MOUSE BRAIN OLIGODENDROGLIAL NUCLEAR RNA POLYMERASES: MODIFICATION OF FUNCTION BY NARCOTICS

by

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PROLOGUE

To speak the truth is the most difficult thing in the world; and one must study a great deal and for a long time in order to be able to speak the truth. The wish alone is not enough. To speak the truth one must know what the truth is and what a lie is, and first of all in onself.

G.I. Gurdjieff as told to P.D. Ouspensky in *In Search of the Miraculous*

ABSTRACT

Specific aspects of the involvement of macromolecular synthesis in opiate tolerance-dependence development were investigated. Previous research had demonstrated that chronic morphine treatment induced alterations in the acidic proteins, protein kinase, and phosphorylation in mouse brain oligodendroglial chromatin. RNA polymerases from oligodendroglial nuclei were solubilized, partially purified, and resolved on DEAE-Sephadex into multiple forms. The fractionation of RNA polymerase (RNAP) by the methods reported yielded three RNA polymerases, I, II, and III. The degrees of purification relative to total brain homogenate were 47-, 118- and 27-fold, respectively. These RNA polymerases were shown to be DNA-dependent and not to contain significant ribonuclease activity. They were characterized relative to the effects on their activities of α-amanitin, ammonium sulfate, magnesium, manganese, synthetic poly dAdT, pH, and temperature. The several properties of the RNA polymerases which were examined were within the limits of those described in other systems. It was thus possible to conclude that these brain enzymes were RNA polymerases I, II, and III. The affinities of RNAP I and II for the substrates ATP and UTP were shown not to follow Michaelis-Menten kinetics. Polyacrylamide gel electrophoresis of RNAP I and II under non-denaturing conditions indicated the presence of one or

more contaminant proteins in each of the polymerases. Chronic morphine pellet implantation was shown to decrease the specific activity of RNA polymerase I. This effect was shown to be reversed by naloxone pellet preimplantation and thus to be narcotic specific. This change in RNAP I was also shown not to result from acute administration of morphine sulfate (MS) nor from *in vitro* MS or β-endorphin. Chronic morphine treatment was also shown to shift the optimal assay pH for RNAP I to 8.5 from the 8.0 optimum for naive and placebo. The decrease in specific activity of RNAP I was shown not to occur in mouse liver. This brain effect was also shown to revert to normal after cessation of drug treatment. The RNA polymerase I fraction eluted from a single DEAE-Sephadex column was sub-fractionated by a second DEAE-Sephadex chromatography into five consistently separable activity peaks. These peaks were shown to differ in their sensitivities to α-amanitin and in their relative preferences for poly dAdT. Two of these peaks were shown to be lowered in specific activity by chronic morphine and the remaining peaks to be unaltered by the treatment. RNA polymerases II and III were shown not to be altered in specific activity following acute, chronic, or *in vitro* morphine. However, the optimum Mg^{+2} concentration for RNA polymerase II function was shown to be lowered in tolerance-dependence. This effect was demonstrated to revert to normal after cessation of drug treatment. The

Mn^{+2}/Mg^{+2} activity ratios of both RNAP II and III from morphine tolerant-dependent animals were demonstrated to be higher than those of placebo controls. These enzymes were also shown to be stimulated two-fold by the endogenous opioid peptide β-endorphin at 10 uM concentration. With the addition of cAMP-independent protein kinase, a correlation was seen between RNA polymerase activity and phosphorylation for RNAP I and II, but not for RNAP III, suggesting that the nuclear protein kinase may be regulating RNAP I and II through a phosphorylation mechanism. Evidence for biochemical adatation as a mechanism of opiate tolerance-dependence development is discussed.

INTRODUCTION

The phenomena of narcotic tolerance and physical dependence have caused countless problems for mankind and have been the subject of much scientific inquiry. In considering the nature of this tolerance and dependence, a simple, plausible model for the mechanism of tolerance-dependence development becomes obvious.

The organism adapts to external stimuli through its homeostatic mechanisms. It is thus easy to hypothesize that narcotic tolerance development is some adaptive response to maintain homeostasis in the constant presence of the perturbing narcotic. Extension of this hypothesis, to consider the situation arising upon abrupt withdrawl of the narcotic, predicts an abrupt alteration in the state of the organism, a withdrawl syndrome. Several such hypotheses have been advanced by various groups (1, 2, 3, 4, 5).

Current dogma describes an opiate agonist binding to a cell surface receptor which causes some internal effect in the cell (6). Some intracellular sequence of events must then occur which eventually leads to the total opiate effect. Presumably, then, in the continued constant presence of the drug, the cell's homeostatic mechanisms return net cell function toward its pre-drug condition.

Our knowledge of biochemistry and molecular biology tells us that these mechanisms are at least in part protein-dependent.

While several enticing examples of changes in intracellular conditions have been supplied, the exact pertinent aberrations remain unidentified. However, disruptions of certain fundamental cellular processes have been shown to prevent or to attenuate the development of the opiate tolerant-dependent state. Such studies indicate that each of these processes is therefore necessary for, and might play a role in, tolerance-dependence development.

Two examples of processes which have been so implicated are RNA synthesis at the level of transcription and protein synthesis at the level of translation. The RNA synthesis inhibitors actinomycin D (7, 8, 9, 10) and 8-azaguanine (11, 12) have been shown to block morphine tolerance-dependence development in rats and mice, while 6-mercaptopurine and 5-flourouracil attenuate opiate tolerance development (13, 14). Such studies have the intrinsic problems of the effects of the additional agents themselves. Actinomycin D causes widespread metabolic disturbances and increases the uptake of morphine into the brain (10). Even in the best-controlled experiments of

this type, questions thus remain as to the validity of the conclusion.

Total brain RNA synthesis has been measured following acute and chronic morphine treatments,and the total of these studies is equivocal (15, 16, 17). Important but small changes in the total amount of brain RNA could be at best difficult to measure, while large changes in a few specific RNA species would likely not be observed. It has been shown (16) that H-uridine incorporation is decreased in brain following chronic morphine and that ribonuclease specific activity is also decreased (18). Datta and Antopol have also demonstrated (19) that RNA synthesis is lowered in whole nuclei. The study of the individual aspects of RNA synthesis has been necessary in order to ascertain whether some part of this process is, in fact, being altered by chronic opiate administration.

Nuclear chromatin has been isolated from several fractions of brain cell nuclei, and these have been assayed for their abilities to act as templates for the DNA-dependent RNA polymerase of E. Coli strain K-12. (20). It has been reported that the template activities of the chromatin isolated from a particular fraction of nuclei from brains of morphine tolerant-dependent mice are increased relative to the placebo control. This chromatin has been reported to show increases in the amounts of certain specific

acidic proteins and no changes in the amounts of histones relative to the placebo control (21).

Oguri et al. (22) observed that the specific activity of nuclear cAMP-independent protein kinase increased following chronic morphine treatment. This protein kinase was shown to phosphorylate the acidic proteins of the chromatin with remarkable specificity for the phosphorylation of those same acidic proteins observed to be increased following chronic morphine treatment. Hook et al. (23) have further purified this protein kinase and have identified the single specific form (of the multiplicity of kinases present) increased in specific activity. These observations indicate that some change is occuring following chronic morphine treatment which results in a very specific alteration in the composition and function of this chromatin.

When considering the in vivo processes of transcription and how they might be altered, the need arises of separating effects on template availability from those on RNA polymerase functions. It has been suggested that RNA polymerase plays an important role at the transcriptional level, and that it is also the site of action for many therapeutic and toxic agents (24). Thus, the endogenous RNA polymerase has been studied in order to elucidate its role in the transcriptional processes that have been

implicated in the development of morphine tolerance-dependence.

The observations of Lee and Oguri (20, 22) have been found in a highly purified, morphologically homogenous nuclear fraction from mouse brain which has been identified as containing primarily oligodendroglial nuclei. These alterations in chromatin and in protein kinase specific activity have not been observed in other nuclear fractions nor in a mixture of total brain nuclei (25). Because these very interesting and specific alterations in nuclear and chromatin composition and function have been observed only in this particular, highly purified fraction of oligodendroglial nuclei, this nuclear subpopulation has been selected as the source of the RNA polymerase described in this dissertation.

RNA polymerases from many eukaryotic organisms have been studied (26). The enzymes have been purified from several mammalian tissues, e.g. calf thymus (27), liver (28), and mouse myeloma (29). Multiple forms of the RNA polymerases have been detected in most of the systems studied. Very little work has been done on this enzyme from brain tissue. However, the brain RNA polymerases have been shown to be affected by the administration of both opiates and ethanol (30). This dissertation describes the

isolation and purification of the three DNA-dependent RNA polymerase (I, II, and III) from mouse brain oligodendroglial nuclei. Identification of the three enzymes and a sub-fractionation of RNA polymerase I are reported. Chronic morphine treatment is demonstrated to decrease significantly the specific activity of the peak I enzyme, and this is shown to be the result of effects on two specific sub-types of this enzyme. The Mg^{+2} requirement of RNA polymerase II is shown to be lowered by chronic morphine. The Mn^{+2}/Mg^{+2} activity ratios are also shown to be altered in morphine tolerant-dependent animals, as is the pH optimum of RNA polymerase I.

Positive regulation of the RNA polymerases I and II via phosphorylation by the endogenous protein kinase described by Hook et al. (23) is demonstrated. It is suggested that regulation of the phosphorylated state of RNA polymerase may account for some of the observed effects of morphine. Evidence for biochemical adaptation as a mechanism of opiate tolerance-dependence development is discussed.

The evidence presented here, in addition to the studies described above (20, 21, 22, 23, 25), constitute a large addition to the body of evidence implicating the role of glial cells in opiate tolerance-dependence development.

MATERIALS

Animals: ICR mice (male, 23-27g) were supplied by Simonsen Laboratories, Gilroy, CA, and were housed by the UCSF animal care facility.

Chemicals: All chemicals were reagent grade. Biochemicals which were not so designated were obtained as "A" grade. Most reagents were from Mallinckrodt, St. Louis, MO, "AR" series. Those which were not are listed below.

From Calbiochem, La Jolla, CA: Ultrol Tris, dithiothreitol, adenosine 5'-triphosphate (ATP), calf thymus DNA, actinomycin D.

From Sigma, St. Louis, MO: bovine serum albumin (BSA), bentonite, Coomasie blue G-250, Coomasie blue R, N,N,N',N',-tetramethylethylenediame (TEMED), acrylamide, N,N-bis-methylene acrylamide, ammonium persulfate, bromophenol blue.

From PL Biochemicals, Milwaukee, WI: polydeoxyadenylate-deoxythymidylate alternating copolymer (poly dAdT), cytidine 5'-triphosphate (CTP), guanosine 5'-triphosphate (GTP), uridine 5'-triphosphate (UTP).

From Boehringer Mannheim USA, Indianapolis, IN: α-amanitin.

From ICN, Chemical & Radioisotope Division, Irvine, CA: adenosine 5'-triphosphate (2,8- ^{3}H), sp. act. 27.7 Ci/mmole; uridine 5'-triphosphate (5,6-^{3}H), sp. act. 27 Ci/mmole; adenosine 5'-triphosphate (gamma- ^{32}P), sp.act. 10-20 Ci/mmole.

From Pharmacia Fine Chemicals, Piscataway, NJ: diethylaminoethyl-Sephadex A-25.

METHODS

Preparation of Oligodendroglial Nuclei:

Oligodendroglial nuclei were prepared according to the method of Oguri et al. (22). Mice were sacrificed by decapitation, and whole brains were immediately removed for immersion in ice-cold 0.25 M sucrose TKM (Tris-HCl, 50mM, pH 7.4 @ 4°C; KCl, 25 mM; $MgCl_2$, 5mM). The mean wet weight of the mouse brains was 0.4 g; the brains were homogenized in 0.8 ml of 0.25 M sucrose TKM per mouse brain in a Thomas tissue grinder, motor driven on low speed, ten strokes. The homogenate was filtered through four layers of cheesecloth. An equal volume of 2.3M sucrose TKM solution was added to the filtered homogenate. This hypertonic homogenate was then layered above 2.3M sucrose TKM in a polypropylene centrifuge tube. This preparation was then subjected to ultracentrifugation at 4° C in a Beckman SW 27 swinging bucket rotor for 90 min at 27,000 r.p.m. (140,000 X g max). A typical preparation would consist of the brains of 75 mice, approximately 30 g wet weight, which would produce approximately 150 ml of the hypertonic homogenate. The lower layer in each tube would contain 9 ml of 2.3 M sucrose TKM; 27 ml of homogenate could thus be layered above in each of the six 37 ml capacity tubes accomodated by the SW27 rotor.

The supernatants were drawn off from the tubes by suction, and the pellets were suspended in 0.25 M sucrose TKM, 5 ml per tube. These suspensions were combined, and the nuclei were collected by centrifugation at 4°C in a Sorvall SS-34 rotor for 20 min at 18,000 r.p.m. (42,000 x g max). Oguri et al (22) reported that nuclei prepared by this method were 97% homogenous on the basis of morphology. The nuclei were reported as being small, dense, and darkly staining and were identified according to the criteria of Austoker et al. (31) as being oligodendroglial in their origin. The preparation was reported to yield nuclei representing 10% of total brain DNA.

Solubilization of RNA Polymerases:

The solublization, separation, and assay of the RNA polymerases were by methods modified from those described by Kedinger et al. (27) and by Schwartz et al. (29) for studies of RNA polymerases from calf thymus and from mouse myeloma cells respectively. The pellet of oligodendroglial nuclei was suspended in 5.0 ml of TEMDG (25) + 30 buffer. This buffer was similar to those employed by Kedinger et al (27) and by Schwartz et al. (29) and consisted of: Tris-HCl, 50 mM, pH 7.9 at 4°C; ethylenediamine tetracetic acid (tetrasodium salt), 1 mM; magnesium chloride, 4 mM; dithiothreitol, 1 mM; glycerol, 25% v/v; ammonium sulfate, 30 mM. In this original

nomenclature, "TEMDG (25) + X" refers to the buffer described above except that the buffer is at a concentration of X mM ammonium sulfate. This nuclear suspension was adjusted to 0.33 M ammonium sulfate by addition of 3M ammonium sulfate solution, pH 7.9. The suspension was mixed by inversion five times, and the result was the production of an extremely viscous mixture.

This hypertonic suspension was then sonicated by means of a Branson model 140D sonifier using the microtip immersed less than 1 cm below the surface of the suspension. Sonication was conducted at the limit of intensity for the microtip in 15s bursts. The suspension was maintained in ice throughout the sonication, and each 15s burst was followed by a 30s pause. The number of bursts varied from four to six; the endpoint of sonication was the attainment of a clear and freely flowing solution.

This sonicated solution was subjected to centrifugation in a Beckman SW65 Ti swinging bucket rotor for 45 min at 45,000 rpm (220,000 x g). The supernatant solution containing the solubilized RNA polymerases was concentrated to 3.0 ml in a Schleicher and Schuell model 100 collodion bag (molecular weight exclusion 25,000). This concentrated solution was dialyzed against 200 volumes of TEMDG (25) + 30 overnight.

Preparation of DEAE-Sephadex A-25 Column:

DEAE-Sephadex A-25 anion exchange resin was suspended in TEMDG (25) + 1000. The suspension was stirred at 4°C for 24 hr, the resin was allowed to settle, and then the liquid was decanted. Additional TEMDG(25)+1000 was added, stirred, etc., three times more. In this manner, the resin was prepared as the sulfate form by exchange from the chloride salt as supplied by the manufacturer. The resin thus prepared was then similarly treated with TEMDG(25)+30 in order to bring the concentration of free ammonium sulfate in the suspension toward 30 mM. This process was essential because changes in salt concentration decreased the flow rates attainable when the resin was packed into columns.

