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A Special Form of Deoxyribonucleic Acid Dependent Ribonucleic Acid Polymerase from Oocytes of *Xenopus laevis*. Isolation and Characterization†

John Wilhelm, Dino Dina, and Marco Crippa*

ABSTRACT: DNA-dependent RNA polymerase activity has been extracted in low salt from *Xenopus laevis* ovaries. The readily soluble enzyme has been purified 200-fold by DEAE-cellulose, phosphocellulose, and Sepharose 6B chromatography. The purified enzyme shows general elution and enzymatic properties similar to form A (I) from other tissues but can be distinguished by two main criteria: it is almost completely inhibited by high levels of α-amanitin and transcribes preferentially native DNA. An analysis of the activities in liver and kidney nuclei has shown that they contain only the known A (I) and B (II) forms of the enzyme, leading to the conclusion that the enzyme described below is probably a special form localized in the ovary. By microdissection techniques it has been found that this enzyme is localized in the nucleus of the oocytes and it is therefore called "oocyte-soluble polymerase." Germinal vesicles of stage 6 oocytes contain a large amount of RNA polymerase, which has been calculated to be roughly 10^5 times larger than a liver cell. In addition to a small amount of form B (II) polymerase (10–15% stage 6 oocytes contain predominanty the special oocyte soluble polymerase, whereas form A (I), if present, must represent less than 5% of the total RNA polymerase activity. This large amount of enzyme exists at a time when the oocyte nucleus also contains the amplified rDNA cistrons, and the possibility that the polymerase is involved in rDNA transcription is discussed.

During oogenesis the amphibian oocyte accumulates large quantities of different RNA species for later use during early embryonic development. Several biochemical events connected with the synthesis of the different RNA species are now well established.

An interesting situation exists in fully grown *Xenopus laevis* oocytes which contain a large store of ribosomes. The synthesis of the ribosomal RNA (rRNA)[1] components (28, 18, and 5 S) during oogenesis is achieved by different and rather intriguing processes.

The *Xenopus* diploid genome has at least 54,000 5S RNA genes (Brown and Weber, 1968) which are active during the entire period of oogenesis (Mairy and Denis, 1971). On the contrary, there are only 900 rRNA genes per diploid genome. The oocyte, however, selectively replicates its rDNA complement and then transcribes the amplified genes very actively but only in a temporally limited period.

We were interested in studying the properties of the RNA polymerases[1] operating inside of the oocyte during these events. In eukaryotic systems different forms of RNA polymerase have been separated (Roeder and Rutter, 1969; Kedinger *et al.*, 1970; Jacob *et al.*, 1970) and purified (Chambon *et al.*, 1970; Blatti *et al.*, 1970; Weaver *et al.*, 1971; Chesterton and Butterworth, 1971a,c; Gissinger and

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[1] Abbreviations used are: DEAE, diethylaminoethyl; RNA polymerase, nucleotide triphosphate:RNA nucleotidyltransferase (EC 2.7.7.6); EDTA, ethylenediaminetetraacetate; rRNA, ribosomal ribonucleic acid.

Chambon, 1972). The two main forms A (I) and B (II) and a third nuclear form (III), found in certain cases (Roeder and Rutter, 1969, 1970; Blatti *et al.*, 1970; Ponta *et al.*, 1972; Doenecke *et al.*, 1972), have been separated by DEAE chromatography and characterized.[2]

In vivo, form A (I) is localized in the nucleolus and is thought to be related to the synthesis of rRNA whereas form B (II) is nucleoplasmic and is probably involved in the synthesis of nuclear and messenger RNAs (Chambon *et al.*, 1970; Jacob *et al.*, 1970; Pogo *et al.*, 1967).

In the present paper we report the isolation of a special form of RNA polymerase from *Xenopus* ovarian tissue. This enzyme, in contrast to the A (I) and B (II) forms isolated from *Xenopus* liver, prefers double-stranded DNA as a template and shows intermediate sensitivity to α-amanitin. It represents about 70% of the total enzyme activity found in whole ovaries and at least 90% of the polymerase in oocytes (stage 6). A small amount (10%) of polymerase form B (II) is observed in oocytes but no detectable form A (I). This special enzyme activity, called *soluble oocyte polymerase* in this paper, is localized in germinal vesicles, but its function is as yet undetermined.

Materials

Some α-amanitin was kindly supplied by Professor Wieland, Heidelberg, and the rest was purchased from C. H. Boehringer, Ingelheim, Germany. Tritiated UTP was from New England Nuclear and unlabeled nucleotide triphosphates were from Fluka AG, Buchs, Switzerland. Tris base and ammonium sulfate were Schwarz/Mann enzyme grade, DEAE- and phosphocellulose were Whatman DE-52 and P-11, respectively, and Sephadex and Sepharose were from Pharmacia Fine Chemicals.

Xenopus laevis were purchased from the South African Snake Farm, Fish Hoek, South Africa.

Methods

Solutions used were: homogenization buffer, 15% glycerol–20 mM Tris-HCl (pH 7.9)–0.1 mM dithiothreitol–0.1 mM EDTA; diluting buffer, same but 30% glycerol; DEAE buffer, 30% glycerol–20 mM Tris-HCl (pH 7.9)–50 mM $(NH_4)_2SO_4$–3 mM $MnCl_2$–0.1 mM dithiothreitol–0.1 mM EDTA; phosphocellulose buffer, DEAE buffer without $MnCl_2$; Sepharose buffer, DEAE buffer but with 100 mM $(NH_4)_2SO_4$; storage buffer, 50% glycerol–20 mM Tris-HCl (pH 7.9)–3 mM $MnCl_2$–0.1 mM dithiothreitol; incubation mixture for RNA polymerase activity 20 mM Tris-HCl (pH 7.9)–0.5 mM ATP, GTP, CTP–0.005 mM [^{3}H]UTP (5 μCi/ml)–0.1 mM dithiothreitol. The salt and divalent cation vary according to the particular experiment as described.

General Procedures. All preparative procedures are done in ice and chromatographic columns (except Sepharose 6B) are run and collected at −10° in an LKB 7000 refrigerated fraction collector. The enzyme fractions are stored in 50% glycerol at −20° or in liquid N_2 (Kedinger *et al.*, 1972). Every ammonium sulfate precipitation is done at 50% saturation (0°). All water is quartz distilled deionized. Protein concentration is determined by the Lowry procedure (Lowry *et al.*, 1951) with parallel blanks to correct for the interference of the glycerol buffers.

