SEDIMENTATION OF A DNA-POLYMERASE COMPONENT FROM BACTERIAL EXTRACTS*

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The cessation of *in vivo* DNA synthesizing capacity in bacteria following protein synthesis inhibition has been described (1,2,3,4). When inhibited cells were broken open, the extracts were found to possess the enzymes needed to incorporate all four deoxyribonucleotides into an acid insoluble DNA polymer (5). Thus the kinases and DNA-polymerase required for *in vitro* DNA synthesis (6,7) appeared to be present in cells devoid of *in vivo* DNA synthesizing capacity. Since renewed protein and RNA synthesis were necessary to reinitiate what appeared to be the next "round" of DNA replication (8), it had been suggested that the newly synthesized protein and/or RNA component was necessary to "trigger" the next replication cycle which, once initiated, ran to completion (5,8,9). In this laboratory experiments have been started to determine the nature of such a control mechanism if it indeed exists. To initiate this study, it was decided to determine whether polymerase and its DNA primer could be obtained from cell extracts without the added complication of chemical precipitation. Through use of density gradient sedimentation it has been found possible to sediment a polymerase containing component from bacterial extracts prepared from growing cultures. In these extracts the polymerase appears to be associated with DNA.

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METHODS

Log phased Escherichia coli $15T^{-}A^{-}U^{-}$ (1-2 liters of culture) were harvested, washed once, and the resultant cell pellet ground with powdered alumina. The disrupted cells were extracted with 5-8 ml of TSM (Tris 0.01M, Succinate 0.004M, Mg acetate 0.01M) buffer, pH 7.4 and the extract subjected to a low speed (3000 rpm) centrifugation for ten minutes to remove coarse debris and whole cells. The resulting extract was centrifuged twice at 7000 x g for 10 or 30 min. to further remove cells and alumina. One ml of final extract was layered on top of a 5-20% sucrose gradient (sucrose was dissolved in a TSM solution buffered at pH 8.2). Centrifugation was at 25,000 rpm in a Spinco SW 25 swinging-bucket rotor. At the end of the run the tube bottom was punctured and one ml fractions collected.

A unit of polymerase or H^3-thymidine (H^3-Tdr) incorporating activity is defined as that activity resulting in the incorporation of 1 mμ mole of labeled substrate into acid insoluble material during a 20 minute incubation at 37°C. The complete assay mixture of H^3-Tdr or labeled deoxyriboside triphosphate incorporation activity consisted of the following: 0.02 ml of glycine buffer (1M, pH 9.2); 0.02 ml each of the triphosphates of deoxyadenosine, deoxyguanosine and deoxycytosine (500 mμ moles/1 ml) except where the analogous labeled triphosphate was substituted; 0.02 ml $MgCl_2$ (0.01M); 0.03 ml of 2-mercaptoethanol (0.01M); 0.02 ml of thymus DNA previously heated to 100°C. for 5 minutes (0.5 mg/ml); 0.01 ml ATP (100 μ moles/ml); 0.01 ml phosphoglyceric acid (0.05M); and 0.2 ml of enzyme fraction. The final volume was made to 0.40 ml with H_2O.

The H^3-TTP was prepared enzymatically, with E. coli extract, from H^3-Tdr (1,900 μC/μ mole) and isolated by ion exchange chromatography using the method of Hurlbert (10). P^{32}-dGTP (about 2×10^6 cpm/μ mole) was prepared enzymatically from P^{32}-dGMP using a soluble liver enzyme preparation followed by

boiling (11). Analysis of the soluble material by paper chromatography showed that more than 90% of the dGMP had been converted to dGTP.**

A method similar to that of Bollum (12) employing paper discs for rapid measurement of radioactivity in the acid insoluble material was used in these studies. Following incubation the reaction mixture was rapidly cooled and 400 μg of carrier DNA added (2.0 mg/ml). 60 μl of mixture was applied to a 25 mm diameter Whatman #4 filter paper disc which was then immersed in cold 5% TCA. The procedure for washing the discs was identical to that described by Bollum. In our experiments, counting of the dried discs was accomplished by immersion of the discs into scintillation fluid contained in screw cap glass vials which were subsequently counted in the Tri-Carb liquid scintillation spectrometer. In the double labeling experiments to be described, H^3-Tdr was added to intact growing cells to identify cellular DNA, while P^{32} labeled substrate was used for polymerase assay.

RESULTS AND DISCUSSION

Density gradient sedimentation of E. coli extract for 3-1/2 hours at 25,000 rpm moved the enzyme complex (Tdr and TMP kinase and polymerase) needed for Tdr incorporation into DNA as a rather broad, slowly sedimenting band near the top of the tube. On a specific activity basis (units activity/per μg protein) fraction #20 was five times more effective than the whole extract. The ribosomes (pellet through fraction #10, see Gros, et al. (13)) possessed little, if any, Tdr incorporating activity.

