because a covalently bound ligase-AMP complex (6) and a DNA-AMP complex (7) are formed during the interaction between the enzyme and DNA containing single strand breaks. On prolonged storage in solution, the DNA ligase I dissociates into an active species of one-half the original size (5).

In addition to DNA ligase I, a second DNA ligase activity is present in calf thymus cell extracts (2). This enzyme, which is referred to as DNA ligase II, also requires ATP, but it has different fractionation properties and is more heat-labile than DNA ligase I. Each of the separated enzymes is active in a standard DNA joining assay and can catalyze the conversion of circular DNA molecules containing single strand breaks to a covalently closed circular form. Here we show that antibodies to purified DNA ligase I do not inhibit DNA ligase II, that both enzymes are present in cell nuclei, and that they are found in all mammalian cells and tissues investigated.

**EXPERIMENTAL PROCEDURE**

Reagents—Pronase was obtained from Calbiochem and incubated in 0.05 M Tris-HCl (pH 7.5) at a concentration of 11 mg/ml for 2 hours at 37° before use. Trvovin (code TRTPCK) and chymotrypsin (code CDI) were purchased from Worthington. Phage T4-induced DNA ligase was purified according to Weiss (8). Phosphocellulose (P 11) was prepared according to Bernardi (9). Sarkosyl was obtained from Geigy, Sephadex G-150 from Pharmacia Fine Chemicals, Freund's complete adjuvant from Difeo, and Trasylol (basic proteinase inhibitor) from Bayer.

**Assay**—DNA ligase activity was measured by the conversion of 5'-P-phosphomonoester termini in DNA containing single strand breaks into a form that remains acid-insoluble after incubation with alkaline phosphatase (10). [5'-32P]Phosphoryl-DNA was prepared according to Weiss et al. (11). Reaction mixtures (150 μ1) contained 10.5 μmol of Tris-HCl (pH 7.5), 1.5 μmol of MgCl₂, 0.15 μmol of dithiothreitol, 0.05 μmol of EDTA, 0.05 μmol of ATP, 1.5 μg of [5'-32P]phosphoryl-DNA (initially 30,000 cpm), and a limiting amount of enzyme (<0.002 ligase unit). With crude enzyme fractions (cell extracts and ammonium sulfate fractions), the reaction mixtures were supplemented with 15 μmol of NaCl to suppress nuclease activity (12). When antibodies were added, the enzyme-containing reaction mixtures (without ATP and [5'-32P]phosphoryl-DNA) were first incubated for 5 min at 0° with a rabbit γ-globulin fraction (up to 600 μg of protein), followed by addition of the ligase substrate and ATP and transfer to 20°. Preincubation of ligases with antibodies for longer time periods or at higher temperatures (10 min at 23 or 37°), or both, did not cause increased inhibition of ligase activity. The reaction mixtures were incubated for 30 min at 20°. The reactions were stopped by addition of 4.5 μmol of EDTA, 10.5 μmol of Tris-HCl (pH 8.5), 120 μg of Sarkosyl, and 23 μg of preincubated pronase, followed by incubation for 60 min at 37°. Determinations of the alkaline phosphatase-resistant 32P in the DNA then were performed according to Weiss et al. (10). Under the conditions used, 1 ligase unit catalyzes the conversion of 1 nmol of 32P to phosphatase resistance.

**Cells and Tissues**—Bovine tissues were obtained from newly slaughtered calves (less than 6 months old) at the local slaughterhouse. The tissues were packed in ice and used for enzyme preparations within 2 hours. Rabbit spleens were obtained from locally killed animals. Cells from the mouse TAH₃ ascites tumor were a gift from Professor G. Klein, Karolinska Institutet, Stockholm. This ascites tumor was originally derived from a spontaneous mammary carcinoma. A human placenta was obtained immediately after delivery at a local hospital.

**Subcellular Distribution**—Cell nuclei were prepared from calf thymus according to Gottesman and Canellakis (13). The sucrose/CaCl₂-containing supernatant solution after the first low speed centrifugation was further subdivided by differential centrifugation into a mitochondrial fraction (pelleted by 11,000 x g for 20 min), a fraction containing smaller particles (pelleted by 100,000 x g for 2 hours), and a...
cytoplasmic fraction. The mitochondria were further purified by isopycnic sucrose gradient centrifugation (14), and mitochondria were also prepared from calf liver by the same procedures. Cell nuclei were directly extracted as described below for disintegrated whole tissue, and the extract was fractionated and analyzed for DNA ligase activities. Purified mitochondria were disrupted by ultrasonic treatment in a fractionator, and the fractionated supernatant was used for DNA ligase analysis.