The resin thus prepared was packed into a column 0.9 cm in diameter to a bed height of 14 cm, a total column volume of 9 ml. The resin was equilibrated by washing with at least 90 ml of TEMDG(25)+30.

Application and Elution of RNA Polymerases:

The dialyzed solubilized RNA polymerases were applied to the DEAE-Sephadex A-25 columns. Application of the samples was at approximately 0.01 mg protein per ml bed volume, a level well below that of 2 mg protein per ml bed

volume used by Schwartz et al. (29). This supplied assured additional resin capacity to allow for variations in protein content of applied solutions. After the applied solution had entirely entered into the resin, the column was washed with two volumes of TEMDG(25)+30. RNA polymerase activities were eluted by a discontinuous ammonium sulfate gradient. Two volumes of each of TEMDG(25)+225, TEMDG(25)+275, and TEMDG(25)+1000 were applied to and eluted from the columns. Fractions equal to one third of the column volume were collected. For most studies, those fractions containing RNA polymerase activity for each elution condition were combined. The columns were usually eluted with the aid of a peristaltic pump attached to the tubings leading from the bottoms of the columns. The maximum flow rate which was attained was 24 ml per hour; most elutions were at slower rates.

Protein Determinations:

Protein concentrations were determined either by a modification of the method of Lowry et al (32) or of the method of Bradford (33). In the Lowry method, sample or sample plus water were measured to a total volume of 0.72 ml. To this was added 0.08 ml of 1M NaOH. These samples were incubated at 45°C for 45 min. To these samples was added 2 ml of Lowry reagent "C": 2 ml of 1% sodium potassium tartrate plus 2 ml of 0.5% $CuSO_4 5H_2O$ plus 2%

sodium carbonate to a total volume of 100 ml. Reagent "C" was prepared immediately prior to use from the three stock solutions. After the addition of reagent "C", the samples were allowed to sit at room temperature for 30 min. At this time, 0.2 ml of Folin-Ciocalteau reagent was added to each tube with immediate vortexing. Folin-Ciocalteau reagent was prepared immediately prior to use by 1:1 dilution of reagent supplied by Harleco. These samples were allowed to sit at room temperature, for development of their blue color, for 20 min. The optical absorbance of each sample for the wave length 700 nm was measured in a Gilford model 220 or a Zeiss model PM2D spectrophotometer. Protein concentrations were determined according to comparisons with absorbances of samples containing known amounts of bovine serum albumin (BSA) which were prepared for each set of samples assayed.

TEMDG(25)+X buffers showed a strong reactivity with the reagents employed in this assay. The changes in absorbances of these buffers were not linear with respect to changes in amounts of buffer added. The absorbances of samples containing these buffers were also of such intensity that small amounts of protein could not be detected: a standard curve for BSA in TEMDG(25)+30 could not reliably be constructed over the range of five to fifty micrograms. Thus, it was necessary to dialyze the samples against distilled water prior to Lowry assay so

that the buffer constituents could be removed. Dialysis was performed quantitatively: a measured amount of sample was placed in each dialysis bag, and the volume present after dialysis was determined. Since the protein samples were thus in distilled water at the time of Lowry assay, the control zero protein sample was 0.72 ml water. BSA standard solution was also prepared in water.

The large numbers of samples to be assayed for protein content in some studies necessitated the development of a method which could be performed without the laborious quantitative dialysis procedure. The method of Bradford (33) was ideal for this situation in that Bradford reported little or no interference in the assay by any of the TEMDG(25)+X buffer components. The reagent was prepared as described (33) except that anhydrous methanol replaced the absolute ethanol. 100 mg of Coomasie Blue G-250 was dissolved in 50 ml of spectrophotometric grade anhydrous methanol; the dye is very difficult to dissolve in ethanol. Glass distilled water and 100 ml of phosphoric acid were added to a total volume of 1 liter. This solution was filtered through a Whatman no. 1 filter paper and used within two weeks. A sample volume of 0.1 ml and 2 ml of reagent were combined and allowed to sit at room temperature for 30 min. At the end of this time, the absorbances of the samples at the wavelength 595 nm were measured in a Zeiss model PM2D spectrophotometer

relative to a zero protein control sample containing 0.1 ml TEMDG(25)+30. Protein standards were prepared containing BSA in TEMDG(25)+30. Comparisons of the dialysis/Lowry and Bradford methods indicated nearly identical protein determinations for the two methods for the RNA polymerase fractions.

RNA Polymerase Assay:

The RNA polymerase assay was a modification of methods reported by Lee et al (20), by Kedinger et al (27) and by Schwartz et al (29). The RNA polymerase assay measured the incorporation of ^{3}H-labelled UTP into TCA-insoluble precipitates. The assay medium contained: Tris-HCl, 100mM (pH 7.9, @ 37^{o}C); $(NH_4)_2SO_4$, 35 mM; dithiothreitol (DTT), 1 mM; EDTA, 1 mM; $MgCl_2$, 4 mM; $MnCl_2$, 2 mM; bentonite, 20 ug/ml; ATP, GTP and CTP, 1 mM each; UTP, 0.01 mM; [^{3}H]-UTP, 15 uCi (sp. act. 27.3 mCi/m-mole), DNA, 50 ug (calf thymus, Sigma Co., St. Louis, MO); enzyme fraction and water to make up the total of 0.5 ml. The reaction mixture was incubated at 37^{o} C for 30 min and stopped by addition of 2 ml of 10% TCA-PPi (Trichloroacetic acid + 3% sodium pyrophosphate). Bovine serum albumin, 0.15 mg, was added to the mixture and allowed to stand in ice for 15 min. The precipitate was washed on a Whatman GFC filter with 8 ml of 5% TCA-PPi five times and rinsed with cold 95% ethanol. The samples

were counted in 9 ml Scintiverse LSC mixture in a Beckman LS-100 scintillation counter. One unit of enzyme activity equalled 1 pmole UMP incorporated into TCA-insoluble material in 30 min. The assays performed typically incorporated less than 0.1% of total UTP present.

Other RNA Polymerase Assay Conditions:

Studies of the RNA polymerases under various conditions required modifications of the assay media and/or procedures. In all cases, except for the assessment of ribonuclease presence, the assay medium was modified prior to addition of enzyme fraction. When an agent was simply to be added to the medium, e.g., morphine sulfate or α-amanitin, this was performed without alteration of the concentrations of the other components of the medium. In the studies utilizing synthetic poly dAdT as template, calf thymus DNA was deleted from the medium and replaced with the poly dAdT.

In studies of the ion dependent kinetics of the RNA polymerases, the RNA polymerases were usually dialyzed against TEMDG(25)+30, as described above. Thus, in the studies of the varying of ammonium sulfate and magnesium chloride concentrations, the beginning low concentrations of the salts could be 15 and 2 mM, respectively, if the enzyme fractions were diluted 1:1 by addition of an equal

volume of assay medium as in the usual procedure. Lower concentrations could be obtained by further dilution; higher concentrations were attained by addition of concentrated solutions to the individual assay tubes. In studies of the manganese dependencies of the RNA polymerases, it was necessary to dialyze the enzyme fractions against TEMDG(25)+30 buffer which did not contain magnesium chloride in order to prevent expression of magnesium-stimulated activity.

Studies of the temperature dependencies of the RNA polymerases required the preparation of separate buffers, and thus assay media, for each temperature to be studied. This was necessary because the tris-HCl buffer equilibrium pH became more basic as temperature decreased. Thus, tris-HCl buffers were prepared at pH7.9 at each temperature studied.

For determination of the pH optima for RNA polymerase activities, the basic pH required addition of glycine to increase the buffering capacity of tris at high pH. Tris-glycine-HCl buffers were thus prepared at the designated pH's at 37^{o}C.

In the studies for the estimation of the Km's of the substrates ATP and UTP, the particular nucleotide under study contained the tritium label present at the standard

concentration 30 uCi/ml. Additional unlabelled nucleotide was added to attain higher substrate concentrations. Only in the studies of Km of ATP was ^{3}H-ATP utilized. In all other studies, ^{3}H-UTP was the substrate whose incorporation was measured. In these studies of Km, the three nucleotides not under examination were present at 1 mM concentrations as usual.

Diphenylamine assay for deoxyribonucleic acid:

DNA concentrations were determined by the diphenylamine assay of Leyva and Kelley (34). An equal volume of 0.4M perchloric acid was added to each sample in a plastic centrifuge tube, and these were incubated at 4^{o} C for 1 hour. The samples were then centrifuged at 10,000 X g for 30 min at 4^{o}C. The supernatants were decanted, and the pellets received 0.25 ml of 1 M perchloric acid. This was followed by incubation at 70^{o}C for 1 hr. Incubation times in this procedure were double those reported (34) because plastic tubes were used. The assay tubes were cooled to room temperature for 1 hr, and 0.5 ml of freshly prepared chromogenic reagent was then added. Chromogenic reagent was 0.1 ml of 2% v/v acetaldehyde plus 20 ml diphenylamine solution (1.5 g diphenylamine plus 100 ml glacial acetic acid plus 1.5 ml concentrated sulfuric acid). The assay tubes were sealed with parafilm and incubated at 37^{o} C overnight. The tubes were then centrifuged at 10,000 x g

for 10 min. The absorbances of the supernatants at 600 nm were then measured in a Zeiss model PM2D spectrophotometer. DNA standards in TEMDG(25)+30 and TEMDG(25)+30 buffer blanks were prepared simultaneously with the samples for assay and contained also 0.1 mg bovine serum albumin to resemble more closely the enzyme samples themselves; this was as suggested (34).

Polyacrylamide Gel Electrophoresis:

Polyacrylamide gel electrophoresis was conducted under non-denaturing conditions according to the method of Maizel (35) or according to a modification of this method as described by Beckman and Frenkel (36). It had been reported by Schwartz & Roeder (37) that the RNA polymerases could be resolved electrophoretically using 5% polyacrylamide gels and non-denaturing conditions. Their system, modified from those of Laemmli (38) and Maizel (35), described a 10 cm resolving gel beneath a 3% stacking or spacer gel prepared at basic pH.

The resolving gel solution contained: acrylamide, 5%; N, N -bis-methylene acrylamide, 0.133%; tris-HCl, 0.375M, pH 8.9; N,N,N',N'-tetramethylethylenediamine (TEMED), 0.5% v/v; ammonium persulfate, 0.05%. In the modified method (36), the resolving gels were the same as above with the

additional component 0.1 mg/ml calf thymus DNA. The solutions were prepared such that ammonium persulfate was added last. Following this final addition, the solution was immediately transfered by pipette to 5 mm i.d. X 18 cm gel tubes to a solution height of 10.5 cm. Distilled water was then layered over each to produce a flat top to each gel. When polymerization was complete, generally after 30 min, the water layer was removed and the stacking gel solution then applied.

The stacking gel solution contained: acrylamide, 3%; N,N'-bis-methylene acrylamide, 0.08%; tris, 0.47 M; phosphoric acid, 0.256 M; TEMED, 0.5% v/v; ammonium persulfate, 0.1%. As above, ammonium persulfate was added last, and the stacking gel solution was immediately applied. Generally, 1.2 ml of stacking solution was applied to the top of each resolving gel. Stacking gels never contained DNA.

Enzyme fractions were dialyzed in TEMDG (25)+30 buffer and applied to gels. Enzyme fractions applied were typically 1.0 ml with an addtional 0.02 ml of 0.5% bromophenol blue solution thoroughly mixed into each sample. Electrode buffer (0.6% tris, 2.88% glycine) was layered over the samples and in both upper and lower chambers. Electrophoresis was conducted in a Hoeffer Scientific model GT-6 apparatus. This apparatus accomodated gel tubes up to 20 cm in length and was equipped with a

central cooling core. Water was circulated through the core from a bath maintained at 2°C. The gel apparatus was also packed in ice up to the level of the top of the upper chamber.

Current was applied to the gels at 1 mA per gel until the blue dye fronts had completely entered all the resolving gels. The current was then increased to 3 mA per gel until the dye fronts neared the ends of the gels. The gels were removed from the tubes with glass-distilled water, and the location of each dye front was marked by a small nick in the gel.

Gels which were to be stained were then subjected to the sequential staining/destaining process of Fairbanks (39). The process consisted of soaking the gels in each of four solutions for 8-12 hr. Solution 1: isopropanol, 25% v/v; Coomasie blue R, 0.05%; glacial acetic acid, 10% v/v. Solution 2: isopropanol, 10% v/v; Coomasie blue R, 0.005%; glacial acetic acid, 10% v/v. Solution 3: Coomasie blue R, 0.0025%; glacial acetic acid, 10% v/v. Solution 4: glacial acetic acid, 10% v/v. Gels received four equilibrations with the final solution 4.

Gels which had been polymerized in the presence of 0.1 mg/ml calf thymus DNA were either stained and destained as described above, or they were assayed for RNA polymerase

activity. The RNA polymerase assay was similar to that described above and was based on that developed for DNA polymerase (36). The only source of DNA template was within the gel itself along with the enzyme The gels were placed in media which supplied all of the other usual assay components, including ^{3}H-UTP, at concentrations calculated to be identical with normal when the volume of the gel was included as part of the total volume. These were then incubated at 37^{o}C for 60 min. Those gel regions which contained active RNA polymerase molecules thus would now also contain RNA fragments into which ^{3}H-UMP had been incorporated.

The media were then decanted, and the gels were submerged in ice-cold 10% TCA-PPi and placed in ice. After 1 hr, the solution was decanted and replaced by 5% TCA-PPi, and the gels were stored at 4^{o}C for 8-16 hr. The 5% TCA-PPi solutions were changed twice daily for periods of 7 to 10 days. The gels were then partially frozen on dry ice and sliced into 1 mm slices by means of a Mickle gel slicer (Brinkman Instruments) (40). The slices were placed in individual scintillation counting vials (7 ml capacity). Slices were dissolved by addition of 0.2 ml of 30% hydrogen peroxide to each vial. The vials were then sealed with caps and incubated at 60^{o}C overnight (41). 4 ml of Scintiverse was then added to each vial, and the ^{3}H

content of each sample was measured in a Packard PL Tri-Carb liquid scintillation counter.

Some gels were scanned at wavelengths of 500 nm or of 280 nm in a Gilford model 2520 gel scanner attached to a Gilford model 220 spectrophotometer. Gels which were to be scanned at 280 nm were prepared without the bromophenol blue tracking dye; the dye showed strong absorption at this wavelength. In these cases, two gels were prepared identically except that only one contained tracking dye. The dye in the one gel was used to indiciate the dye fronts of both gels in the pair.

Morphine Tolerance-Dependence Induction:

Mice were rendered tolerant-dependent to morphine by the method of Way et al (42), which was a modification of the method of Huidobro and Maggiolo (43). A pellet containing 75 mg of morphine base was implanted subcutaneously in the backs of the mice for 68-72 hr. Control animals received placebo pellets. Way et al. (42) and Wei and Way (44) demonstrated that tolerance and physical dependence reached a maximum after 3 days implantation.

Lee et al. (20) demonstrated that implantation of a 10 mg naloxone pellet 2 hr prior to the morphine pellet implantation prevented the development of analgesic

tolerance. Their procedure was followed for experiments designed to show narcotic specificity by this naloxone reversal. Four experimental groups of mice were implanted with two pellets: two placebo pellets implanted 2 hr apart, a placebo pellet followed by a morphine pellet, a naloxone pellet followed by a placebo pellet, and a naloxone pellet followed by a morphine pellet.

Way et al. (42) demonstrated that removal of morphine pellets after 68 hr implantation induced withdrawal syndrome which could be measured within 4 hr after pellet removal. In order to determine whether observed effects of chronic morphine treatment were reversible in the same manner as tolerance and physical dependence, pellets were removed as described by Ho et al. (45).

