N. (1975a), *Nature (London) 255,* 533.
Kedes, L. H., Cohn, R. H., Lowry, J. C., Chang, A. C. Y., and Cohen, S. N. (1975b), *Cell 6,* 359.
Liu, L. F., and Wang, J. C. (1975), *Biochim. Biophys. Acta 395,* 405.
Syres, I., Maxam, A., Cohn, R. H., and Kedes, L. H. (1977), *Cell 9,* 495.
Thomas, M., White, R., and Davis, R. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 2294.
White, R., and Hogness, D. (1977), *Cell* (in press).
Wu, M., and Davidson, N. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 4506.
Wu, M., Holmes, D. S., Davidson, N., Cohn, R., and Kedes, L. H. (1976), *Cell 9,* 163.

Novikoff Hepatoma Deoxyribonucleic Acid Polymerase. Identification of a Stimulatory Protein Bound to the β-Polymerase†

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ABSTRACT: The Novikoff hepatoma DNA polymerase-β sediments as a 7.3S form in crude extracts but during purification sediments as a 4.1S form (after diethylaminoethyl-Sephadex chromatography) or as a 3.3S form (after DNA-cellulose chromatography). If 0.25 M ammonium sulfate or 0.5 M NaCl is included in the sucrose gradients, the 7.3S form sediments at 3.3 S; after removal of the salt, it sediments again at 7.3 S, indicating the reversibility of the aggregation phenomenon. By careful adjustment of ionic strength in the gradient, four distinct and reproducible forms of the enzyme sedimenting at 7.3, 5.8, 4.1, and 3.3 S can be generated. The isoelectric point of the DNA polymerase also changes during purification; the 7.3S form has a p*I* of 7.5, while the 4.1S form isoelectrically focuses at a pH of 8.5. During DNA-cellulose chromatography, the Novikoff β-polymerase is separated from a stimulatory factor designated as Novikoff factor IV. Factor IV is a protein as shown by its sensitivity to protease and resistance to nucleases. It is responsible for converting the 3.3S enzyme to the 4.1S form since the 3.3S homogeneous DNA polymerase-β sediments at 4.1 S in the presence of factor IV. Factor IV confers stability to the polymerase in low ionic strength buffers as well as stability to heat denaturation. Factor IV has the ability to increase the activity of the 3.3S homogeneous polymerase by about fourfold.

The β class of DNA polymerases (EC 2.7.7.7) from higher eukaryotes is characterized, among other properties, by a relatively small size (see recent reviews by Loeb, 1974; Bollum, 1975; Weissbach, 1975). In mammalian cells, homogeneous β-polymerases obtained from calf thymus (Chang, 1973), human KB cells (Wang et al., 1974), Novikoff hepatoma (Stalker et al., 1976), and guinea pig liver (Kunkel, Tcheng, and Meyer, in preparation) have molecular weights of 32 000 to 45 000. In crude cellular extracts or partially purified preparations, β-polymerases have been shown, under low salt conditions, to sediment in sucrose gradients as a 6–8S complex of proteins or to filter through Sephadex gels as an aggregate form (Hecht, 1973a,b; Hecht and Davidson, 1973; Lazarus and Kitron, 1973; Probst, 1974; Wang et al., 1975). They can be converted to a 3–4 S or low-molecular-weight form if appropriate salt concentrations are included in the sucrose gradients or Sephadex buffers. This aggregate form may represent a native conformation of proteins involved in DNA metabolism in vivo, particularly in light of recent studies with prokaryotic systems (Geider and Kornberg, 1974; Schekman et al., 1975; Hendler et al., 1975) which indicate that DNA replication and repair involve multienzyme complexes. We have previously purified to homogeneity and characterized the DNA polymerase-β from the Novikoff hepatoma (Stalker et al., 1976; Mosbaugh et al., 1976) and have identified three proteins isolated from this tumor which markedly and specifically stimulate this enzyme (Probst et al., 1975). In order to determine whether these stimulatory proteins are part of a polymerase complex and to identify other proteins involved in DNA synthesis, we are attempting to resolve, purify, and characterize the individual proteins found in the 6–8S β-polymerase complex. In the present report we (a) describe the changes in sedimentation behavior and isoelectric pH of the Novikoff hepatoma DNA polymerase-β during purification; (b) identify a new stimulatory protein, factor IV; and (c) provide evidence that factor IV binds to the homogeneous β-polymerase and is probably one of the components of the 6–8S complex. An abstract of this work has been published (Stalker et al., 1975).

Experimental Procedure

Materials

All chemicals were of analytical or reagent grade. Unlabeled deoxyribonucleoside triphosphates, phenylmethyanesulfonyl fluoride, and calf thymus DNA were purchased from Sigma. Enzyme grade ammonium sulfate, BSA,[1] and [^{3}H]dTTP were obtained from Schwarz/Mann. New England Nuclear Corp.

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[1] Abbreviations used are: BSA, bovine serum albumin; dNTP, unlabeled deoxyribonucleoside triphosphates; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; PMEG buffer, 0.02 M potassium phosphate at pH 7.5, 0.005 M β-mercaptoethanol, 0.001 M EDTA, and 10% (w/v) glycerol; PMG buffer, PMEG with EDTA omitted; Cl_3AcOH, trichloroacetic acid; TMEG, 0.02 M Tris-HCl at pH 8.0, 0.005 M β-mercaptoethanol, 0.001 M EDTA, 10% (w/v) glycerol; TME buffer, TMEG with glycerol omitted; BBOT, 2,5-bis[2-(5-*tert*-butylbenzoxazolyl)]thiophene.

was also a source for [^{3}H]dTTP. Pancreatic DNase I (EC 3.1.4.5), trypsin (EC 3.4.4.4), soybean trypsin inhibitor, ribonuclease A (EC 2.7.7.16), and *Escherichia coli* alkaline phosphatase (EC 3.1.3.1) were obtained from Worthington Biochemicals. DNA-cellulose was prepared according to Alberts and Herrick (1971) and activated DNA according to Loeb (1969) except that, after DNase treatment, the nuclease was removed by phenol extraction. Novikoff hepatoma DNA polymerase-β was purified as previously described (Stalker et al., 1976). Fractions II, III, V, and VI were used in the experiments to be described.

