Hydrolysis of Endogenous Diacylglycerol and Monoacylglycerol by Lipases in Rat Brain Microsomes†

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ABSTRACT: [^{14}C]Fatty acids were injected into brains of 18-day-old rats and the labeled microsomes were isolated and treated with phospholipase C. This resulted in a degradation of the membranal phospholipids, thus forming radioactively-labeled, endogenous diacylglycerol. Hydrolysis of this diacylglycerol by the microsomal lipases was followed by incubating the membranes at pH 7.4 and 4.8. After phospholipase C treatment and further incubation at pH 7.4, part of the endogenous diacylglycerol was degraded by the membranal alkaline diacylglycerol lipase. The monoacylglycerol thus formed was further degraded by the membranal monoglyceride lipase. Pretreatment of the membranes with phospholipase C at pH 7.4, followed by incubation at pH 4.8, resulted in a further decrease in the amount of diacylglycerol accompanied by concommitant increases in monoacylglycerol and free fatty acids. These experiments verify the existence of two diacylglycerol lipases in rat brain microsomes, one with an alkaline and the second with an acidic pH optimum, but of only one monoacylglycerol lipase with an alkaline pH optimum (Cabot, M. C., and Gatt, S. (1976), *Biochim. Biophys. Acta 431*, 105–115). This system provides a method for the study of membrane-bound lipolytic enzymes acting on endogenous substrate, thereby obviating the use of exogenous dispersions of lipid substrates.

A previous paper reported on the presence of two diacylglycerol lipases in rat brain microsomes (Cabot and Gatt, 1976). One enzyme had an optimal pH at about 8.0 and the second at 4.8. In the present communication, the existence of these enzymes was verified by employing microsomes which had been labeled by intracerebral injection of [^{14}C]fatty acids and then treated with phospholipase C in order to promote formation of endogenous diacylglycerol, the substrate for these lipases.

The ease with which brain lipids can be labeled via intracerebral injection of radioactive fatty acids has been amply demonstrated (Gatt, 1963; Sun and Horrocks, 1971; Yau and Sun 1973, 1974). Sun and Horrocks (1971) and Yau and Sun (1973) found rapid incorporation of [^{14}C]palmitic and [^{14}C]oleic acids into the lipids of mouse brain with the phosphoglyceride fraction being maximally labeled 80 min after intracerebral injection of the fatty acids. Phospholipase C specifically hydrolyzes the phosphoryl-base portion of glycerophosphatides and results in the formation of diacylglycerols (MacFarlane, 1948). This enzyme is thus a useful tool for manipulating the lipid composition of intact membranes. Stahl (1973) employed a purified preparation of phospholipase C and studied its activity with respect to brain microsomal membrane phospholipids. Phospholipase C, through its generation of endogenous membrane-bound diacylglycerols, has also proven useful in the study of biosynthetic reactions of the phospholipids (Van Schijndel et al., 1973; McMurray, 1975). More specifically, phospholipase C treatment of rat erythrocyte membranes has led to the identification of lipolytic activity, as was documented in the study by Michell et al. (1973).

The combined techniques of intracerebral injection and phospholipase C treatment of the isolated rat brain microsomes have been utilized here to verify the presence of three lipases associated with the microsomal fraction of rat brain. The presence of these enzymes was reported (Cabot and Gatt, 1976) using dispersions of mono- and diacylglycerol.

Materials and Methods

[1-^{14}C]Palmitic acid (57.9 mCi/mmol) and [1-^{14}C]oleic acid (54 mCi/mmol) were products of Amersham. [16-^{14}C]Palmitic acid (53 mCi/mmol) and [10-^{14}C]oleic acid (54 mCi/mmol) were purchased from CEA-France. Bovine albumin (fraction V), phospholipase C (*Clostridium welchii*), and phospholipase A_2 (*Crotalus adamanteus*) were purchased from Sigma Chemical Co. All organic solvents used were distilled or of analytical grade.

Eighteen-day-old male rats were injected intracerebrally with equimolar amounts of ^{14}C-labeled palmitic and oleic acids (99% radioactively pure) in the following manner derived essentially from the method of Yau and Sun (1974). Hexane solutions of palmitic and oleic acids were mixed and the solvent was evaporated under a stream of nitrogen. The residue was neutralized with 1 equiv of NaOH and emulsified in 15% (w/v) bovine serum albumin. Two groups of five rats were injected with mixtures of either the carbonyl-labeled fatty acids or the distally-labeled fatty acids. Each rat was injected with 25 μL containing 1 μCi of palmitic acid and 1 μCi of oleic acid using a 50 μL Hamilton luer-tip syringe fitted with a 27-gauge needle.

