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Identification of Retinal Isomers Isolated from Bacteriorhodopsin†

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ABSTRACT: The purple membrane of *Halobacterium halobium* contains the protein bacteriorhodopsin which resembles the visual pigment, rhodopsin, in many aspects. The isomeric configurations of its chromophore, retinal, were studied by a combination of methylene chloride extraction and analysis by high-pressure liquid chromatography. The light-adapted form bR_{570}^{LA} yields solely *all-trans*-retinal, while the dark-adapted form of bacteriorhodopsin, bR_{560}^{DA}, yields a mixture of 13-cis and all-trans with a ratio of ~1:1. The photointermediate M_{412} in a membrane modified by ether at high NaCl concentration also yields an approximately 1:1 mixture of 13-*cis*- and *all-trans*-retinals, while a similar M_{405} species produced by illumination in 2 M guanidine hydrochloride at high pH yields mainly 13-*cis*-retinal. These results indicate that the photochemical cycle of bR_{570}^{LA} may involve an isomerization of the retinal chromophore from the all-trans to the 13-cis form.

The first detected reaction in the bleaching of rhodopsin is the isomerization of the chromophore from 11-*cis*- to *all-trans*-retinal, according to Wald (1968). This notion has been generally accepted (Honig and Ebrey, 1974; Ebrey and Honig, 1975; Rosenfeld et al., 1977), even though some objections have recently been raised (Warshel, 1976; Fransen et al., 1976). The purple membrane protein, bacteriorhodopsin, of the bacterium *H. halobium* undergoes a photoreaction with intermediates very similar to those of rhodopsin. However, when bacteriorhodopsin in its light-adapted form is illuminated, a cycle is initiated which returns the pigment spontaneously to its original state without detachment of the chromophore, i.e., *all-trans*-retinal (Oesterhelt et al., 1973; Jan, 1975).

The light-adapted form of the pigment denoted bR_{570}^{LA} absorbs maximally around 570 nm. When kept in the dark or under dim red light, a species absorbing at ~560 nm called dark-adapted pigment bR_{560}^{DA} (Lozier et al., 1975) is formed, which reverts rapidly in light to bR_{570}^{LA}. Extraction of the retinal as the oxime from bR_{560}^{DA} yielded a mixture of 13-*cis*- and *all-trans*-retinal (Oesterhelt et al., 1973). However, Jan (1975) considered the all-trans isomer to be an artifact since she found that very rapid extraction at low temperature yielded only the 13-*cis*-oxime, and that upon standing isomerization to all-trans occurred.

If a suspension of bR_{570}^{LA} in salt solution is treated with ether, a reaction cycle intermediate designated M_{412} is accu-

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mulated in rather high photosteady state concentrations. Jan (1975) attempted to extract the chromophore of this intermediate and found only *all-trans*-retinal in the extract. Since this was the same isomer of retinal found in bR_{570}^{LA}, it has been generally assumed that the photoreaction cycle of bacteriorhodopsin does not involve isomerization of the chromophore around carbon-carbon double bonds.

The apparent absence of isomerization during the photoreaction cycle of light-adapted bacteriorhodopsin is surprising in view of the similarities to the photoreactions of visual pigments, and recently it has been stated (Rosenfeld et al., 1977) that some significant geometrical change in the chromophore should take place. We have therefore reinvestigated the problem using solvent extraction and high-pressure liquid chromatography (HPLC), a technique that has been successfully applied for the identification of chromophores in visual pigments without using light or heat during extraction (Crouch et al., 1975; Pilkiewicz et al., 1977). This is in contrast to the published investigations in which the cationic detergent CTAB[1] and hydroxylamine were used to extract the chromophore as the oxime and the isomers were identified by TLC.

Materials and Methods

H. halobium R_1 was grown and the purple membrane isolated as described (Oesterhelt and Stoeckenius, 1974). The membrane was stored in sealed glass vials as a suspension in basal salt (BS), i.e., the growth medium without the nutrients (Stoeckenius and Rowen, 1967). The purple membrane contains bacteriorhodopsin as the only protein. All experiments were carried out on the purple membrane and not on isolated bacteriorhodopsin. After treatment with ether or Gdn·HCl as used here (see below), the membrane lipids are still present but their association with the pigment has not been established.