Removal of the pellets required new incisions at the site of the pellet and sometimes the scraping of the tissue to remove all adhering pellet substance. These new incisions were closed with Clay-Adams wound clips. Mice were sacrificed 10 days after the pellet removal. The 10 day period was greater than three times the length of time allowed for the induction of alterations produced by morphine. All procedures were duplicated in mice receiving placebo pellets. The mortality rate resulting from these procedures was less than 1%, negligible relative to untreated animals.

Acute Morphine Sulfate Injections:

Mice were injected intracerebroventricularly (icv) with 0.005 ml either of 0.9% sodium chloride or of 2 mg/ml morphine sulfate (MS) in 0.9% sodium chloride. The 23-27g mice receiving morphine thus were injected with 0.01 mg of morphine sulfate (46). Mice were sacrificed one hour after injection. Subjective observations of the behavior of the animals were recorded and indicated that the saline injections resulted in slight sedation while MS induced running and straub tails after 20-30 min. MS animals exhibited a noticeable lack of struggling when being held for decapitation; saline controls showed the usual amount of struggling.

Second DEAE-Sephadex A-25 Chromatography:

In some studies, the RNA polymerase activity eluted by TEMDG(25)+225 was dialyzed against 100 volumes of TEMDG(25)+30 for 1 hour, four times. This dialyzed fraction was then applied to a DEAE-Sephadex A-25 column identical to the first column. RNA polymerase activity was eluted from this second column by a linear ammonium sulfate gradient. The gradient was generated by a Pharmacia GM-1 gradient maker using TEMDG(25)+30 as the starting buffer and TEMDG(25)+500 as the buffer added in the gradient maker. Gradients designed in this manner

were developed until half consumed. Deviations from linearity which were observed to occur in the final fractions when gradients were fully developed could thus be avoided. The conductivities of fractions eluted from the column were measured by use of a Radiometer Copenhagen model CDM 2d. Measurement of the conductivities of TEMDG(25)+X buffers indicated that the increase in conductivity was directly proportional to the increase in ammonium sulfate concentration, i.e., a linear relationship, over the range of TEMDG(25)+30 to TEMDG(25)+1000.

Preparation of Liver RNA Polymerases

Studies of liver RNA polymerases were conducted in a manner closely resembling that used for the brain enzymes so that the two could be compared under the most similar conditions. The method for preparation of oligodendroglial nuclei reported by Oguri *et al*. (22) was a modification of the method of Blobel and van Potter (47) for liver nuclei. Thus, the methods were already very similar.

Six mouse livers, weighing approximately 2g each, were used for each preparation. The livers were weighed and homogenized in 2 ml of 0.25 M sucrose TKM per gram of liver. The homogenate was filtered and the total volume

determined. Twice this volume of 2.3 M sucrose TKM was added and mixed. This hypertonic homogenate was layered over 9 ml of 2.3 M sucrose and submitted to ultracentrifugation as described above for the brain nuclei. The liver nuclei were collected, solubilized, and dialyzed as described above.

The soluble activity, dialyzed in TEMDG (25) + 30, was applied to a 0.9 cm X 14 cm column of DEAE-Sephadex A-25 which had been prepared as described above. The RNA polymerase activities were eluted from this column by a linear gradient of ammonium sulfate generated by a Pharmacia GM-1 gradient maker. The gradient was developed from TEMDG (25)+30 to TEMDG (25)+500. The total elution volume was 12 column volumes; fractions equal to 25% of the column volume were collected. Weaver et al (28) reported that the liver RNA polymerases could be separated on this resin by a linear salt gradient.

RNA polymerase assays were conducted as described above. Protein concentrations were determined by the Lowry method (32) following quantitative dialysis of the samples as described above.

Protein Kinase Phosphorylation of RNA Polymerases

Protein kinase was isolated and purfied from mouse brain oligodendendroglial nuclei and supplied by Vivian Y.H. Hook of the Department of Pharmacology, University of California, San Francisco. The method of preparation was as reported by Hook et al. (23), and is briefly summarized below. The oligodendroglial nuclei were prepared and the protein kinase activity was solubilized by sonication in high salt medium by the identical methods described above for the RNA polymerases. The sonicated supernatant was subjected to 40% ammonium sulfate precipitation, and the precipitate was resuspended, dialyzed against TEMD (nomenclature as above), and applied to a phosphocellulose column. Protein kinase activities were eluted by a linear gradient of sodium chloride, resulting in resolution of four peaks of PK activity. The first-eluting and major peak of activity was cAMP-independent and was shown to be increased 25% in specifc activity following chronic morphine treatment (23). This peak was the protein kinase employed in these experiments.

Ten ug of RNA polymerase I, II, or III was preincubated with varying amounts of the major peak of cAMP-independent nuclear protein kinase with final concentrations of 50 mM potassium phosphate buffer pH 6.5 or 7.4, 0.3 mM EGTA, 10 mM NaF, 10 mM $MgCl_2$, 20 uM ATP, 2 uCi γ-^{32}P- ATP per tube

for 5 min at 30 °C. At the end of the 5 min preincubation, the tubes were placed on ice, and the RNA polymerase assay medium was immediately added to each tube to attain the final concentrations: 0.1 M Tris-HCl, pH 7.9 at 37°C, 0.1 mg/ml calf thymus DNA, 1 mM ATP, 1 mM CTP, 1 mM GTP, 0.02 mg/ml bentonite, 5 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM EDTA, 1 mM DTT, and H-UTP. The samples were incubated for 30 min at 37 °C and the reaction stopped with 5 ml of 10% TCA-3% Na-PPi. The samples were washed and counted as described above for the RNA polymerase assay except that two channels of the scintillation counter were utilized, ^{3}H and ^{32}P.

RESULTS

RNA POLYMERASES FROM NAIVE MICE

RNA Polymerase Purification:

RNA polymerase was solubilized by procedures described in Methods. The pellet of oligodendroglian nuclei was resuspended in TEMDG (25) + 30. The suspension was adjusted to 0.33 M ammonium sulfate, pH 7.9. This suspension was then sonicated by using a Branson model W140D Sonifier (microtip, setting 5) for 15-sec intervals until the suspension was no longer clouded and nearly free-flowing (F2). The suspension was then centrifuged in a Beckman SW 65 rotor at 220,000 g for 50 min. The supernatant fraction (F3) was concentrated to 3.0 ml in a collodion bag with aspiration. This concentrated solution (F4) was dialyzed against TEMDG (25) + 30. DEAE-Sephadex (A-25) was suspended in TEMDG (25) + 1000 and washed with 10 vol. TEMDG (25) + 30. The dialyzed F5 solution was applied to the DEAE-Sephadex column at about 10 ug protein/ml bed volume. The column was then washed with two column volumes of TEMDG (25) in 30 mM $(NH_4)_2$ SO_4. The RNA polymeras activities were eluted by ammonium sulfate concentrations increased in steps: two column volumes each of TEMDG (25) + 225, TEMDG (25) + 275 mM, and TEMDG

(25) + 1000 were applied. Fractions equal to one-third the column volume were collected. These fractions (as well as F1 to F5) were dialyzed to TEMDG (25) + 30. These dialyzed fractions were then assayed for RNA polymerase activity. Protein concentrations were determined according to the method of Lowry et al. (32) after the samples were dialyzed against distilled water.

The results of the solubilization and purification are summarized in Table 1. In each assay, a limited amount of the fraction was used in order to insure linearity of enzyme activity. The percentage yield of nuclei from the total brain homogenate is low, a recovery of 10 per cent of total brain homogenate DNA (22). Thus, the initial fractionation and sonication (F2) resulted in an activity equal to 15 per cent of that from total brain homogenate (F1). The ammonium sulfate elution profile on the DEAE-Sephadex column is shown in Fig. 1. Three enzymatic activities, referred to as peak 1, peak 2 and peak 3, were detected with different ammonium sulfate concentrations, 0.225, 0.275 and 1 M, respectively. The degrees of purification relative to whole brain homogenate were 47-, 118- and 27-fold, respectively.

Table 1

PURIFICATION OF RNA POLYMERASE FROM OLIGODENDROGLIAL NUCLEI OF MOUSE BRAIN *

FRACTION	TOTAL ACTIVITY (UNITS)	TOTAL PROTEIN (MG)	SPECIFIC ACTIVITY (UNITS/MG PROTEIN)	PURIFICATION FACTOR	ACTIVITY RECOVERY (%)
F1	8298	2630	3.16	1	100
F2	1286	25.8	49.8	16	15
F3	859	18.3	46.9	15	10
F4	320	4.2	76.9	24	4
DEAE-SEPHADEX	234	3.5			3
PEAK 1	67	0.77	147+	47	1
PEAK 2	80	0.47	373+	118	1
PEAK 3	56	1.73	85+	27	1

*EXPERIMENTAL DETAILS OF FRACTION PREPARATION AND DESIGNATION ARE DESCRIBED IN THE TEXT.

+SPECIFIC ACTIVITIES OF PEAKS 1, 2, AND 3 REPRESENT FRACTIONS OF MAXIMUM SPECIFIC ACTIVITY WITHIN EACH PEAK (AS SHOWN IN FIGURE 1).

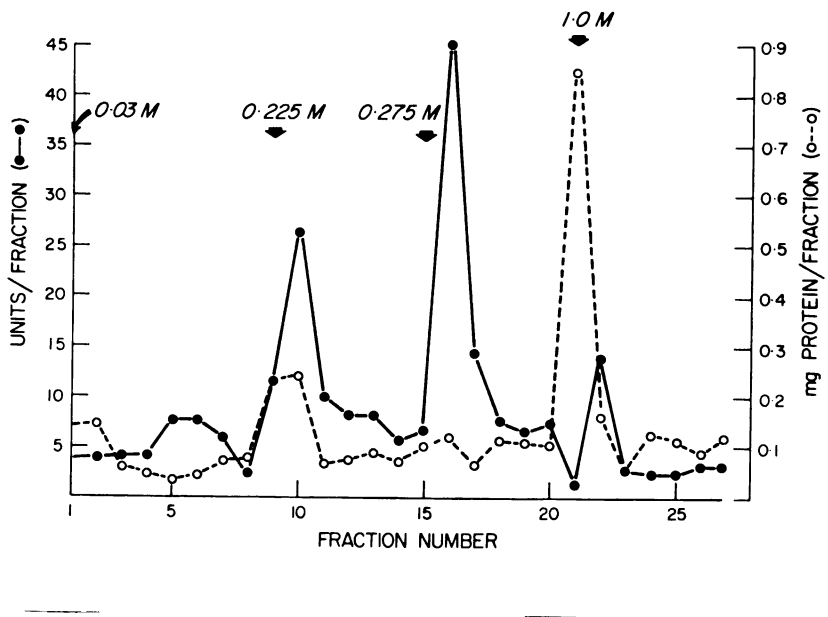


Figure 1. DEAE-Sephadex A-25 elution profile of oligodendroglial nuclear RNA polymerases. Elution was by $(NH_4)_2SO_4$, pH 7.9. Enzyme was applied at 0.03 M $(NH_4)_2SO_4$, and elution was by increasing steps of $(NH_4)_2SO_4$ concentration. Arrows indicate first fractions containing $(NH_4)_2SO_4$ at the concentrations indicated. Fraction volumes of one-third column volume were colected and assayed for enzyme activity (●—●) and protein concentration (o---o) as described.

RNA Polymerase Characterization:

The RNA polymerase activities in peaks 2 and 3 depended on the addition of exogenous DNA (Table 2). These enzymes were thus clearly DNA-dependent. Without exogenous DNA added, there was essentially no UTP incorporation. Peak 1, however, showed only partial dependence on additional DNA. This was likely due to the contamination of this fraction by endogenous DNA. The peak 1 fraction contained DNA at 69 ug/ml, while peaks 2 and 3 contained 14 and 21 ug/ml, respectively. When actinomycin D was added to the medium at the concentration 25 ug/ml, in the presence of exogenous calf thymus DNA, 18% of peak 1 activity remained. Actinomycin D has been shown to inhibit DNA-dependent RNA polymerase activity by preventing the binding of the enzyme to the template (48). The peak 1 RNA polymerase could thus also be concluded to be DNA-dependent.

In order to assess the ribonuclease contamination of the RNA polymerase fractions, the inhibitory effect of actinomycin D (as shown in Table 2) was utilized as described in methods. Following incubation of each polymerase in standard conditions for 15 min, actinomycin D was added to a final concentration of 25 ug/ml. Incubation was then continued for an additional 15 min. Table 3 shows the relative increase in UMP incorporation

TABLE 2

DNA DEPENDENCIES OF RNA POLYMERASE FRACTIONS FROM

DEAE - SEPHADEX CHROMATOGRAPHY

RNA POLYMERASE PEAK	SPECIFIC ACTIVITY (UNITS/MG PROTEIN) WITHOUT EXOGENOUS DNA	SPECIFIC ACTIVITY (UNITS/MG PROTEIN) WITH 100 UG/ML CALF THYMUS DNA	ENDOGENOUS DNA CONTENTS (UG/ML)	% OF CONTROL ACTIVITY WITH 25 UG/Ml ACTINOMYCIN D
1	21.3	50.9	69	18
2	3.4	239	14	7
3	0	64.8	21	9

TABLE 3

ASSESSMENT OF APPARENT RIBONUCLEASE ACTIVITIES

% CHANGE IN H-UMP INCORPORATION IN 15 MINUTE ADDITIONAL INCUBATION PERIOD WITH 25 UG/ML ACTINOMYCIN D	
PEAK I RNA POLYMERASE	+4%
PEAK II RNA POLYMERASE	+3%
PEAK III RNA POLYMERASE	+9%

SAMPLES WERE INCUBATED IN THE NORMAL MANNER TO SYNTHESIZE RNA. ACTINOMYCIN D WAS ADDED AND INCUBATION CONTINUED FOR 15 MINUTES. NO DECREASE IN UMP INCORPORATION AFTER THIS PERIOD INDICATES NO SIGNIFICANT RIBONUCLEASE CONTAMINATION.

during the second 15 min incubation. The three RNA polymerases showed slight increases in UMP incorporation during this period. The absence of a decrease in TCA-insoluble ^{3}H activity as a result of this continued incubation indicated that ribonuclease contaminations of the peaks 1, 2 and 3 RNA polymerases were insignificant. The question to be considered in this assessment was operational, and the presence of significant amounts of product-destroying activity was not observed. All assays were conducted in the presence of bentonite, a ribonuclease inhibitor (49), and ribonuclease activity would not be expected to be observed. Similar experimental studies of brain homogenate did, however, reveal a 10% decrease in TCA-insoluble ^{3}H during the second incubation. This method thus was capable of indicating ribonuclease presence in a preparation known to contain ribonuclease (18).

Among the distinguishing properties of the three RNA polymerases typical of eukaryotes have been their relative sensitivities to inhibition by the toxin α-amanitin from the mushroom amanita phalloides (27, 28, 29). RNA polymerase II has typically been observed to be extremely sensitive, polymerase III to show moderate sensitivity and polymerase I to be insensitive to the toxin (50). The activity in peak 2 was most sensitive to α-amanitin (Table 4). In the presence of a low concentration of α-amanitin,

TABLE 4

SENSITIVITIES OF RNA POLYMERASES TO α-AMANITIN

RNA POLYMERASE	% of control of remaining activities	
	+ α-Amanitin (10 ng/ml)	+ α-Amanitin (5 ug/ml)
PEAK 1	73.5	36.0
PEAK 2	29.5	4.5
PEAK 3	76.5	45.5

RNA polymerase peaks 1, 2, and 3 eluted from DEAE-Sephadex A-25 at ammonium sulfate concentrations 0.225 M, 0.275 M, and 1.0 M, respectively, as described in Methods.

10 ng/ml, the activity was reduced to 29.5 per cent of the control. Higher concentration of the toxin, 5 ug/ml, inhibited virtually all the enzyme activity.