The animals were sacrificed after 90 min and the brains were rapidly removed and rinsed in ice-cold 0.32 M sucrose. Subcellular fractionation was carried out as described previously (Cabot and Gatt, 1976). The microsomal pellet was washed once in 0.32 M sucrose, sedimented at 100 000*g*, then resuspended in 0.32 M sucrose, and stored at −20 °C. In order to reduce the amount of free fatty acids present in the microsomes, an amount equal to 10 mg of protein was incubated at 37 °C for 10 min in 40 mM Tris[1]-HCl, pH 7.4, containing 25 mg/mL bovine serum albumin. The microsomes were resus-

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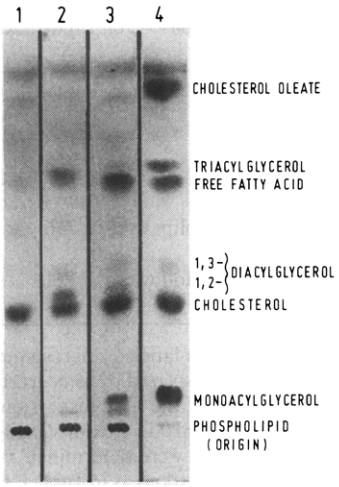

FIGURE 1: Thin-layer chromatography of lipids from rat brain microsomes. Microsomes (750 μg of protein) were incubated and the lipids were extracted and chromatographed as described under Materials and Methods. Lane 1: 1-h incubation in the presence of 1 mM $CaCl_2$, 25 mM Tris-HCl, pH 7.4. Lane 2: incubated as above but including 200 μg of phospholipase C. Lane 3: same as 2 with continued incubation for 1 additional h at pH 4.8. Lane 4: mobilities of reference lipids.

pended in 0.32 M sucrose, sedimented at 100 000*g*, and again suspended in sucrose for storage. The above procedure resulted in a recovery of about 12% of the radioactivity in the lipid moiety of rat brain. Approximately 10% of the incorporated radioactivity was located in the microsomal fraction.

Assay and Analyses. Labeled microsomes (0.75–2.0 mg of protein) were incubated at 37 °C, in a final volume of 1 mL for varying time periods in 25 mM Tris-HCl, pH 7.4, 1.0 mM $CaCl_2$, and, if introduced, 200 μg of phospholipase C. Changing the pH of the reaction mixture was accomplished by adding 0.2 mL of 0.4 M sodium acetate buffer, pH 4.8. The reactions were terminated and the lipids were extracted by a modified procedure of Bligh and Dyer (1959) in which the methanol contained 2% acetic acid. The chloroform phase was prepared for thin-layer chromatography as previously described (Cabot and Gatt, 1976). The lipids were dissolved in chloroform–methanol (2:1) and aliquots were applied to silica gel G thin-layer plates. Authentic standards were used in the identification of all lipids. The plates were first run in a solvent system containing petroleum ether (bp 40–60 °C)–diethyl ether–glacial acetic acid (60:40:1). The chromatograms were dried in an atmosphere of nitrogen, a line was etched 3 cm above the origin, and the plates were redeveloped to the etch line in diethyl ether. This facilitated further migration of monoacylglycerol from the origin. Reaction products were also developed in a solvent system containing chloroform–acetone–glacial acetic acid–methanol (72.5:25:0.5:2) on boric acid impregnated silica gel plates (Thomas et al., 1965). Lipids were visualized in iodine vapors and areas were scraped into scintillation vials containing 10 mL of scintillator (Cabot and Gatt, 1976). The percent recovery of isotope from thin-layer plates ranged between 99 and 105%.

For the experiment described in Figure 1, unlabeled microsomes from adult rat brain were incubated as described above. Aliquots of the reaction lipids were separated on thin-layer plates according to the method of Skipski et al. (1965).

[1] Abbreviation used: Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

Phospholipase A_2 degradation of labeled microsomal lipids was carried out by direct application of lipid to the origin of a thin-layer chromatographic plate followed by application of phospholipase A_2 (*Crotalus adamanteus*) according to the method of Goerke et al. (1971). Chromatograms were developed in chloroform–methanol–water (65:25:4). This solvent system was also used to separate and identify the phospholipid components of labeled microsomal preparations. Protein was determined according to the method of Lowry et al. (1951).