Preparation of Ovarian RNA Polymerases. Whole adult ovaries are removed from anesthetized females and rinsed in isotonic media. The tissue is homogenized in 2–3 vol of homogenization buffer in loose-fitting Teflon-glass motorized homogenizers at slow speed. The homogenate is filtered through two layers of gauze and the yolk is pelleted by centrifugation at 400–500*g* for 10 min. The supernatant is made 50 mM $(NH_4)_2SO_4$ by the addition of 4 M $(NH_4)_2SO_4$ and the solution is centrifuged 220,000*g*-hr.

Chromatin. With the addition of salt to 50 mM, the chromatin is pelleted as a layer above the black pigment granules on the bottom of the tube. The crude chromatin is resuspended in a small volume (about one-fourth of the original volume) of DEAE buffer containing 300 mM $(NH_4)_2SO_4$. The solution is sonicated (6 × 15 sec at full power on the Branson Sonifier) until the viscosity is lost and centrifuged 220,000*g*-hr. The supernatant is diluted to 50 mM $(NH_4)_2SO_4$ in DEAE buffer, batch absorbed to a volume of DEAE-cellulose equal to half the starting material, eluted stepwise, and precipitated with $(NH_4)_2SO_4$. The pellets are redissolved in storage buffer.

50 mM Supernatant. The supernatant is made 3 mM $MnCl_2$ and the precipitate centrifuged and discarded. The soluble enzyme is stirred for 30 min with a volume of settled DEAE-cellulose, equilibrated in DEAE buffer, equal to three times the original volume. The suspension is washed with DEAE buffer on a Büchner funnel and the enzyme eluted with 200 mM $(NH_4)_2SO_4$ in DEAE buffer. The protein is precipitated with $(NH_4)_2SO_4$, collected by centrifugation, and redissolved in storage buffer.

Preparation of Liver RNA Polymerases. Freshly excised livers are homogenized in 0.25 M sucrose–20 mM Tris-HCl (pH 7.9)–10 mM $MgCl_2$–25 mM KCl (Seifart *et al.*, 1972). The nuclei are recovered by centrifugation at 800*g* for 10 min, the supernatant being subsequently used as the source of liver "C" enzyme (see below). The nuclei are purified through sucrose, resuspended in DEAE buffer with 300 mM $(NH_4)_2SO_4$ (half the original volume), sonicated to reduce the viscosity (8 × 15 sec), and centrifuged 220,000*g*-hr. The A (I) and B (II) forms are subsequently separated on DEAE-cellulose by the procedure of Kedinger *et al.* (1972). The centrifuged supernatant is diluted to 100 mM $(NH_4)_2SO_4$ with DEAE buffer minus $(NH_4)_2SO_4$ and batch absorbed to a settled volume of DEAE-cellulose equal to twice the original liver weight. Form A (I) is recovered from the 100 mM filtrate by ammonium sulfate precipitation and form B (II) is eluted from the DEAE-cellulose at 300 mM $(NH_4)_2SO_4$ and also ammonium sulfate precipitated. Both fractions are redissolved in storage buffer. The separation of the two forms is checked by assays with 0.1 μg/ml of α-amanitin. If necessary, the fractions are rechromatographed on DEAE-cellulose; otherwise they are further purified by phosphocellulose chromatography.

The cytoplasmic supernatant is further centrifuged at 48,000*g* for 30 min. The resulting postmitochondrial fraction is ultracentrifuged 400,000*g*-hr and the final supernatant is batch absorbed to 2 vol of DEAE-cellulose equilibrated in DEAE buffer. The enzyme is eluted at 200 mM $(NH_4)_2SO_4$, and precipitated with ammonium sulfate. The resulting liver cytoplasmic form of RNA polymerase is further purified by DEAE- and phosphocellulose chromatography.

Chromatographic Procedures. DEAE- and phosphocellulose columns are prepared and run as described by Weaver *et al.* (1971) and Gissinger and Chambon (1972). Fractions in

[2] Two kinds of nomenclature are currently used to identify the multiple forms of RNA polymerase in eukaryotes. One uses alphabetic letters such as A, B, and C while the other employs Roman numerals such as I, II, and III. In the present paper we use letters to identify the different enzymes, putting in parentheses the corresponding Roman number of the alternative nomenclature: A (I), B (II).

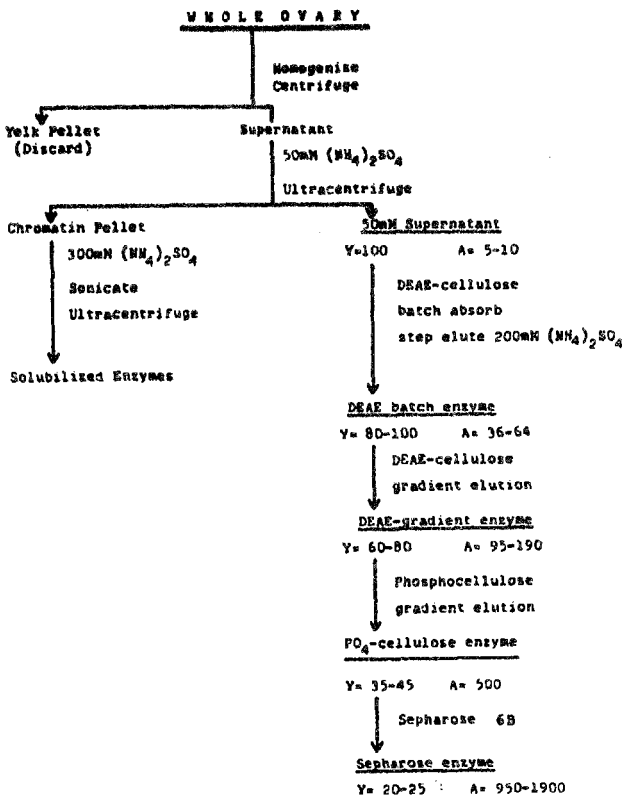

FIGURE 1: Flow chart of RNA polymerase purification from *Xenopus* ovaries: Y, yield of enzyme at each step; A, specific activity of the enzyme in units per milligram of protein. The numbers represent the range of values obtained from several preparations.

storage buffer are diluted to 30% glycerol and the ammonium sulfate is adjusted to 50 mM by the use of a conductivity meter. $MnCl_2$ (3 mM) is included in all DEAE-cellulose steps, but is omitted in samples chromatographed on phosphocellulose, although $MnCl_2$ is added to the tubes in the fraction collector. The ammonium sulfate concentration of column fraction assays is adjusted to 20–50 mM, or about 100 mM for B enzymes. Sepharose 6B columns (1.6 × 90 cm) are loaded with 3 ml or less of DEAE- and phosphocellulose purified enzyme and eluted with Sepharose buffer at 15 ml/hr. The enzyme-containing fractions are concentrated by ammonium sulfate precipitation.