The relative α-amanitin inhibitions for peak 1 and peak 3 were not as clearly distinct. α-Amanitin, at 10 ng/ml, inhibited both peaks to about the same degree. When the α-amanitin concentration was increased to 5 ug/ml, the activities for peak 1 and peak 3 were inhibited to 36 and 45.5 per cent of control, respectively. It was, therefore, difficult to identify peak 1 and peak 3 according to α-amanitin sensitivity. Peak 2 was most sensitive to α-amanitin and, thus, it appeared that peak 2 represented the RNA polymerase responsible for HnRNA. Hager et al. (51) reported the identification of RNA polymerase I in Saccharomyces cerevisiae which was also sensitive to α-amanitin, but which exhibited characteristics common to most RNA polymerase I forms which have been studied. These authors strongly urged that multiple criteria be utilized for the identification of the multiple RNA polymerases.

Eukaryotic RNA polymerases have been shown to require the presence of several inorganic ions for their activity. The relative activities in the presence of these ions in some cases have been used as criteria for identifying the multiple forms. Very few thorough reports of the ion

dependencies of the RNA polymerases have been published. Rather, historically significant distinguishing characteristics have been emphasized in most reports. The most completely studied system to date has been the mouse myeloma tumor cell line MOPC 315 (29). The ion-dependent kinetics of RNA polymerases I, II, and III from these cells have been shown to differ relative to varying concentrations of ammonium sulfate, magnesium chloride, and manganese chloride. For comparison with this well-studied system and for the supplication of additional information by means of which to identify the brain RNA polymerases, similar studies were conducted in this brain system.

The responses of the peaks 1, 2 and 3 RNA polymerases to various concentrations of ammonium sulfate in the assay medium are represented graphically in Figure 2. Two particular characteristics of the responses are immediately apparent: the three polymerases showed distinct patterns; the peak 3 RNA polymerase exhibited a biphasic response. The optimal concentrations for polymerases 1, 2 and 3 of 0.07M, 0.03M, and 0.015/0.115M, respectively, did not correspond closely with those shown for MOPC 315 (29). A review of RNA polymerase studies by Roeder (50), which included unplubished results, showed that the optima pictured in Figure 2 were within the limits demonstrated elsewhere. Particularly

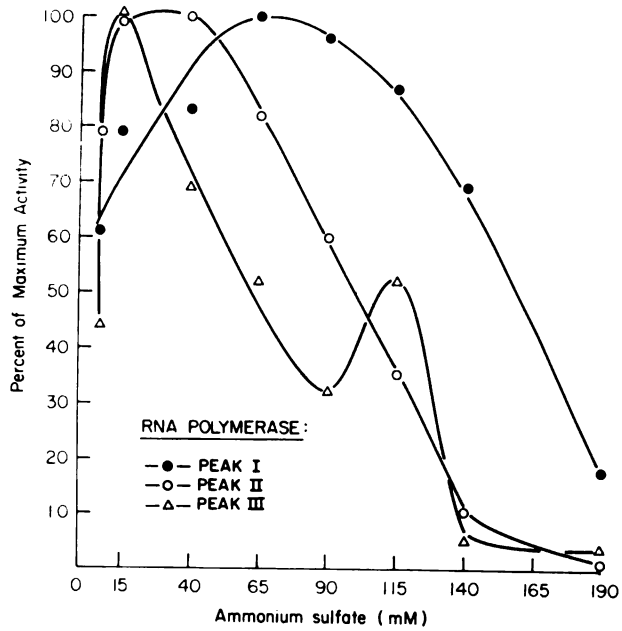


Figure 2. Ammonium sulfate - stimulated activities of RNA polymerases: ●—● peak 1; o-o peak 2; △—△ peak 3. RNA polymerases prepared and assayed as described in Methods.

characteristic was the biphasic response of the peak 3 RNA polymerase. The appearance of two optima was both distinct to RNA polymerase III and a requirement for its definitive identification.

The magnesium-stimulated activities of the three RNA polymerases assayed in the absence of magnanese are shown in Figure 3. The optimal magnesium concentrations of 10 mM and 8 mM for the peaks 1 and 2 polymerases, respectively, were identical to those reported for MOPC 315 RNA polymerases I and II (29). The peak 3 RNA polymerase did not show such a similar correspondence. The MOPC 315 system was described as containing two RNA polymerase III activities, referred to as IIIA and IIIB. The III A activity did not reveal a true optimal magnesium concentration. The III B activity was maximal at 8-10 mM magnesium. The peak 3 RNA polymerase optimum of 6 mM thus was dissimilar to both RNA polymerases IIIA and IIIB from MOPC 315 (29).

The manganese-stimulated activities of the peaks 1, 2, and 3 RNA polymerases are shown in Figure 4. As described in Methods, the RNA polymerases were dialyzed prior to assay against TEMDG(25)+30 which did not contain the usual magnesium. These activities were thus measured in the absence of magnesium. The optimal concentrations of 4, 4, and 6 mM manganese for peaks 1, 2, and 3 activities,

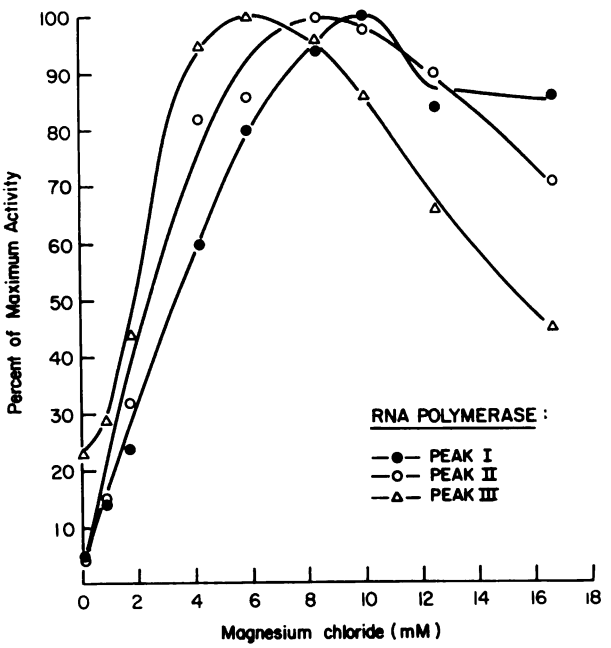


Figure 3. Magnesium-stimulated activities of RNA polymerases: ●—● peak 1; o—o peak 2; △—△ peak 3. Assays performed in absence of Mn^{+2}. RNA polymerases prepared as described in Methods.

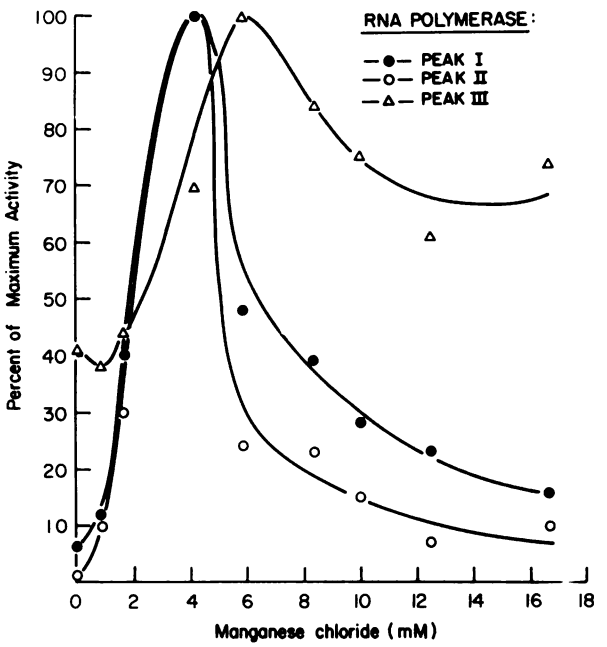


Figure 4. Manganese-stimulated activities of RNA polymerases: ●—● peak 1; o-o peak 2; △—△ peak 3. Assays performed in absence of Mg^{+2}. RNA polymerases prepared as described in Methods.

respectively, were totally unlike those of the MOPC 315. In the latter system, all RNA polymerases exhibited maximal activity at the concentration of 2 mM manganese. This dissimilarity further serves to emphasize the unique nature of this system.

Assessment of the magnesium and manganese optima made possible the measurement of another documented distinguishing criterion. Roeder (50) reported that the ratios of manganese- to magnesium-stimulated activities were distinct among the RNA polymerases. These were the ratios of activities at respective concentrations of Mn^{+2} and Mg^{+2} which showed greatest activity when enzymes were assayed in the presence of only one of the divalent cations. The Mn^{+2} /Mg^{+2} activity ratios reported for RNA polymerases I, II, and III were 1, 5-10, and 2, respectively (50). The ratios observed for the peaks 1, 2 and 3 RNA polymerases were 1.1, 9.9, and 1.7, respectively. There was clearly excellent concordance between the predicted and the observed values.

The final distinguishing characteristic to be considered was the relative preference of each enzyme for the transcription of two different DNA templates. RNA polymerase forms had been shown to transcribe the synthetic template polydeoxyadenylate-deoxythymidylate alternating copolymer, referred to as poly dAdT. This

template was double-stranded DNA consisting only of the two bases in precise alternating sequence. Relative activities were measured with concentrations of 0.1 mg/ml calf thymus DNA or 0.05 mg/ml poly dAdT. These template concentrations were shown to yield maximal activity for each of the peaks 1, 2 and 3 RNA polymerases and were identical to those employed by Schwartz et al. (29). The ratios of the activities, comparing poly dAdT/DNA, for peaks 1, 2, and 3 were 2.5, 1.9, and 5.2, respectively. The expected ratios (50) for RNA polymerases I, II, and III were 1-2, 0.5-1, and 5, respectively. These ratios were thus similar to those predicted.

Table 5 summarizes the four principal distinguishing characteristics as measured for this brain RNA polymerase system. The characteristics of these RNA polymerases 1, 2, and 3 fall within the limits of those reported in other eukaryotic systems. Thus, it was concluded that the peaks 1, 2, and 3 RNA polymerases, prepared as described, respectively each contained primarily the RNA polymerases I, II, and III which have been demonstrated in other systems to transcribe 18S and 28S rRNA's, hnRNA (precursor to mRNA), and tRNA and 5S rRNA, respectively. The peaks 1, 2, and 3 RNA polymerases will, therefore, be subsequently referred to as RNA polymerases I, II, and III. This will be abbreviated as RNAP I, RNAP II, and RNAP III, according to the common practice.

TABLE 5

GENERAL PROPERTIES OF RNA POLYMERASES FROM

MOUSE BRAIN OLIGODENDROGLIAL NUCLEI

RNA POLYMERASE	PEAK 1	PEAK 2	PEAK 3
α-AMANITIN[a] SENSITIVITY	MODERATE	HIGH	MODERATE
OPTIMAL ACTIVITY,[b] CONCENTRATION AMMONIUM SULFATE	0.07 M	0.03 M	0.015 M and 0.115 M
ACTIVITY RATIO,[c] Mn^{+2}/Mg^{+2}	1.1	9.9	1.7
ACTIVITY RATIO,[d] poly dAdT/DNA	2.5	1.9	5.2

Legend for Table 5

GENERAL PROPERTIES OF RNA POLYMERASES FROM

MOUSE BRAIN OLIGODEDROGLIAL NUCLEI

RNA polymerase peaks 1, 2, and 3 eluted from DEAE-Sephadex A-25 at ammonium sulfate concentrations 0.225 M, 0.275 M, and 1.0 M, respectively, as described in Methods.

a Results as shown in Table 5

b Results as shown in Figure 2.

c Ratio of activities at respective concentrations of Mn^{+2} and Mg^{+2} which showed greatest activity; as shown in Figures 3 and 4 for this determination only, enzymes were assayed in the presence of only one of the divalent cations.

d Ratio of activities at 0.05 mg/ml poly dAdT and at 0.1 mg/ml calf thymus DNA; these concentrations of template showed greatest activities.

Additional studies were carried out for the elucidation of properties of the RNA polymerases which were likely to be important in further studies of the enzymes. Specifically, these were the determinations of optimal temperature and pH for assay of each RNAP, the estimations of the Km's of the substrates, and assessments of the relative purities of the RNAP's.

Figure 5 shows the relative activities of the three RNAP's when assayed for 30 min at various temperatures. The optimum temperature observed for each RNAP was physiological 37°. Both RNAP I and RNAP II showed broad optima in the range of 30°-37° while RNAP III activity did not show as broad an optimum.

The three RNA polymerases showed maximal activity at the assay pH of 8.0, as seen in Figure 6. RNAP II exhibited a distinct optimum at pH 8.0. RNAP I activity was not inhibited to as great an extent by increasing the pH to 8.5 and 9.0. RNAP III activity did not decrease greatly when the pH was lowered to 7.5. The reactions catalyzed by the three enzymes are chemically very similar (in fact, they might easily be considered identical). Thus, the differences in their inhibitions by changes in pH away from the 8.0 optimum should not be due to alterationns of the reaction equilibria.

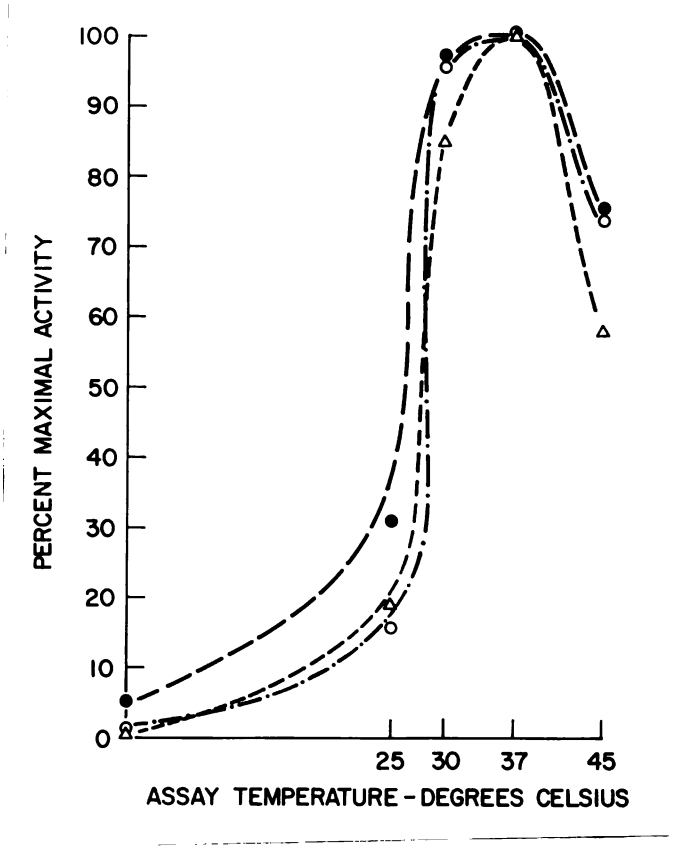


Figure 5. Effects of different assay temperatures on activities of RNA polymerases: ●-● I; o-o II; ▲-▲ III. Separate Tris-HCl buffers for pH 7.9 at each temperature and RNA polymerases were prepared as described in Methods.