The specific activity of the endogenous diacylglycerol was determined in the following manner. Labeled membranes were incubated for 10 min with phospholipase C using the method described above. The reaction was terminated and lipids were extracted by the method of Bligh and Dyer (1959). Aliquots of the total lipid extract were applied to acid-washed thin-layer chromatographic plates of silica gel H. The chromatogram was developed in a solvent system containing benzene–diethyl ether–ethanol–acetic acid (50:40:2:0.2) (Allan and Michell, 1975b), and the areas containing diacylglycerol were eluted with chloroform–methanol (2:1). Aliquots of this extract were taken along with a silica gel blank for quantitation (Marsh and Weinstein, (1966) and determination of radioactivity.

Results

When microsomes of adult rat brain were incubated at pH 7.4 with phospholipase C, diacylglycerol was formed by degradation of the membranal phospholipids. This endogenous diacylglycerol was further split by the microsomal lipases, the product being mostly glycerol and free fatty acid, with only little accumulation of monoacylglycerol. Figure 1, lane 2, shows this effect. When the incubation was repeated and, after phospholipase C treatment, the pH was lowered to 4.8, the following changes occurred. The density of the diacylglycerol spot diminished and the amount of free fatty acid increased (Figure 1, lane 3), but now spots of monoacylglycerol are clearly visible. In Figure 1, lane 1, the lipid profile of microsomes incubated at pH 7.4 without phospholipase C shows the presence of cholesterol and phospholipids, but no neutral glycerides and only traces of free fatty acids. The above supports, qualitatively, the findings of the former paper in which dispersions of diacyl- or monoacylglycerol were used as substrates for the microsomal lipases (Cabot and Gatt, 1976). In that paper, we reported the presence of diacylglycerol and monoacylglycerol lipases with pH optima at about 7–8 and a diacylglycerol lipase but no monoacylglycerol lipase with a pH optimum at 4.8.

Table I represents quantitative data of an experiment using labeled microsomes of immature rat brain, prepared by intracerebral injection of carbonyl-labeled fatty acid. At pH 7.4, 28% of the endogenous diacylglycerol was degraded (4507 dpm of phospholipid was degraded, but only 3252 dpm of diacylglycerol was recovered). Only minute quantities (134 dpm) of the product, monoacylglycerol, accumulated, most of it having been further degraded to glycerol and free fatty acid by the microsomal monoacylglycerol lipase (Cabot and Gatt, 1976). When phospholipase C treated microsomes were further incubated at pH 4.8, the amount of diacylglycerol degraded was about 60%. The decrease in diacylglycerol at the acid pH was accompanied by a simultaneous increase in monoacylglycerol.

In order to establish the authenticity of monoacylglycerol, an aliquot of these reaction lipids was separated on boric acid impregnated silica gel plates. The counts corresponding to monoacylglycerol on the silica gel G plates were recovered in

TABLE I: Distribution of Radioactivity in Labeled Microsomes before and after Treatment with Phospholipase C.[a]

Procedure	Disintegrations of ^{14}C/min				
	PL	MG	DG	TG	FFA
Immediate extraction	5456	112	332	302	235
2-h incubation at pH 7.4	5425	80	364	277	543
As above, with phospholipase C	918	214	3616	350	1680
1-h incubation at pH 7.4 with phospholipace C, followed by 1-h incubation at pH 4.8	868	1180	1703	400	2756

[a] Labeled microsomes (1 mg of protein containing 7130 dpm in the lipid moiety) were prepared, washed with albumin, and incubated at 37 °C for the times and at the pH values indicated. The lipids were isolated and their radioactivities were determined. Details of the preparation of microsomes, the incubation system, and isolation of the lipids appear under Materials and Methods. Abbreviations used are: PL, phospholipid; MG, monoacylglycerol; DG, diacylglycerol; TG, triacylglycerol; FFA, free fatty acid.

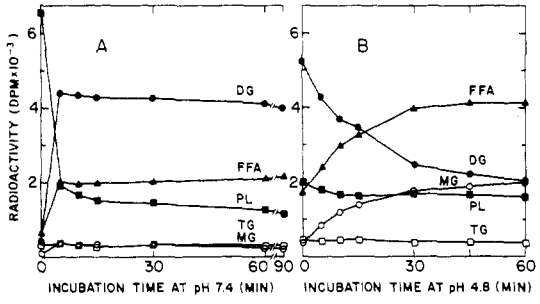

FIGURE 2: The change in lipid composition of microsomes pretreated with phospholipase C. Labeled microsomes, amounts equal to 1.5 and 2.0 mg of protein, for the experiments of (A) and (B), respectively, were incubated in the presence of phospholipase C for the times and at the pH indicated, according to the procedure outlined under Materials and Methods. Zero time of (B) represents the lipid profile of microsomes incubated for 10 min with phospholipase C at pH 7.4. Radioactivities were determined as detailed under Materials and Methods.

the monoacylglycerol area of the borate-impregnated plates.