Methods

DNA Polymerase Assay. Standard reactions were carried out in 12 × 75 mm glass tubes in 125-μL volumes containing 25 mM Tris-HCl[2] (pH 8.4), 5 mM β-mercaptoethanol, 7 mM magnesium acetate, 0.5 mM EDTA, 50 mM NaCl, 0.015 mM each of dATP, dCTP, dGTP, and [^{3}H]dTTP (sp act., 325 mCi/mmol), 15% (w/v) glycerol, 100 μg/mL activated calf thymus DNA, and 25 μL of the enzyme fraction (generally 0.01–0.3 unit). Incubation was carried out in stoppered tubes for 60 min at 37 °C. After incubation, 100-μL samples were pipetted onto filter paper disks which were dropped into cold 10% Cl_3AcOH. The disks were processed for liquid scintillation counting as described previously (Meyer and Keller, 1972), and radioactivity was measured in a Packard Tricarb or Beckman liquid scintillation spectrometer using 0.4% 2,5-bis[2-(5-*tert*-butylbenzoxazolyl)]thiophene (BBOT) in toluene as the scintillator at an efficiency of 16–26%. When assaying column fractions or sucrose gradient fractions, the [^{3}H]dTTP was used undiluted at 0.3 μM (54 Ci/mmol). This is referred to as a "limited substrate assay". Any changes from these conditions are noted in the legends to the figures. One unit of DNA polymerase activity is defined as the incorporation of 1 nmol of total nucleotide into DNA per h at 37 °C.

Stimulatory Protein Assay. To detect stimulatory activity, two sets of standard DNA polymerase assay tubes were prepared as described above. One set was used to detect endogenous DNA polymerase activity; to the second set, a small amount of homogeneous DNA polymerase-β (fraction VI) was added as a source of exogenous enzyme. Any activity in excess of the endogenous plus exogenous activity is considered to be due to the presence of stimulatory factors. One unit of stimulatory activity is defined as that which will cause a 100% increase in the exogenous DNA polymerase activity (Probst et al., 1975). In all cases, 25 μL of the fraction being tested was used in the stimulatory assay, and the total reaction volumes were kept at 125 μL.

Alkaline Phosphatase Assay. Each assay tube contained 0.10 M Tris-HCl (pH 8.0), 1.0 mM *p*-nitrophenyl phosphate, and 25 μL of the fraction tested in a final volume of 0.5 mL. After 30 min incubation at 37 °C, 0.5 mL of 0.10 M NaOH was added and the absorbance determined spectrophotometrically at 410 nm.

Sucrose Gradient Centrifugation. Gradients of 5–20% sucrose containing 20 mM Tris-HCl (pH 8.0), 5 mM β-mercaptoethanol, 1.0 mM EDTA, and 0.5 mg/mL BSA were prepared. To the top of each 5.0-mL gradient, 100 μL of sample was applied and centrifugation carried out for 18 h at 35 000 rpm in a Spinco SW 50L rotor at 4 °C. Each gradient contained 1.0 μL of *E. coli* alkaline phosphatase (6.3 S, 80 000 mol wt) as an internal marker. After centrifugation, the bottoms of the tubes were punctured, seven drop fractions were collected, and assays for DNA polymerase and alkaline phosphatase were performed. The *s* values and approximate molecular weights were determined relative to the alkaline phosphatase marker according to Martin and Ames (1961). In some cases, salts were added to the gradients; these are given in the legends to the figures. In each gradient, the recovery of activity was greater than 93% of that applied.

Isoelectric Focusing. Isoelectric focusing was carried out in a 110-mL LKB type 8101 isoelectric focusing column. To 5 mL of Novikoff hepatoma fractions II, III, and V were added glycine to a final concentration of 1%. A 3–50% sucrose gradient containing the sample and 2.5% ampholytes (pH 7–10) was poured into the unit. Samples were focused for 36 h at 4 °C, and 2.3-mL fractions were collected. The pH of each fraction was read, and a small aliquot of each was assayed for DNA polymerase activity as described above. In all experiments, greater than 97% of the applied activity was recovered after focusing.

Maintenance of the Tumor. The Novikoff hepatoma was maintained in Holtzman rats by intraperitoneal injection. The ascites fluid was harvested 6–7 days postinjection and the cells were pelleted by centrifugation at 12 000*g* for 10 min. The pellet was resuspended in 4 volumes of 0.01 M Tris-HCl at pH 7.0 and 0.002 M $MgCl_2$. After hemolysis of the erythrocytes, the tumor cells were collected by centrifugation, washed in Tris–Mg, and used for enzyme and stimulatory protein purification as described previously (Probst et al., 1975; Stalker et al., 1976).

Other Methods. Protein concentrations were determined by the Lowry procedure (Lowry et al., 1951), and by the fluorescamine technique (Böhlen et al., 1973). DNA concentrations were measured by the diphenylamine reaction (Burton, 1956).

Results

Changes in Molecular Weight of the Novikoff Hepatoma DNA Polymerase-β during Purification. The Novikoff enzyme, like many other β-polymerases, sediments in low ionic strength buffers as a 6–8S form in crude extracts or after ammonium sulfate precipitation (fraction II) as shown in Figure 1a. During further purification, however, the enzyme sediments as a smaller molecular weight form: after DEAE-Sephadex (fraction III), the enzymes sediments at 4.1 S (Figure 1b) and after DNA-cellulose chromatography (fraction VI) at 3.3 S (Figure 1c).

