# Partial Purification of "ω" Protein from Calf Thymus†

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ABSTRACT: Proteins which relax supercoiled DNA, called "ω" proteins, are thought to be involved in DNA replication. Calf thymus is a plentiful source of "ω" protein activity. It has been extensively purified and has been characterized as behaving similarly to other eukaryotic "ω" proteins in completely relaxing either positively or negatively supercoiled DNA, requiring a salt concentration of about 0.2 *M* NaCl or KCl, and not requiring $Mg^{2+}$. A transient nick must occur but could not be detected. A new assay for "ω" activity is described which is rapid and sensitive, and depends on the fluorescence enhancement of ethidium intercalating duplex DNA.

Proteins which relax supercoiled ccc-DNA[1] were originally isolated from *Escherichia coli* (Wang, 1969) and from mouse cells (Champoux and Dulbecco, 1972). These "ω" proteins,[2] by changing the topological winding number of ccc-DNA, effectively combine the activity of a nuclease and ligase, which suggested roles in replication and transcription. We describe a rapid and inexpensive assay for "ω" protein which we have used in the characterization and partial purification of "ω" protein from calf thymus. Calf thymus seemed a particularly suitable tissue for the large-scale purification of "ω" protein since it contains a great deal of activity in crude extracts and can be obtained in large quantities.

Our assay for "ω" activity originated from a simple assay for clc-DNA (Morgan and Paetkau, 1972; Coulter et al., 1974). In essence by raising the pH to around 11.6 duplex DNA can still be detected very sensitively (as little as 0.1 μg), by the enhanced fluorescence of ethidium bromide which specifically intercalates duplex DNA. At these high pH's any short intramolecular base pairing of denatured DNA is destabilized and it assumes a true single-stranded conformation. Thus clc-DNA is simply and quantitatively assayed by heating the ethidium bromide (pH 11.6) assay mixture and measuring the amount of fluorescence returning. At this high pH, ccc-DNA also gives a quantitative return of fluorescence (at lower pH's a denatured form of ccc-DNA is obtained) and this has been exploited as a sensitive assay for measuring breaks introduced into ccc-DNA by either nucleases or chemical means (Morgan and Pulleyblank, 1974) (see Figure 1). Since ethidium bromide unwinds DNA on intercalating, the more negatively supercoiled ccc-DNA will bind more ethidium bromide. Therefore if the highly negatively supercoiled PM2 phage ccc-DNA is completely relaxed by "ω", there is a 33% decrease in fluorescence both before and after heat. This contrasts with the nuclease action on PM2 ccc-DNA which gives rise to an increase in fluorescence of about 30% before heat since the nicked molecules have no topological constraints on the binding of ethidium bromide and a total loss of fluorescence after heat since the strands can separate. This is all summarized in Figure 1 in which also the effect of ligase action is indicated. The advantages of this assay for "ω" is that it is rapid and inexpensive.

## Experimental Procedure

*PM2 DNA.* Bacteriophage PM2 was obtained by the method of Salditt et al. (1972). Purification of the bacteriophage was taken to the first CsCl centrifugation. The DNA was extracted by the phenol–sodium dodecyl sulfate procedure of Mitra et al. (1967). The yields of ccc-DNA improved when the phenol was substituted by a 3:1 mixture of chloroform and 1-butanol. This method requires more extractions (4 or 5) than the standard phenol method (2) for removal of protein. Up to 200 $OD_{260}$ units of PM2 DNA were obtained from a single 30-l. batch of lysate. The DNA was usually greater than 90% ccc-DNA with a little contaminating oc-DNA as determined by the ethidium bromide fluorescence assay (Morgan and Pulleyblank, 1974). It was extensively dialyzed against 10 m*M* Tris-HCl (pH 8) and 0.1 m*M* EDTA, and stored frozen.