RNA Polymerase Assay. Assays are performed in a volume of 200 μl (100 μl with purified enzyme) in the incubation mixture (see section on solutions) for 15 min at 30°, the optimal temperature for these enzymes *in vitro*. The incubation mixture, divalent cation, ammonium sulfate, and DNA are added separately and vary according to the experiment. Normally, incubations include 1.5 mM $MnCl_2$ and 30 μg/ml of bulk *Xenopus* DNA. Incubations are stopped by the addition of cold 1% $Na_4P_2O_7$ (pH 8) and trichloroacetic acid to 5%. The precipitate is collected and washed on Whatman GF/C glass fiber filters and scintillation counted.

One unit of enzyme activity under these conditions incorporates 1 pmol of [^{3}H]UTP into acid-precipitable product.

Preparation of DNA. Xenopus laevis bulk DNA was prepared from erythrocytes as described by Brown and Weber (1968). High molecular weight DNA (~35 × 10^6 daltons) was prepared from ovaries of *Xenopus* tadpoles with an initial digestion with proteinase K and subsequent purification as outlined by Gross-Bellard *et al.* (1973).

Determination of Nucleases in the Enzyme Preparation (Sepharose Enzyme). For DNase determination 10 μg of enzyme (Sepharose fraction) was incubated with 25,000 cpm of ^{3}H-labeled supercoiled polyoma DNA (sp act. ~2 × 10^5 cpm/μg) for 30 min at 30°. To determine the extent of contamination by RNase, the same amount of enzyme was incubated either with 30,000 cpm of [^{3}H]rRNA or with 10,000 cpm of purified polysomal ^{3}H-labeled 12S mRNA from *Xenopus* embryos. Both RNAs had a specific activity higher than 10^5 cpm/μg. The incubation mixture contained 1.5 mM Mn^{2+} and 20 mM Tris (pH 7.9) to reproduce the standard RNA polymerase assay conditions. The total incubation volume was 100 μl. The reaction was stopped by making the mixture 1% sodium dodecyl sulfate and 2 mM EDTA. The samples were layered onto a 15–30% sucrose gradient in 20 mM Tris (pH 7.5), 1% sodium dodecyl sulfate, and 0.1 mM EDTA and centrifuged at 22° in a SW 41 Spinco rotor. Controls incubated under the same conditions but without enzyme were run in parallel gradients.

Results

Purification. DNA-dependent RNA polymerase can be obtained from whole *Xenopus* ovary by the high salt solubilization of the enzyme from a crude chromatin preparation (Tocchini-Valentini and Crippa, 1970). Whole ovaries are homogenized in a salt-free glycerol buffer (homogenization buffer) and the yolk is pelleted by low-speed centrifugation. No RNA polymerase activity could be solubilized from or detected in this yolk pellet. Ammonium sulfate (4 M) is added to the supernatant to a final concentration of 50 mM and a chromatin pellet is recovered after ultracentrifugation (see Methods and Figure 1). However, the polymerase activity recovered from this chromatin pellet represents only 5–10% of the total ovarian polymerase: the remainder is soluble even in low salt and is found in the 50 mM supernatant at this step. This easily soluble RNA polymerase activity has been further purified and characterized.

Properties of the 50 mM Supernatant. Enzyme activity estimates in this fraction are difficult, due to the high protein concentration (10 mg/ml) and to the presence of unidentified factors partially inhibiting the enzyme activity. However, as the DNA dependency is usually over 80%, some indicative estimates of the total and specific activity have been made (Figure 1). When this enzyme fraction is tested with native and denatured templates, it shows a preference for double-stranded DNA (see paragraph on enzyme specificity), a property which is retained throughout the purification procedure. All estimates of the inhibition in the presence of low and high α-amanitin concentrations are made impossible by the low specific activity of this crude fraction and by the poor reproducibility of the assays.

DEAE-Cellulose. When the 50 mM supernatant is made 3 mM $MnCl_2$, a precipitate is formed which, however, contains very little enzyme activity and which can be removed by centrifugation. The supernatant is batch absorbed to DEAE-cellulose, eluted, and ammonium sulfate precipitated (see Methods).

This step yields an enzyme which is about tenfold purified and can be assayed in a linear way up to a protein concentration of 0.5 mg/ml without any inhibitory effect. The enzyme recovered accounts for more than 100% of the original 50 mM supernatant activity and this activation is probably explained by the removal of nonspecific inhibitors. The DNA dependence is absolute and the preference for native over denatured template is about twofold.

When assayed in the presence of α-amanitin, the redissolved enzyme showed about 5% inhibition at 0.1 μg/ml while it was