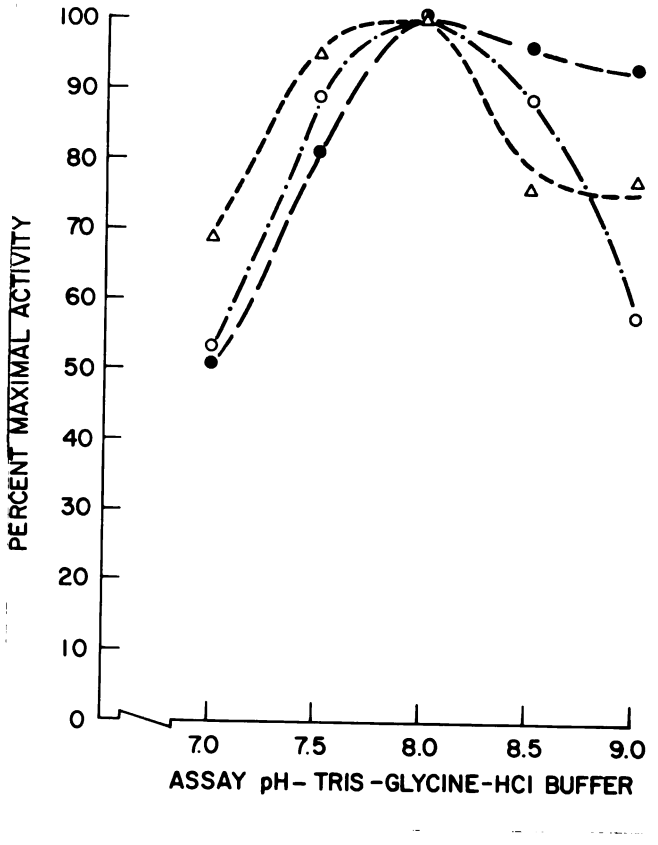


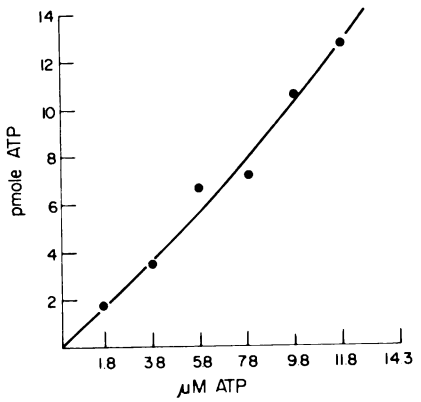
Figure 6. Effects of different assay pH on activities of RNA polymerases: ●-● I; o-o II; △—△ III. Separate Tris-glycine-HCl buffers for each pH at 37°C and RNA polymerases were prepared as described in Methods.

In the studies heretofore described, the RNA polymerase assay measured the incorporation of ^{3}H-UTP into RNA as ^{3}H-UMP. The affinities of the three RNA polymerases for the UTP substrate were studied for the purpose of estimation of the Km for each enzyme. For comparison, similar studies were conducted on the substrate ATP. This substrate was selected because of the base pair relationship of adenine and uridine in nucleic acids and because in this manner a purine as well as a pyrimidine ribonucleotide would be examined.

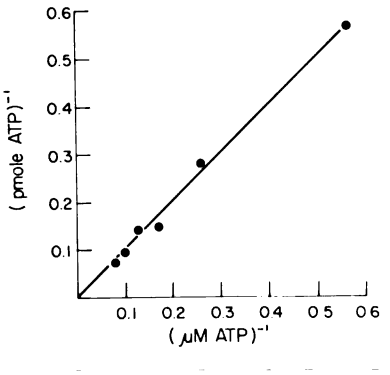
For the study of UTP, ^{3}H-UMP incorporation was measured. The results of these studies are shown in Figures 7 and 8 for RNAP I and RNAP II, respectively. At the low substrate levels utilized, RNAP III activity could not be measured. As seen in Figure 1, very little of this activity was eluted from the DEAE-Sephadex column.

Figures 7 and 8 show that for both substrates for both enzymes, the Michaelis plots were not hyperbolic. In Figures 8A and C it is seen that the RNAP II showed a sigmoidal response relative to increasing substrate concentrations. The resultant Lineweaver-Burke plots of these values were not linear. It was thus not possible to derive Km values from the results shown in Figures 7 and 8.

FIGURE 7



A



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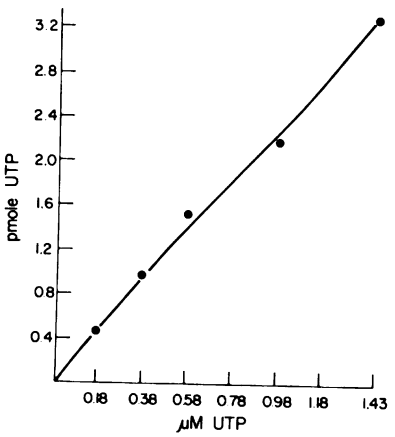
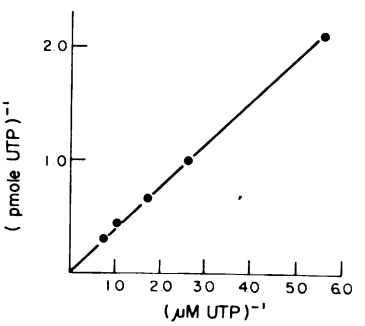


C

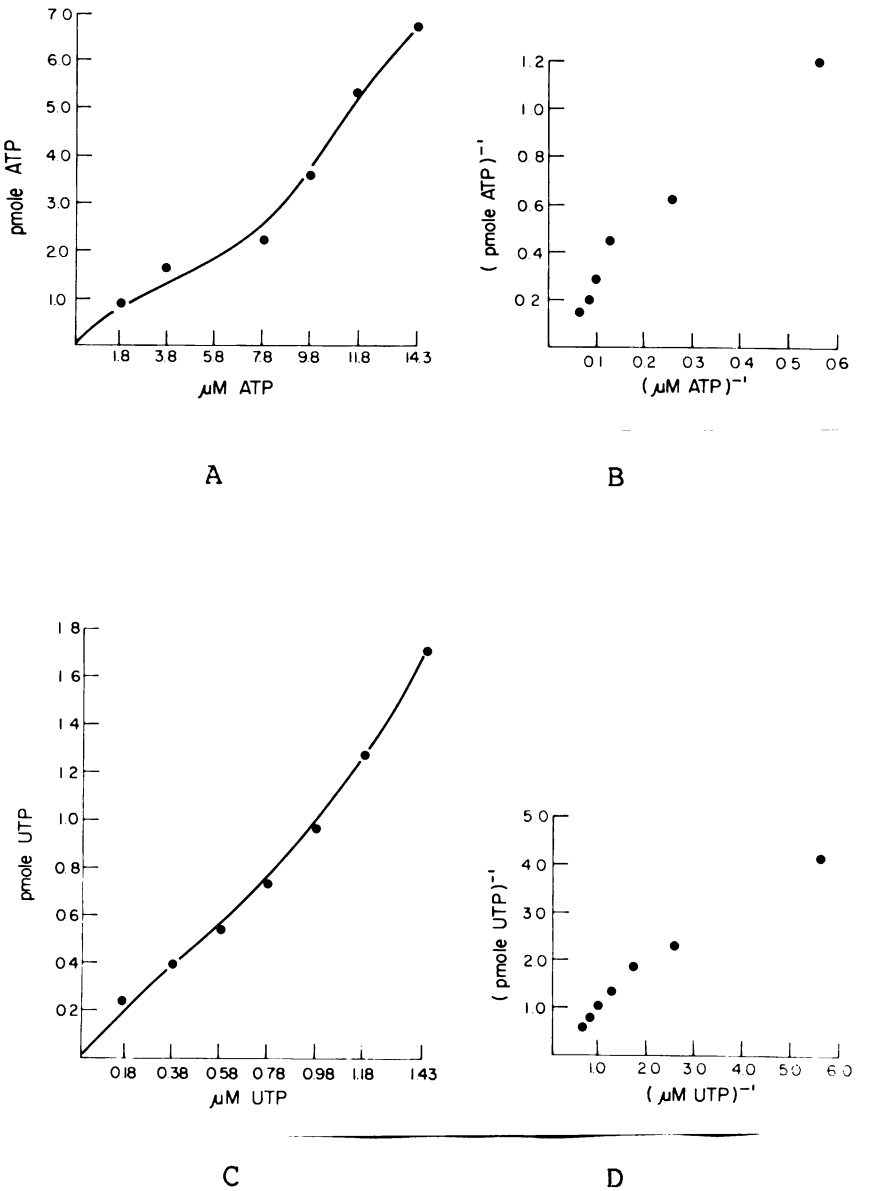


D

Figure 7. Substrate affinities of RNA polymerase I for ATP and UTP. A: Michaelis plot for ATP; ^{3}H-ATP as labelled nucleotide. B: Lineweaver-Burke plot of data shown in Figure A. C: Michaelis plot for UTP; ^{3}H-UTP as labelled nucleotide. D: Lineweaver Burke plot of data shown in Figure C. Assays performed as described in Methods.

Figure 8. Substrate affinities of RNA polymerase II for ATP and UTP. A: Michaelis plot for ATP; ^{3}H-ATP as labelled nucleotide. B: Lineweaver-Burke plot of data shown in Figure A. C: Michaelis plot for UTP; ^{3}H-UTP as labelled nucleotide. D: Line-weaver Burke plot of data shown in Figure C. Assays performed as described in Methods.

FIGURE 8


pmole ATP
7.0
6.0
5.0
4.0
3.0
2.0
1.0
1.8 3.8 5.8 7.8 9.8 11.8 14.3
μM ATP
A
(pmole ATP)⁻¹
1.2
1.0
0.8
0.6
0.4
0.2
0.1 0.2 0.3 0.4 0.5 0.6
(μM ATP)⁻¹
B
pmole UTP
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.18 0.38 0.58 0.78 0.98 1.18 1.43
μM UTP
C
(pmole UTP)⁻¹
5.0
4.0
3.0
2.0
1.0
1.0 2.0 3.0 4.0 5.0 6.0
(μM UTP)⁻¹
D

These patterns suggest cooperativity of enzyme-substrate association, but the complexity of this system (template, enzyme, four substrates) precludes any conclusions of this nature based solely on these studies. If, for example, the rate determining step of RNA synthesis were initiation, apparent cooperativity might be observed when none existed. In such a case, increasing the substrate concentration may stimulate initiation. Once transcription was initiated, the substrate incorporation might be expected to follow Michaelis -Menten kinetics. The order of association and dissociation of the various components is unknown, and thus many explanations are possible.

In order to identify the presence of additional contaminating proteins in the RNAP fractions, polyacrylamide gel electrophoresis was conducted in non-denaturing conditions. As stated in the Methods, it had previously been demonstrated (37, 52) that the RNA polymerases would enter a 5% polyacrylamide gel in the absence of sodium dodecyl sulfate (SDS). These workers had also shown that addition of SDS dissociated the RNA polymerases into six (or more) subunits which could be observed in the SDS gel. For the assessment of relative purity, the SDS system would thus be unsuitable because the appearance of multiple proteins would be expected in every case, regardless of the fractions' purities.

As described in the Methods, the 5% polyacrylamide gels were prepared containing 0.1% calf thymus DNA. Above these were layered 3% stacking gels of volume 20% greater than the sample volumes. Gels were run in duplicates. Several protocols for the duplicate gels were followed in various experiments. In early experiments, both samples contained tracking dye; one gel of each pair was assayed for RNA polymerase activity; the other gel was submitted to the Fairbanks staining procedure (39) for detection of proteins. While RNAP I, and II activities were measured in discrete positions in the assayed gels, no proteins could be detected in the stained gels. RNAP III activity could not be detected in the assayed gels. The net measured activities of RNAP I and II in their respective gels were less than 5% of the total activity applied. The small amount of RNAP III activity eluted from the DEAE-Sephadex column would make impossible the detection of this enzyme's activity in the light of the loss of 95% of activity as a result of this procedure. It was observed that migrations in each pair of gels were identical: at all times throughout electrophoresis, the dye fronts were observed to be at the same relative positions.

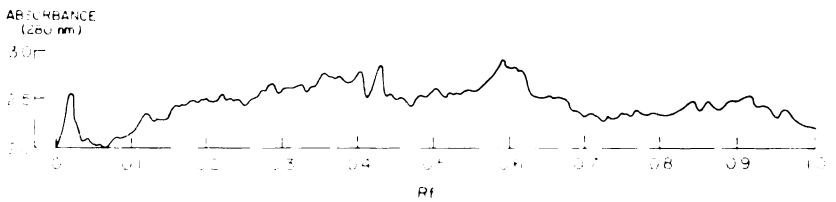
An alternative protocol, based on these observations, was developed wherein only one sample of each pair contained bromophenol blue tracking dye. Following electrophoresis, the gel which did not contain the dye was subjected to

spectrophotometric scan at 280nm. Bromophenol blue was observed to have a strong absorbance at this wavelength, precluding such scans of gels containing the dye. Following the scan, a three minute procedure, both gels of each pair were assayed as described in Methods. Figures 9 and 10 show the results of this procedure for RNAPI and RNAPII, respectively. These figures show the relative migrations (expressed as Rf) of RNA polymerase activity and of protein. It can be seen in these figures that the absorbances of the gels were very strong at 280nm. The DNA contained in the gels exhibited an absorption maximum at 262nm; this absorbance was still very high at 280nm.

The RNAP I activity was observed at Rf 0.6. A small increase in the absorbance at 280 nm can also be seen at this position indicating the presence of protein. The RNAP II activity was observed at Rf 0.73. The spectrophotometric tracing indicated that protein was also present in this position. These activities were the only reproducible identifiable activities observed. It can be seen that in both RNAP I and II fractions, there was apparently contaminating protein present at Rf 0.3 - 0.4. In addition, the RNAP II contained a significant amount of protein at Rf 0.9.

The RNAP I and II fractions did not contain significant amounts of each other's activity, nor was protein observed

FIGURE 9


Rf

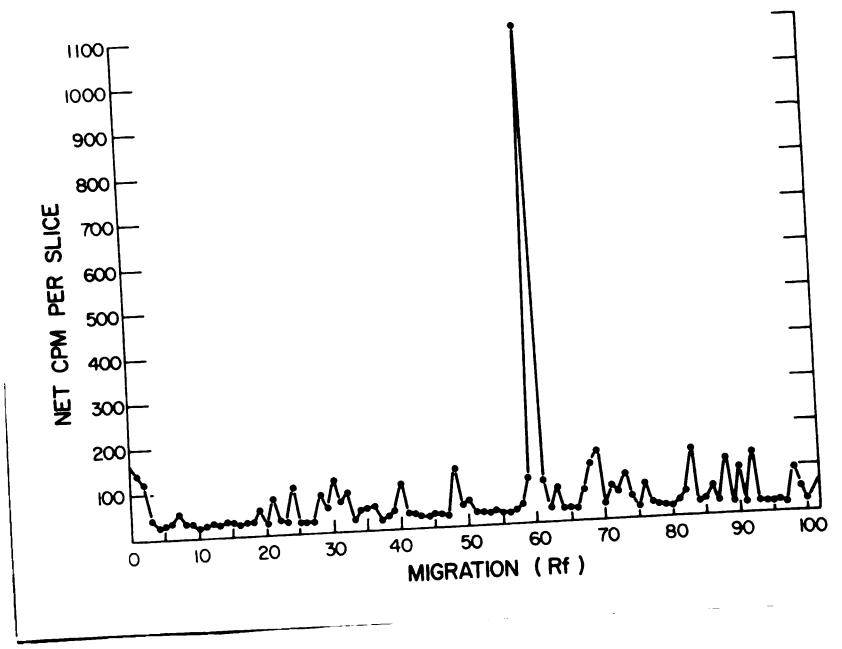
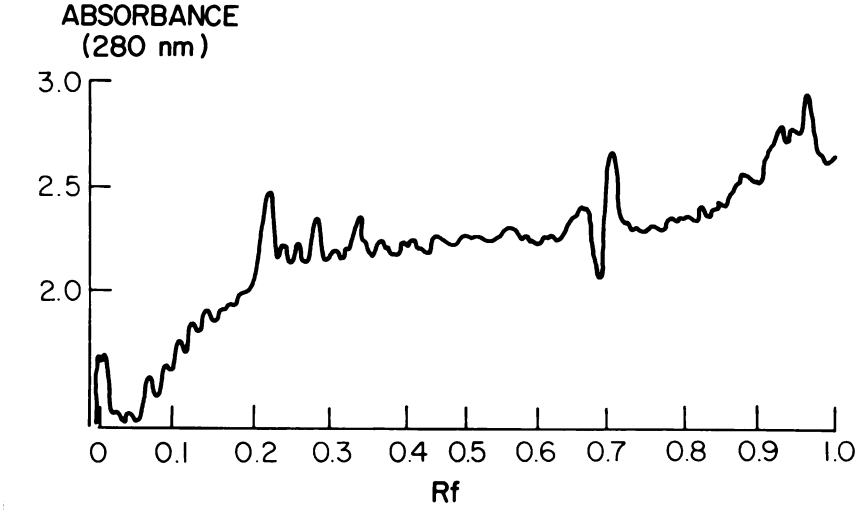
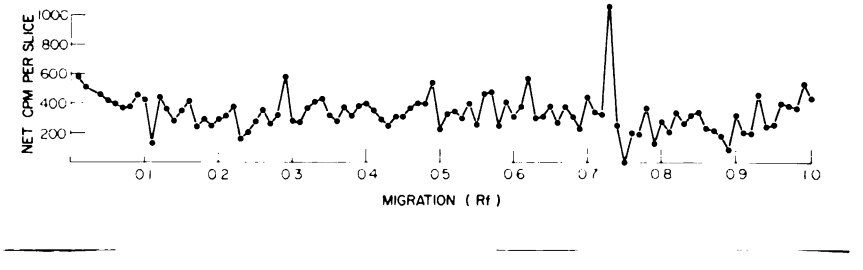

NET CPM PER SLICE
1100
1000
900
800
700
600
500
400
300
200
100
0
10
20
30
40
50
60
70
80
90
100
MIGRATION (Rf)

Figure 9. Polyacrylamide gel electrophoresis of RNA polymerase I under non-denaturing conditions. Gels were prepared, developed, assayed and sliced as described in Methods. ●-● ^{3}H incorporated per slice. —— Absorbance at 280 nm as described in Methods.