*Analytical Ultracentrifugation.* Sedimentation velocity runs were in 2.83 *M* CsCl at 44000 rpm at 20°C with 8-min photographs using a Vinograd type 1 band forming center piece (Gray et al., 1971). The sedimentation coefficients were calculated using $s^0 = d(\log r)/dt \times 27480$ where the coefficient 27480 included $1/\omega^2$ and correction coefficients for the density and viscosity of the medium.

*Polyacrylamide Gel Electrophoresis.* Acrylamide was purified by mixing a 3 w/v solution with activated charcoal (5 g/100 ml of acrylamide solution) and filtering under vacuum through a Millipore filter. The dodecyl sulfate gel system of Laemmli (1970) was used. Gels were stained with 0.5% Coomassie Brilliant Blue and destained electrophoretically with the Canalco destainer in 7.5% acetic acid in 30% methanol.

*Fluorometric Procedures.* These were essentially as previously reported (Morgan and Pulleyblank, 1974). The ethidium bromide (pH 11.6) solution contains 0.5 μg/ml of ethidium bromide, 0.1 m*M* EDTA, and 20 m*M* potassium phosphate made up to pH 11.6. The precise pH has been difficult to determine and was earlier stated as pH 12. However, even with the Beckman 39301 electrodes (accurate to pH 14) we have found considerable variation de-

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[1] Abbreviations used are: ccc-DNA, covalently closed circular DNA; oc-DNA, open circular (nicked) DNA; clc-DNA, covalently linked complementary DNA (rapidly renaturing).

[2] We use the term "ω" protein here for any activity which changes the topological winding number of covalently closed circular DNA. Until more is known about the mechanism of "ω" action, those working in the field have agreed that it is premature to give it a systematic name.

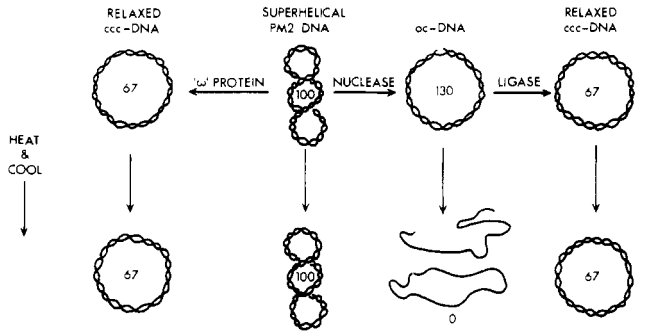


FIGURE 1: A diagrammatic representation of the effects of various treatments on the fluorescence enhancement of ethidium bromide intercalated into DNA. The structures indicate the state of the DNA prior to addition of ethidium bromide, which will convert all the ccc-DNAs into positively supertwisted molecules. The numbers give the relative fluorescence above the blank of the different DNA species using PM2 ccc-DNA from the virion as the starting material. It has a high negative superhelical density. The fluorescence measurements were made under the alkaline conditions described in Experimental Procedures.

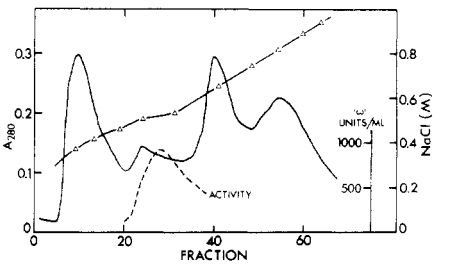


FIGURE 2: Elution profile of "ω" from phosphocellulose. (See Purification of "ω" for details.) The activity was eluted with a NaCl gradient (0.2–1.5 *M*) in 50 m*M* sodium phosphate (pH 6.8). (···) "ω" activity, (—)$A_{280}$, (△) NaCl concentration.

pending on the standardizing buffer as well as the history of the electrode. Experimentally 87.2 g of $K_2HPO_4$ and 15 g of KOH are made up to 500 ml with water (1 *M* in phosphate) and this solution is diluted appropriately. If after heating a denaturable DNA (e.g., T7 DNA) there is more than 1% return of fluorescence the solution should be made more basic. Calf thymus DNA gives about 1.5% return of fluorescence after heat most probably due to palindromic DNA. Due to the possible interference of the intense fluorescence from the ink of felt marking pens, tubes should not be marked.