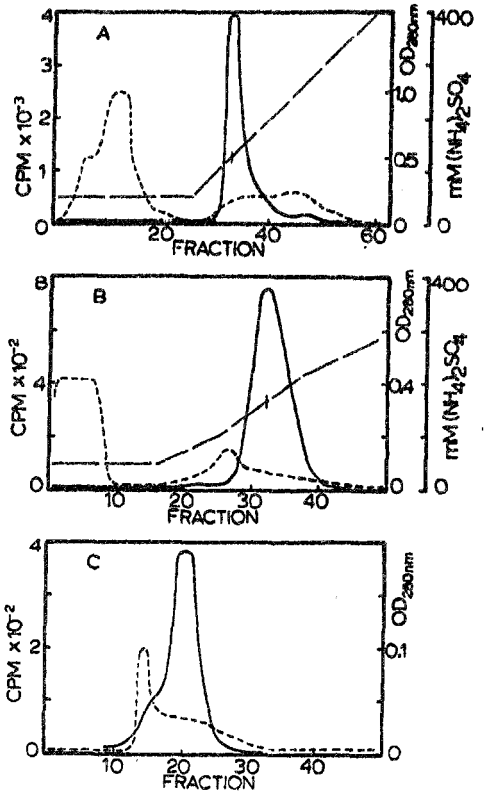

FIGURE 2: Purification of the RNA polymerase solubilized in 50 mM ammonium sulfate from *Xenopus* ovaries. (A) DEAE-cellulose; 200 mg of protein containing 1.2×10^4 units of enzyme in DEAE buffer were loaded onto a 1.2×40 cm column of DEAE-cellulose. The enzyme was eluted with a 300-ml gradient (50–450 mM ammonium sulfate) and 50-μl aliquots of the fractions (10 ml) were assayed. (B) Phosphocellulose; on a 1.2×20 cm column of phosphocellulose was loaded the enzyme from the DEAE-cellulose column dissolved in phosphocellulose buffer. The column was developed with a 200-ml gradient (50–350 mM ammonium sulfate) and 50-μl aliquots of the fractions (6 ml) were assayed for enzyme activity. (C) Sepharose 6B; 3 ml of phosphocellulose-purified enzyme (10–15 mg of protein) dissolved in Sepharose buffer with 400 mM ammonium sulfate was loaded onto a 1.6×90 cm column of Sepharose 6B; 10-ml fractions were collected at 17 ml/hr and 50-μl aliquots were assayed for enzyme activity: (–) [^{3}H]UTP incorporation into acid-precipitable product; (---) $OD_{280\ nm}$; (– – –) ammonium sulfate concentration.

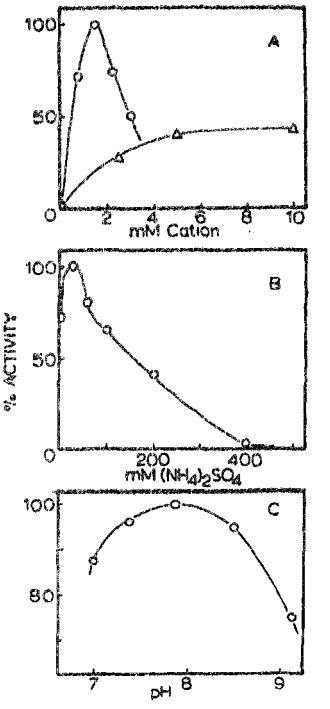

FIGURE 3: Divalent cation, salt, and pH requirements of the purified oocyte polymerase. The enzyme used was purified through the Sepharose 6B step (Figure 1) and assayed by the procedures outlined under Methods. (A) Divalent cation; the assays were performed in the absence of ammonium sulfate at different concentrations of $MnCl_2$ (O) and $MgCl_2$ (Δ); 100% activity = 2.5 units of enzyme. (B) Ammonium sulfate; the enzyme was assayed in the presence of 1.5 mM $MnCl_2$ (O) with different concentrations of ammonium sulfate; 100% activity = 3.5 units of enzyme. (C) pH; the assays contained 1.5 mM $MnCl_2$, 20 mM $(NH_4)_2SO_4$, and 50 mM Tris-HCl adjusted to different pH values; 100% activity = 1.2 units of enzyme.

90% inhibited by high amounts of this drug (250 μg/ml) (see Figure 4). The template specificity and drug inhibition will be described in greater detail later.

Further Purification Procedures. The enzyme obtained after ammonium sulfate precipitation of the DEAE eluate can be stored several days at −20° in storage buffer. The presence of $MnCl_2$ is important for the enzyme stability as well as for obtaining reproducible purification. The small amount of form B (II) present after the first DEAE batch absorption is removed by loading the enzyme onto a DEAE-cellulose column and eluting it with a salt gradient (Figure 2A). The peak of activity eluted at 130–150 mM $(NH_4)_2SO_4$ is ammonium sulfate precipitated, resuspended in Mn^{2+}-free buffer, and loaded onto a phosphocellulose column (Figure 2B). The enzyme eluted by a salt gradient comes out at a concentration of about 180 mM ammonium sulfate. Remaining traces of nucleases (see Methods) are removed when the enzyme is then fractionated on the Sepharose 6B column (Figure 2C).

The final specific activity of the enzyme is 1900 units/mg and represents a 200-fold purification from the 50 mM supernatant. It should be noted that for practical reasons the enzyme is routinely assayed at a limiting concentration of UTP. At saturating levels of UTP the specific activity is increased two- to threefold (E. Long, unpublished observations). Further purification of the enzyme on glycerol gradients has been thwarted due to the instablity of the purified enzyme in all conditions tested. In addition, chromatography on carboxymethylcellulose or hydroxylapatite was unsuccessful, but affinity chromatography on DNA-Sepharose appears more promising.

Properties. Figure 3 shows the results obtained when the purified enzyme was analyzed for divalent cation preference, salt, and pH dependence. There is a twofold preference for Mn^{2+}; moreover Mn^{2+} displays a very sharp optimum at 1.5 mM, while Mg^{2+} has a much broader range (Figure 3A). Ammonium sulfate in the presence of Mn^{2+} is stimulatory (a maximum of 30% at 20–40 mM; Figure 3B) but is not effective with Mg^{2+}. Finally, the optimum pH for the enzyme is near 8.0 (Figure 3C). Thus, it is apparent that chromatographically (Figure 2A–C) and enzymatically (Figure 3A–C) the properties of the enzyme are quite similar and indeed almost identical with those of the form A (I) polymerase previously described (Chambon *et al.*, 1970; Chesterton and Butterworth, 1971a; Seifart *et al.*, 1972).

