Isolation of plasmid DNA from *Agrobacterium* by isopycnic density gradient centrifugation in vertical rotors[1]

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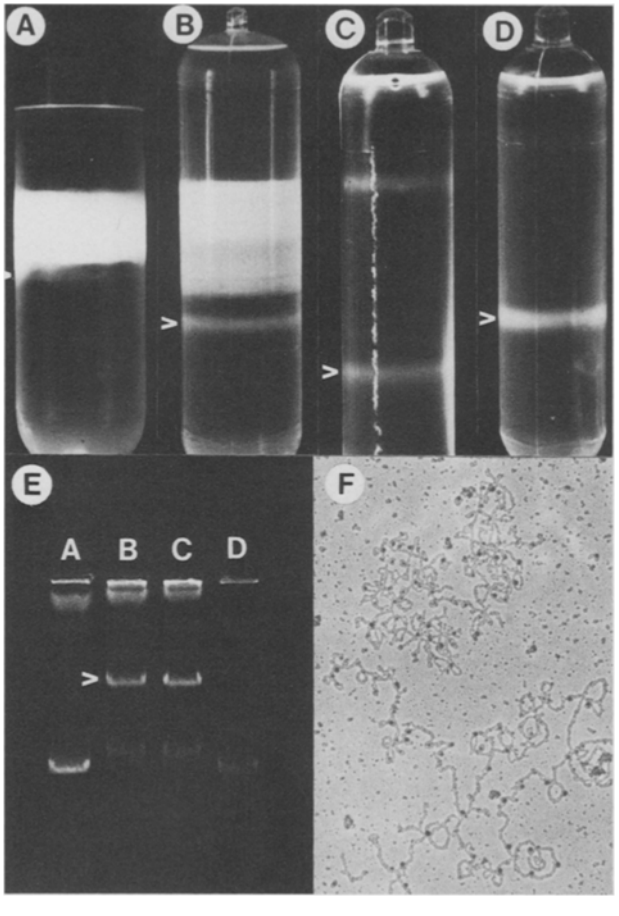
Summary. Fixed angle and vertical rotors are compared in terms of their sample load, run time, and resolution for the preparation of plasmid DNA from *Agrobacterium.*

The production of ultra-pure DNA from eucaryotic and procaryotic cells for genetic analysis and manipulations is costly and time consuming. The tumour-inducing (Ti) plasmid of the crown gall bacterium *Agrobacterium tumefaciens* is a potential vector in the genetic engineering of plants[2,3] since part of the Ti plasmid (the T-DNA) becomes integrated into the nuclear fraction of transformed cells[4]. As with other single copy, non-amplifiable, and high molecular weight ($> 10^8$ Daltons) plasmids, it is difficult to isolate in quantity. Published isolation methods using fixed angle rotors involve centrifugation times approaching 70 h[5]. However, the use of vertical rotors for the caesium chloride density gradient step reduces the centrifugation time to 12–16 h, allows more crude lysate DNA to be loaded onto the gradient, and improves the separation of plasmid from chromosomal DNA.

Materials and methods. Agrobacterium tumefaciens (octopine strain, ACH5) was cultured, lysed[6], and DNA from a 3L culture concentrated by centrifuging (29,000 rpm, 3 h) onto 3.0 ml glycerol cushions in a Beckman Ti 45 (6×94.0 ml) fixed angle rotor. Following removal of the supernatant, the DNA-glycerol layers (10.0–15.0 ml) were resuspended in 85.0 ml volumes of Tris buffer (0.05 M Tris-HCl, 0.02 M NaEDTA, pH 8.0), and recentrifuged over fresh glycerol. Bottom fractions (10.0 ml) from each tube were pooled, and the DNA concentration measured[7]. 2 sets of caesium chloride/ethidium bromide gradients were constructed containing 6.5–103.9 µg/ml of total DNA, 500 µg/ml of ethidium bromide and a final density of 1.56 g/ml. Centrifugation was compared in a Beckman Ti 70 fixed angle (8×38.5 ml) and a Beckman VTi 50 (8×38.5 ml) vertical rotor (Quick-seal tubes). Tubes were centrifuged (38,000 rpm, 20 °C) in a Beckman L5-65B centrifuge fitted with an ω^2t integrator for up to 60 h using slow acceleration (setting 4) and slow brake. Samples were examined after 12, 16, 20, 36, 48 and 60 h for the appearance of a narrow plasmid band beneath the chromosomal band. DNA bands were visualised (UV-light, 366 nm), gradients photographed, and interband distances (bottom of chromosomal band to top of plasmid band) measured from prints. Plasmid was collected in as small a volume as possible (1.5–2.0 ml/tube). Bands from the angle rotor were pooled, as were those from the vertical rotor. In some cases the pooled