The denaturation-extraction procedure, used to isolate the chromophore from the protein, consisted of emulsifying a sample of purple membrane suspension with an equal volume of CH_2Cl_2 through a 14-gauge syringe needle at a specified temperature and letting the emulsion stand for a specific time. The sample was then centrifuged in an SS-34 rotor at 12 000 rpm for ~10 min (longer or shorter times do not affect isomer distribution) at temperatures below 4 °C. The CH_2Cl_2 layer was dried over Na_2SO_4 at 0 °C followed by rotatory evaporation below room temperature. The residue was transferred with CH_2Cl_2 into a pointed tube, evaporated over N_2 to dryness, and finally taken up in a total of 20 μL of CH_2Cl_2 for analysis by HPLC (Pilkiewicz et al., 1977). All operations, including HPLC, were carried out under dim red light to prevent retinal isomerization.

Dark-Adapted Purple Membrane (bR_{560}^{DA}). The purple membrane was used as a suspension in either basal salt solution or distilled water. To ensure dark adaptation, the purple membrane was stored in the dark at 2 °C for at least 24 h prior to the extraction procedures. Formation of the bR_{560}^{DA} species was verified by the absorption maximum at 560 nm.

Light-Adapted Purple Membrane (bR_{570}^{LA}). For identification of the chromophore contained in the light-adapted membrane, the suspension in basal salt solution was irradiated with a 150-W bulb through two Corning filters (No. 3-64, $\lambda > 540$ nm) at a distance of 10 cm, at 0 °C for 20 min, emulsified with CH_2Cl_2, and analyzed by HPLC. An absorption spectrum taken prior to emulsification ensured that this irradiation procedure converted all the bR_{560}^{DA} into bR_{570}^{LA}.

The 412-Nanometer Intermediate (M_{412}). To obtain the retinal isomer from M_{412}, the bR_{570}^{LA} or bR_{560}^{DA} pigment was diluted with basal salt (Oesterhelt and Stoeckenius, 1974) and then briefly sonicated to keep aggregation minimal. Fresh ether was bubbled repeatedly through the suspension to give an ether-saturated aqueous phase covered with a layer of ether (Oesterhelt and Hess, 1973). (If more vigorous methods are used to obtain ether-saturation, apparently denatured protein collects at the interface and anomalous chromatograms result.) The mixture was then cooled to −1 to −2 °C and irradiated with the same apparatus used for obtaining the bR_{570}^{LA} species, i.e., light of wavelength greater than 540 nm. Under these conditions, the purple color of bR_{570}^{LA} fades into the yellow color of M_{412} with no trace of the original purple color present. The color of the suspension changes from purple to yellow and vice versa in a matter of seconds when light is supplied or withheld, a characteristic behavior of the M_{412} intermediate. To ensure extraction of only the M_{412} species without bR_{570}^{LA}, the suspension was emulsified with CH_2Cl_2 while still being irradiated. This is possible since, as shown in control experiments, the light of $\lambda > 540$ nm entering the apparatus will not isomerize the free retinal.

The 405-Nanometer Intermediate (M_{405}). We have observed that, in the presence of guanidine hydrochloride, alkaline pH, and light, a spectral species of bacteriorhodopsin with a broad absorption maximum centered around 405 nm accumulates. Since this species bears striking resemblance to the M_{412} (the solution turns from purple to yellow on exposure to light and reverts in the dark to purple), investigation of its retinal chromophore was undertaken. To obtain retinal from M_{405}, bR_{560}^{DA} in basal salt solution was pelleted and resuspended in 2 M guanidine hydrochloride, and its pH was adjusted with 0.5 M Na_2CO_3 to ca. 9.8. Under these conditions and in the presence of light, the bacteriorhodopsin bleached reversibly to the species with a broad absorption around 405 nm, which we designate M_{405}. For extraction and identification of the chromophore contained in the bleached membrane, the same procedure and apparatus used for the M_{412} form were applied.

Preparations of Oximes from bR_{560}^{DA}. Since the hydroxylamine procedure employed in previous investigations (Oesterhelt et al., 1973; Jan, 1975) depends on a chemical equilibrium which is affected by pH, temperature, and numerous other factors, the results obtained were checked by the present CH_2Cl_2/HPLC method. The retinal oximes were prepared by following the procedure of Oesterhelt et al. (1973); that is, the purple membrane was incubated with 400 mM NH_2OH·HCl in 20 mM CTAB–basal salt emulsion (pH 7) at specified temperatures and times. At this point, the oximes were extracted and analyzed using the CH_2Cl_2/HPLC method.