*Assay for "ω" Activity.* The solution of "ω" to be assayed from column fractions was diluted as necessary (see below for the definition of activity units) with 0.2 *M* NaCl and 10 m*M* Tris-HCl (pH 8) and 1–5 μl was added to 30 μl of 0.2 *M* NaCl, 2 m*M* EDTA, 10 m*M* Tris-HCl (pH 8), and ccc-PM2 DNA at 1 $A_{260}$. After 15 min at 20°C, 2 ml of ethidium bromide (pH 11.6) solution was added and the fluorescence measured at 22°C before and after heating to 98°C for 2 min. For pure ccc-DNA the readings are the same, but the small contamination of oc-DNA usually present leads to a decrease. Complete relaxation of ccc-PM2 DNA leads to a 33% decrease in the after heat fluorescence which measures only ccc-DNA. One unit of activity was operationally defined as the amount required to completely relax 1.5 μg of ccc-PM2 DNA (the amount present in 30-μl assay mixture) in 15 min at 24°C. Since the kinetics are neither linear nor exponential, exact quantitation is uncertain. A series of concentrations is assayed, with the end point taken where no further decrease in fluorescence enhancement occurs.

An additional problem in the quantitation of "ω" is that it is inactivated when the protein concentration in the reaction mixture falls below 1 μg/ml (possibly because of adsorption to the glass walls of the reaction vessel). The effect is partially but not completely overcome by the addition of 300 μg/ml of gelatin to the reaction mixture (see Results).

Addition of large amounts of the lysine-rich histone (KAP) could artifactually decrease the fluorescence even at pH 11.6 due to the binding of KAP to DNA with consequent exclusion of ethidium. However this was only a serious problem in the isolation of "ω" protein from salmon testes (B. Eskin, unpublished data), and where suspected the reaction of DNA with "ω" was stopped by the addition of 3 μg of trypsin (stored as a 1 mg/ml of solution in 1 m*M* HCl) followed by a 10-min incubation at 24°C. Under these conditions the activity of "ω" is destroyed almost instantaneously, and the histones are degraded to a point where they no longer interfere with the fluorescence enhancement of DNA.

## Results

*Purification of "ω".* The purification of "ω" protein from calf thymus is considered in general terms later. All operations were at 4°C, 100 g of calf thymus (stored frozen at −20°C) was homogenized at full speed for 1 min in a Sorvall Omni Mixer cup with 500 ml of 0.1 *M* NaCl, 0.05 *M* sodium phosphate buffer (pH 6.0), and 1 m*M* EDTA (buffer A). Chromatin and connective tissue debris were collected by centrifugation at 5000 rpm for 10 min in a Sorvall GSA rotor. The supernatant was discarded and the pellet was washed by three cycles of homogenization with 500 ml of buffer A and centrifugation. It was then resuspended in 300 ml of 0.1 *M* NaCl, 0.05 *M* sodium phosphate (pH 6.8), 1 m*M* EDTA containing 1 m*M* benzylsulfonyl fluoride in ethanol (this reagent is also commonly referred to as phenylmethanesulfonyl fluoride) in order to inhibit nuclear proteases. After incubation for 15 min at room temperature 55 mg of iodoacetamide dissolved in 5 ml of water was added (1 m*M* final concentration) and the incubation was continued for a further 45 min. The chromatin was then pelleted by centrifugation and washed once with 500 ml of 0.1 *M* NaCl in 0.05 *M* sodium phosphate (pH 6.8)–1 m*M* EDTA (buffer B). The pellet was resuspended in 500 ml of buffer B; 500 ml of 2 *M* NaCl–0.05 *M* sodium phosphate was added with rapid stirring. The exceedingly viscous chromatin solution was sheared in 50-ml batches in a small Omni mixer cup at full speed for 15 sec and the sheared solution was added with rapid stirring to 7 l. of 0.05 *M* sodium phosphate (pH 6.8). A stringy precipitate forms which was collected on a glass rod and homogenized with 300 ml of buffer B; 300 ml of 1.6 *M* NaCl–0.05 *M* sodium phosphate (pH 6.8) was then added with rapid stirring followed by 60 g of poly(ethylene glycol) 6000 to precipitate the DNA. After centrifugation at 10000 rpm for 10 min in the Sorvall GSA rotor, the supernatant was saved ("ω" fraction 1). In this experiment, as in previous ones, a second extraction of the DNA pellet was carried out with 2 *M* (final concentration) NaCl. This extract contains about the same amount of "ω" activity as the first one.