Separation of DNA Ligases—Calf and human tissues were processed in 100-g amounts, while the rabbit and mouse material was analyzed by the same methods but at a smaller scale (1 to 4 g). Here, the fractionation of 100 g of tissue is described. All buffers contained 0.01 M 2-mercaptoethanol, centrifugations were for 15 min at 16,000 × g, and all buffer solutions were equilibrated at 0 to 4°C if not otherwise stated.

The tissue was cut in pieces and washed with 200 ml of 0.1 M NaCl/0.05 M Tris-HCl (pH 7.4/1 mM EDTA). The tissue fragments were homogenized in a Waring Blendor with 500 ml of the same buffer by treatment at full speed three times for 30 s. Between the treatments, the homogenizer was chilled in an ice bath for 2 min. 1 The homogenate was gently stirred for 60 min at 0°C. After centrifugation, the supernatant (Fraction I, 505 ml) was diluted with 1 volume of 1 mM EDTA. To the diluted extract, 0.1 volume of 5% streptomycin sulfate was slowly added with continuous stirring, and the mixture was gently stirred for an additional 30 min. The precipitate was removed by centrifugation and discarded. To the supernatant (Fraction II, 1000 ml), 213 g of cold ammonium sulfate was added slowly, and the resulting suspension was brought to pH 7.5 by addition of 1 M Tris base. After 30 min, the precipitate was removed by centrifugation. Additional ammonium sulfate (160 g/liter) was added to the supernatant, and the resulting suspension was again neutralized and left for 30 min. After centrifugation, the supernatant was discarded. The precipitate was suspended in 50 ml of 0.015 M potassium phosphate (pH 7.2)/1 mM EDTA and dialyzed against this buffer for 10 to 14 hours. The dialyzed material (Fraction III, 100 ml) was applied to a phosphocellulose column (4 × 10 cm) equilibrated with the same buffer. The column was washed with 250 ml of this buffer, and the adsorbed protein was subsequently eluted with 0.8 M NaCl/0.015 M phosphate buffer (pH 7.2). 2 The eluate (Fraction IV, 40 to 80 ml) was applied to a hydroxyapatite column (2 × 7 cm) equilibrated with 0.015 M phosphate buffer (pH 7.2). 3 The column then was eluted with potassium phosphate buffer (pH 7.2) as follows: 160 ml of 0.05 M buffer (Fraction Va), 400 ml of 0.14 M buffer (Fraction Vb), and 160 ml of 0.40 M buffer (Fraction Vc). Figure 1 shows the elution pattern and indicates how the fractions were pooled for assay. The recovery of DNA ligase activity in the phosphocellulose and hydroxyapatite chromatography steps were approximately 70% and 60%, respectively.

Further Purification of DNA Ligase I—Calf thymus DNA ligase I was further purified by applying Fraction Vb, isolated from 900 g of calf thymus, to a phosphocellulose column (2.2 × 20 cm) equilibrated with 0.015 M potassium phosphate buffer (pH 7.2/1 mM EDTA) and dialyzed against this buffer for 10 to 14 hours. The dialyzed material (Fraction III, 100 ml) was applied to a phosphocellulose column (4 × 10 cm) equilibrated with the same buffer. The column was washed with 250 ml of this buffer, and the adsorbed protein was subsequently eluted with 0.8 M NaCl/0.015 M phosphate buffer (pH 7.2). The eluate (Fraction IV, 40 to 80 ml) was applied to a hydroxyapatite column (2 × 7 cm) equilibrated with 0.015 M phosphate buffer (pH 7.2). The column then was eluted with potassium phosphate buffer (pH 7.2) as follows: 160 ml of 0.05 M buffer (Fraction Va), 400 ml of 0.14 M buffer (Fraction Vb), and 160 ml of 0.40 M buffer (Fraction Vc). The elution pattern is shown in Figure 1. The elution profile indicates how the fractions were pooled for assay.

The recovery of DNA ligase activity in the phosphocellulose and hydroxyapatite chromatography steps were approximately 70% and 60%, respectively.