High-Pressure Liquid Chromatography (*HPLC*). To separate and analyze the retinals and retinal oximes obtained from the various CH_2Cl_2 extracts of bacteriorhodopsin, high-pressure liquid chromatography was employed. A Waters Associates 6000-psi HPLC system equipped with a septum injector and a Laboratory Data Control 350 nm detector was used with either μ-Porasil or ten μ-Lichrosorb 60 columns. The solvent systems were 2% ether–hexane (v/v) for retinals, and 10% for the retinal oximes on the μ-Porasil columns,[2] and 7%

[1] Abbreviations used: CTAB, cetyltrimethylammonium bromide; BS, basal salt; Gdn·HCl, guanidine hydrochloride; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography.

[2] The use of μ-CN bonded HPLC columns is recommended for application of the methylene chloride technique to rhodopsins because the column can be safely used in the presence of detergents (Pilkiewicz et al., 1977). However, the μ-CN column is not necessary for bacteriorhodopsin because the sample extract contains fewer polar contaminants.

TABLE I: Retinals Present in bR_{560}^{DA}, bR_{570}^{LA}, M_{412}, and M_{405} Bacteriorhodopsin Species.[a]

Expt	Species	Conditions	Area Ratio 13-cis/all-trans	% Composition[b] 13-cis	all-trans
1-3	bR_{560}^{DA} (dist. H_2O)	0 °C, CH_2Cl_2 left 1 min	0.97 (av)	56	44
4-6	bR_{560}^{DA} (basal salt)	0 °C, CH_2Cl_2 left 1 min	1.1 (av)	59	41
7	bR_{560}^{DA}	0 °C, CH_2Cl_2 left 1 h, 45 min	0.85	53	47
8	bR_{560}^{DA}	RT, CH_2Cl_2 left 1 h, 45 min	0.85	53	47
9	bR_{560}^{DA}	0 °C, NH_2OH-CTAB left 10 min, then CH_2Cl_2	1.12	60	40[c]
10	bR_{560}^{DA}	0 °C, NH_2OH-CTAB left 30 min, then CH_2Cl_2	0.75	50	50[c]
11	bR_{560}^{DA}	RT, NH_2OH-CTAB left 1 hr 45 min, then CH_2Cl_2	0	0	100[c]
12	bR_{570}^{LA}	0 °C, $h\nu$ ($\lambda > 540$) CH_2Cl_2	0	0	100
13-15	M_{412}	0 °C, ether $h\nu$ ($\lambda > 540$) CH_2Cl_2	1.12 (av)	60	40
16	M_{412} bR_{570}^{LA}	0 °C, ether $h\nu$ ($\lambda > 540$) 2.5 min in dark then CH_2Cl_2	0.14	16	84
17-19	M_{405}	0 °C, 2 M Gdn·HCl, pH ~9.8, $h\nu$ ($\lambda > 540$), CH_2Cl_2	9.6 (av)	93	7
20	M_{405} bR_{570}^{LA}	0 °C, 2 M Gdn·HCl, pH 9.8, $h\nu$ ($\lambda > 540$) 5 min in the dark, then CH_2Cl_2	0.25	25	75

[a] Each series of experiments were repeated several times with the same results. [b] Corrected for detector response (ϵ's at 350 nm). [c] Approximate from area of combined *syn*- and *anti*-oxime peaks.

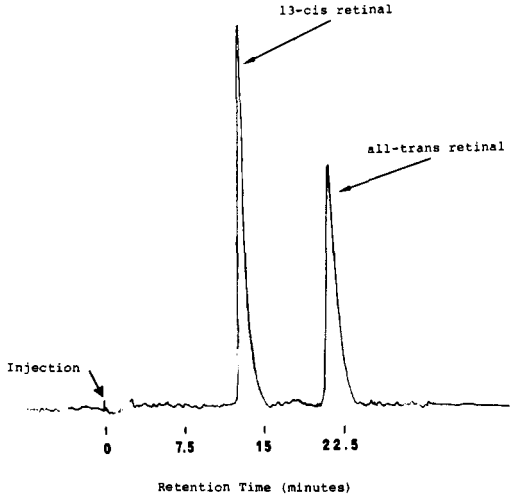

FIGURE 1: HPLC trace of extraction of bR_{560}^{DA} pigment Table I, experiment 4 on μ-Porasil column, 4 mm i.d. × 30 cm, flow rate = 2.0 mL/min, and solvent is 2% ether in hexane.