There are, however, two distinct properties of the enzyme purified from *Xenopus* ovaries which make it quite easily distinguishable from polymerase A (I): (1) sensitivity to the mushroom toxin α-amanitin and (2) different template specificity. These two properties, as discussed before, are already present after the very first purification step (DEAE-cellulose

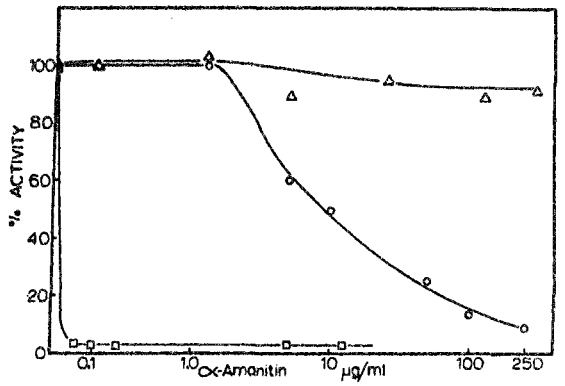

FIGURE 4: Effect of α-amanitin on *Xenopus laevis* RNA polymerase. RNA polymerase was fractionated and partially purified (see Methods and Figure 1) from *Xenopus* liver nuclei and ovary. α-Amanitin sensitivity was assayed using optimal conditions for each enzyme (Figure 3 and Table I): (△) liver A (I) purified through the DEAE-cellulose step; 100% activity = 0.75 unit; (○) oocyte soluble enzyme purified through the Sepharose 6B step; 100% activity = 1.1 units; (□) liver B (II) purified through the DEAE-cellulose step; 100% activity = 0.5 unit; ovarian B (II) polymerase gives the same result.

batch absorption) and are not changed during the entire procedure. Indeed, if a portion of the 50 mM supernatant, before the DEAE absorption, is chromatographed directly on Sepharose 6B (10 ml applied to a 2.6 × 100 cm column) the enzyme eluted (tenfold purified) shows similar recovery and identical enzymatic characteristics as the DEAE-cellulose treated enzyme, which indicates that the enzyme properties are independent of the purification techniques employed.

(1) α-AMANITIN SENSITIVITY. Figure 4 shows the results obtained when the ovary RNA polymerase and the polymerase A (I) and B (II) extracted and purified from *Xenopus* liver are assayed in the presence of increasing concentrations of α-amanitin. The liver A (I) enzyme, as previously reported by other authors (Seifart *et al.*, 1972; Kedinger *et al.*, 1972), is insensitive to the drug even at concentrations above 250 μg/ml, whereas the liver B (II) enzyme is completely inhibited at 0.1 μg/ml. The ovarian enzyme is unaffected by α-amanitin up to a concentration of 1 μg/ml but it is progressively inhibited at higher concentrations. The inhibition is independent of divalent cation, salt (0–50 mM $(NH_4)_2SO_4$), or template (single- or double-stranded DNA). The final inhibition at 250 μg/ml is 80–90%. This pattern of sensitivity does not result from the presence in our enzyme preparation of another α-amanitin binding protein interfering with the assay since in a control experiment the *Xenopus* liver form A (I) and B (II) polymerases, mixed with the oocyte-soluble enzyme, still showed the expected α-amanitin sensitivity.

(2) TEMPLATE SPECIFICITY. The lack of *in vitro* specificity of all the eukaryotic RNA polymerases so far isolated in transcribing different purified DNA templates has been reported by many laboratories. The existence of a few factors modifying the activity of eukaryotic enzymes has been shown (Stein and Hausen, 1973; Seifart *et al.*, 1973; Di Mauro *et al.*, 1972). It is also known that some of the enzymes purified from different sources transcribe better single-stranded templates and have a certain affinity for single-stranded nicks (from this point of view they behave exactly as the "core" *Escherichia coli* RNA polymerase) (Hinkel *et al.*, 1972). In fact, forms A (I) and B (II) isolated from *Xenopus* liver and form B (II) from *Xenopus* ovaries show the same kind of preference (Table I). This lack of specificity suggests that these enzymes initiate either at nicks or free ends while being unable to recognize any promotor-like site. Unlike form A (I) and B (II) polymerases from *Xenopus*, the oocyte-soluble RNA polymerase transcribes optimally high molecular weight unnicked DNA (~35 × 10^6 daltons, double stranded) and shows, under all the conditions tested, a striking preference for double-stranded templates (D. Dina and M. Crippa, manuscript in preparation).

Figure 5A shows the two curves obtained with native and denatured low molecular weight DNA in the presence of increasing salt concentrations. The DNA was used in saturating amounts as judged by the optimal incorporation of [^{3}H]UTP into the Cl_3CCOOH-insoluble product. It appears that the lower salt concentrations allow a better selection for native DNA. For this reason the following experiment has been run with no salt added to the incubation mix.

Figure 5B shows a saturation curve of 1 unit of enzyme by increasing amounts of native and denatured low molecular weight DNA (10^6 daltons, double stranded). It is clear that even low amounts of DNA give a good level of activity. However, the preference for native DNA increases as saturating conditions are approached, indicating a preference of the enzyme for certain sites that are provided as more DNA is added. On the basis of the increasing preference for native DNA it is then reasonable to conclude that these sites are somehow "specific." The two main properties of the oocyte-soluble polymerase in transcribing DNA, that is, the preference for native DNA and the ability to transcribe high molecu-

TABLE I: Properties of Some RNA Polymerases from *Xenopus laevis*.[a]

Source	Localization	Type	DEAE-Cellulose Elution mM $(NH_4)_2SO_4$	Mn^{2+}/Mg^{2+}	Optimal $[(NH_4)_2SO_4]$ (mM)	α-Amanitin % Inhibition Low	α-Amanitin % Inhibition High	DNA Template[b] Denatured/ Native
Liver	Nuclear	A (I)	130	2.8	20	0	0	2.2
Liver	Nuclear	B (II)	240	20	100	100		2.2
Liver	Cytoplasmic	C	130	2.3	20	0	70	2.1
Ovary	Nuclear	B (II)	240	10		100		
Oocyte	Nuclear	Soluble	130	2.3	30	0	92	0.60

[a] The DNA-dependent RNA polymerases of liver, ovary, and oocyte were purified by DEAE-cellulose and phosphocellulose chromatography as outlined under Methods. The partially purified fractions were assayed in parallel experiments to those shown in Figures 3–5. α-Amanitin concentrations: low, 0.1 μg/ml; high, 250 μg/ml. [b] Assays with low molecular weight DNA at saturation.