The precipitated protein was collected by centrifugation at 40,000 × g for 30 min, suspended in 1 ml of 1 M NaCl/0.05 M Tris-HCl (pH 7.4/1 mM EDTA), and dialyzed for 2 hours against the same buffer (Fraction VI, 2 ml). Fraction VI then was chromatographed on a Sephadex G-150 column (1.2 × 87 cm) equilibrated with the same buffer. Fractions were assayed for DNA ligase activity, and active fractions (Kav = 0 to 0.15) were pooled (Fraction VII, 11 ml) and concentrated by dialysis against a saturated ammonium sulfate solution. The ammonium sulfate-precipitated Fraction VII was recovered by centrifugation. It could be stored at -10°C in precipitate form without loss of activity. For antibody induction, aliquots of this enzyme were suspended in a 10-fold volume of 1 M NaCl/0.05 M Tris-HCl (pH 7.4/1 mM EDTA), dialyzed against the same buffer for 6 hours, heated for 5 min at 4°C, and left for 20 min at 0°C. A small precipitate was removed by centrifugation, and the supernatant was dialyzed against 1.0 M NaCl/0.05 M Tris-HCl (pH 7.4) for 3 hours (Fraction VIII). The approximately 1000-fold purified preparation of DNA ligase I was used for induction of antibodies. Fraction VIII is not homogeneous but should be free from DNA ligase II, as the last purification steps were primarily employed to remove any traces of the latter enzyme. The purification of calf thymus DNA ligase I is summarized in Table I.

**Immunization Procedures**—Fraction VIII material (500 μl containing 1 mg of protein) was thoroughly emulsified with 500 μl of Freund's complete adjuvant and injected into multiple small aliquots intraperitoneally into a rabbit. The treatment was repeated four times with a time interval of 4 to 6 weeks between each injection, because DNA ligase I was not effectively inhibited by the antiserum obtained after only two injections. Twelve days after the last injection the animal was bled from the marginal ear vein. Collected blood was incubated at 20°C for 60 min, followed by 5 hours at 4°C, and clotted blood was removed by low speed centrifugation. To obtain a partly purified γ-globulin fraction, ammonium sulfate was added to 40% saturation. The resulting precipitate was collected, dissolved in 0.15 M NaCl/0.01 M potassium phosphate buffer (pH 7.0) and dialyzed against the same buffer for 16 hours. The ammonium sulfate precipitation and dialysis steps were repeated once. γ-Globulin from nonimmunized rabbits was obtained in the same fashion. These partly purified γ-globulin fractions were employed in all experiments. Sheep serum containing antirabbit IgG was a gift from Dr. B. Andersson. The γ-globulin fraction from this sheep serum was purified as above.

**RESULTS**

**Specificity of DNA Ligase I Antiserum**—To study the specificity of the antiserum obtained, calf thymus DNA ligase I and II (Fractions Vb and Vc) were concentrated by ammonium sulfate precipitation and separately chromatographed on Sephadex G-150 as described (Ref. 2; see also the Fraction VI → VII purification step in this work). This purification step was necessary to obtain DNA ligase II essentially free from DNA ligase I. The DNA ligase I fraction obtained had a specific activity three times higher than that of the DNA ligase II fraction, and either of the two ligases could catalyze the conversion of a maximum of 30% of the total 32P in the ligase substrate to an alkaline phosphatase-resistant form. The DNA ligase then were assayed under the standard conditions in reaction mixtures containing 10 -3 unit of DNA ligase (2.5 μg of DNA ligase I or 7 μg of DNA ligase II) and increasing amounts of γ-globulin from immunized or nonimmunized rabbits. As shown in Figure 2, DNA ligase I was inhibited to more than 98% by addition of the DNA ligase I antiserum to a protein concentration of 0.6 mg/ml. Addition of a corresponding γ-globulin fraction from nonimmunized rabbit (referred to below as control serum), or from the immunized rabbit prior to immunization, did not cause significant inhibition (<5%) at protein concentrations up to 4 mg/ml. DNA ligase II was not inhibited by addition of either the ligase I antiserum or the control serum at protein concentrations up to 4 mg/ml (Fig. 2). As a further control, the phage T4-induced DNA ligase was assayed with and without antiserum under the same conditions. This microbial enzyme was not detectably inhibited by the DNA ligase I antiserum (less than 5% inhibition by 0.6, 2, or 4 mg/ml of antiserum). When DNA ligase I and II were mixed in different proportions in the same reaction mixture, ligase activity equivalent to the amount of DNA ligase I added was inhibited by addition of antiserum, while activity corresponding to ligase II was unaffected. In a typical experiment, a reaction mixture containing 11 x 10^-4 unit of ligase I and 4 x 10^-4 unit of ligase II yielded 16 x 10^-4 unit of ligase activity in the presence of control serum (2 mg/ml), and 4.5 x 10^-4 unit of ligase activity in the presence of ligase I antiserum (2 mg/ml). The lack of inhibition of DNA ligase II by the DNA ligase I antiserum strongly indicates that DNA ligase II is a separate