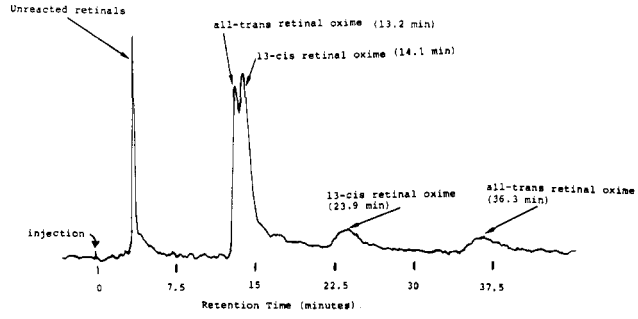

FIGURE 2: HPLC trace of retinal oximes, Table I, experiment 9, on μ-Porasil column, 4 mm i.d. × 30 cm, flow rate = 2 mL/min, and solvent is 10% ether in hexane. Note that there are two peaks for each oxime of a retinal isomer. (Retention times are shown in parentheses.)

ether–hexane for retinals on the Lichrosorb 60. The retinal isomers present in the extracts were identified by comparison with known standards.

HPLC analysis traces of retinals and retinal oximes are shown in Figures 1 and 2. The areas under the curves were measured both by the cut-and-weigh method and a planimeter, while the percent compositions found in Table I were obtained by dividing the areas by their respective ϵ_{350} values: 40 000 for the all-trans and 30 000 for the 13-cis isomer (Becker et al., 1971). In the case of the oximes, each retinal gave two peaks corresponding to its syn and anti isomers, and the combined areas of these two peaks were used for an estimate of the retinal ratios.

Results

The extractions of retinal and retinal oximes from light- and dark-adapted purple membrane were repeated several times at various temperatures and time schedules in order to check for artifacts arising from isomerizations in the extracts. The extraction of the bound retinal from the dark-adapted species (bR_{560}^{DA}) in both distilled water and basal salt solution yielded the same approximate ratio of ca. 1:1 13-*cis*- to *all-trans*-retinal (experiments 1–6). In order to ensure that no isomerization occurred during the extraction, the temperature and time in which the bR_{560}^{DA} was left in the methylene chloride (CH_2Cl_2) solvent were changed. In experiments 7 and 8, it can be seen that the ratios of retinals were similar to the first experiments, indicating no isomerization.

In contrast to these results, extraction of the retinals of bR_{560}^{DA} as the oximes (experiments 9–11) led to isomerization. The amounts of *all-trans*-retinal increase significantly with changing temperature and time, indicating that a 13-cis to all-trans conversion occurs during the NH_2OH/CTAB procedure. As shown in Figure 2, and in agreement with the literature (Oesterhelt et al., 1973), each retinal oxime gave peaks corresponding to the syn and anti isomers. The intensity of the early peak with a retention time of 3.5 min, due to unreacted free retinal, decreases with increased reaction time or temperature.

In experiment 12, only *all-trans*-retinal is extracted from the light-adapted pigment bR_{570}^{LA} in agreement with the results of both Oesterhelt et al. (1973) and Jan (1975).

The extraction of the intermediate M_{412} from ether–basal salt suspension (experiments 13–15) yielded an average 13-cis

to all-trans ratio of 1.12, which is close to that observed in the dark-adapted form. Samples obtained from experiments starting from different lots of bacteriorhodopsin all gave approximately the same ratios. These ratios were obtained whether the starting material was in the bR_{560}^{DA} or the bR_{570}^{LA} form prior to treatment with ether and light. When M_{412} is placed in the dark for a few minutes, the reformation of the bR_{570}^{LA} species occurs and extraction of the suspension (experiment 16) yields predominantly *all-trans*-retinal, indicating the gradual transition from the ~1:1 13-cis/all-trans composition of M_{412} to the totally all-trans composition of bR_{570}^{LA}. With longer periods of time in the dark, the formation of dark-adapted bacteriorhodopsin may occur, leading to the presence of 13-*cis*-retinal.