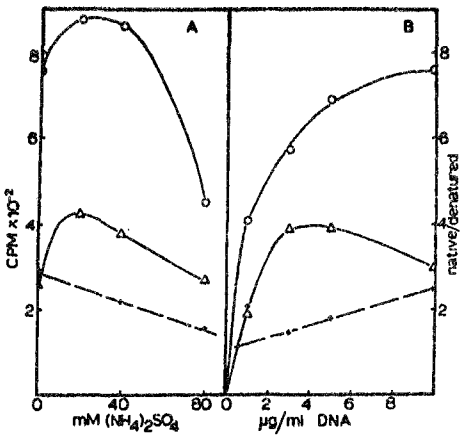

FIGURE 5: (A) Effect of salt on the transcription of native and heat-denatured DNA. DNA was denatured by incubation for 15 min at 105°. After cooling in ice it was added in saturating amounts to the assays containing increasing concentrations of $(NH_4)_2SO_4$. In a parallel experiment native DNA has been used: (O–O) native; (Δ–Δ) denatured; (+–+) native/denatured. (B) Saturation of the enzyme by native and denatured DNA; 1 unit of Sepharose-purified enzyme was assayed with amounts of DNA varying between 1 and 10 µg/ml. DNA was denatured as described above. The template size, 10^6 daltons double stranded, was determined by electron microscopy: (O–O) native; (Δ–Δ) denatured; (+–+) native/denatured.

lar weight intact templates, should be relevant to obtain a good degree of fidelity in an *in vitro* transcriptional system. Work is in progress to characterize the affinity of the enzyme for different templates and to analyze the RNA products.

Organ Specificity and Cellular Localization. Having purified and partially characterized this new RNA polymerase from *Xenopus* ovaries, other tissues were checked for the presence of the enzyme. In *Xenopus* liver nuclei, two species of RNA polymerase were characterized and found similar to those isolated by Roeder *et al.* (1970). In addition, it was possible to detect in the liver cytoplasm an enzyme with intermediate α-aminitin sensitivity, similar to enzyme "C" described recently by Seifart *et al.* (1972) in rat liver.

Table I shows the basic properties of the different RNA polymerase activities isolated from *Xenopus* liver compared to ovarian enzymes. It is immediately apparent that the partially α-amanitin sensitive liver "C" enzyme does not show the same template specificity as the soluble oocyte polymerase. Moreover, the soluble oocyte enzyme is localized in the nucleus of the oocyte (see Table IV), whereas the liver "C" enzyme is recovered in the postmicrosomal supernatant and is undetectable in purified nuclei. Therefore, it looks more as if the liver "C" and the oocyte-soluble enzyme could be two different enzymes which show similar α-amanitin sensitivity patterns. The results summarized in Table II clearly show that the recovery of the oocyte-soluble enzyme does not depend on the extraction conditions. At the same time, *Xenopus* liver nuclei do not yield any trace of an enzyme with such α-amanitin and template properties, either after low or high salt extraction. It seems therefore that the extraction conditions do not select for a minor species of RNA polymerase nor do they cause any artificial interconversion of the classical nuclear A (I) and B (II) RNA polymerases into the special form which is characteristic of the oocyte.

A further search for an RNA polymerase with intermediate α-amanitin sensitivity in *Xenopus* kidney disclosed the presence of the well-characterized forms A (I) and B (II); however, no partially α-amanitin sensitive activity could be detected.

LOCALIZATION IN THE OVARY. Most of the mass of the ovary

TABLE II: Enzyme Recovery with Different Extraction Conditions.[a]

Source	Extraction mM $(NH_4)_2SO_4$	Total Act. Units per g of Tissue	Enzyme Amounts (Units) A (I)	B (II)	Special Form[b]
Whole ovary	50	160	<5	12	148
	300	178	24	18	136
Liver nuclei	50	15	9	6	0
	300	40	28	12	0

[a] Whole ovarian tissue was extracted with 50 or 300 mM $(NH_4)_2SO_4$ as described under Methods. Frog liver nuclei were extracted with high salt (see Methods) or low salt (Chesterton and Butterworth, 1971b). The ultracentrifuge supernatants were absorbed to DEAE-cellulose at 50 mM and step eluted at 300 mM $(NH_4)_2SO_4$. The eluates were assayed for sensitivity to 0.1 µg/ml of α-amanitin (form B (II)) and to 250 µg/ml of α-amanitin. The activity insensitive at 250 µg/ml and preferring denatured DNA is considered to be form A (I). These assays were performed at limiting UTP; under nonlimiting conditions the total activity is increased two- to threefold, but the enzyme properties are not affected. [b] This form, found in the ovaries, has been named "oocyte-soluble polymerase" (see introductory statement).

of an adult *Xenopus* female is contributed by the oocytes in various stages of development. However, many other cells are also present and their number exceeds by far the number of the oocytes. For instance, each oocyte is surrounded by continuous sheet of follicle cells in a ratio of approximately 2000 follicle cells per oocyte. Having therefore established that the ovarian enzyme is unique to this organ, it seemed appropriate to determine in which of the ovarian cells the enzyme is localized.

To do this, we needed to solubilize and recover the total RNA polymerase activity from a limited number of manually dissected cells. Thus, to recover both the soluble and chromatin-bound enzymes, instead of pelleting the chromatin by ultracentrifugation in 50 mM ammonium sulfate (Figure 1), the post-yolk (500*g*) supernatant was immediately raised to 300 mM ammonium sulfate, sonicated briefly, and ultracentrifuged as usual. The supernatant was mixed with DEAE-cellulose and diluted to 50 mM. The total enzyme was eluted at 300 mM ammonium sulfate and precipitated. The redissolved enzyme was assayed at two concentrations of α-amanitin (0.1 and 250 µg/ml) and for template preference in the absence and presence of 250 µg/ml of α-amanitin. The enzyme inhibited at 0.1 µg/ml of α-amanitin is considered to be form B (II) (Table I). Since the inhibition of the easily soluble ovarian enzyme by α-amanitin at 250 µg/ml is never 100% (Figure 4 and Table I), we characterized the residual 10–15% of the activity by its template preference using the following rationale. If the polymerase in the presence of high concentrations of α-amanitin prefers native DNA as a template (denatured/native = 0.60 ± 0.05), it is considered to be soluble oocyte polymerase activity. Higher ratios are considered to be indicative of the existence of polymerase A(I) which is not inhibited by α-amanitin (Figure 4) but prefers denatured DNA by more than twofold (Table I). It must be stressed that these ratios are obtained with standard assay conditions of 20–30 mM $(NH_4)_2SO_4$ and low molecular weight DNA (Figure 5).