Figure 10. Polyacrylamide gel electrophoresis of RNA polymerase II under non-denaturing conditions. Gels were prepared, developed, assayed and sliced as described in Methods. ●-● ^{3}H incorporated per slice. —— Absorbance at 280 nm as described in Methods.

FIGURE 10


ABSORBANCE
(280 nm)
3.0
2.5
2.0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Rf


NET CPM PER SLICE
1000
800
600
400
200
01
02
03
04
05
06
07
08
09
10
MIGRATION (Rf)

in the reciprocal positions. Their relative affinities for DEAE ion exchange resin indicated that RNAP II was more strongly anionic than RNAP I. The further migration of RNAP II was likely due to this property. By extension of this analogy, RNAP III would be expected to exhibit migration even greater than Rf 0.73. The protein present in the RNAP II fraction, migrating at Rf 0.9, could be due to RNAP III presence. However, no significant activity was observed in this position. No identification of the protein(s) observed at Rf 0.3 - 0.4 was possible.

RNA POLYMERASES FROM MORPHINE TOLERANT-DEPENDENT MICE

Chronic morphine pellet implantation resulted in lower specific activity of the peak I RNA polymerase, as shown in Table 6. The specific activities of the peak I RNA polymerase in the morphine tolerant-dependent groups were 24-57% fo those of the placebo controls in various experiments. The specific activities of the peaks II and III enzymes were not significantly affected by chronic morphine treatment in any of the experiments.

In order to ascertain whether this effect of chronic morphine treatment was narcotic specific, a 10 mg pellet of the morphine antagonist naloxone was implanted two hr prior to implantation of a morphine pellet. This pre-implantation of the antagonist has been shown to block entirely the development of morphine tolerance (20) as measured 72 hr later.

Four experimental groups of mice were implanted with two pellets as described in the Methods. Each experimental group was in turn composed of three groups of 25 mice each. This resulted in a total of 12 groups. The specific activities of the peak I RNA polymerases eluted from the 12 columns are shown in Table 7 as the mean values for each treatment. It can be seen that the

TABLE 6

SPECIFIC ACTIVITIES OF RNA POLYMERASES

IN CHRONIC MORPHINE-TREATED MICE

	SPECIFIC ACTIVITY: PMOLE UMP INCORPORATED/MG PROTEIN		
RNA POLYMERASE	PEAK I	PEAK II	PEAK III
PLACEBO	14.41 ± 1.23	22.88 ± 1.65	4.53 ± 0.94
MORPHINE	3.50 ± 1.05*	24.39 ± 2.02	4.12 ± 1.05

Mice were implanted with morphine or placebo pellets for 68-72 hr and sacrificed; RNA polymerases were prepared as described in Methods. Three groups of 25 mice each were implanted, sacrificed, and prepared simultaneously for both treatments; enzymes were assayed in triplicate. Proteins were determined by the method of Lowry et al. (32) as described in Methods. Specific activities are mean ± standard deviation for the nine values attained for each enzyme.

*$P < 0.0025$ by Student's t test; peaks II and III not significant at the 0.05 level.

TABLE 7

EFFECT OF NALOXONE PRETREATMENT ON THE ACTIVITY OF PEAK I RNA POLYMERASE AFTER CHRONIC MORPHINE PELLET IMPLANTATION

IMPLANTATION	SPECIFIC ACTIVITY PMOLES UMP INCORP./MG PROTEIN ± S.D.
PLACEBO-PLACEBO (PP)	11.36 ± 0.76*
PLACEBO-MORPHINE (PM)	5.09 ± 1.02*
NALOXONE-PLACEBO (NP)	15.13 ± 5.27
NALOXONE-MORPHINE (NM)	33.6 ± 19.9

Mice were implanted with two pellets, 2 hr apart as described in Methods. Specific activities are means ± standard deviation for nine values as in Table 7.

*P< 0.001 by Student's t-test; NP-NM not significant, P> 0.1; PP-NP not significant, P>0.2.

naloxone-placebo treatment did not result in a significant change in the specific activities relative to the placebo-placebo controls. The specific activities from mice receiving a placebo and a morphine pellet were 45% of those from mice receiving two placebo pellets, a significant difference. The naloxone pretreatment blocked the observed morphine effect on RNA polymerase I since the peak I specific activity was no longer depressed as it was with morphine alone. It is interesting to note that the naloxone-morphine treatment resulted in a mean of specific activities higher than that for the naloxone-placebo controls, but this result was not statistically significant. The elevation of specific activities by naloxone-morphine treatment to levels greater than those observed for the naloxone-placebo controls has been substantiated by further exeperiments, as have been the coincidentally large standard deviations within both groups, but it has not been possible to demonstrate statistical significance. However, this curious result clearly is a reversal of the effect of morphine in this system.

Morphine sulfate, when injected acutely icv one hour prior to sacrifice, did not change the specific activities of the peaks I, II, and III RNA polymerase (Table 8).

TABLE 8

Specific Activities of RNA Polymerases in

Acute Morphine - Treated Mice

	SPECIFIC ACTIVITY: pmole UMP incorporated/mg Protein		
RNA Polymerase	Peak I	Peak II	Peak III
Saline	11.31 ± 1.69	31.65 ± 11.34	0.925 ± 0.567
Morphine	10.47 ± 8.22	48.7 ± 16.88	1.605 ± 0.532

Mice were injected with 5 ug morphine sulfate icv or with saline and sacrificed 2 hr after injection. RNA polymerases were prepared as described in Methods. Three groups of 25 mice each were injected, sacrificed, and prepared simultaneously for both treatments; enzymes were assayed in triplicate. Proteins were determined by method of Lowry *et al.* (32) as described in Methods. Specific activities are mean ± standard deviation for the nine values attained for each enzyme.

Results not significant ($P>0.05$) by Students t-test for peaks I, II, and III RNA polymerases.

The altered specific activity of RNA polymerase I which resulted from chronic morphine treatment was not asspciated with any alteration of its kinetics relative to ammonium sulfate, Mg^{+2}, or Mn^{+2} concentrations in the assay medium. However, in Table 9, it can be seen that morphine tolerance-dependence development was associated with alterations in the functions of both RNA polymerases II and III. The optium Mg^{+2} ion concentrations for RNAP II was shifted to 6 mM from the 8 mM optimum of the placebo control. The optimum Mg^{+2} concentration for this enzyme isolated from naive mouse brains was also 8 mM. Figure 11 shows that the altered Mg^{+2} response was consistent over a wide range of concnetrations. It can also be seen from Table 9 that chronic morphine treatment resulted in higher ratios relative to the placebo controls of Mn^{+2}- and Mg^{+2}-stimulated activities for RNA polymerases II and III. By comparison with Table 5, it is clear that the RNAP I and RNAP III placebo control values for the Mn^{+2}/Mg^{+2} activity ratios were comparable to those of the respective RNA polymerases from naive animals. However, there was a notable effect of the placebo treatment on this ratio for RNA polymerase III, but the Mg^{+2} and Mn^{+2} concentration optima for this enzyme in the placebo group were identical to those found in the naive animals.

TABLE 9

GENERAL PROPERTIES OF RNA POLYMERASES FROM CHRONIC MORPHINE-TREATED MICE

RNA POLYMERASE	PEAK I		PEAK II		PEAK III	
	MORPHINE	PLACEBO	MORPHINE	PLACEBO	MORPHINE	PLACEBO
OPTIMAL ACTIVITY, CONCENTRATION AMMONIUM SULFATE	0.07 M	0.07M	0.03 M	0.03 M	0.03 M	0.03M
OPTIMAL ACTIVITY, CONCENTRATION MAGNESIUM CHLORIDE	12 mM	12 mM	6 mM	8 mM	8 mM	8 mM
OPTIMAL ACTIVITY, CONCENTRATION MANGANESE CHLORIDE	4 mM	4 mM	4 mM	4 mM	4 mM	4 mM
ACTIVITY RATIO,* Mn^{+2}/Mg^{+2}	1.2	1.2	2.4	1.3	2.6	1.6
OPTIMAL ACTIVITY, ASSAY TEMPERATURE	37	37	37	37	37	37
OPTIMAL ACTIVITY, ASSAY pH	8.5	8.0	8.0	8.0	8.0	8.0

*As described in Table 1.

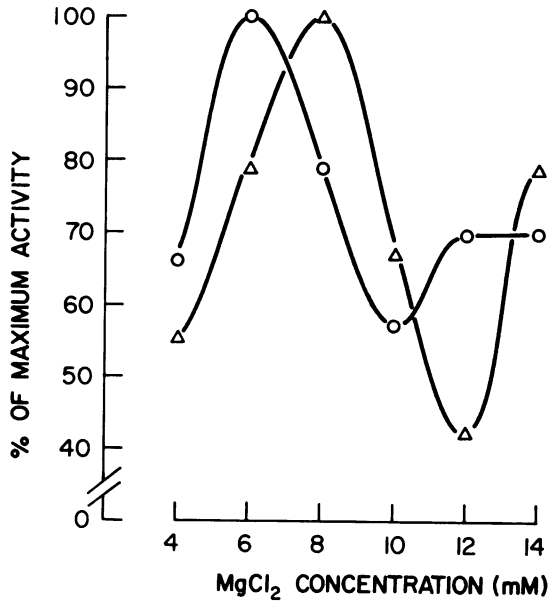


Figure 11. Magnesium-stimulated activity of RNA polymerase II following chronic morphine treatment. Assay performed in absence of Mn^{+2}. RNA polymerase II prepared as described in Methods from brains of: o—o morphine tolerant-dependent mice; Δ—Δ placebo-treated mice. Morphine tolerant dependent activity at maximum was 114% of maximum placebo activity. Specific activities of morphine tolerant-dependent and placebo control under normal assay conditions, as described in the methods, were 12.4 and 12.7 units/mg protein, respectively.

Chronic morphine pellet implantation did not result in any shift of the optimal assay temperatures for RNA polymerases I, II, and III (Table 9) away from the 37 seen in the naive animals (Figure 5). The treatment did, however, result in altered function of RNAP I relative to assay pH (Figure 12). The pH response of the placebo enzyme can be seen to be very similar to that of the naive RNAP I shown in Figure 6 with the optimum at pH 8.0. The RNAP I isolated from morphine tolerant-dependent mouse brains exhibited optimal activity at pH 8.5.

As shown in Figures 7 and 8 described above, and the RNAP I and II did not obey Michaelis Menten kinetics relative to the substrates ATP and UTP. The Michaelis plots showed the kinetics to be sigmoidal rather than hyperbolic. Thus, it was not possible to derive Km constants for these substrates and these enzymes. The incorporation of ^{3}H-UMP, used in the studies described, was similarly examined for RNAP I and II isolated from chronic morphine - and placebo - treated mouse brains. The sigmoidal behavior was not altered by the drug treatment. The decrease in specific activity which was observed for RNAP I following morphine tolerance-dependence induction thus was not a manifestation of an alteration in the enzyme's affinity for the labelled UTP precursor substrate.

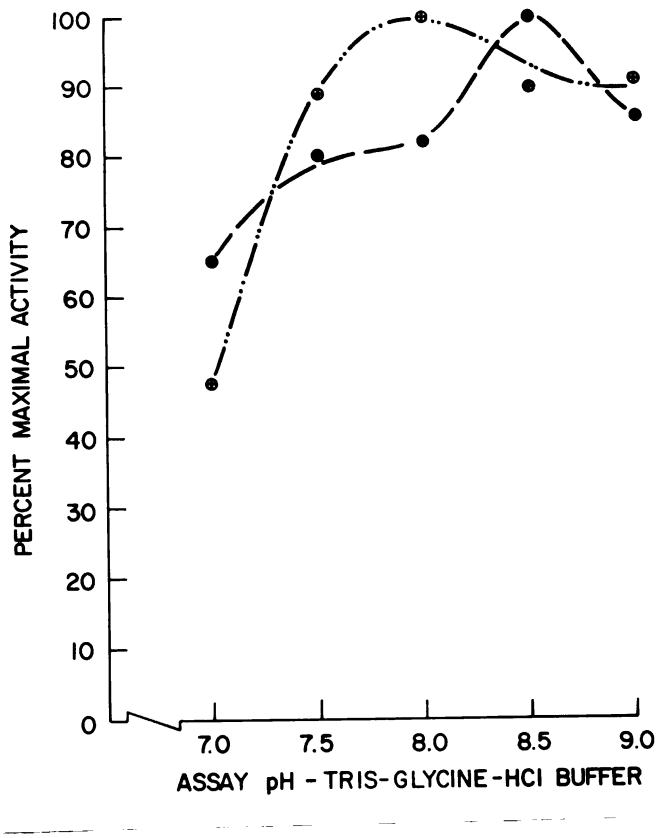


Figure 12. Effects of different assay pH on activity of RNA polymerase I following chronic morphine treatment. RNA polymerase I prepared as described in Methods from brains of: ●--● morphine tolerant-dependent mice; ●···—···● placebo-treated mice.

Similar conclusions cannot be drawn for the several observed differences in optimal conditions for activity after chronic morphine, however, the relationships between varied magnesium, manganese, or pH and the affinity for UTP substrate remain unexplained. It is possible that no such relationships exist, but the possibility prevents further conclusions. Nonetheless, it was observed that the sigmoidicity was not altered by chronic morphine in the cases of both RNAP I and RNAP II when these enzymes were examined at standard conditions of pH, magnesium, and manganese.

The RNA polymerases did not show altered activities when morphine sulfate was included in the medium at the concentration of 0.1 mM. When similarly examined, the opiod peptide β-endorphin was observed to stimulate RNAP II and RNAP III at concentrations of 1 uM (Figure 13). A two-fold increase in activity was observed for these enzymes at 10 uM β-endorphin. RNAP I was not affected by the peptide. Neither met-enkephalin nor the pituitary peptide ACTH were observed to affect any of the RNA polymerases over the same range of concentrations (1 nM-10 uM).