Experiments 17–19 reveal the overwhelming predominance of 13-*cis*-retinal in the intermediate absorbing at 405 nm (M_{405}). Under the conditions given, 93% of the extracted chromophore is the 13-cis isomer. That such a high percentage of the less stable 13-cis isomer can be obtained supports the reliability of the present extraction procedure. The 7% *all-trans*-retinal may be inherent in the 405-nm species; however, it may also result from the reformation of bR_{570}^{LA} and/or the occurrence of minor isomerization during extraction. When M_{405} was allowed to stand in the dark for several minutes (experiment 20), extraction of the suspension yielded ca. 75% *all-trans*-retinal, indicating the gradual transition from the 13-cis chromophore of M_{405} to the all-trans chromophore of bR_{570}^{LA}.

Discussion

To summarize, the bR_{570}^{LA} gives exclusively all-trans, bR_{560}^{DA} and M_{412} give approximately 1:1 mixtures of 13-cis and all-trans, whereas M_{405} gives predominantly 13-cis. Leaving the suspension of M_{412} and M_{405} in the dark affords all-trans as the major isomer.

In previous studies on bR_{560}^{DA}, the chromophores were extracted with CTAB–NH_2OH–salt for 90 min at room temperature, and the extracted chromophores were then identified by TLC. Oesterhelt et al. (1973) thus obtained 100% all-trans isomer from the light-adapted and a mixture of all-trans (major component) and 13-cis (minor component) from the dark-adapted pigment. Although they did not obtain a *cis*- to *trans*-retinal ratio, the overall results are corroborated by the current studies. In a similar experiment with CTAB–NH_2OH–salt, Jan (1975) noticed that isomerization of the chromophore in the extract occurred at room temperature and that extractions at 0 °C for 10 min minimized this isomerization. Thus, she concluded that the chromophore of the dark-adapted pigment was solely 13-cis.

The conflict between the previous and current results may be resolved by a comparison of the two procedures. The previous experiments consisted of incubation in CTAB–NH_2OH–salt solution (opsin denaturation), extraction of oxime with $CHCl_3$, freeze-drying, and TLC analysis. The present experiments demonstrate that thermal isomerization of 13-*cis*- to *all-trans*-retinal oxime occurs to a considerable extent during the course of oxime formation even at 0 °C. However, no significant isomerization is observed in the CH_2Cl_2 denaturation–extraction procedure as seen in experiments 7 and 8 as well as by the following results: 13-*cis*-retinal was added to a basal salt suspension of bR_{560}^{DA} at 0 °C and the mixture was emulsified with CH_2Cl_2. Immediate work-up with HPLC gave a 13-*cis*/*all-trans*-retinal ratio of about 4.7. When the mixture was left for 2 hr at 0 °C with frequent emulsification (syringe), the 13-cis:trans ratio was still ca. 4.3.

Another objection with the previous method is that, in the CTAB–NH_2OH incubation stage, the denaturation could have been incomplete. In experiment 11, the purple membrane solution was still deep blue even after the 1 h and 45 min incubation before the vigorous CH_2Cl_2 emulsification; hence, it is conceivable that the subsequent $CHCl_3$ extraction performed in the previous studies did not extract the entire population of chromophores. Moreover, oxime formation is not quantitative, and the rate is probably dependent on the retinal isomers. The free retinal peak in Figure 2 shows that the reaction is not complete after 10 min at 0 °C, and, although the peak eventually disappeared, with longer incubation times, a 13-cis to trans conversion also took place. If bR_{560}^{DA} indeed consisted of two chromophores, i.e., 13-*cis*- and *all-trans*-retinals, then Jan's detection of only 13-*cis*-retinal for bR_{560}^{DA} could be explained by a faster extraction rate for the 13-cis species.

For the M_{412} species, Jan found only *all-trans*-retinal or retinal oxime when she extracted the chromophore from the ether–basal salt preparation. The extraction as the oxime meets with the same objections raised above for the bR_{560}^{DA} extraction, while the extraction as the retinal with CTAB–HCl–$CHCl_3$ is rather slow and isomerization to the all-trans form may well have occurred.

M_{412} and M_{405} are probably analogous intermediates in the reaction cycles. The pigment undergoes a photoreaction cycle accompanied by a release and uptake of protons as indicated by a decrease in pH when the M_{405} accumulates in the light (R. Henselman, unpublished). Because the return of the pigment from M to bR is much slower in the Gdn·HCl modified form than in the ether–BS modified form, the CH_2Cl_2 extract from the Gdn·HCl preparation may be a truer reflection of the composition of M. The much higher yield of 13-*cis*-retinal in this preparation therefore may be an indication that the chromophore of M is 13-*cis*-retinal and that an isomerization around this double bond occurs in the photoreaction cycle of bacteriorhodopsin. Consistent with this interpretation is the observation that in the photoreaction cycle a decrease of light-induced dichroism has been observed, which has a time constant similar to the rise time of M_{412} (Lozier and Niederberger, 1977).