The change in lipid composition of the microsomal membranes as a function of time was followed by incubating microsomes (made radioactive using distally-labeled fatty acids) at pH 7.4 in the presence of phospholipase C (Figure 2A). During the first 5 min, the radioactivity in the phospholipid fraction decreased by 74% with corresponding stoichiometric increases of radioactivity in the diacylglycerol and fatty acid fractions. Quantitation of the data shows that only about 80% of the phospholipid degraded is accounted for by diacylglycerol formed; the rest was degraded by the alkaline lipases to glycerol and free fatty acid. Further incubation, at pH 7.4 continued to 90 min, resulted in relatively little further changes in the radioactivities of the diacylglycerol, free fatty acid, phospholipid, or monoacylglycerol. This was of interest as it was similar to the time course obtained for the hydrolysis of a sonically irradiated dispersion of diacylglycerol incubated with microsomes at pH 8.0 (Cabot and Gatt, 1976).

The above contrasts with the results of an incubation carried out at pH 4.8, following the initial 10-min incubation at pH 7.4 with phospholipase C (Figure 2B; the time course of the initial 10 min at pH 7.4 is not shown in Figure 2B). At this acid pH, the radioactivity of the diacylglycerol which accumulated during the 10 min at pH 7.4 decreased rapidly during the first 30 min and was accompanied by a stoichiometric increase in the radioactivities of the free fatty acid and monoacylglycerol fractions. There was practically no decrease in the phospholipid radioactivity during the incubation at pH 4.8. The radioactivity in the triacylglycerol fraction, at both pH 7.4 and 4.8, did not change throughout the entire duration of the experiment. The radioactivity in the membranes used in this experiment was determined by thin-layer chromatography and found to occur mainly in phosphatidylcholine (61%, inclusive of phosphatidylserine and phosphatidylinositol), phosphatidylethanolamine (18%), and the remaining 21% composed of lysophosphatidylcholine, sphingomyelin, phosphatidic acid, neutral lipids, and fatty acids. Phospholipase A_2 treatment of the lipids extracted from labeled microsomes provided data that indicated an approximate 1:1 distribution of radioactivity in the *sn*-1 and *sn*-2 positions of the glycerol moiety. These data, obtained from thin-layer chromatograms, were the average of three experiments. The specific radioactivity of the membrane diacylglycerol formed by the action of phospholipase was about 11 000 dpm/μmol.

TABLE II: Action of Phospholipase C on Lipids Extracted from Labeled Microsomes.[a]

Procedure	Disintegrations of ^{14}C/min		
	PL	DG	FFA
Control[b]	2795	140	129
Incubated 60 min with phospholipase C at pH 7.4	736	2495	372
Incubated as above for 10 min followed by 60 min at pH 4.8	721	2378	180

[a] Lipids were extracted from labeled microsomes by the method of Bligh and Dyer (1959) and dispersed by sonic irradiation in 10 mM Tris buffer, pH 7.4. Aliquots of the lipid dispersion were incubated with phospholipase C (200 μg/mL) in 1.0 mM $CaCl_2$ as noted below. Radioactivities of the products were determined as outlined under Materials and Methods. Abbreviations as in Table I. [b] Control denotes the lipid profile of the nonincubated dispersion.

In order to determine if the phospholipase C preparation contributed to the changes in lipid composition other than removal of the phosphoryl-base group, lipids were extracted from labeled membranes, dispersed, and incubated with *Clostridium welchii* under the conditions described in Table II. As compared to an unincubated control, only a very small increase in the free fatty acid radioactivity was noted when lipids were incubated at either pH 7.4 or 4.8. Furthermore, dissimilar to the results with intact microsomes, the diacylglycerol level remained practically unchanged at pH 4.8.