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1Small amounts of tissue were instead disintegrated in a glass hand homogenizer. Control experiments with calf thymus showed that the amounts of DNA ligase I and DNA ligase II extracted were approximately the same after either disintegration method.
Table I

Purification of DNA ligase I from calf thymus

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein concentration ( \text{mg/ml} )</th>
<th>Specific activity ( \text{ligase units/mg protein} )</th>
<th>Total activity ( \text{ligase units} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Crude extract</td>
<td>19.5</td>
<td>0.906</td>
<td>530</td>
</tr>
<tr>
<td>II Streptomycin supernatant</td>
<td>4.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III Ammonium sulfate</td>
<td>4.1</td>
<td>0.800</td>
<td>310</td>
</tr>
<tr>
<td>IV Phosphocellulose</td>
<td>1.9</td>
<td>0.60</td>
<td>250</td>
</tr>
<tr>
<td>V Hydroxyapatite</td>
<td>0.70</td>
<td>1.2</td>
<td>160</td>
</tr>
<tr>
<td>VI Second phosphocellulose</td>
<td>11.2</td>
<td>5.2</td>
<td>105</td>
</tr>
<tr>
<td>VII Sephadex G-150</td>
<td>1.1</td>
<td>5.5</td>
<td>75</td>
</tr>
<tr>
<td>VIII Heat treatment( ^a )</td>
<td>2.0</td>
<td>5.7</td>
<td>45</td>
</tr>
</tbody>
</table>

\( ^a \) Protein was determined in Fractions I to III by the biuret reaction and in Fractions IV to VIII by ultraviolet absorption.

\( ^b \) In assays of Fractions I to III the reaction mixture was supplemented with NaCl (to 0.1 \( \text{M} \)). The values in the table have been corrected for the 50% inhibition caused by this addition (see Footnote 2 in the text).

\( ^c \) The data on Fractions I to IV include ligase II activity and are therefore approximately 10% too high.

\( ^d \) Heat treatment of Fraction VII was performed in aliquots suitable for immunization. The data given here for Fraction VIII represent the sum of these aliquots.

The results above do not exclude the possibility that the two DNA ligases could be antigenically related, as the rabbit antiserum might interact with DNA ligase II without causing inhibition of enzyme activity. To investigate this point, the rabbit antibodies against DNA ligase I (600 \( \mu \text{g} \) of protein) were incubated together with purified goat anti-rabbit IgG (820 \( \mu \text{g} \) of protein) in 50 \( \mu \text{l} \) of 0.15 \( \text{M} \) NaCl/0.01 \( \text{M} \) sodium phosphate (pH 7.0) at 0°C for 60 min. A visible precipitate was formed, which was removed by centrifugation. The supernatant solution had a ligase I-inhibiting activity (amount needed for 50% inhibition) 6-fold lower than that of rabbit antibodies incubated in an identical fashion but without goat anti-rabbit IgG. Thus, the goat antiserum had apparently precipitated 80 to 85% of the DNA ligase I antibodies. If an enzymatically active complex between DNA ligase II and rabbit anti-ligase I antibodies was formed, it should also be precipitated by the goat anti-rabbit IgG under the same conditions. Therefore, DNA ligase II (6 \( \times \) 10\(^{-3} \) unit) was first incubated with either ligase I antibodies or rabbit control serum (600 \( \mu \text{g} \) of protein) at 0°C for 30 min. Anti-rabbit IgG from goat (820 \( \mu \text{g} \) of protein) then was added, and the incubation was continued at 0°C for 60 min. A visible precipitate was again formed, which was removed by centrifugation, and the supernatant solutions were assayed for DNA ligase activity. There was no significant difference (<10%) in ligase activity between the supernatant solutions from DNA ligase II incubated with ligase I antibodies or control serum, showing that no DNA ligase II had been co-precipitated with the rabbit antibody-goat anti-rabbit IgG complex. In separate control experiments, it was found that goat anti-rabbit IgG did not affect either DNA ligase I or ligase II activity at the concentrations used here. It is concluded that DNA ligase I and DNA ligase II do not seem to be antigenically related.