TABLE III: Quantitative Estimation of RNA Polymerases in Ovary and Ooctyes.[a]

Tissue	Units of Oocyte	α-Amanitin % Inhibition Low	α-Amanitin % Inhibition High	DNA Template Denatured/Native α-Amanitin −	DNA Template Denatured/Native α-Amanitin +
Whole ovary		8	75	0.71	0.79
Ovary without stage 5 and 6 oocytes		15	64	1.2	1.54
Stage 6 with follicles	0.12	10	84	0.57	0.65
Stage 6 defolliculated	0.12	10	81	0.63	0.57

[a] Stage 6 oocytes were manually collected (stage 6 follicu-lated, 0.6 ml, 500 oocytes; stage 6 defolliculated, 0.45 ml, 400 oocytes; whole and stripped ovary, 0.5-ml volume) and homogenized in 3 vol of homogenizing buffer and the enzyme prepared as described in the text, except the $MnCl_2$ was 1 mM throughout the procedure. α-Amanitin concentrations: low, 0.1 μg/ml; high, 250 μg/ml. DNA template preference was measured in the absence (−) and presence (+) of α-amanitin at 250 μg/ml. Assays were adjusted to 20–30 mM $(NH_4)_2SO_4$. The ovary minus stage 5 and 6 oocytes is prepared by manually removing stage 5 and 6 oocytes (stripping). The remaining ovarian tissue then consists of stage 1–4 oocytes and is enriched relative to the whole ovary for connective tissues.

TABLE IV: Localization of RNA Polymerase in Oocytes.[a]

Expt	No. of Germinal Vesicles	Total Enzyme Units	Units/Vesicle	% Total Activity GV Soluble	GV Pellet	GV Medium	Enucleated Oocyte
1	460	39	0.085	87	2	11	
2	250	94	0.37	89		11	
3	300	39	0.13	76		20	4
4	250	22	0.088	75	2	23	

[a] Germinal vesicles were manually removed from defolliculated oocytes (stage 6) and placed in Barth medium until all were collected (2–3 hr). The medium (GV medium) was removed and assayed. The germinal vesicles were homogenized in 0.5–0.8 ml of diluting buffer containing 300 mM $(NH_4)_2SO_4$, mixed for 0.5 hr and centrifuged at 30,000*g* for 10 min. The pellet (GV pellet) was resuspended in 200 μl of the same buffer for assays, and the supernatant (GV soluble) was identified as the oocyte soluble enzyme. The supernatant was routinely assayed at 10× dilution (30 mM salt in the final assay). In one experiment (3) enucleated, defolliculated oocytes were collected and homogenized in 2 ml of homogenizing buffer, and the 50 mM supernatant was prepared (Figure 1) and assayed. The differences in the quantitative recovery of enzyme per vesicle probably reflect the biological variability of different oocytes for different individual females as observed by Schorderet-Slatkine (1972).

On this basis, the results shown in Table III present evidence against the existence of A (I) form polymerase in oocytes. That is, the 10–20% of enzyme activity of stage 6 oocytes remaining in the presence of 250 μg/ml of α-amanitin still definitely prefers native DNA template. We note also that the enzyme recovered from both folliculated and defolliculated stage 6 oocytes is quantitatively and qualitatively the same, indicating the negligible contribution of enzyme from follicle cells. The presence of "A (I)" in whole ovary is seen by the slightly decreased sensitivity to α-amanitin and concomitant increase in single-stranded preference. This situation is more evident with a piece of ovary "stripped" of all large (stages 5–6) oocytes, thus enriching the amount of connective tissue.

INTRACELLULAR LOCALIZATION. Since this newly characterized ovarian polymerase seemed to exist specifically in the oocytes, we wanted to determine if the enzyme was found in the nucleus. The oocyte is a cell which lends itself very nicely to this kind of investigation, since the large oocyte nucleus (germinal vesicle) can be manually removed with very little or no cytoplasmic contamination. In the same procedure a nucleus-free cell can be obtained with no danger of nuclear leakage.

The results of such experiments are shown in Table IV. The enzyme solubilized from germinal vesicles accounts for over 75% of the activity recovered, but the germinal vesicle residue (membranes chromatin) and the enucleated cytoplasm together contain less than 5% of the polymerase. A certain amount (11–23%) of the enzyme leaks into the Barth medium used to store the germinal vesicles during isolation, a reasonable result considering the easy solubility of the enzyme as well as the possible breakage of germinal vesicles. It is apparent from these experiments that the RNA polymerase is localized intranuclearly and can be obtained with similar yields, both from germinal vesicles (Table IV) and intact oocytes (Table III).

Discussion

The results we have obtained suggest that *Xenopus laevis* ovaries contain a special form of DNA-dependent RNA polymerase with unique properties. The enzyme is easily soluble and is inhibited by α-amanitin when the drug is used in high amounts (Figure 4) and preferentially transcribes native DNA. These properties make it unlikely, although do not absolutely disprove, that we are dealing with the previously described nuclear A (I), B (II), or III RNA polymerase. Indeed the special oocyte polymerase can be differentiated from *Xenopus* liver polymerase A (I) by α-amanitin sensitivity (Figure 4) and template preference (Table I, Figure 5), and from B (II) by these characteristics as well as cation and salt optima and DEAE chromatographic elution (Table I). Another form of RNA polymerase (III) has been observed in sea urchin embryos (Roeder and Rutter, 1969, 1970), in *Xenopus laevis* embryos (Roeder *et al.*, 1970), and in *Xenopus* liver (Reeder and Roeder, 1972). The described form III has a different salt optimum and elutes at high ionic strength (0.30–0.35 M ammonium sulfate) from DEAE-Sephadex. The relationship between the enzyme that we describe and form III is, however, still unclear, since form III has been detected only after DEAE-Sephadex chromatography and never after DEAE-cellulose chromatography (Sergeant and Krsmanovic, 1973). Indeed, when the purified oocyte-soluble polymerase is chromatographed on DEAE-Sephadex (unpublished observations) two broad peaks of activity can be obtained, one eluting at very low ionic strength and the other at approximately 0.3 M. The significance of the apparent fractionation of the oocyte-

soluble polymerase obtained on DEAE-Sephadex is, however, still in doubt since both peaks have exactly the same properties, *i.e.*, salt optimum (40 mM ammonium sulfate), sensitivity at high concentrations of α-amanitin (80% inhibitions at 250 μg/ml), and preference for native over denatured DNA. Finally, the possibility of mitochondrial origin is ruled out by the completely different properties reported for the mitochondrial enzyme (Wu and Dawid, 1972) as well as by the nuclear localization of the oocyte polymerase.