Generation of Intermediate Forms of Novikoff Hepatoma β-Polymerase. By careful adjustment of ionic strength in the gradient, Novikoff fraction II β-polymerase can be made to sediment at a lower *s* value and several reproducible forms of the enzyme can be obtained (Figure 2). In order to determine the exact salt concentration at which the aggregate dissociates, sucrose gradients were run with different salts and at different concentrations. Four different forms of the enzyme could be generated (Figure 2) with sedimentation values of 7.3 S (Figure 2a, no salt), 5.8 S (Figure 2b, 0.01 M ammonium sulfate), 4.1 S (Figure 2c, 0.03 M ammonium sulfate), and 3.3 S (Figure 2d, 0.25 M ammonium sulfate; or 0.5 M NaCl, data not shown). The 7.3S form is identical with that seen in crude extracts or ammonium sulfate fractions of the β-polymerase (fractions I and II), while the 4.1S form is similar to the polymerase after hydroxylapatite chromatography (fraction V). The 3.3S form is similar to the homogeneous enzyme (fraction VI). The 5.8S form does not correspond to DNA

[2] Buffers are routinely prepared as 1 M stock solutions and pH adjustments made at 20 °C. Corrections for temperature and dilution coefficients have not been made.

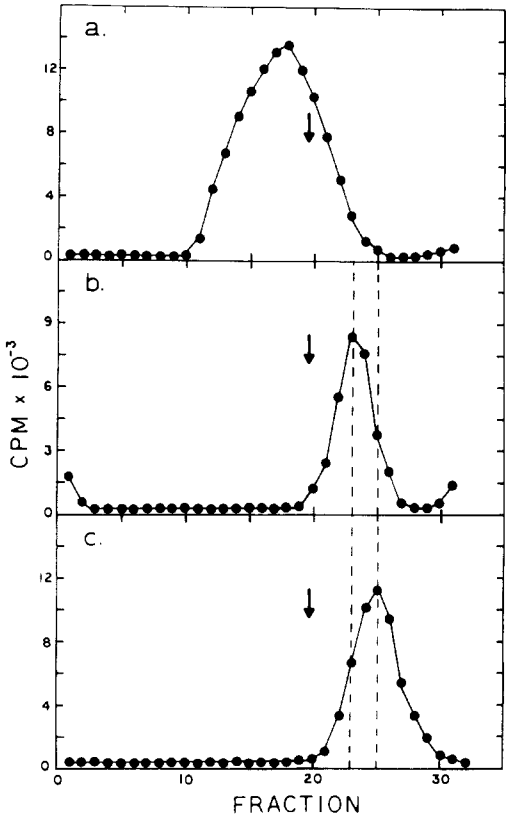

FIGURE 1: Sucrose gradient centrifugation of Novikoff DNA polymerase-β at different stages of purification. Five-milliliter gradients of 5–20% sucrose containing 20 mM Tris-HCl (pH 8.0), 5 mM β-mercaptoethanol, 1.0 mM EDTA, and 0.5 mg/mL BSA were overlaid with a 100-μL sample of enzyme to which 1 μL of *E. coli* alkaline phosphatase had been added as a marker. Centrifugation was carried out for 18 h at 35 000 rpm in a Spinco SW 50L rotor at 4 °C. After centrifugation, the tubes were punctured and seven drop fractions collected from the bottom. Each fraction was measured for DNA polymerase activity in a limited substrate assay as described in Methods. The position of the alkaline phosphatase marker (6.3 S) is indicated by an arrow. Sedimentation is from right to left in all gradients. Figure 1a is the ammonium sulfate fraction (fraction II, Stalker et al., 1976) which sediments as a broad peak from 6 to 8 S with the peak tube at 7.3 S.[3] The crude extract (fraction I, data not shown) gives an identical pattern. Figure 1b is the hydroxylapatite fraction (fraction V) in which the enzyme sediments at 4.1 S. Fraction III in the purification scheme (DEAE-Sephadex step) yields a similar sedimentation pattern (data not shown). Figure 1c is the homogeneous polymerase (DNA-cellulose step, fraction VI) which sediments at 3.3 S.

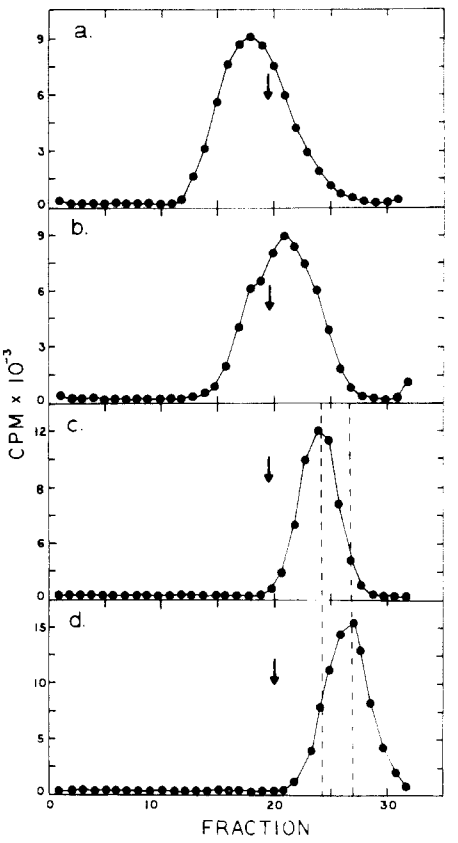

FIGURE 2: Sucrose gradient centrifugation of fraction II Novikoff DNA polymerase-β in the presence of varying salt concentrations. Experimental conditions were the same as described in the legend to Figure 1, except that ammonium sulfate was included in each gradient at the concentration listed below. (a) No salt (the enzyme sediments at 7.3 S); (b) 0.01 M ammonium sulfate (the peak sediments at 5.8 S with a shoulder at 7.3 S); (c) 0.03 M ammonium sulfate (the peak sediments at 4.1 S); (d) 0.25 M ammonium sulfate (the peak sediments at 3.3 S). The alkaline phosphatase marker is indicated by the arrow.

polymerase at any step in purification and suggests that perhaps two polypeptides found in the 7.3S form are simultaneously removed during DEAE-Sephadex chromatography.