In order to determine the reversibility of some of the effects of chronic morphine treatment which had been observed, post-withdrawal animals were studied. RNA

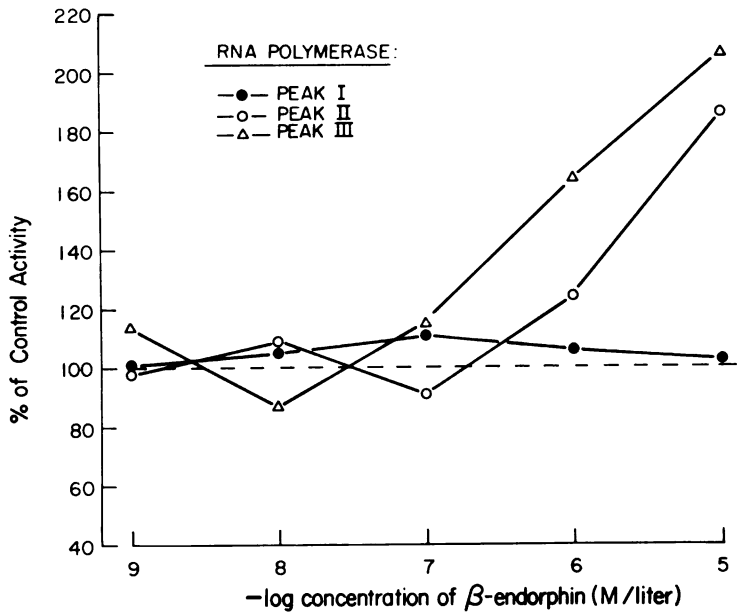


Figure 13. Effects of -endorphin on activities of RNA polymerases: ●—● I; o—o II; Δ— Δ III. RNA Polymerases prepared and assayed as described in Methods.

polymerases were prepared from brains of mice which had been implanted with morphine or placebo pellets. The pellets were left in place for the usual 72 hr and then removed. Ten days after removal of the pellets, the mice were sacrificed. Ho et al. (45) reported that, for example, body weight returned to normal within 72 hr of pellet removal. The delay of 240 hr, being more than thrice this length of time, thus produced mice well beyond the stage of withdrawl syndrome measured by Ho (45).

The specific activities and magnesium concentration optima were studied in this post-withdrawal system. No differences in these parameters were observed between the morphine and placebo-treated animals. The decrease in specific activity of RNAP I and the change in magnesium optimum of RNAP II thus were both effects which ceased to be observed after disappearance of the tolerant-dependent and withdrawal states. Complete investigations of the time course of onset and decline of these effects were not conducted, and further conclusions should be limited in the light of previous reports of varying time courses for onset and disappearance of the various effects which have been studied (53). The return to normal of these criteria after 240 hr did however, indicate that these were not protracted effects of morphine tolerance-dependence induction.

SECOND CHROMATOGRAPHY OF RNA POLYMERASE I

RNA Polymerase I From Naive Mice:

Multiple forms of RNA polymerase I have been reported in mouse myeloma cells and Bombyx mori (37), in Xenopus laevis (54), and in Drosophila (55). In order to ascertain whether the lowered specific activity of the peak I RNA polymetase was a general effect on all forms of the RNA polymerase I or a specific effect on one of the sub-types of this enzyme, a second DEAE-Sephadex chromatography was carried out. The peak I RNA polymerase was dialyzed and applied to a second DEAE-Sephadex column, as described in the Methods. RNA polymerase activity was eluted by a linear ammonium sulfate gradient, and the conductivities of the fractions collected were measured.

The results of one such experiment can be seen in Figure 14. Five activity peaks could be separated consistently, corresponding to fractions 21, 23, 25, 29 and 33 in the figure. It can also be seen that α-amanitin-resistant activity was detected at conductivity less than 7 mmho. The activity in the peaks eluting at 7 to 9 mmho were observed not to be inhibited by α-amanitin at the low concentration of 10 ng/ml. When poly dAdT was substituted for calf thymus DNA as template, only the activity in the

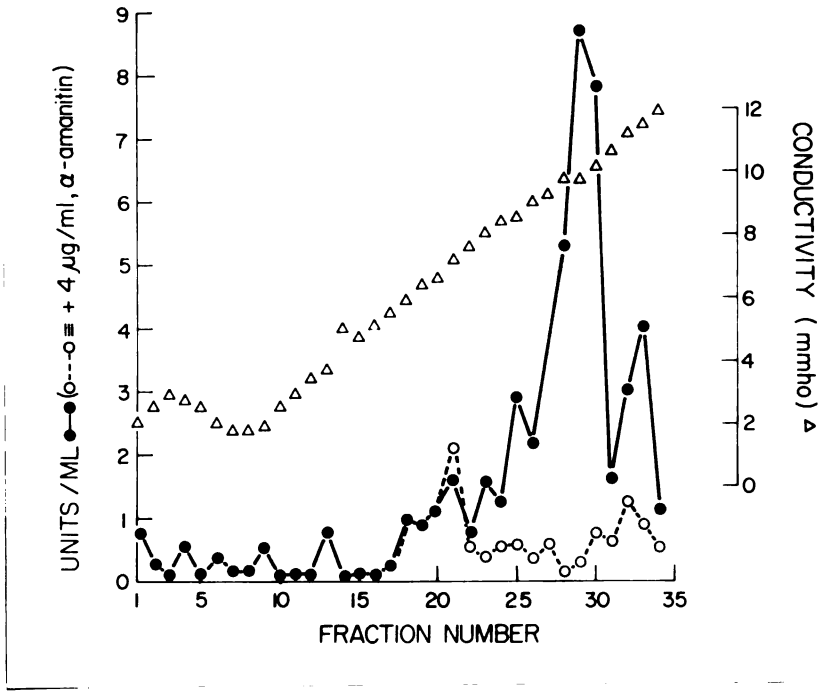


Figure 14. Second DEAE-Sephadex A-25 chromatography of RNA polymerase I. Fractions were eluted by a linear gradient of ammonium sulfate as described in Methods.

principal peak was affected. This activity was stimulated 2.5-fold. The presences of activities of intermediate α-amanitin sensitivity suggested the presence of RNA polymerase III in the elution. Such a presence had been previously reported (56). The activity in these peaks was not stimulated five-to ten-fold by poly dAdT, as described above for RNAP III. The activities in the peaks at fractions 21, 23, 25, and 29 exhibited, by these criteria, characteristics which were within the limits of those for RNA polymerase I Their characteristics were not appropriate for RNAP II or RNAP III. (These have been discussed in great detail in an earlier section.) As multiple forms of RNA polymerase I, the enzymes may exhibit different functional capabilities. The activity in the last-eluting peak (fraction 33 in the figure), being sensitive to α-amanitin and affected by poly dAdT, could represent RNAP II activity eluted with RNAP I by the discontinuous gradient of the first chromatography.

RNA Polymerase I From Morphine-Treated Mice:

The RNA polymerase I fractions from morphine tolerant and placebo groups were similarly dialyzed and applied to second DEAE-Sephadex columns for linear gradient elution. Figure 15 shows that when the fractions were compared according to their conductivities, to indicate comparable eluting concentrations of ammonium sulfate, it was seen

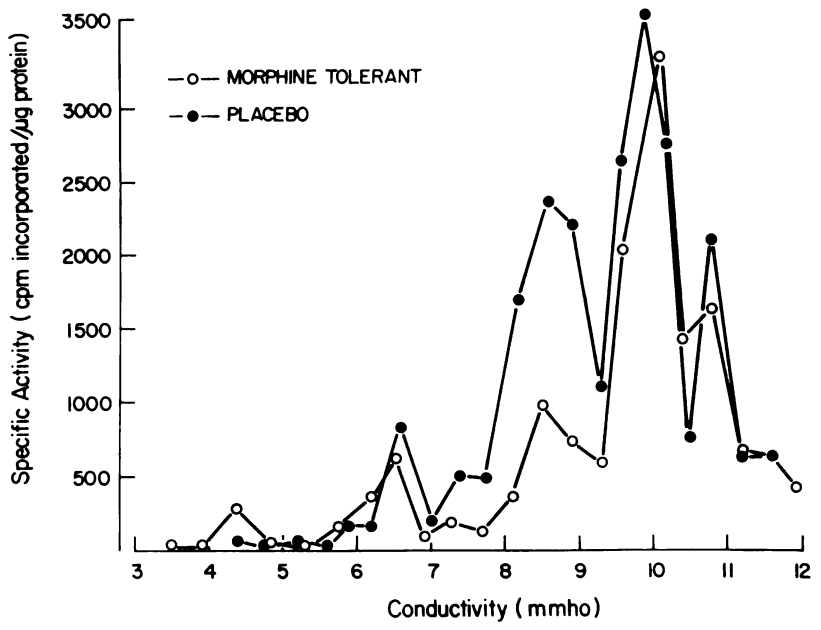


Figure 15. Specific activities of fractions eluted from a second DEAE-Sephadex A-25 column chromatography of RNA polymerase I. Fractions were eluted by a linear gradient of ammonium sulfate as described in Methods. Fractions are shown according to the conductivity of each for comparison of RNA polymerases from: o—o morphine tolerant-dependent mice; ●—● placebo-treated mice. Protein measurements were performed according to Bradford (33).

that the RNA polymerase specific activities of the morphine tolerant and placebo groups were comparable through most of the gradient, except for fractions in one section of the gradient, conductivity 7.0 mmho to 9.3 mmho. Two activity peaks were discerned in this section of the gradient. Thus, the lower specific activity of the peak I RNA polymerase which was observed after chronic morphine treatment was due to lower specific activities of these two sub-types of RNA polymerase I. The activity eluted from the column containing the morphine tolerant-dependent sample was normalized relative to the placebo control on the basis of the respective totals of activities applied to the two columns. Once normalized in this manner, the activities eluted between 7.0 and 9.3 mmho were compared. The morphine tolerant-dependent fractions in this gradient region contained 180 units of activity, an amount one third greater than the 135 units in the corresponding placebo control fractions. Analogous comparison of the amounts of protein in these fractions, normalized on the basis of total amounts of protein applied, showed that the morphine tolerant-dependent fractions contained 240 ug protein, 50% more protein than the 160 ug in the placebo control fractions. The lower specific activities in these fractions of the morphine tolerant-dependent sample were thus associated both with higher activities and with greater protein concentrations.

LIVER RNA POLYMERASES

Weaver et al. (28) demonstrated that liver RNA polymerases could be prepared and separated on a DEAE-Sephadex A-25 column by elution with a linear ammonium sulfate gradient. In order to determine whether the observed decrease in specific activity of RNA polymerase I was an effect of morphine common to cells of all organs, the liver RNA polymerases were examined. As described in Methods, the preparation of liver nuclei and RNA polymerases was nearly identical to the methods employed for the brain enzymes except that the elution from the DEAE-Sephadex A-25 column was by a linear gradient of ammonium sulfate.

Figure 16 showns that this method resulted in separation of multiple forms of liver RNA polymerases. RNA polymerases I, II, IIIA and IIIB were separated. As reported by Weaver et al. (28) for rats, the mouse liver nuclei contained a much greater proportion of RNA polymerase I than did the brain oligodendroglial nuclei.

Liver nuclei and RNA polymerases were prepared from morphine and placebo implanted mice and separated on DEAE-Sephadex columns as above. Figure 17 shows that when the fractions were compared according to their conductivities, to indicate comparable eluting concentrations of ammonium sulfate, it was seen that the

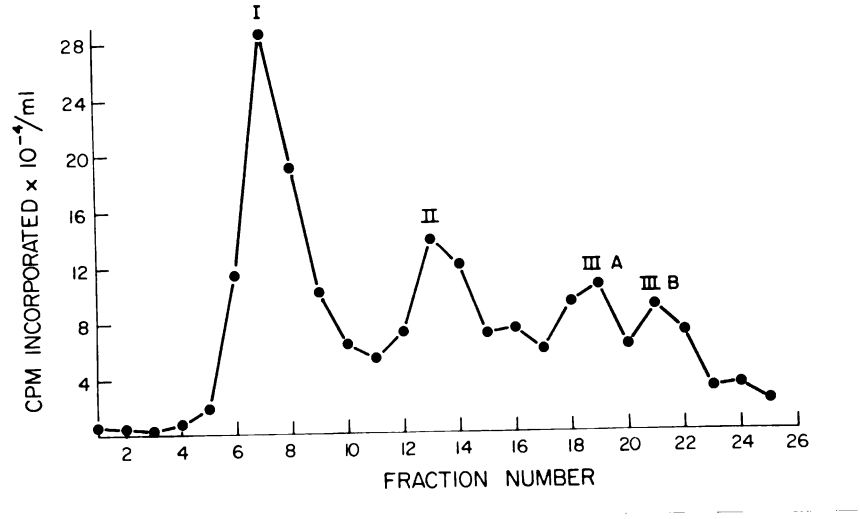


Figure 16. DEAE-Sephadex A-25 chromatography of liver RNA polymerases. Preparation, application and elution of RNA polymerases were as described in Methods. Fractions were eluted by a linear gradient of ammonium sulfate as described in Methods. Enzyme assay was as described in Methods.

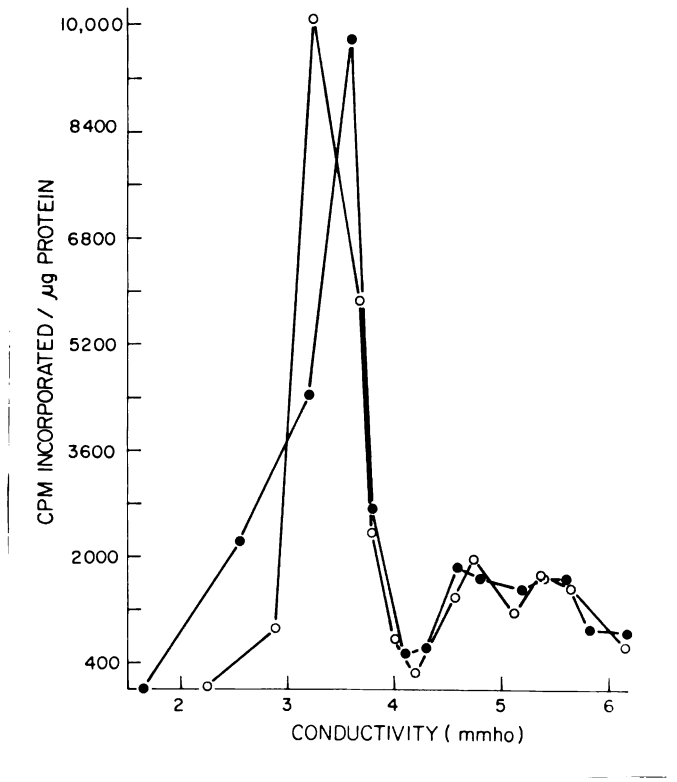


Figure 17. Specific activities of fractions eluted from a DEAE-Sephadex A-25 column chromatography of liver RNA polymerases. Fractions were eluted by a linear gradient of ammonium sulfate as described in Methods. Fractions are shown according to the conductivity of each for comparison of RNA polymerases from: ●—● morphine tolerant-dependent mice; o—o placebo-treated mice. Protein measurements were performed by the Lowry method (32) described in Methods.

RNA polymerase specific activities of the morphine tolerant and placebo groups were comparable throughout the gradient. Chronic morphine treatment did not alter the specific activities of the liver RNA polymerases, indicating that the decrease in specific activity of brain RNA polymerase I was not a general phenomenon.

PROTEIN KINASE PHOSPHORYLATION OF RNA POLYMERASES

It has been suggested (57, 58) that protein kinase (PK) may act as a positive regulator of RNA polymerases. Calf thymus RNA polymerases were shown to be stimulated by preincubation with homologous PK (57, 58) and to be inhibited by preincubation with non-homologous alkaline phosphatase (57). These studies indicated that RNA polymerases were phosphate acceptors and that the phosphorylated forms of the enzymes were more active. In order to establish phosphorylation of the brain RNA polymerases as a possible mechanism of their positive regulation, protein kinase preincubations were carried out. As described in Methods, the RNA polymerases I, II, and III were incubated with homologous PK for 5 min under phosphorylating conditions. RNA polymerase assay medium was then added, and the RNAP assay was subsequently conducted in the usual manner.