We conclude from these observations the C–C double bond isomerization of the retinal may well occur during the photoreaction cycle of bacteriorhodopsin, as suggested by Rosenfeld et al. (1977). They also propose that a rigid reversal during the cycle to the original *all-trans*-retinal is not unlikely based on potential energy surface arguments. It should, however, be kept in mind that we are characterizing the retinal after its interaction with the protein has been destroyed and that the retinal extracted into the CH_2Cl_2 may differ from the form in which it is present in the intact pigment. Namely, the retinal in bR_{560}^{DA} may indeed be a 1:1 mixture of 13-cis and all-trans, or alternatively, it could adopt an "intermediate shape" which is neither 13-cis nor all-trans and that this intermediate shape relaxes to the 13-cis and all-trans species upon cleavage of the retinal–protein bond. The same reservation holds for an interpretation of the results obtained by extracting the M species.

On the other hand, the possibility that the 13-cis isomer found is due to a rapid dark adaptation of the pigment modified by ether or Gdn·HCl appears to be ruled out by the observation that, when a short time is allowed for relaxation of the M species, in the dark, the amount of the all-trans isomer increases beyond that found in the dark-adapted pigment. Both

M_{412} and M_{405} are modified preparations and caution must be exercised in extrapolating these results to the photoreaction cycle of the intact purple membrane.

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Studies on the Subunit Structure of Wheat Germ Ribonucleic Acid Polymerase II†

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ABSTRACT: We have previously presented a rapid, high yield method for the large scale purification of homogeneous RNA polymerase II from wheat germ (Jendrisak, J. J., and Burgess, R. R. (1975), *Biochemistry 14,* 4639), and we now report a detailed study of its subunit structure. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate indicates that polypeptides with molecular weights of 220 000, 140 000, 40 000, 27 000, 25 000, 21 000, 20 000, 17 800, 17 000, 16 500, 16 000, and approximately 14 000 are associated with the enzyme. Two-dimensional polyacrylamide gel electrophoresis, by which the subunits were separated in the first dimension in the presence of 8 M urea at pH 8.7 and in the second dimension in the presence of 0.1% sodium dodecyl sulfate, indicates that the 40 000 molecular weight component is composed of two nearly identical polypeptides and that the low molecular weight components (≤40 000) are acidic proteins except for the 25 000 molecular weight polypeptide.

In every eukaryotic system examined, multiple nuclear RNA polymerases have been detected, and due to recent advances in methodology for the purification of these enzymes (Schwartz and Roeder, 1975; Greenleaf and Bautz, 1975; Buhler et al., 1974; Valenzuela et al., 1976) there has been rapid progress in elucidation of the complex physical structure in those instances where sufficient amounts of homogeneous protein have been available for analysis. We have recently described a method for the rapid, large-scale purification of milligram quantities of homogeneous RNA polymerase II from wheat germ (Jendrisak and Burgess, 1975). This enzyme is inhibited by low concentrations of α-amanitin (<0.1 μg/mL) and is probably the enzyme involved in the synthesis of heterogeneous nuclear RNA. The ease of purification and availability of large quantities of wheat germ RNA polymerase II make it attractive for further studies on the physical properties of eukaryotic RNA polymerase and for possible enzyme reconstitution from isolated subunits. We have examined in detail the polypeptide subunit composition of this enzyme in order to establish criteria for enzyme purity and as prerequisite information for further studies on subunit function and reconstitution.

Experimental Procedures

Materials. The following electrophoresis reagents were purchased from Bio-Rad: acrylamide, methylenebisacrylamide, ammonium persulfate, tetraethylmethylenediamine and sodium dodecyl sulfate. Tris[1] base and glycine were purchased from Sigma. Coomassie brilliant blue and urea (Ultrapure) were purchased from Schwarz/Mann. The following protein molecular weight markers were obtained from Worthington: yeast pyruvate kinase, ovalbumin, bovine pancreatic chymotrypsinogen, and *Escherichia coli* β-galactosidase. Bovine serum albumin was purchased from Miles; hemoglobin and

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[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.