For unknown reasons the "ω" in this second extract prepared from chromatin which has been treated with protease inhibitors has not been successfully chromatographed. The "ω" fraction 1 was diluted with 3 volumes of 0.05 *M* sodi-

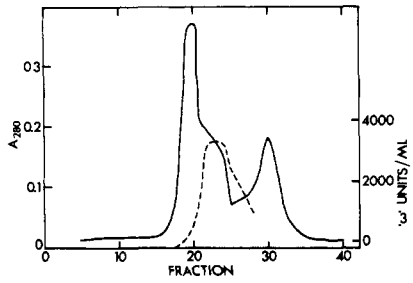


FIGURE 3: Elution profile of "ω" from Sephadex G-100 (Details under Purification of "ω".) (- - -) Activity of "ω", (—)$A_{280}$.

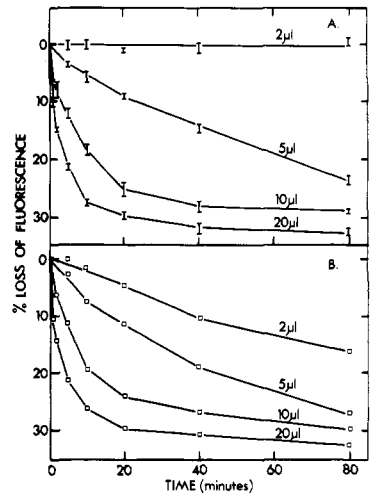


FIGURE 4: The kinetics of relaxation of PM2 DNA by "ω" protein. (A) To 0.32 ml of 0.2 *M* NaCl, 10 m*M* Tris-HCl (pH 8), 2 m*M* EDTA, and 1 $A_{260}$ PM2 DNA at 10° was added 2, 5, 10, or 20 μl of purified (G100) "ω" protein; 50-μl samples were added to 2 ml of pH 11.6 ethidium bromide assay mixture at the times indicated. The readings are those obtained after heating (only ccc-DNA detected). (B) As for A but with 0.3 mg/ml of heated gelatin added to the reaction.

um phosphate (pH 6.8)–1 m*M* EDTA (final NaCl concentration 0.2 *M*) and a 30 ml packed bed volume of Whatman P11, previously equilibrated against the same buffer, was added. The suspension was stirred rapidly for 1 hr and the phosphocellulose was collected by decantation and filtration, washed briefly with 0.05 *M* sodium phosphate (pH 6.8), 1 m*M* EDTA, and 0.2 *M* NaCl (starting buffer), and resuspended in 50 ml of the same. The slurry was layered over a previously prepared column (150 ml packed bed volume) of P11 and the activity was eluted from the column with a 1-l. linear salt gradient from 0.2 to 1.5 *M* NaCl–0.05 *M* sodium phosphate (pH 6.8). The elution profile is shown in Figure 2.