Presence of DNA Ligase I and II in Different Tissues—Several different tissues and cells from different mammals were surveyed for DNA ligase I and II activity. Due to their relatively high DNA ligase activity, crude cell extracts from calf thymus could be directly assayed in a quantitative fashion in the presence of neutral salt (0.1 \( \text{M} \) NaCl) in the reaction mixture.\( ^e \) Thus, addition of 45 \( \mu \text{g} \) of a thymus extract (Fraction

\( ^e \) The addition of NaCl serves to inhibit nuclease activity (12). It also causes a 2-fold inhibition of either ligase I or ligase II (2); the data have been corrected for this salt inhibition. At the Mg\(^{2+} \) concentration
I) to the standard reaction mixture yielded $2 \times 10^{-4}$ unit of ligase activity. After simultaneous addition of 2.0 or $4.5 \times 10^{-4}$ units of purified ligase II, respectively, together with $2 \times 10^{-4}$ unit of Fraction I, 4.0 or $7.5 \times 10^{-4}$ units of ligase activity were found. When ligase I antibodies were added to the latter mixtures, 2.1 and $5.3 \times 10^{-4}$ units of ligase activity, respectively, were observed. On addition of ligase I antibodies to crude thymus extracts, approximately 90% of the total ligase activity was inhibited. It is concluded that thymus cell extracts contain approximately 10 times higher amounts of ligase I than ligase II activity. Different batches of calf thymus have yielded extracts containing 0.003 to 0.010 ligase unit per mg of protein. This variation may depend on the age of the animals.

Tissue extracts from several sources other than calf thymus contained considerably less DNA ligase activity, and it was therefore necessary to perform a partial purification and separation of the ligases prior to quantitative estimations. The procedures were first investigated with calf thymus extracts. When cell extracts were purified by streptomycin treatment, ammonium sulfate fractionation, and phosphocellulose chromatography as described under "Experimental Procedures," the proportion of ligase I to ligase II in the different fractions was not markedly changed. In control experiments on the discarded fractions, only very small amounts of DNA ligase activity could be detected in the material precipitated with streptomycin (after extensive dialysis against a buffer containing 1 M NaCl), in the ammonium sulfate side fractions, and in the material not adsorbed to phosphocellulose, and such ligase activity was inhibited by the ligase I antiserum. On subsequent hydroxyapatite chromatography, all detectable DNA ligase was adsorbed, and a separation of ligase I and ligase II was obtained on elution as previously observed (2). After hydroxyapatite chromatography, amounts of DNA ligase I approximately 10-fold higher than DNA ligase II were found (Table II), which is consistent with the results obtained with crude cell extracts. Thus, large selective losses of either DNA ligase I or II in the simple purification procedure employed apparently did not occur. The extracts from the other tissues were therefore subjected to the same purification procedure.

Calf spleen and liver, rabbit spleen, human placenta, and mouse ascites tumor cells were studied. The results are summarized in Table II. In all cases, DNA ligase activity was present both in hydroxyapatite Fractions Vb (0.14 M phosphate eluate) and Vc (0.40 M phosphate eluate). All fractions showed an absolute dependence on the addition of a divalent metal ion (Mg$^{2+}$ or Mn$^{2+}$) and ATP to the reaction mixtures, except that traces of ATP-independent activity in some cases were observed in Fraction Vb, presumably due to the presence of ligase-AMP complexes (6). Fractions Vb and Vc were assayed both in the presence and absence of antiserum against ligase I (2 mg/ml of protein). Large variations in the amounts of Fraction Vb ligase activity were found in different tissues, but in all cases this activity was effectively inhibited by addition of the ligase I antiserum (Fig. 3). In comparison with the results obtained on Fraction Vb, the amounts of DNA ligase activity found in Fraction Vc were more similar among the different tissues. The activity in Fraction Vc was in general not inhibited by ligase I antiserum, but with tissues containing much more ligase I than ligase II, a minor part of the activity was in several experiments inhibited by addition of the ligase antiserum. Thus, more than 98% of the ligase activity in Fraction Vb and 10 to 30% of the ligase activity in Fraction Vc from calf thymus were inhibited by the antiserum, while less than 5% of the Fraction Vc activity from calf spleen or liver could be similarly inhibited. The amount of antibody-inhibitable material in Fraction Vc from thymus was ascribed to a small amount of tailing of ligase I from Fraction Vb during hydroxyapatite chromatography. The fraction of DNA ligase eluting in Fraction Vc which was active in the presence of antiserum (2 mg/ml) was considered as DNA ligase II (Table II). With calf tissues, this activity also had the heat lability typical of bovine DNA ligase II (2). Thus, heating of Fraction Vc from calf thymus, spleen, or liver for 5 min at 45°C in 0.3 M NaCl/0.05 M Tris-HCl (pH 7.4)/1 mM EDTA/1 mM dithiothreitol resulted in loss (>95% inactivation) of the ligase active