Changes in Isoelectric Point of the β-Polymerase during Purification. We have previously shown that the p*I* of fraction V Novikoff DNA polymerase-β (4.1 S) is about 8.5 (Stalker et al., 1976). When fraction II (7.3 S) was isoelectrically focused, it surprisingly had a p*I* of 7.5. In contrast, fraction III, which sediments like fraction V at 4.1 S, also had the same p*I*. These data argue indirectly that the 7.3S complex may be composed of distinct proteins, acidic enough to change the p*I* of the complex by a whole pH unit and that the 7.3S form is probably not a self-aggregate.

Identification of Factor IV Stimulatory Protein. During DNA-cellulose chromatography of the β-polymerase, we frequently encountered considerable (40–80%) losses of activity. This suggested that we might be separating another stimulatory protein from the DNA polymerase. Upon assay of the DNA-cellulose fractions with homogeneous DNA polymerase (Figure 3), another stimulatory factor, eluting at 0.15 M NaCl, was found. This peak did not overlap with the DNA polymerase eluting at 0.55 M NaCl. The new stimulatory activity has been designated as Novikoff factor IV. We calculate that adding all of the factor IV to the DNA polymerase fraction VI in the experiment shown in Figure 3 would restore the activity to nearly 100% of that applied to the column.

Stimulation of the Homogeneous Novikoff β-Polymerase by Factor IV. Factor IV has been identified by its ability to stimulate the 3.3S DNA polymerase. Figure 4 illustrates the effect of adding increasing concentrations of factor IV. There is a linear increase in the extent of incorporation with increased amounts of factor IV up to a maximal level of about fourfold increase in activity in an hour incubation. Since factor IV has copurified with the DNA polymerase through five steps, separated from the enzyme on DNA-cellulose and then rechromatographed on hydroxylapatite (see below), the final fraction[4] is highly purified. It is necessary to add BSA to the buffer during purification of the polymerase on DNA-cellulose (Stalker et al., 1976). Our inability, so far, to completely

[3] In crude extracts the enzyme sediments broadly at 6–8 S with the midpoint of the peak at 7.3 S. In subsequent discussions, we will refer to this as the 7.3S form.

[4] Factor IV which has been rechromatographed on hydroxylapatite is designated as factor IV, fraction VII.

TABLE I: Chemical Nature of Novikoff Factor IV.[a]

Preincubation Components I	Preincubation Components II	[^{3}H]dTMP Incorp. (cpm)	% Control Act.
Buffer	Buffer	1 868	100
Trypsin + trypsin inhibitor	Buffer	2 391	128
Factor IV + trypsin	Trypsin inhibitor	2 353	126
Factor IV	Buffer	7 921	424
Trypsin + trypsin inhibitor	Factor IV	10 270	551

[a] To test the chemical nature of factor IV, three sequential incubations were set up as follows. Tubes (12 × 75 mm) containing preincubation components I in a volume of 37.5 μL were incubated for 30 min at 37 °C. Preincubation components II, in a volume of 12.5 μL, were then added and incubation was continued for an additional 30 min. The tubes were placed in an ice bath, and standard DNA polymerase assay components with saturated levels of deoxyribonucleoside triphosphates plus 0.05 unit of Novikoff β-polymerase (fraction VI, 0.9 ng) in a total volume of 75 μL were added to each tube. Incubation was then carried out for 1 h at 37 °C in a total reaction volume of 125 μL as described in Materials and Methods. For tubes containing trypsin 12.5 μg was used, for trypsin inhibitor 25 μg was used, and for factor IV 4.5 units of fraction VII was used. (The amount of protein in the factor IV aliquot was less than 1 μg.)

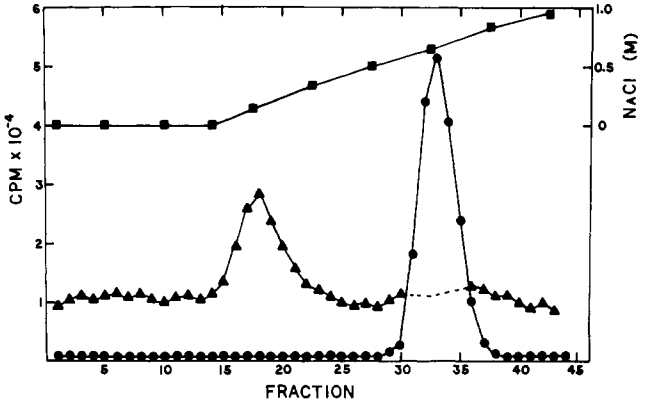

FIGURE 3: DNA-cellulose chromatography of Novikoff DNA polymerase-β. Fraction V DNA polymerase (Stalker et al., 1976) was loaded onto a 0.63 cm² × 5 cm single-stranded DNA-cellulose column equilibrated with TMEG buffer containing 1.0 mg/mL BSA. The column was washed with 40 mL of TMEG–BSA and the enzyme eluted with a 200-mL linear gradient of 0.01–1.0 M NaCl in TMEG–BSA at a flow rate of 30 mL/h. Aliquots of 25 μL of each column fraction were assayed for DNA polymerase (●—●) or for polymerase stimulatory activity (▲—▲) in assay tubes containing 1.0 ng of exogenous, homogeneous DNA polymerase. The DNA polymerase elutes at 0.55 M NaCl and a peak of stimulatory activity, designated factor IV, elutes at 0.15 M NaCl. In the chromatogram shown above, 1.34 mg of fraction V DNA polymerase was used.