"ω" containing fractions from phosphocellulose were pooled and concentrated by ultrafiltration to approximately 3 ml using a PM10 membrane (Amicon Corp., Diaflo Membrane). The concentrate was then applied to a Sephadex G-100 or G-150 superfine column (100 ml bed volume) with 0.5 *M* NaCl–0.05 *M* sodium phosphate (pH 6.8) as the eluting medium; 1.7-ml fractions were collected. The elution profile from Sephadex G-100 is shown in Figure 3. Chromatography on Sephadex G-150 gave better resolution of the "ω" activity from the excluded peak, at the cost of failing to resolve the enzyme peak completely from the residual lysine rich histone KAP. Dodecyl sulfate gel electrophoresis of the active fractions from G-100 showed three main components to be present with molecular weights of 53000, 51000, and 32000. Minor components of higher molecular weight were present. However, these were also present in the inactive excluded peak and so are unlikely to be the enzyme.

*Confirmation that the "ω" Fluorescence Assay Changes the Topological Winding Number of ccc-DNA.* Figure 4 shows the kinetics of relaxation of PM2 ccc-DNA. A maximum 33% drop in fluorescence which was not increased by adding further "ω" is obtained. The drop in fluorescence is that expected for complete relaxation of DNA. This was determined as follows: for 100 arbitrary fluorescence units of supercoiled PM2 ccc-DNA, the fluorescence rises to 130 units on nicking (due to removal of any topological constraint). The fluorescence drops to 67 units on relaxation with "ω". Therefore the relaxed DNA on nicking would show an increase to 130 units or nearly 100% increase. PM2 DNA which had been nicked and resealed with ligase and was therefore presumably relaxed also gave 100% increase in fluorescence on nicking, confirming that the drop in fluorescence with "ω" had given rise to relaxed ccc-DNA. (The increase in fluorescence on nicking ccc-DNA is of course a measure of its superhelical density and will be reported elsewhere.) Furthermore the fluorescence readings were not affected by Pronase or trypsin treatment prior to adding samples to the ethidium bromide assay mixture. Also when ethidium bromide was present in the reaction mixture in sufficient quantity to cause positive supercoiling of ccc-DNA, there was an increase in fluorescence with time (Figure 6) as expected for relaxation of positive supertwisting by "ω", i.e., due to reduction in $\alpha$, the topological winding number. Such an increase in fluorescence would be hard to rationalize on the basis of any compound binding to DNA. Finally, the assay was checked by conventional sedimentation velocity procedures. In Figure 5 ccc-PM2 DNA before and after "ω" treatment was sedimented with oc-DNA in 2.83 *M* CsCl containing 1.5 μg/ml of ethidium bromide. Due to the change in salt concentration from 0.2 *M* NaCl in the assay mixture to 2.83 *M* CsCl in the sedimentation run, "ω" relaxed DNA has negative supercoils induced in it and the ethidium bromide present is just sufficient to remove these and give it a sedimentation coefficient of oc-DNA (Wang, 1969). Calf thymus "ω" activity is not observed in 0.4 *M* NaCl, suggesting that if the drop in fluorescence was due to some unknown salt sensitive component binding to DNA it should not have interacted with the DNA in 2.83 *M* CsCl and a difference in the sedimentation coefficient would not have been observed, contrary to Figure 5. There appears to be no doubt therefore that the fluorescence assay is measuring true "ω" activity.

*Comments on the Purification Procedure for "ω".* A small amount of "ω" can be detected in crude supernatants from calf thymus prepared by homogenization of the tissue in the presence of 2 m*M* calcium chloride and centrifugation to remove nuclei. This "ω" could be cytoplasmic or may result from the disruption of a small fraction of the nuclei during the initial extraction. It can be precipitated with ammonium sulfate between 50 and 67% saturation, but with a large loss of activity. No further attempt was made to purify the "ω" from this source after it was recognized that the chromatin pellet obtained by centrifugation of the homogenate contains much larger quantities of "ω". The activity cannot be released by rehomogenization of the pellet, but is released upon extraction of chromatin with concentrated salt solution, along with histones and some acidic proteins.