The effect of cAMP-independent nuclear protein kinase on RNA polymerase activity and phosphyorylation is shown in Table 10. Protein kinase and RNA polymerases I, II, and III were each added to the medium at respective protein concentrations which had previously been shown to be linear with respect to the activity of each enzyme when assayed alone. Phosphorylation was measured by following ^{32}Pincorporation from γ-^{32}P-ATP Into TCA-precipitated protein. RNA polymerase activity was measured by following ^{3}H-UTP Incorporation into synthesized RNA. Each RNA polymerase was preincubated with protein kinase at pH 6.5 or 7.4.

The activities of RNA polymerases I and II were stimualted with the addition of increasing amounts of nuclear protein kinase; phosphorylation was also increased with the addition of protein kinase. These results suggest a correlation between phosphorylation by protein kinase and stimulation of RNA polymerase I and II activities. However, a given relative increase in phosphorylation was not associated with the same relative increase in RNA synthesis. The RNA polymerase I activity increased to 239% when preincubated with protein kinase at pH 6.5, but the phosphorylation itself was increased only to 164%. Furthermore, it appeared that RNA polymerase I activity was stimulated to a much greater extent than RNA

TABLE 10

PROTEIN KINASE PEAK I PHOSPHORYLATION AND STIMULATION OF HOMOLOGOUS RNA POLYMERASES

	AMOUNT PK ENZYME (UG)	H-UTP INCORPORATION % CONTROL ACTIVITY	P-INCORPORATION % CONTROL ACTIVITY
RNA POLYMERASE I			
pH 6.5	0	100	100
	1.9	199	134
	3.8	239	164
pH 7.4	0	100	100
	1.9	265	141
	3.8	349	161
POLYMERASE II			
pH 6.5	0	100	100
	1.9	109	146
	3.8	104	210
pH 7.4	0	100	100
	1.9	113	249
	3.8	135	254
POLYMERASE III			
pH 6.5	0	100	100
	1.9	102	324
pH 7.4	0	100	100
	1.9	102	302

polymerase II for a given relative increase in phosphorylation.

Stimulation of both RNA polymerase I and II by protein kinase was sensitive to the pH of the preincubation reaction. RNA polymerase I activity was stimulated to a greater degree at pH 7.4 than at pH 6.5, although the extent of phosphorylation appeared to be identical at both pH's. This indicated that the RNA polymerase I enzyme may be affected diffferently or prhaps unstable at lower pH's. RNA polymerase II activity and the corresponding phosphorylation were both more greatly augmented at pH 7.4 than at pH 6.5. RNA polymerase III activity was not stimulated at either pH 6.5 or pH 7.4, even though the phosphorylation was increased to 300% relative to control.

SUMMARY AND DISCUSSION

The fractionation of RNA polymerases (RNAP) by the methods reported yielded three RNA polymerases. The degrees of purification relative to whole brain homogenate were 47-, 118- and 27-fold, respectively. These RNA polymerases have been shown to be DNA-dependent and not to contain significant ribonuclease activity.

The RNA polymerases have been studied and characterized relative to several properties. The RNAP II was extremely sensitive to α-amantin. RNAP III showed intermediate sensitivity to the toxin, as did RNAP I. The sensitivity of RNAP I to α-amanitin was unlike the comparable enzyme from other sources (27, 28, 29), but this was not a unique characteristic (51).

The ammonium sulfate optima for the three enzymes were appropriately within the limits demonstrated elsewhere (50) and RNAP III exhibited the characteristic biphasic response. The magnesium optima for RNAP I and II were identical to those of the corresponding enzymes from MOPC 315. The magnesium optimum for RNAP III was dissimilar to those of both RNA polymerases IIIA and IIIB of the MOPC 315 system. The optimal concentrations of manganese for RNAP I, II, and III were totally unlike those of the MOPC

315. The manganese/magnesium and poly dAdT/DNA activity ratios both were in good concordance with the predicted values for all three RNA polymerases.

The several properties of the RNA polymerases which have been examined are within the limits of those described in other systems. Thus, it was possible to conclude that these brain enzymes are, in fact, RNA polymerases I, II, and III. Because of their similarities to those enzymes studied elsewhere, it can be inferred that these RNA polymerases probably perform similar functions to their counterparts in other tissues and organisms. It has been demonstrated in cultured myeloma cells (59) that RNA polymerases I, II, and III transcribe separate classes of RNA's. These are the 18S and 28S RNA's (rRNA precursors), heterogenous nuclear RNA's (mRNA precursors), and tRNA and 5S rRNA, respectively. This functional classification can become useful in the consideration of the cellular implications of altered function of the RNA polymerases.

Polyacrylamide gel electrophoresis of the RNAP's under non-denaturing conditions, followed by assay of the gels for enzyme activity, revealed a recovery of 5% of applied activity. It thus was not possible to measure RNAP III activity in the gels. Following separate electrophoreses of RNAP I and II, one peak of activity could be observed in each gel. The activities migrated to different

positions, Rf 0.6 and 0.73, respectively. Neither activity was observed in the other gels. Both RNAP I and II did contain contaminating protein at Rf 0.3 - 0.4, and RNAP II contained a significant amount of protein at Rf 0.9. The RNA polymerases I and II showed relative migrations toward the anode which corresponded to their relative affinities for DEAE-Sephadex. The protein which was present at Rf 0.9 could thus represent RNAP III which was eluted from the DEAE-Sephadex with RNAP II by the discontinuous ammonium sulfate gradient. No significant activity was observed at this site in the gels of RNAP II or III, however.

When the affinities of the RNAP's for the substrates ATP and UTP were examined, it was not possible to study RNAP III because of the small amount of enzyme recovered and the low substrate concentrations. The enzyme-substrate affinities for RNAP I and II did not follow Michaelis-Menten kinetics, and it was not possible to derive Km values. The Lineweaver-Burke representations of the substrate incorporations measured for RNAP II were shown not to be linear; this suggested cooperativity of enzyme-substrate association. The processs of transcription is complex and involves enzyme, template, four substrates, and divalent cations. It is thus only possible to report the results of these studies and of the identical studies conducted in drug-treated mice. Further conclusions

concerning the enzyme-substrate associations would require much additional experimentation, whereas the purpose of these studies was to establish the nature of the interaction in order to deduce whether this property was affected by opiate tolerance-dependence induction.

Chronic morphine pellet implantation has been shown to decrease the specific activity of RNA polymerase I. This effect has been shown to be reversed by naloxone pellet preimplantation and thus to be narcotic specific. This change in RNAP I specific activity has also been shown not to result from acute administration of morphine sulfate (MS) nor from in vitro MS or β-endorphin. This effect is thus due to some result of prolonged exposure of the mouse to morphine. Chronic morphine treatment has also been shown to shift the optimal assay pH for RNAP I to 8.5 from the 8.0 optimum for naive and placebo.

The decrease in specific activity of RNAP I which has been demonstrated to occur in mouse brain as a result of chronic morphine treatment has been shown not to occur in mouse liver. This effect is thus not a general phenomenon occuring in all organs and cell types. This brain effect has also been demonstrated to revert to normal after cessation of drug treatment.

The RNA polymerase I fraction eluted from a single DEAE-Sephadex column has been sub-fractionated by a second DEAE-Sephadex chromatography into five consistently separable activity peaks. These peaks have been shown to differ in their sensitivities to α-amanitin and in their relative preferences for poly dAdT. Two of these peaks have been shown to be lowered in specific activity by chronic morphine pellet implantation and the remaining peaks to be unaltered by the treatment. The multiple peaks revealed by this second chromatography were not also observed in the polyacrylamide gels. The loss of 95% of activity which was observed following electrophoresis would suggest that the forms of RNA polymerase I which were eluted by the second chromatography in small quantities should not be expected to be observed in the gel. The single activity observed in the gel thus probably represented the major activity eluted from the column. The broad protein peak observed in the gel, in the position corresponding to the discrete activity peak, may be due to the presences of the less prevalent additional forms. These forms would be expected to show similar migrations as they were eluted from the DEAE-Sephadex by similar conditions.

Craves *et al*. (60) have reported that cell-free protein synthesis by polyribosomes and pH 5 enzyme(s) isolated from brains of morphine tolerant-dependent mice occurred

at greater specific rates than when these components were isolated from placebo control brains. Mixing experiments between the drug and placebo groups have shown that both the polyribosomes and the pH 5 enzyme(s) from the chronic morphine groups contributed to these elevated rates.

It has been suggested (37) that multiple forms of RNA polymerase I may either represent two forms of the same enzyme, active and inactive, which transcribe the same DNA segments or that these are different enzymes which transcribe distinct DNA segments. The observed decrease in specific activity of two forms of RNAP I may reflect a differential solubilization of these forms of RNA polymerase I between the morphine tolerant and placebo control preparations. In such a case, the lower specific activity may be the result of some unidentified protein present in greater concentration in the morphine tolerant preparation.

Lower specific activity of RNA Polymerase I, which synthesizes 18S and 28S rRNA, has been reported in this study. Diminished ribosomal RNA synthesis is consistent with the observed increase in cell-free protein synthesis in morphine tolerance. By definition, net cell function in the tolerant state should be normal in the constant presence of the narcotic. Thus, total translation may be occurring at normal rates due to the combination of

diminished ribosomal RNA synthesis and greater rates of translation. The temporal and causal relationships between these effects remain to be shown.

RNA polymerases II and III have been shown not to be altered in specific activity following acute, chronic, or in vitro morphine. However, the optimum Mg^{+2} concentration for RNA polymerase II function has been shown to be lowered in tolerance-dependence. This effect has been demonstrated to revert to normal after cessation of drug treatment and thus not to be a protracted effect of chronic morphine treatment. In addition, the Mn^{+2}/Mg^{+2} activity ratios of both RNA polymerases II and III from morphine tolerant-dependent animals have been demonstrated to be higher than those of the placebo controls. These enzymes have also been shown to be stimulated two-fold by the endogenous opiod peptide β-endorphin at 10 um concentration.

The morphine-induced shift of the optimal Mg^{+2} concentration for RNA polymerase II to a lower level as reported here is analogous to the shifts reported by Craves (61) for several brain amino acyl synthesases (AAS). The Mg^{+2} activity optima of the leucine, lysine, and tryptophan amino acyl synthetases isolated from morphine tolerant-dependent mouse brains have each been shown to be lower than those of the placebo controls. The

phenylalanine AAS have been shown not to be affected. These observations, taken together, suggest that the intracellular Mg^{+2} concentration may be altered in the morphine tolerant-dependent mouse brain.

Chronic morphine pellet implantation has been reported to elevate chromatin template activities (20) and to increase protein kinase activity in these oligodendroglial nuclei (22). The elevated protein kinase activities were shown to be associated with increased phosphorylation of acidic chromatin proteins, and it has been suggested that such phosphorylation would act as a positive control of gene expression (62). It is possible that elevated chromatin template activity, which may result from increased phosphorylation of non-histone proteins in the nuclei, may also be associated with altered function of the RNA polymerases.

With the addition of cAMP-independent nuclear protein kinase, a correlation was seen between RNA polymerase activity and phosphorylation for RNA polymerases I and II, but not for RNA polymerase III. Stimulation of RNA polymerase I and II activities by nuclear protein kinase was associated with increases in phosphorylation. However, because the protein kinase and RNA polymerase fractions were only partially pure, it is not known if the RNA molecule itself was phosphorylated or some other

molecule was phosphorylated which, in turn, was then able to stimulate RNA synthesis. Nevertheless, this data suggests that the nuclear protein kinase may be regulating RNA polymerase I and II activities through a phosphorylation mechanism.

Since both nuclear protein kinase and RNA polymerase I specific activities are altered after chronic morphine treatment and the optimum concentration of co-factors required for RNA polymerases II and III are also altered (23), it is possible that during morphine tolerance-dependence, regulation of RNA polymerase by protein kinase through phosphorylation is also altered. Costa (63) and Jungman (58) have hypothesized the translocation of cAMP-dependent protein kinase from the cytoplasm to the nucleus. In this hypothesis, when there is a sustained elevation of the cAMP level in the cell, the cAMP binds to the regulatory (R) subunit of the cAMP-dependent cytosolic protein kinase and dissociates the regulatory (R) and catalytic (C) subunits of the kinase. The catalytic subunit is then translocated to the nucleus which results in modification of gene expression through phosphorylation of nuclear proteins.

There is much evidence to indicate a role for cAMP in the internal mediation of the cellular response to the interaction of morphine with the opiate receptor at the

cell surface. Both cAMP and the phosphodiesterase inhibitor theophylline have been shown to antagonize acute morphine analgesia (64). Development of tolerance and physical dependence have been shown to be accelerated by icv. injection of cAMP, even when cAMP is administered 2 hr prior to commencement of the 72 hr morphine pellet implantations (45). These observations imply that there is an elevation of cAMP in certain brain regions during morphine tolerance-dependence. This novel condition of elevated cAMP would result in a number of responses, one of which would likely be the dissociation and translocation of protein kinase. Increased nuclear protein kinase may result in the increased chromatin phosphorylation and template activity, previously reported (20, 22) and may regulate RNA polymerase in a manner as demonstrated in vitro in this report. The alterations in kinetic behaviors observed following chronic morphine may result from changes in the phosphorylated states of the enzymes. Altered affinity for cofactor has been demonstrated to result from phosphorylation in other enzyme systems, for example tyrosine hydroxylase and tetrahydrobiopterin (65).

Modulation of nuclear systems implies some functional alteration in macromolecular synthetic processes, and these are indeed found in morphine tolerance-dependence. Actinomycin D (7) and cycloheximide (66) block tolerance-

dependence development, suggesting that changes at the level of transcription and translation are both required for morphine tolerance-dependence development. No unique "morphine tolerance-dependence protein" has been found. However, changes in the isozyme functions of several important regulatory proteins have been reported. For example, the optimum Mg^{+2}: ATP ratio and temperature for isozymes of aminoacyl-tRNA transferases are altered during chronic morphine treatment (61). Also, the optimum Mg^{+2} concentration for RNA polymerase II activity, the Mn^{+2}/Mg^{+2} ratios of RNA polymerases II and III, and the pH optimum of RNAP I are altered by chronic morphine treatment as shown above. The appearance of these new isozymes whose maximum activities require different optimum concentrations of co-factors suggest that they are the result of adaptational responses to new conditions in their local environments.

In the case of RNAP I, it has been demonstrated that there is a shift in the composition of the RNAP I "family" induced by chronic morphine. A thorough study of the ion dependencies and pH optima of these sub-forms has not yet been possible, but the observed alteration is a transition toward more activity and more protein in the lower specific activity forms. As implied above, the "switching off" of rRNA synthesis, by conversion of RNAP I enzymes to less active forms, operating at the same time as increased

rates of translation, would result in normal total translational rates. Jungman (58) has demonstrated that dephosphorylation by alkaline phosphatase results in decreased activity of calf thymus RNA polymerase. The decrease in specific activity of these specific brain RNAP I forms could be the result of such a mechanism. Phosphorylation states could rapidly be altered *in vivo* and would thus serve the organism well as a means of adaptation.

Evidence has been presented here for a number of altered conditions induced by morphine to which some biochemical systems are capable of adapting. The net effect of the total of these adaptations is normal behavior of the animal or human in the tolerant state in the presence of morphine. Upon removal of the drug, the previously altered conditions revert to their pre-drug states. The biochemical systems which had adapted thus no longer function normally, and the withdrawal syndrome is observed. Biochemical adaptations such as those described here would be secondary or tertiary to morphine's receptor-mediated action *in vivo*. These systems thus would not be expected to respond to morphine *in vitro* or to acute administration of the drug in a manner appropriate to opiate receptor characteristics.

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