![Fig. 2 (left). Effect of DNA ligase I antiserum on calf thymus DNA ligase I and DNA ligase II. Aliquots (0.001 unit each) of DNA ligase solutions were preincubated with different concentrations of ligase I antiserum or control serum prior to assaying. For further details, see "Experimental Procedure." Circles, DNA ligase I; triangles, DNA ligase II; filled symbols, ligase I antiserum; open symbols, control serum.](http://www.jbc.org/)

![Fig. 3 (right). Inhibition of DNA ligase I from different sources by the antiserum against calf thymus DNA ligase I. The experiments were performed as in Fig. 2. •, calf spleen; ○, calf liver; △, human placenta; ▲, mouse ascites tumor; ○, rabbit spleen.](http://www.jbc.org/)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>DNA ligase I</th>
<th>DNA ligase II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units recovered from 1 g tissue</td>
<td></td>
</tr>
<tr>
<td>Calf thymus</td>
<td>0.33</td>
<td>0.024</td>
</tr>
<tr>
<td>Calf spleen</td>
<td>0.040</td>
<td>0.020</td>
</tr>
<tr>
<td>Calf liver</td>
<td>0.031</td>
<td>0.020</td>
</tr>
<tr>
<td>Human placenta</td>
<td>0.013</td>
<td>0.009</td>
</tr>
<tr>
<td>Rabbit spleen</td>
<td>0.014</td>
<td>0.005</td>
</tr>
<tr>
<td>Mouse ascites tumor cells</td>
<td>0.31</td>
<td>0.028</td>
</tr>
</tbody>
</table>

Employed here (0.01 M), there is no significant stimulation of either DNA ligase I or II by addition of Na$^+$ (cf. Ref. 21).
in the presence of the ligase I antiserum, while identical heat treatment only slightly affected (<25% inhibition) the ligase activity in Fraction Vb.

Crude cell extracts (Fraction I) from all cells and tissues examined were assayed in parallel with the hydroxypatite fractions (Vb and Vc). The relative proportions of DNA ligase activity in Fractions I and V appeared to be similar in all cases. Consequently, the low amounts of DNA ligase I detected in several tissues, in comparison with calf thymus, did not seem to be due to a selective loss of this enzyme during purification.

Calf thymus and mouse ascites tumor contained more ligase activity than the other tissues. This was due to high levels of DNA ligase I, while these two sources did not contain significantly larger amounts of DNA ligase II than other tissues. It is noted that the mouse tumor was rapidly proliferating, and that the thymus in young animals contains a large proportion of very rapidly dividing cells (15).

**Subcellular Distribution**—It was previously reported that DNA ligase activity is present both in the nuclei and cytoplasm of mammalian cells (12, 16). In order to study the individual subcellular distribution of DNA ligase I and ligase II, isolated cell nuclei, mitochondria, and cytoplasm from calf thymus, as well as mitochondria from calf liver, were separately processed as described above for whole tissues, and the hydroxypatite Fractions Vb and Vc were assayed for DNA ligase activity. In several experiments, 30 to 60% of the total DNA ligase I activity and 60 to 80% of the total DNA ligase II activity were found in the nuclear fraction. The remaining DNA ligase activity was recovered in the cytoplasmic fraction. The mitochondrial fractions from thymus or liver did not contain detectable amounts of ATP-dependent DNA ligase activity. In control experiments, purified DNA ligase I added to the mitochondrial enzyme fractions could be quantitatively assayed (in the presence of 0.1M NaCl), so the apparent absence of activity in the mitochondrial extracts was not due to interference of nucleases or other inhibitors in the ligase assay. When the cytoplasmic fraction was further separated into a small particle fraction and a cytosol fraction, the large majority (>95%) of the ligase activity was recovered in the cytosol fraction. These data indicate that both DNA ligases are present in the cell nuclei and that neither of them is a predominantly mitochondrial enzyme. We do not know if the ligase activity present in the cytoplasm was due to leakage from the cell nuclei during isolation, or if the enzymes are partly localized in the cytoplasm in vivo.