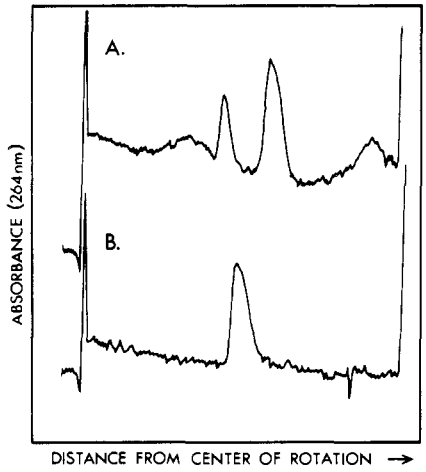


FIGURE 5: Sedimentation profiles of ccc-PM2 DNA with added oc-DNA, as a marker. Sedimentation is from left to right in 2.83 *M* CsCl and 10 m*M* Tris-HCl (pH 8) containing 1.5 μg/ml of ethidium bromide at 44000 rpm. The photographs were taken 40 min after the start of the run. (A) ccc-DNA and oc-DNA before treatment with "ω". (B) ccc-DNA (now relaxed) and oc-DNA after treatment with "ω".

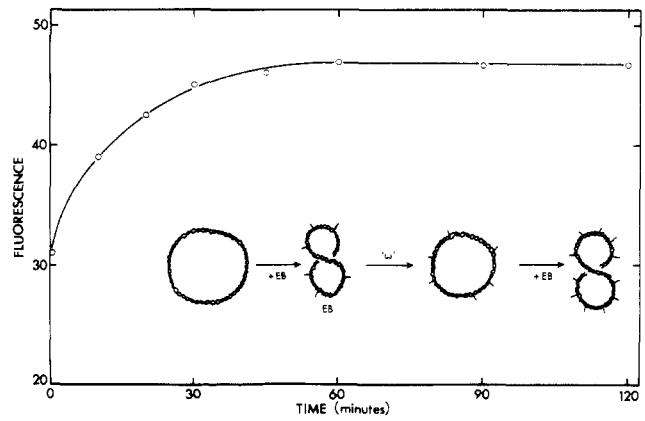


FIGURE 6: Relaxation of positively supertwisted PM2 DNA. To 0.25 ml of the standard "ω" reaction mixture at 37°C containing 0.16 mg/ml of heated gelatin in addition, was added 5 μl of "ω" protein (poly(ethylene glycol) supernatant fraction). Samples (20 μl) taken at 0 and 60 min showed the DNA had completely relaxed. The solution was then made 14 μg/ml in ethidium bromide (EB) and 20 μl samples were added to the alkaline ethidium bromide assay mixture at various times. The fluorescence readings are those after heat treatment and a small correction was made for the extra ethidium bromide added to the assay mixture from the reaction. There is a 50% rise in fluorescence.

The DNA from such an extract can be removed either by high-speed centrifugation of the unsheared viscous suspension, or by the addition of poly(ethylene glycol) (10% w/v) to a sheared solution of chromatin. The latter procedure has the advantage of being rapid and convenient, and of allowing more than one extraction of a single chromatin preparation at differing salt concentration. "ω" appears to be eluted from the chromatin in two distinct steps. The first activity "$\omega^1$" is eluted from the chromatin by 0.7 *M* NaCl, the second "$\omega^2$" by 1.7 *M* NaCl. Reextraction of the chromatin precipitated from 0.7 *M* NaCl with 0.7 *M* NaCl gives an insignificant improvement in yield of "$\omega^1$" implying that the appearance of a second activity in 1.7 *M* NaCl is not a consequence of incomplete elution of "ω". The impression was confirmed when the two activities prepared at 0.5 *M* NaCl and 1.2 *M* NaCl were diluted and applied to phosphocellulose. Elution of the activities from the columns with linear salt gradients gave peaks of activity eluting in the ranges 0.5–0.6 *M* NaCl and 0.74–0.84 *M* NaCl, respectively. In the case of "$\omega^1$" the activity appeared to resolve into two separate activities which were correlated with optical density peaks. The main components of the peaks observed by gel electrophoresis by the method of Panyim and Chalkley (1969) appeared to be fragments of histone KAP formed by autolysis (Bartley and Chalkley, 1972). "$\omega^2$" did not correspond to an optical density peak. The main components present in the mixture were histones, LAK and KAS, and autolysis fragments.