In the previous paper (Cabot and Gatt, 1976), it was shown that *N*-ethylmaleimide inhibits the alkaline lipases, but has only little effect on the acid lipase. This was verified using endogenous substrate as follows. In the first experiment, *N*-ethylmaleimide (5–10 mm) was added to labeled microsomes (1.4 mg of protein) and these were incubated for 5 min at pH 7.4. Phospholipase C was then added and the incubation was continued. The radioactivity of the free fatty acids (which are formed from lipase action of the endogenous diacylglycerol) was only about 40% of that of a control tube that contained no *N*-ethylmaleimide. In the second experiment, microsomes were

incubated at pH 7.4 with phospholipase C, the pH was adjusted to 4.8, *N*-ethylmaleimide was added, and the incubation was continued for 40 min. At 5 and 10 mM *N*-ethylmaleimide, the reduction in the radioactivity of the free fatty acid relative to a control tube was only 7 and 16%, respectively; monoacylglyceride accumulated to an extent of 88% of that of the control tube which had no *N*-ethylmaleimide. This suggests that the acid diacylglycerol lipase was practically unaffected by the *N*-ethylmaleimide.

Labeled membranes were heated at 60 °C for varying time periods following a 5-min preincubation with phospholipase C at pH 7.4. The pH was then adjusted to 4.8 and the incubation was continued for 60 min. This showed an increasing inhibition of the hydrolysis of endogenous diacylglycerol as followed at pH 4.8. A 3-min incubation at 60 °C resulted in an approximate 60% inhibition of the hydrolysis of diacylglycerol.

Discussion

The previously reported microsomal lipases of rat brain which hydrolyzed dioleylglycerol at about pH 8 or 4.8 and monoacylglycerol at the alkaline pH only (Cabot and Gatt, 1976) were assayed by incubating enzyme (microsomes) with a sonically irradiated dispersion of lipid. Activities of membrane-bound lipolitic enzymes from other tissues have been studied by employing the microsomes as such (Biale et al., 1968; Muller and Alaupovic, 1970) or by extraction of the enzyme from the membrane, followed by partial purification (Pope et al., 1966). In the present communication, we assayed the activity of membrane-bound lipases through their action on endogenous diacylglycerol, generated by the addition of phospholipase C. The lipase activity was followed by determining the quantities of the two products, monoacylglycerol and fatty acid. Phosphatidylethanolamine and phosphatidylcholine account for about 34 and 35%, respectively, of the phospholipids of rat brain microsomes (Cuzner et al., 1965). Palmitic and oleic acids are the predominant fatty acids of the phosphatidylcholine fraction (Skrbic and Cumings, 1970). These two acids were therefore employed for labeling, by intracerebral injection, the lipids of rat brain microsomes. Hydrolysis of the microsomal phospholipids by snake venom phospholipase A_2 demonstrated an approximate 1:1 distribution of radioactivity between the *sn*-1 and *sn*-2 positions of the glycerol moiety. This is in accord with the well-established fact that animal glycerides have predominantly saturated fatty acids in the *sn*-1 and unsaturated acids in the *sn*-2 positions, respectively (Lands, 1965). The stoichiometric relationship and the time course suggest that the phospholipases (Woelk et al., 1974; Gullis and Rowe, 1975) or lysophospholipases (Leibovitz-BenGershon et al., 1972) of rat brain are minor contributors to the free fatty acids formed in the experiment illustrated in Figure 2, especially at pH 4.8 (Figure 2B).

The data of Table I and Figures 1 and 2 clearly demonstrate hydrolysis of endogenous diacylglycerol by the microsomal lipases. This is especially marked at pH 4.8 but also occurs at pH 7.4. At the latter pH, monoacylglycerol did not accumulate. This therefore confirms the previous report (Cabot and Gatt, 1974) that rat brain microsomes contain a diacylglycerol lipase and a monoacylglycerol lipase, both with an alkaline pH optimum, as well as a diacylglycerol lipase but no monoacylglycerol lipase with an acidic pH optimum. The time course as well as response to *N*-ethylmaleimide were also in accord with the observations made when exogenous dispersions of glycerides were employed as substrates for the above lipases (Cabot and Gatt, 1976).

In the recent few years, other investigators have employed techniques to enrich membranes with endogenous neutral glycerides (Van Schijndel et al., 1973; McMurray, 1975; Michell et al., 1973; Kanoh and Kimiyoshi, 1973, 1975; Fallon et al., 1975; Allan and Michell, 1975a). In the present study, labeled membranes were treated with phospholipase C. The latter were then used as a source of both enzyme and substrate and verified previous findings on lipases of rat brain microsomes. This might be extended by injecting different fatty acid species to determine acyl chain specificity of the rat brain lipases. Furthermore, selective labeling of the various lipid classes might be controlled by the time duration between injection and sacrifice of the animals (Yau and Sun, 1973).