A and *B* Separation of plasmid DNA from chromosomal DNA in (A) a large capacity Ti 70 fixed angle rotor tube, (B) a large capacity VTi vertical rotor tube. Each tube contained 77.9 µg/ml of total DNA and was centrifuged for 48 h. *C* and *D* Bands formed after re-centrifuging, in small capacity VTi 65 vertical rotor tubes, the plasmid collected from the Ti 70 fixed angle rotor (C) and the VTi 50 vertical rotor (D). A top contaminating chromosomal band is almost absent in D. *E* Sharp bands (channels B and C, arrowed) after gel electrophoresis of plasmid collected from the VTi 50 vertical rotor. Channels A and D, DNA standards (0.5 and 0.1 µg/well respectively). *F* Supercoiled plasmid molecules prepared in the VTi 50 vertical rotor. ×21,200. (Plasmid band arrowed).

Table 1. Effect of DNA concentration on the separation of Ti plasmid from chromosomal DNA in fixed angle and vertical rotors

DNA concentration per tube (mg)	DNA concentration in gradient (µg/ml)	Presence of a distinct plasmid band	
		Ti 70	VTi 50
6.0	155.8	−	+
4.0	103.9	−	+
3.0	77.9	±	+
2.0	51.9	+	+
1.0	26.0	+	+
0.5	13.0	+	+
0.25	6.5	+	+

Centrifugation conditions: 38,000 rpm, 48 h, 20 °C.

Table 2. Effect of centrifugation time on separation of Ti plasmid from chromosomal DNA in fixed angle and vertical rotors

Rotor	Presence of separate plasmid and chromosomal bands after centrifugation (h)						Distance between bands (mm)
	12	16	20	36	48	60	
Ti 70	−	−	−	+	+	+	5.0
VTi 50	+	+	+	+	+	+	12.8

Centrifugation conditions: 38,000 rpm, 20 °C, DNA concentration, 51.9 µg/ml.

preparations were re-centrifuged in the small capacity VTi 65 (8×5.3 ml) vertical rotor. Ethidium bromide and caesium chloride were removed from the plasmid, the latter characterized by horizontal gel electrophoresis and electron microscopy[6], and the DNA concentration measured[7].

Results. The efficiency of the Ti 70 fixed angle and VTi 50 vertical rotors for Ti plasmid preparation can be compared, since both have a similar maximum radius and hold tubes of similar dimensions and capacities. The smaller VTi 65 is useful for estimating the purity of plasmid prepared in the larger volume Ti 70 and VTi 50 rotors. At DNA concentrations higher than 77.9 µg/ml, a plasmid band was not always visible in the Ti 70 rotor after centrifugation. However, in the vertical rotors, with the same and higher concentrations of DNA, separation of plasmid from chromosomal DNA was always achieved, and it was possible to use up to 155.8 µg/ml of total DNA without overloading the gradient (table 1). When a DNA concentration was used at which the separation of plasmid from chromosomal DNA could be achieved repeatedly in both rotors (51.9 µg of total DNA/ml of gradient), it was found that in the vertical rotors equilibrium was reached with distinct plasmid and chromosomal bands present after 12–16 h of centrifugation. However, in the fixed angle rotor, equilibrium was not achieved until 36–48 h (table 2). Resolution in the vertical rotors was superior to that in the fixed angle rotor (table 2; fig. A and B). Plasmid prepared in the fixed angle Ti 70 rotor usually contained 20–30% contaminating chromosomal DNA (fig. C), which increased when the gradient carried more than 50 µg/ml of total DNA. In contrast, plasmid prepared in the VTi 50 rotor contained little or no chromosomal DNA after re-centrifugation (fig. D), and was at least 70% supercoiled as judged by gel electrophoresis (fig. E) and electron microscopy (fig. F). Plasmid yield was routinely 50 to 80 µg/l of bacterial culture.