Several experiments were performed to investigate if additional DNA ligase activity could be extracted from calf thymus by varying the extraction procedures. When the initial extraction time (1 hour) was extended, no increase in either DNA ligase I or ligase II could be detected. Extraction of calf thymus by the methods used by Chang and Bollum (17) or extraction of disintegrated calf thymus with a buffer containing 4 M NaCl, followed by fractionation in an aqueous polymer two-phase system to remove nucleic acids (18), also did not increase the yield of either DNA ligase I or II. Consequently, it would appear that both DNA ligases are efficiently extracted from disintegrated thymus cells by the standard procedure used in this work.

**Attempts at Interconversion**—We have previously reported (2) that DNA ligases I and II could not be interconverted by storage in solution, by salt precipitation, by freezing, or by adenylylation. However, these studies did not completely rule out that the smaller and more labile DNA ligase II could be generated from ligase I by dissociation or proteolysis, and further experiments to investigate this possibility have now been performed. To exclude that ligase II was generated during hydroxypatite chromatography, the hydroxypatite fraction of calf thymus DNA ligase I (Fraction Vb) was concentrated by ammonium sulfate precipitation, dialyzed against 0.8M NaCl/0.015M potassium phosphate (pH 7.2)/0.01M 2-mercaptoethanol, and rechromatographed on hydroxypatite as described under "Experimental Procedure." Fraction Vb contained all DNA ligase activity recovered (78% yield), and this DNA ligase was completely inhibited (>98% inhibition) by the ligase I antiserum. Fraction Vc contained no detectable DNA ligase activity (<2% of the activity applied to the column). Further, exclusion of the phosphocellulose chromatography step in the purification procedure did not affect the yields of either DNA ligase I or ligase II on subsequent hydroxypatite chromatography.

Pedrali-Noy et al. (3) have shown that the large majority of DNA ligase activity in extracts from a human heteroploid line, EUE, is due to an enzyme with the size of DNA ligase I. On storage of crude enzyme fractions at 0° for 20 days, or after extensive purification, small amounts of DNA ligase activity with approximately one-half the molecular weight of DNA ligase I were generated. These data are consistent with the dissociation of a dimeric protein into active monomers, the slow dissociation of a tightly bound non-ligase protein from the active ligase, or generation of a smaller form by proteolysis. As the size of these ligase I "monomers" are similar to that of DNA ligase II, it seemed possible that ligase II could be identical with the ligase I monomers. We have repeated and confirmed the results of Pedrali-Noy et al. (3). Thus, on gel filtration of several preparations of DNA ligase I (Fraction Vb) on Sephadex G-150, small and variable amounts (0 to 30%) of the ligase activity were sometimes recovered as a distinct peak with a Kav value of 0.24, separated from the majority of the activity which chromatographed as high molecular weight DNA ligase I (Kav = 0.09, Ref. 2). This calf thymus DNA ligase I monomer, purified by gel filtration, was studied with regard to stability and sensitivity to DNA ligase antibodies. It was completely inhibited by the addition of ligase I antibodies (0.6 mg/ml) to the reaction mixture, and thus showed similar sensitivity in this regard to the high molecular weight form of DNA ligase I.

Further, the ligase I monomer had the same heat stability in solution as the high molecular weight form of DNA ligase I, and also had the same relative activity in the pH 6 to 7 range as the larger form. In all these respects, it differs completely from DNA ligase II (2). We conclude that the DNA ligase I monomer discovered by Pedrali-Noy et al. (3) and DNA ligase II are two different proteins.