The possibility of conversion of form A (I) or B (II) polymerase into the oocyte special enzyme during the extraction and purification procedure also seems unlikely. First, the recovery of the enzyme was not affected by the extraction conditions (low or high salt) of ovaries or oocytes and no appearance of an enzyme with properties similar to the oocyte-soluble form was ever observed when the same extraction conditions were used with liver nuclei. Second, enzyme recovery and properties were unchanged by extraction in the presence of proteolytic inhibitors (unpublished observations), as observed in certain cases (Weaver *et al.*, 1971; Kedinger and Chambon, 1972). Finally, the properties of the enzyme do not change during any step of the purification procedure.

This special RNA polymerase has been found only in ovarian tissue (Tables I and II) and is localized in the nuclei (germinal vesicles) of the oocytes (Table IV). Stage 6 oocytes contain about 10–15% form B (II) polymerase but the soluble polymerase seems to be the predominant form present (Table III). Although the presence of a form A (I) polymerase of the kind found in liver (*i.e.*, completely insensitive to α-amanitin and with a preference for denatured DNA) cannot be excluded by the criteria used here, it seems to represent less than 5% of the total RNA polymerase activity present in the oocyte.

A low amount of forms A (I) and B (II) polymerase can be detected in homogenates of total ovary and can be recovered in the chromatin pellet. Indeed, these two forms had been previously solubilized from ovarian chromatin and separated on DEAE-cellulose (Tocchini-Valentini and Crippa, 1970). Since the polymerase eluting at low ionic strength, identified as form A (I), was at that time not assayed at high α-amanitin concentration, it is impossible to say how much of the enzyme eluting at low ionic strength was oocyte-soluble polymerase as described here.

The soluble RNA polymerase activity present in the oocyte represents indeed a very conspicuous amount of enzyme; it should be stressed that the enzyme recovered from 100 germinal vesicles of stage 6 oocytes has an activity comparable to that of 1 g of liver tissue, leading to the conclusion that the activity per nucleus must be at least 10^5 times greater in the oocyte than in the liver cell. Similar high levels of RNA polymerase activity have been found in *Xenopus laevis* oocytes (Roeder *et al.*, 1970; Roeder, 1972) and in *Rana pipiens* (Wasserman *et al.*, 1972). After DEAE-Sephadex chromatography these investigators could identify three forms (I, II, and III) of RNA polymerase present in approximately equal amounts. We are unable at this time to reconcile their findings observed after DEAE-Sephadex with the results obtained with our present extraction and purification procedures. This discrepancy is currently under investigation.

Some important questions can now be raised about the biological functions of the high amount of this unique enzyme in oocyte nuclei. The storage hypothesis, *i.e.*, the presence of high levels of RNA polymerase in oocyte for use during early embryogenesis, formulated by Roeder *et al.* (1970) and Wasserman *et al.* (1972) cannot be discussed in relation to our current results. We want to suggest, in fact, an additional possibility.

A very peculiar template situation is present in the amphibian oocyte. In the germinal vesicle of stage 4 oocytes about 10^6 cistrons coding for rRNA are present and are actively transcribed up to stage 5–6 (Davidson *et al.*, 1964; Scheer, 1973). One would expect that a very large amount of RNA polymerase is required to transcribe this template. It is extremely difficult to calculate the number of molecules we actually extract from each germinal vesicle as both the final specific activity of the enzyme and the efficiency of the crude assay are unknown, but the large amounts of oocyte-soluble polymerase we observed are in good agreement with the situation existing *in vivo*.

The preference of oocyte-soluble polymerase for native DNA templates is a necessary requirement to obtain asymmetric transcription *in vitro*. The large amounts of enzyme that we find appear to be correlated with the presence of the amplified ribosomal cistrons. This important property of the enzyme and its association with the existence of a specific template make the system very suitable for studies on *in vitro* transcription, both from the point of view of fidelity and of regulation.

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Purification and Characterization of a B_6-Independent Threonine Dehydratase from *Pseudomonas putida*†

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ABSTRACT: L-Threonine dehydratase [EC 4.2.1.16] has been purified to electrophoretic and ultracentrifugal homogeneity from *Pseudomonas putida* in 9% yield. Although the enzyme is inhibited by several common carbonyl-attacking reagents, an exhaustive search for pyridoxal phosphate failed to demonstrate the presence of significant amounts of this coenzyme. The *Pseudomonas* enzyme also differs from threonine dehydratases isolated from other sources in that it possesses a much lower specific activity toward threonine and exhibits a more acid pH optimum. It is, however, able to utilize L-serine as an alternate substrate, is strongly inhibited by L-isoleucine, and undergoes a time-dependent inactivation with serine, properties shared by most established biosynthetic-type threonine dehydratases. Preliminary evidence suggests the existence of a dehydroalanyl residue as a component of the active site. This conclusion rests on the identification of tritiated alanine from hydrolysates of enzyme which had been inactivated by reduction with NaB^3H_4. The failure to obtain a satisfactory yield of [^{3}H]alanine, however, requires that the possibility of an alternate coenzyme form not be dismissed.

The dehydration and subsequent deamination of threonine to α-ketobutyrate by threonine dehydratase [L-threonine hydrolyase (deaminating), EC 4.2.1.16] has long been recognized to require the participation of pyridoxal 5′-phosphate as coenzyme. This requirement has been deemed absolute regardless of whether the enzyme is functioning in the biosynthesis of isoleucine or in threonine catabolism. In all instances where purified threonine dehydratases have been examined, an absorption maximum in the range of 404–415 nm has been observed, indicative of an aldimine linkage between pyridoxal-P and the ϵ-amino group of a lysine residue (Umbarger, 1973).

As part of a study concerned with the biosynthesis of the α-ketobutyrate prosthetic group of urocanase in *Pseudomonas putida*, it became necessary to characterize this organism's threonine dehydratase in order to determine if its properties were consistent with an involvement in the formation of the α-ketobutyrate coenzyme. The results presented here concern only the more general properties exhibited by this enzyme; a later report will present findings having to do with the question of urocanase biosynthesis. The *Pseudomonas putida* threonine dehydratase has been found to contain no pyridoxal-P, although it shares a number of other properties characteristic of most threonine dehydratases. Presumably as a direct result of this lack of pyridoxal-P, the enzyme possesses a rather low catalytic activity. Catalysis appears to be mediated through the participation of a different coenzyme which has been tentatively suggested to be a dehydroalanine residue.

Materials and Methods

Strains and Culture Conditions. Pseudomonas putida A.3.12 was obtained from the American Type Culture Collection

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