When a 14 hour run was analyzed for H^3-Tdr incorporation activity, it had in large measure disappeared. Since it was possible that a separation of polymerase from the Tdr phosphorylating enzymes had occurred, polymerase activity was assayed with its immediate substrate, H^3-TTP. The polymerase activity was found only in the first (bottom) fractions with the greatest activity in the pellet. In this experiment, about 41% of whole extract polymer-

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ase activity was found in the pellet. In repeat experiments, yields as high as 68% were obtained in the pellet.

The pellet activity is characteristic of the deoxyribonucleotide polymerizing enzyme (6,7). If the three deoxyriboside triphosphates were omitted from the assay mixture, then 10% or less P^{32}-dGTP was incorporated as compared to the complete reaction mixture. Omitting ATP and phosphoglyceric acid produced a 22% reduction in dGTP incorporation into acid insoluble material. Incubation of the labeled acid insoluble material with DNAase brought about release of the radioactivity in acid soluble form.

Chemical analysis of the pellet indicated the presence of DNA as well as the expected ribosomal RNA. Since most of the fractions obtained from the gradient contained insufficient DNA for chemical analysis, DNA of the growing cells was labeled to determine its distribution in the gradient. Accordingly, 100 μC of H^3-Tdr was added to the 1-1/2 l. of culture, growing in a medium containing a limiting concentration of thymine (2 μg/ml), about 10 minutes prior to harvesting.

In Table I are presented data showing that polymerase activity was only found in association with cellular DNA. A 3-1/2 hr. centrifugation sedimented a small portion of DNA and polymerase, the remainder moving together as a broad band, indicating extreme heterogeniety of the components. A 14 hour centrifugation usually sedimented most of the polymerase and labeled DNA (Table II). In addition a preincubation with DNAase (Nutritional Biochem. Corp., 1 x crystallized), which converted the DNA to an acid soluble state, brought about an almost complete loss of polymerase activity in the pellet subsequently obtained (Table II). DNAase activity was found primarily in the top two fractions. RNAase (Boehringer, 5 x crystallized) pretreatment also brought about an extensive solubilization of both DNA and polymerase although the pellet activity was not as greatly altered. While the nature of the RNAase action is unknown, it is apparent that there was DNAase activity since the DNA was acid solubilized. In repeat experiments the DNAase effect was always the same whereas the RNAase effect was variable, however following

TABLE I

Distribution of polymerase and DNA following density gradient sedimentation of bacterial extract for 3-1/2 hrs.

Fraction	Cellular DNA* cpm	Units Polymerase**
Pellet	3,540	4.48
1	240	0.19
3	250	0.26
5	265	0.33
7	480	0.46
9	675	0.76
11	885	1.28
13	1,330	1.58
15	1,510	2.26
17	1,590	2.21
19	1,500	2.37
21	1,310	1.96
23	715	1.32

* The growing bacteria were labeled with H^3-Tdr 10 min. prior to harvesting. DNA of the enzyme preparations was assayed for by counting tritium label in the acid insoluble component. Values are for radioactivity of 0.1 of fraction.

** Polymerase was assayed by determining incorporation of P^{32}-dGTP into DNA as described in text. All values corrected for cross contamination of one isotope by the other during the double channel counting on the scintillation counter.

the latter treatment, a positive correlation of pellet activity with the presence of DNA was always found. The RNAase preparation was found not to contain significant DNAase activity. It is possible that an endogenous DNAase, both in an active and latent form (14), was present in the extract, and that the latter form was activated by the RNAase. The nature of the loss in total polymerase activity found in the fractions after DNA solubilization is unknown.

These findings do not allow a decision as to whether the DNA-polymerase component is an artifact formed upon cell disruption (but showing some specificity of combination since thymidine kinase and the bulk of the soluble proteins are not precipitated with it), or whether it reflects a physiological state existing *in vivo*. The latter possibility is of interest in light of the

puzzling fact that in mammalian cells DNA-polymerase has been found associated with the cytoplasm and not with the nucleus (15) while most *in vivo* studies indicate that DNA is replicated in the nucleus (16). Experiments are underway to determine whether altered DNA synthesizing states of the intact cell leads to a change in the polymerase-DNA association.

TABLE II

Sedimentation of polymerase and its association with DNA

Fraction	Pretreatment of whole extract*					
	15 min., 37°C.		Plus DNAase (10 μg)		Plus RNAase (10 μg)	
	DNA** cpm	Units Polymerase**	DNA cpm	Units Polymerase	DNA cpm	Units Polymerase
Pellet	7,780	13.0	94	1.06	500	2.29
1	317	0.54	23	0.29	30	0.27
3	169	0.36	7	0.23	13	0.29
5	97	0.34	12	0.27	13	0.29
7	107	0.34	8	0.28	13	0.03
9	98	0.31	12	0.30	7	0.29
11	98	0.28	10	0.28	7	0.37
13	108	0.31	8	0.34	7	0.47
15	92	0.29	12	0.43	7	0.51
17	95	0.19	7	0.82	7	0.32
19	108	0.19	7	1.01	30	0.22
21	125	0.33	7	0.61	10	0.35

* Extracts were incubated at 37°C. for 15 min. with or without additions as shown. Following incubation they were cooled and layered over sucrose gradient for sedimentation study as outlined in text.

** See Table I for explanation.

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