Proteins can sometimes be proteolytically cleaved into fragments that retain biological activity. In the present context, it could be proposed that DNA ligase II was generated when all antigenic determinants were digested away from DNA ligase I by proteases in the cell extract, while catalytic activity was preserved. In attempts to check this model, we have treated purified calf thymus DNA ligase I (Fraction Vb, 0.5 mg/ml of protein) in 0.05M Tris-HCl (pH 7.4)/0.003M CaCl2/0.001M dithiothreitol, with trypsin (0.007 mg/ml, 15 min, 37°) or chymotrypsin (0.007 mg/ml, 15 min, 37°), to cause 19 versus 79% inactivation. The proteolytic reactions were stopped by addition of Trasylol (0.007 mg/ml). In a separate
control experiment it was found that Trasylol did not inhibit either DNA ligase I or ligase II. When the partly inactivated DNA ligase I fractions were assayed in the absence and presence of ligase I antibodies, all enzymatic activity was still completely suppressed by the antiserum. Thus, we were unable to generate DNA ligase activity insensitive to ligase I antibodies by limited treatment of purified DNA ligase I with proteolytic enzymes.

**DISCUSSION**

The present data, taken together with our earlier results (2), strongly indicate that two different DNA ligases are present in mammalian cells. With the enzymes from calf tissues, it has been shown that: (a) DNA ligase I and II show different chromatographic properties on hydroxypatite and Sephadex G-150; (b) DNA ligase I is more stable on storage at 0°C in a variety of buffers and is also considerably more resistant to heat than DNA ligase II; (c) both enzymes have pH optima close to pH 7.8, but DNA ligase I retains 60 to 70% of its maximal activity when assayed at pH 6.4, while DNA ligase II only has 10% of its maximal activity at the lower pH value; (d) a rabbit antiserum against DNA ligase I inhibits this enzyme completely, but does not affect DNA ligase II; (e) an apparently "monomeric" form of DNA ligase I, which is generated on prolonged storage in solution, retains its characteristic heat stability and the property of being inhibited by the ligase I antiserum and is therefore not identical with DNA ligase II; (f) attempts to interconvert the two DNA ligases by a variety of treatments, including limited treatment with proteolytic enzymes, were consistently negative; (g) the relative proportions of the two ligases are different among different tissues. However, final proof that DNA ligase I and II are two separate enzymes will require the isolation of mutants of mammalian cells with defective DNA ligases, or determinations of the amino acid sequences of the enzymes.

The amounts of DNA ligase II activity recovered were relatively similar among different mammalian cells and tissues, while larger variations were observed in the amounts of DNA ligase I activity. We find high levels of DNA ligase I in extracts from calf thymus and mouse ascites tumor cells, but relatively low levels in calf liver, calf spleen, rabbit spleen, and human placenta extracts. Thus, DNA ligase I apparently dominates in tissues with a high proportion of dividing cells and may be present at increased levels as a function of cell proliferation. In agreement with this notion, regenerating rat liver has been found to contain higher levels of DNA ligase I but similar levels of DNA ligase II in comparison with normal rat liver. Neither of the ligases has the almost exclusive localization to the thymus found for the DNA terminal transferase (19). The mammalian DNA ligases studied in other laboratories (1, 3, 20) have usually been isolated from growing cells and tissues and have the general properties of DNA ligase I. A possible exception is the DNA ligase from rat liver nuclei recently purified by Zimmerman and Levin (21), which has the size and strongly reduced activity below pH 7 typical of a DNA ligase II.

It is unknown why mammalian cells have two different DNA ligases. Calf thymus DNA ligase I or DNA ligase II, at the concentrations studied here, do not show detectable RNA ligase activity in a joining assay employing 5'-32P-labeled poly(dI)-poly(dC), so neither of these DNA ligases seems to be identical with the RNA ligase found in the cytoplasmatic fraction from mammalian cells (22, 23). It may be of significance in the present context that two forms of E. coli DNA ligase apparently exist in vivo (24, 25).

Several physiologically directed studies have appeared on the amounts of DNA ligase activity in cell extracts from lymphocytes stimulated by phytohemagglutinin (26), in rat liver during regeneration (27, 28) and after administration of cycloheximid (29), in rat kidneys after tumor induction with N-nitrosodimethylamine (30), in mouse cells after infection with polyoma, SV40, or vaccinia virus (1, 12), and in DNA repair deficient cells from Xeroderma pigmentosum patients (31). It seems possible that similar experiments, performed under conditions where DNA ligase I and II are separately measured, may provide further insight into the function of these enzymes.

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Mammalian DNA ligases. Serological evidence for two separate enzymes.
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