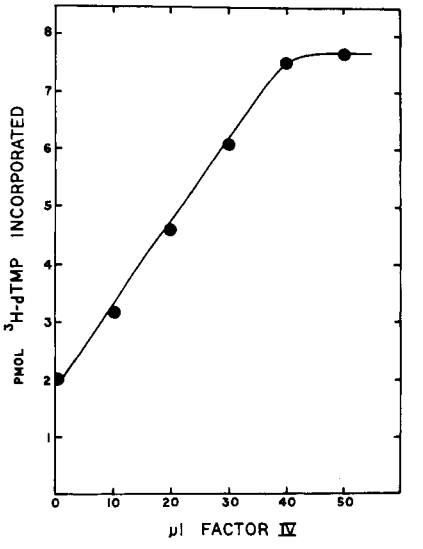

FIGURE 4: Stimulation curve for factor IV with fraction VI DNA polymerase. DNA polymerase activity was measured as described in Methods using 0.5 ng of fraction VI DNA polymerase in each tube. Factor IV was added at the concentration given in the figure, and incubation was carried out for 1 h at 37 °C in final reaction volumes of 125 μL. The exact quantity of factor IV protein and its stoichiometry to DNA polymerase has not been accurately determined because of the inability, as yet, to separate factor IV from BSA.

separate this BSA from factor IV, coupled with the extremely small amounts of factor IV protein, has prevented us from accurately determining the specific activity, purity, and stoichiometry of polymerase binding of the factor. Experiments in progress are aimed at these questions.

Chemical Nature of Novikoff Factor IV. Factor IV is a protein as evidenced by its sensitivity to trypsin (Table I). Preincubation of factor IV with an excess of trypsin for 30 min leads to a complete loss of stimulatory activity. It is curious that the combination of trypsin plus soybean trypsin inhibitor gave a slight (28%) increase in DNA polymerase activity over the buffer control. The reason for this is not known. When factor IV was preincubated with ribonuclease A or DNase I, in experiments similar to those shown in Table I, there was no loss of stimulatory activity. Factor IV is not dialyzable. We have not, as yet, been able to identify any specific enzymatic activity associated with this protein.

Evidence that Factor IV is Normally Bound to the β-Polymerase. Several lines of evidence indicate that factor IV is the component removed in the conversion of the 4.1S form to the 3.3S polymerase. (1) At the previous step in purification (hydroxylapatite chromatography), there is no evidence for stimulatory activity by the usual exogenous DNA polymerase assay (Figure 5a). When factor IV is separated from the DNA polymerase on DNA-cellulose and then rechromatographed on hydroxylapatite, the factor now elutes at a unique position (Figure 5b). These data are explicable if the factor is bound to the polymerase and thereby cochromatographs with it on hydroxylapatite. Factor IV activity cannot be detected at this point since it is saturated with DNA polymerase in the hydroxylapatite fraction and contributes to the endogenous DNA polymerase activity. When freed of polymerase, factor IV now binds directly to the hydroxylapatite column and can be detected in the effluent fractions. (2) Addition of factor IV to the 3.3S β-polymerase reconstitutes a 4.1S form of the enzyme detectable in sucrose gradients as shown in Figure 6. (3) Factor IV restores stability to the 3.3S enzyme. As discussed in the

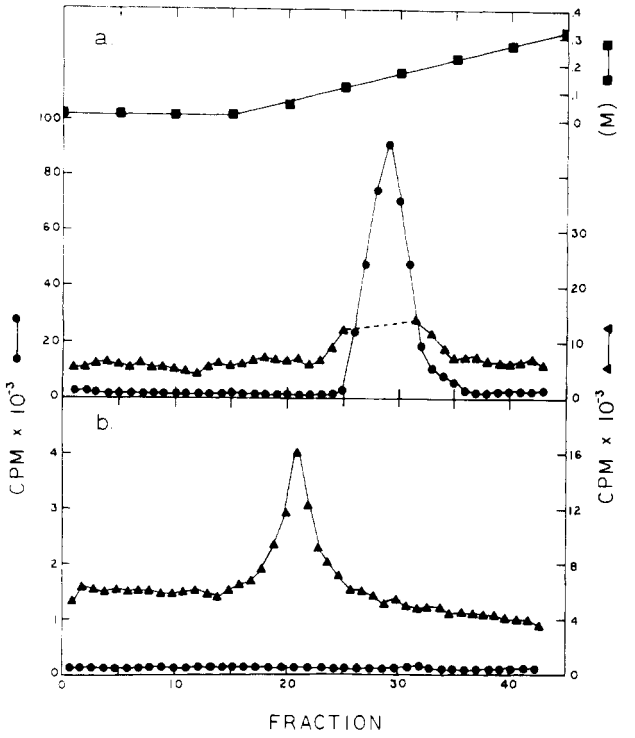

FIGURE 5: Hydroxylapatite chromatography of Novikoff DNA polymerase-β and Novikoff stimulatory factor IV. A 0.63 cm^2 × 8 cm column of hydroxylapatite was equilibrated with PMG buffer. (a) A sample of fraction IV DNA polymerase-β (Stalker et al., 1976) which contains factor IV bound to the enzyme was applied and the column was washed with 50 mL of PMG buffer. The sample was eluted with a 300-mL linear gradient of 0–0.45 M potassium phosphate in PMG at a flow rate of about 25 mL/h. DNA polymerase and stimulatory protein assays were carried out with limited substrate assays using 25-μL aliquots of each column fraction as described in Methods. Stimulatory protein assays contained 1.0 ng of exogenous, homogeneous DNA polymerase-β. The DNA polymerase (●—●) elutes as a single peak at 0.15 M. When assayed for stimulatory activity, none is detected, although there is a slight rise over the polymerase peak. Because of the large amount of polymerase in the peak, accurate measurements of stimulatory activity cannot be made as indicated by a dashed line across the peak. (b) A sample of factor IV, separated from the DNA polymerase on DNA-cellulose, has been rechromatographed on the hydroxylapatite column. Factor IV now elutes in a unique position at 0.07 M (▲--▲). No polymerase activity is detectable.