The above technique provides membranes which have only enzyme, only substrate (if the membrane is first heated to inactivate the enzyme and then treated with phospholipase C), or both enzyme and substrate. It thus permits studying the action of a membrane-bound lipolytic enzyme on an endogenous or exogenous substrate. Even more important, it permits investigation of the interaction of a membrane-bound enzyme with a substrate that resides in the same or in another membrane. It thus opens the possibility of investigating intra- and intermembrane enzyme–substrate interaction, a cardinal problem of lipid enzymology in the intact cell.

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Purification Using Polyethylenimine Precipitation and Low Molecular Weight Subunit Analyses of Calf Thymus and Wheat Germ DNA-Dependent RNA Polymerase II†

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ABSTRACT: DNA-dependent RNA polymerase II from calf thymus has been successfully purified using polyethylenimine precipitation. Thus, 5–6 mg of nearly homogeneous RNA polymerase II (>96% pure) can be prepared from 1 kg of calf thymus with three chromatography steps following extraction and precipitation of the enzyme from the polyethylenimine pellet. This procedure eliminates the high salt extraction of chromatin previously used in purification of this enzyme and makes possible the large scale preparation of mammalian RNA polymerase II. Calf thymus polymerase II prepared by this method is greater than 90% form IIb and consists of ten different subunits having the following molecular weights: 180 000; 145 000; 36 000; 25 000; 20 000; 18 500; 16 000; 15 000; 12 000; 11 500. The homologous enzyme isolated from wheat germ is greater than 90% form IIa and contains subunits of the following molecular weights: 206 000; 145 000; 44 000–47 000; 24 500; 21 000; 19 000; 17 000; 14 000; 13 500. The wheat germ and calf thymus enzymes exhibit similar subunit structures, but the molecular weights of individual subunits are clearly different between the enzymes. Wheat germ RNA polymerase II is 50% inhibited by 0.271 μg/mL of α-amanitin, a level 30-fold higher than that found for calf thymus RNA polymerase II. These enzymes are further distinguished by the absence of antigenic cross reactivity.

Detailed physical and chemical characterization of the eukaryotic tripartite transcriptive system has been impeded by the difficulty of obtaining large quantities of purified RNA polymerases I, II, and III. Although significant amounts of these polymerases can be isolated from yeast (Valenzuela et al., 1976a; Bucher et al., 1974), the subunit structure and catalytic properties of these enzymes are markedly different from their mammalian counterparts (Valenzuela et al., 1976b; Bucher et al., 1976; Biswas et al., 1975). Thus for in vitro studies of chromatin and viral DNA transcription it would be desirable to use mammalian RNA polymerases rather than the bacterial and plant polymerases that have been used previously. Recently Jendrisak and Burgess (1975) have successfully employed Polymin P, a polyethylenimine (Zillig et al., 1970), for the purification of milligram quantities of RNA polymerase II from wheat germ. This methodology avoids high salt sonication and ultracentrifugation and is conducive to large scale enzyme preparation. This paper describes the first application of polyethylenimine fractionation to the purification of RNA polymerase II from a mammalian tissue. This procedure results in high yields of this enzyme and is more suitable for scaleup than previously used methods (Weaver et al., 1971; Kedinger et al., 1972; Weil and Blatti, 1975). The subunit structure, antigenic determinants, and α-amanitin sensitivity of calf thymus RNA polymerase II are compared with those of wheat germ RNA polymerase II. In addition, using lower porosity gels, we report the presence of several smaller subunits or components of calf thymus RNA polymerase II not previously observed in this enzyme.

Materials and Methods

Materials. Wheat germ from General Mills was stored at 4 °C. Fresh frozen calf thymus was obtained from Dubuque Park, Dubuque, Iowa, and maintained at −70 °C until use.

All biochemicals were reagent grade. Tritium labeled UTP (specific activity, 16 Ci/mmol) was obtained from Schwarz/Mann, α-amanitin and dithiothreitol were purchased from Calbiochem, calf thymus DNA (grade I) was from Sigma Chemical Co., and bovine serum albumin was from Miles Laboratories. Polyethylenimine (30%) was obtained from Aldrich Chemical Co. Nonidet-P-40 was a gift of Shell Chemical Co.

Solutions. All buffers were prepared from reverse-osmosis purified deionized water. Dithiothreitol was prepared as a 0.1 M stock solution and was added immediately before use. Buffer

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