*Inhibition of Proteolysis.* Sodium bisulfite, the most commonly used inhibitor of nuclear proteases, is unsuitable for use in ion-exchange chromatography since it oxidizes rapidly in air, causing the pH of the buffer solutions to decrease with time. Other protease inhibitors were examined for suitability using the solubilization assay of Panyim et al. (1968). Mercuric chloride was particularly effective in preventing the solubilization of chromatin, but its use leads to a complete loss of "ω" activity. Benzylsulfonyl fluoride and iodoacetimide are also quite effective and do not appear to inhibit the activity of either "ω" as long as they remain bound to chromatin. However, they partially inhibit isolated "$\omega^2$". Treatment of the washed chromatin with a combination of the two inhibitors has been incorporated into the isolation procedure. On several occasions precipitates were observed when poly(ethylene glycol) extracts were diluted for application to phosphocellulose. It was found that this could be prevented if the prepared chromatin was dissolved in 1 *M* NaCl and then precipitated by dilution to 0.15 *M* final salt concentration. Under these conditions the "ω" coprecipitates with nucleohistone, leaving much of the acidic chromatin protein in the supernatant. Chromatography of "$\omega^1$" on phosphocellulose after these treatments gives a single peak of activity, not corresponding to the main protein peaks. At this stage in the purification the main component observed by gel electrophoresis is still histone KAP. Separation of the activity from the remaining histone KAP was achieved by concentration followed by chromatography on Sephadex G-100 or G-150 (Figure 3).

*Characteristics of "ω" Protein.* The catalytic properties of "$\omega^1$" and "$\omega^2$" are quite similar. Both are most active at 0.2 *M* NaCl and completely inhibited by 0 or 0.4 *M* NaCl. (Trout testis "ω" is active in 0.6 *M* NaCl; data of Dr. B. Eskin.) They also relax both negative and positive supercoils. The assay for relaxation of positive supercoils is shown in Figure 6, ethidium being added to the reaction mixture to induce positive supertwists. It is possible that both enzymes are in fact identical, but as isolated are bound to different histone fractions. This possibility was suggested by the strong specific interactions that occur between the histone fractions, and some acidic chromatin proteins (Hnilica, 1972).

At this stage it would be unwise to try to interpret the kinetics in terms of any model until technical problems related to "ω" stability and possible losses on the reaction vessel have been overcome. Figure 4 shows the kinetics of relaxation at 10°C and the effect of "ω" concentration and added gelatin. The rate of the reaction is approximately ⅕th that at 25°C. Optimal rates are found at around 37°C. Although quantitation of "ω" through the purification procedure is not presently possible, the apparent activity in the G-100 fraction is of the order of 1% of the crude extract.

## Discussion

Little evidence is available on the mechanism of "ω" ac-

tion. Wang (1971) showed that the *E. coli* enzyme acts gradually, not by a one hit mechanism. This has also been demonstrated for the eukaryotic enzyme (Champoux and Dulbecco, 1972). These observations rule out a simple nuclease followed by ligase mode of action. Wang (1971) proposed that the transient nicking of the DNA was a result of "ω" phosphodiester bond formation with concomitant breakage of the DNA backbone. We have unsuccessfully attempted to trap such an intermediate with hydroxylamine, or with antibiotics interfering with replication by unknown mechanisms (e.g., nalidixic acid and camptothecin).

The "ω" protein from calf thymus is very similar to other eukaryotic "ω's" (Champoux and Dulbecco, 1972; Baase and Wang, 1974), in contrast to *E. coli "ω"* (Wang, 1971). The physiological role is still uncertain and the only approach would seem to be the isolation of conditional lethal mutants. The assay we have developed is sensitive enough to detect "ω" activity in $10^5$ stimulated lymphocytes or a 2-mm diameter colony of *E. coli,* which should allow the screening of large numbers of mutants very rapidly.

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