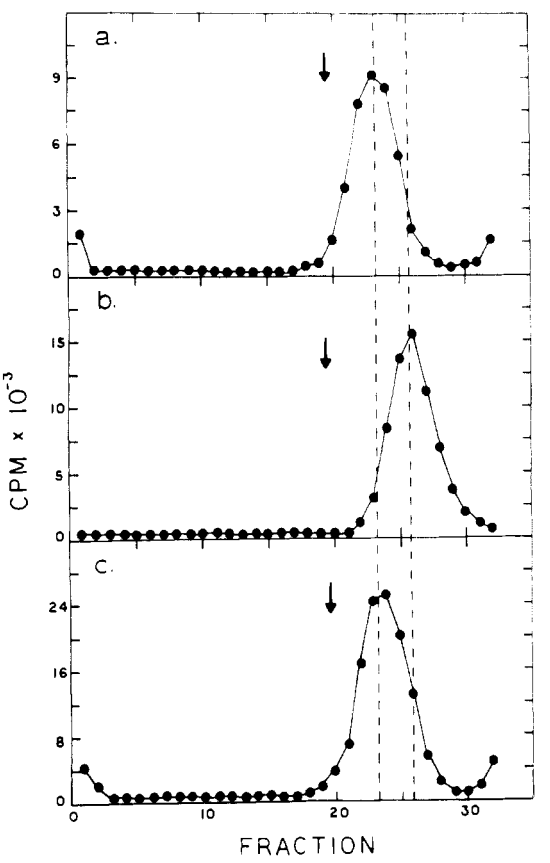

FIGURE 6: Reconstitution of the 4.1S form of Novikoff hepatoma DNA polymerase-β using factor IV. Sucrose gradients were prepared without salt as described in the legend to Figure 1. (a) The sedimentation profile of fraction V DNA polymerase which sediments at 4.1 S. (b) The sedimentation profile of fraction VI DNA polymerase which sediments at 3.3 S. (c) The sedimentation profile obtained when an aliquot of fraction VI polymerase is mixed with an excess of factor IV, dialyzed into TME buffer, and run on the gradient. The peak sediments at 4.1 S. The arrow indicates the alkaline phosphatase marker.

preceding paper (Stalker et al., 1976), fraction V (4.1 S) enzyme is quite stable, but fraction VI (3.3 S) enzyme is unstable in low ionic strength buffers. For example, if fraction V polymerase is dialyzed into TMEG buffer and stored at 4 °C, it retains greater than 98% of its activity after 24 h. Fraction VI polymerase, under the same conditions, loses more than 90% of its activity. If a 400-μL sample of factor IV is added to 4 ng of fraction VI polymerase in a final volume of 1.0 mL and *then* dialyzed into TMEG, virtually all of the activity remains after 24 h in the low ionic strength buffer. (4) Factor IV confers heat stability to the β-polymerase as shown in Figure 7. At 40 °C the 3.3S polymerase has a half-life of about 6 min. Factor IV, on the other hand, is remarkably stable with 97% activity remaining after 15 min under these conditions. Fraction V DNA polymerase (4.1 S) is more stable than the fraction VI enzyme and has a half-life of about 13 min. When excess factor IV is mixed with fraction VI polymerase, the stability of the enzyme is nearly identical with that of fraction V enzyme. The data again suggest a direct interaction of factor IV with the polymerase. In all reaction tubes for the experiment shown in Figure 7, the volume and buffer were identical, and the protein concentration was adjusted to 1.0 mg/mL with BSA in order to eliminate nonspecific protein protection as an explanation for the stability. For comparison, the heat stability of fraction VI polymerase was tested in the presence of DNA. Figure 7 shows that DNA, at the concentration used in our standard assay, stabilizes the enzyme even more efficiently than does factor IV. It also indicates that, under DNA synthesis conditions, the homogeneous DNA polymerase is not particularly unstable.

Discussion

The data reported here indicate that the β-polymerase from Novikoff hepatoma is extractable as a complex of proteins which sediment as a 7.3S form. If changes in s value represent loss of protein rather than conformational change, this complex would be predicted to have at least four polypeptides which can be sequentially removed by careful adjustment of ionic strength. Two of these have been identified as the 3.3S β-polymerase polypeptide and Novikoff stimulatory protein IV which together reconstitute the 4.1S form of the enzyme. Although the generation of intermediate forms between the 6–8S and 3S polymerase has been reported in other laboratories (Hecht, 1973b; Lazarus and Kitron, 1973; Yoshida et al., 1974; Morioka and Terayama, 1974), the present report is the first to identify and purify a specific protein, other than the polymerase, in such a complex. We are currently attempting to isolate the remaining proteins constituting the 5.8S and 7.3S