Discussion. The aim of this investigation was to speed up the large scale isolation of plasmid DNA from *A. tumefaciens* whilst maintaining a low level of contaminating chromosomal DNA and other macromolecules. Using the procedure described, the total DNA from 1.0 g wet wt of bacteria could be processed in a single large capacity vertical rotor tube, and plasmid bands collected after 12 h. If an adequate plasmid concentration step[8] is included in the isolation procedure, then the plasmid DNA can be re-centrifuged several times in a small volume vertical tube to give an ultra-pure preparation in the same time that it would take to achieve a single isopycnic separation in a fixed angle rotor.

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Effect of free fatty acids and cholesterol in vitro on liver plasma membrane-bound enzymes

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Summary. The effect of cholesterol and fatty acid treatment in vitro was tested on rat liver plasma membrane-bound enzymes and lipid fluidity. The observed alterations of membrane fluidity affect both (Na^+-K^+)-ATPase and Mg^{2+}-ATPase activities but not 5′-nucleotidase; basal adenylate cyclase as well as its hormonal sensitivity were differentially affected by changes of membrane microenvironment.

Recent evidence points to the role of lipids in the modulation of plasma membrane-bound enzymatic activities and hormonal responses[2–5]. Alterations in phospholipid content and fatty acid composition as well as changes in cholesterol levels, observed in vivo and in vitro, affect the function of membrane enzymes[6] which in turn are mainly dependent on the fluidity of the membrane microenvironment[5,7].

Few reports have been published so far as to the dependence of liver plasma membrane-bound enzymatic activities on the in vivo or in vitro alteration of membrane lipid composition[6,8]. In this experimental framework, the observations to be reported herein deal with the in vitro modulation of rat liver plasma membrane fluidity as related to the activity of plasma membrane-bound (Na^+-K^+) and Mg^{2+}-ATPase (EC 3.6.1.4 and 3.6.1.3 respectively), 5′-nucleotidase (EC 3.1.3.5) and adenylate cyclase (EC 4.6.1.1).

Materials and methods. Liver plasma membranes were isolated from male Sprague-Dawley rats (150 g, av. b.wt), fed ad libitum, according to Ray[9] as previously reported[10]. Fatty acids dissolved in absolute ethanol were added to plasma membrane suspended in 0.05 M Tris HCl (pH 7.5) at a final concentration of 1 µmole/3 mg membrane protein in a volume of 1 ml, the final ethanol concentration being 1%. Cholesterol was dissolved as above and added to the membrane suspension at a final concentration of 100 µg/3 mg membrane protein. Fatty acid and cholesterol treatment of isolated liver membranes was carried out for 10 min at 20 °C[11] whereas controls were treated with ethanol at a final concentration of 1% for the same time; this gave a 15% decrease in basal activity detectable only in the case of the cyclase assay[5].

Fatty acid- or cholesterol-treated membranes were washed twice with the Tris buffer and resuspended to the initial protein concentration in the same buffer. The measured cholesterol enrichment, estimated as already reported[12], was about 25% ($p < 0.001$) being the cholesterol content 70 ± 3.6 and 93 ± 5.3 (mean ± SD, $n = 4$) µg/mg membrane protein in control and treated membranes respectively. The