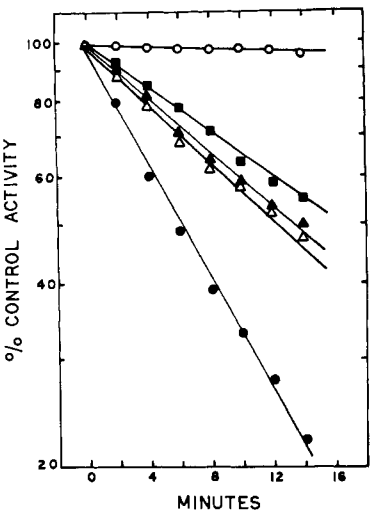

FIGURE 7: Heat inactivation of Novikoff DNA polymerase-β. In each sample tested, the final incubation volume was adjusted to 1.0 mL in TMEG-0.01 M NaCl. Protein was kept constant by adding enough BSA to bring the final concentration of protein to 1.0 mg/mL. Each sample was incubated in a stoppered tube at 40 °C. At the times indicated in the figure, 25-μL aliquots were removed, placed in assay tubes, and chilled in an ice bath. When all samples were collected, 100 μL of DNA polymerase assay components or stimulatory assay components (containing 0.5 ng of DNA polymerase-β fraction VI as the exogenous enzyme) were added, and assays were carried out as described in Methods. The values in parentheses below are the amounts of each component used in the 1.0-mL inactivation samples. (●—●) Fraction VI DNA polymerase (4 ng); (○—○) factor IV (400 μL); (▲—▲) fraction V DNA polymerase (0.17 μg); (△—△) fraction VI DNA polymerase (4 ng) plus factor IV (400 μL); (■—■) fraction VI DNA polymerase (4 ng) plus activated DNA (100 μg).

forms. It should be noted that the evidence for factor IV in the complex is circumstantial at present. Direct evidence will require the isolation of the complex intact from cell extracts and identification of its components in polyacrylamide gels.

Since the α-polymerases from several sources have been reported to form self-aggregates under low ionic conditions (Holmes et al., 1974, 1975; Yoshida et al., 1974; Matsukage et al., 1975; Sedwick et al., 1975; Craig and Keir, 1975), the question naturally arises as to whether the 7.3S form of β-polymerase is simply self-aggregation (see, for example, Lazarus and Kitron, 1973; Hecht, 1975). Our data from the Novikoff system argue against this. We have been unable to obtain aggregates of either the 3.3S or 4.1S forms of the enzyme under any condition of pH or low ionic strength (see Figure 1b, 1c, and 6). Similarly, the purified guinea pig liver β-polymerase is unable to undergo self-aggregation (Kunkel, Tcheng, and Meyer, unpublished data). The only other comparably pure β-polymerase for which data are available is the KB enzyme. Wang et al. (1975) have reported the separation of a factor, needed for aggregation, from the β-polymerase on Sephadex G-200. They could obtain aggregation of the 45 000 KB enzyme only when certain, enzymatically inactive, Sephadex fractions were added back to the monomer. The argument against self-aggregation is further strengthened, albeit indirectly, by our observation that the p*I* of the 7.3S form is considerably different than that of the 4.1S form, suggesting the presence of some protein(s) in the complex more acidic than the polymerase unit. While we cannot, at this time, eliminate the possibility of two β-polymerase units in the aggregate, the data certainly indicate that other proteins are involved.

The molecular weight of the homogeneous Novikoff hepatoma β-polymerase at 32 000 (Stalker et al., 1976) is considerably lower than that of comparably pure enzymes from calf thymus (Chang, 1973) or human KB cells (Wang et al., 1974) at 43 000–45 000. This raises the interesting possibility that factor IV is normally a part of the β-polymerase which has been proteolytically cleaved. The calf thymus enzyme is much more stable and has a threefold higher specific activity than the Novikoff β-polymerase. Addition of excess levels of factor IV to the Novikoff enzyme provides not only a relatively stable polymerase and a molecular weight more comparable to that of the calf thymus enzyme, but also the specific activity of the complex is about the same as that of the calf β-polymerase. To test the possibility of proteolysis, we have added the serine protease inhibitor phenylmethanesulfonyl fluoride to a batch of Novikoff hepatoma cells and purified the β-polymerase by the usual procedure but including 1.0 mM phenylmethanesulfonyl fluoride in all of the buffers. We did not find any difference in the enzyme in the presence or absence of the protease inhibitor. It may very well be that there is a distinct difference between rodent enzymes and other mammalian β-polymerases. In our laboratory, we have made a comparative study of the Novikoff polymerase with that purified from another rodent. We find the guinea pig liver β-polymerase to be a 3.3S enzyme; it also has a factor IV-like protein associated with it (Kunkel, Tcheng, and Meyer, in preparation). A small 2.5S polymerase has been reported for murine tissues (Matsukage et al., 1974).

Until we purify and examine the proteins found in the 7.3S complex, we cannot rule out the possibility that the aggregate form of the enzyme involves fortuitous, nonspecific binding of cellular proteins, particularly since the β-polymerase is a rather basic protein. It is, nevertheless, tempting to speculate that these proteins are involved in DNA synthesis in a meaningful way. The observation that factor IV stimulates the polymerase strengthens this argument as do the data from prokaryotic systems where *E. coli* DNA polymerase III (Schekman et al., 1974; Wickner and Kornberg, 1974) and possibly DNA polymerases I and II (Hendler et al., 1975) have been shown to function as a complex of proteins.

References

Alberts, B., and Herrick, G. (1971), *Methods Enzymol. 21,* 198.

Böhlen, P., Stein, S., Dairman, W., and Undenfriend, S. (1973), *Arch. Biochem. Biophys. 155,* 213.

Bollum, F. J. (1975), *Prog. Nucleic Acid Res. Mol. Biol. 15,* 109.

Burton, K. (1956), *Biochem. J. 62,* 315.

Chang, L. M. S. (1973), *J. Biol. Chem. 248,* 3789.

Craig, R. K., and Keir, H. M. (1975), *Biochem. J. 145,* 225.

Geider, K., and Kornberg, A. (1974), *J. Biol. Chem. 249,* 3999.

Hecht, N. B. (1973a), *Biochim. Biophys. Acta 312,* 471.

Hecht, N. B. (1973b), *Nature (London), New Biol. 245,* 199.

Hecht, N. B. (1975), *Biochim. Biophys. Acta 383,* 388.

Hecht, N. B., and Davidson, D. (1973), *Biochem. Biophys. Res. Commun. 51,* 299.

Hendler, R. W., Pereira, M., and Scharff, R. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 2099.

Holmes, A. M., Hesselwood, I. P., and Johnston, I. R. (1974), *Eur. J. Biochem. 43,* 487.

Holmes, A. M., Hesselwood, I. P., and Johnston, I. R. (1975), *Nature (London) 255,* 420.

Lazarus, L. H., and Kitron, N. (1973), *J. Mol. Biol. 81,*

529.
Loeb, L. A. (1969), *J. Biol. Chem. 244,* 1672.
Loeb, L. A. (1974), *Enzymes, 3rd Ed. 10,* 173.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236,* 1372.
Matsukage, A., Bohn, E. W., and Wilson, S. J. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 578.
Matsukage, A., Bohn, E. W., and Wilson, S. J. (1975), *Biochemistry 14,* 1006.
Meyer, R. R., and Keller, S. J. (1972), *Anal. Biochem. 46,* 332.
Morioka, K., and Terayama, H. (1974), *Biochem. Biophys. Res. Commun. 61,* 568.
Mosbaugh, D. W., Kunkel, T. A., Stalker, D. M., Tcheng, J. E., and Meyer, R. R. (1976), *Nucleic Acids Res. 3,* 2341.
Probst, G. S. (1974), Ph.D. Dissertation, University of Cincinnati.
Probst, G. S., Stalker, D. M., Mosbaugh, D. W., and Meyer, R. R. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1171.
Schekman, R., Weiner, A., and Kornberg, A. (1974), *Science 186,* 987.
Schekman, R., Weiner, J. H., Weiner, A., and Kornberg, A. (1975), *J. Biol. Chem. 250,* 5859.
Sedwick, W. D., Wang, T. S.-F., and Korn, D. (1975), *J. Biol. Chem. 250,* 7045.
Stalker, D. M., Mosbaugh, D. W., and Meyer, R. R. (1975), *J. Cell Biol. 67,* 416a.
Stalker, D. M., Mosbaugh, D. W., and Meyer, R. R. (1976), *Biochemistry 15,* 3114.
Wang, T. S.-F., Sedwick, W. D., and Korn, D. (1974), *J. Biol. Chem. 249,* 841.
Wang, T. S.-F., Sedwick, W. D., and Korn, D. (1975), *J. Biol. Chem. 250,* 7040.
Weissbach, A. (1975), *Cell 5,* 101.
Wickner, W., and Kornberg, A. (1974), *J. Biol. Chem. 249,* 6244.
Yoshida, S., Kondo, T., and Ando, T. (1974), *Biochim. Biophys. Acta 353,* 463.

Purification, Properties, and Partial Structure Elucidation of a High-Molecular-Weight Glycoprotein from Cervical Mucus of the Bonnet Monkey (*Macaca radiata*)†

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ABSTRACT: A high-molecular-weight glycoprotein has been purified from the cervical mucus of the bonnet monkey (*Macaca radiata*). The glycoprotein was shown to be homogeneous by electrophoresis, sedimentation equilibrium, and N-terminal group determination, and to contain 19% protein, 19% D-galactose, 18% *N*-acetyl-D-galactosamine, 15% *N*-acetyl-D-glucosamine, 11% L-fucose, 10% sialic acid, and 1% sulfate groups, corresponding to about 1800 amino acid residues and 400 carbohydrate side chains of about 9 monosaccharides. The carbohydrate chains are linked to the peptide backbone through *N*-acetyl-D-galactosamine and serine (or threonine) residues. Reduction with dithiothreitol and alkylation with iodoacetic acid reduced the molecular mass from 1 to 0.5×10^6 daltons and produced subunits having the same size, charge, and N-terminal amino acid. Electrophoretic studies suggested the presence of disulfide bonds between two chains of the glycoprotein. Degradation with alkaline borohydride gave, after fractionation on Bio-Gel P-2, fractions containing L-fucose, D-galactose, *N*-acetyl-D-galactosaminitol, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, and sialic acid in the ratio of 1.0:3.0:1.0:1.0:1.3:1.0. Further fractionation by electrophoresis and paper chromatography gave a charged fraction representing 13% of the original glycoprotein. Enzymic degradation and methylation studies indicated the presence of the structure α-Gal-(1→3)-[Fuc(1→2)]-Gal-(1→4)-GlcNAc, linked to a core component containing *N*-acetyl-D-galactosaminitol.

Cervical mucus is a hydrophilic, gel-like substance which contains soluble proteins and inorganic ions and is classified as "epithelial mucin" (Pigman et al., 1974; Pigman and Moschera, 1973; Montgomery, 1970; Spiro, 1970). From crude mucus collected from pregnant animals at estrus, Gibbons (1959) has isolated a bovine cervical mucoid consisting of 75–80% carbohydrates and 20–25% amino acid residues; the sialic acid residues were shown to be one of the factors controlling the physical properties of the mucin, and various carbohydrate contents of the cervical secretion have been ob-

† From the Laboratory for Carbohydrate Research, Department of Biological Chemistry and Medicine, and the Gynecology Service, Harvard Medical School, Massachusetts General Hospital, Boston, Massachusetts 02114. *Received September 2, 1976.* This is publication No. 721 of the Lovett Memorial Group for the Study of Diseases Causing Deformities, Harvard Medical School of the Massachusetts General Hospital. This investigation was supported by a grant from the United States Public Health Service (AM-03564). Part of this work has been described in preliminary communications (Hatcher et al., 1973; Hatcher and Jeanloz, 1974a,b). Amino Sugars Series CIX. Cervical Glycoprotein of the Bonnet Monkey, part III; for parts I and II, see Hatcher and Jeanloz, 1974a,b.

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