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Analytical Techniques for Cell Fractions

VI. Multiple Gradient-Distributing Rotor (B-XXI)[1]

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The use of angle-head centrifuges for high-speed isopycnic banding of nucleic acids (1, 2), viruses (1, 3), and subcellular components including microsomes and glycogen (3, 4) offers the possibility of banding as many as twelve samples in one rotor. With rapidly diffusing materials such as cesium chloride, relatively steep gradients may be formed in a short time by diffusion (3). However, if slowly diffusing substances such as sucrose, potassium citrate or tartrate, dextran, or serum albumin are to be used, or if very shallow gradients are required, then simple diffusion between two layers may require a prohibitively long period of time.

In previous studies (5), a distributor head was used to distribute one gradient evenly among several swinging-bucket tubes during rotation. In this communication we describe a distributor rotor (designated B-XXI) which apportions a single liquid gradient uniformly among twelve Oak Ridge No. 30 polycarbonate tubes (6) during rotation at 2000 rpm. The technique is useful when a particle suspension or homogenate is to be subdivided on the basis of sedimentation rate into a number of fractions each one of which is to be further fractionated by isopycnic zonal centrifugation by the so-called "s-ρ" method (3).

PRINCIPLE OF OPERATION

A liquid stream flowing into a spinning cup will be evenly divided between a series of equally spaced apertures as it flows out of the cup

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under centrifugal force. By connecting the apertures to a series of angle-head centrifuge tubes, a single density gradient may be *apportioned* equally among them.

The gradient distributor rotor consists of a standard Beckman No. 30 preparative angle-head rotor with the distributor head securely attached by the radiation handle. The central gradient-receiving annular groove connects through 12 evenly spaced delivery spouts into the openings of the polycarbonate tubes (Fig. 1).

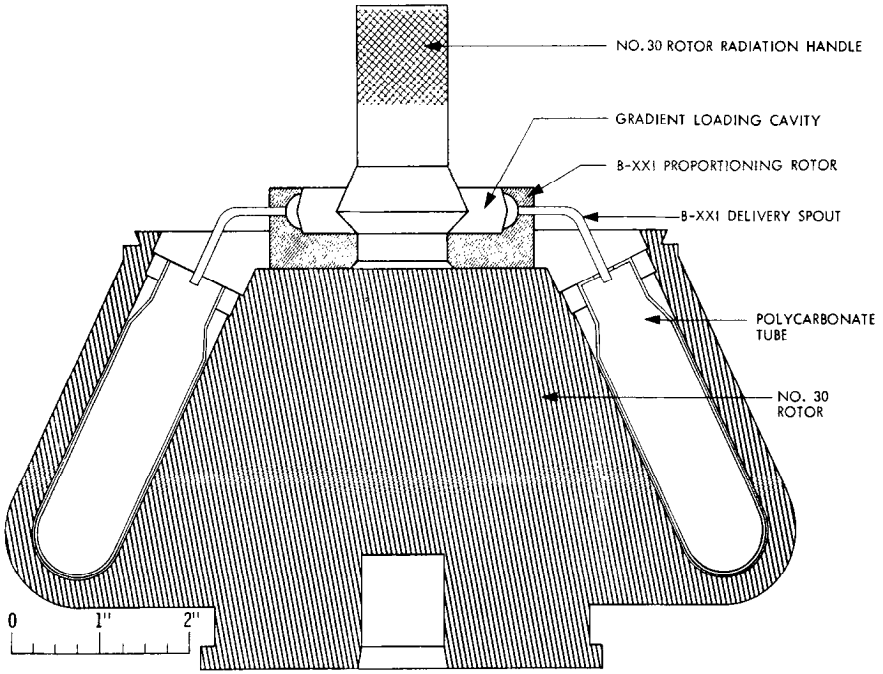

FIG. 1. Multiple gradient-distributing assembly. The diagram shows the combination of the B-XXI proportioning rotor and a standard Beckman No. 30 preparative angle-head rotor. The gradient solution is fed into the gradient loading cavity in the B-XXI rotor, where it is distributed to the tubes in the No. 30 rotor through the delivery spouts by centrifugal force. To scale.

The tubes are loaded while the rotor is spinning in a model L preparative ultracentrifuge. The lid of the centrifuge must be left open so that the gradient inflow line can be positioned in the gradient loading cavity of the B-XXI distributor rotor. The entire assembly is shown in Figure 2 with the nozzle of the gradient inflow line properly positioned in the gradient loading cavity. The gradient may be made previously by any one of a variety of methods and stored in a vertical cylinder that can be drained from the bottom by a gradient inflow line. When the rotor has attained a

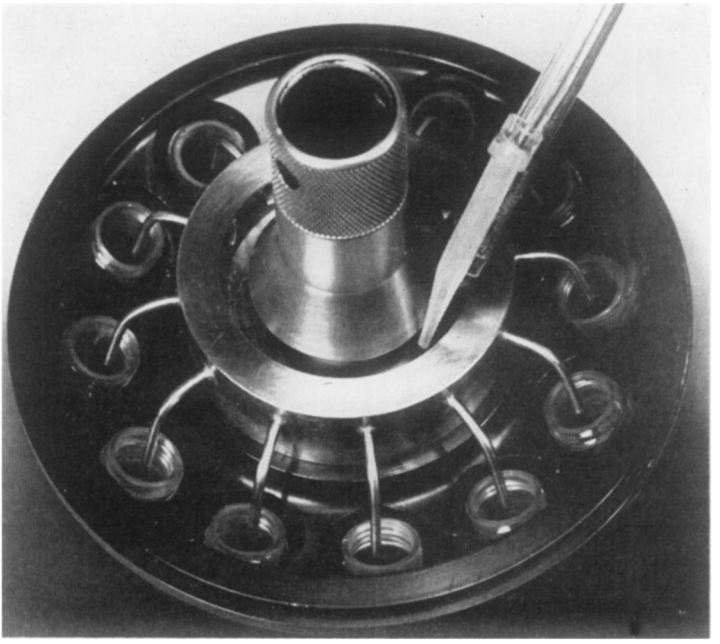

FIG. 2. Gradient-distributing assembly with gradient feed line in position.

speed of 2000 rpm, the gradient solution is introduced as a continuous stream, beginning with the densest portion of the gradient. The distribution of the gradient should require less than 5 min. When the rotor has coasted to a stop, the tubes are removed from the No. 30 rotor and are ready for use. Samples to be fractionated by isopycnic zonal centrifugation may then be layered on top of the gradients in the tubes with a syringe or a pipet.

RESULTS

To test the uniformity of the gradient distribution, three separate density gradient solutions—cesium chloride, potassium citrate, and sucrose—were used. Each had a total volume of 240 ml. A 5 ml overlay of a dilute rat liver homogenate was added by hand to the 20 ml gradient in each tube. These were spun at 24,000 rpm in a preparative ultracentrifuge for 1 hr or less. If the gradient solutions had distributed equally among 12 tubes of each set, the subcellular particles should band in the same position in all the tubes of each set. At the end of centrifugation, each set of 12 tubes was photographed in a special banding camera (6).

As shown in Figure 3, the even alignment of the rat liver bands indicates the reproducibility of the gradient in sets of tubes. In Figure 3 (upper) the cesium chloride density gradient extended from 1.10 to

1.82 gm/ml. After 30 min at 24,000 rpm, two distinct bands are observed, the lower being glycogen (4) at density 1.62, the upper being largely cell membranes and mitochondria. Figure 3 (middle) depicts the banding pattern of the rat liver centrifuged in 1 hr in a potassium citrate gradient

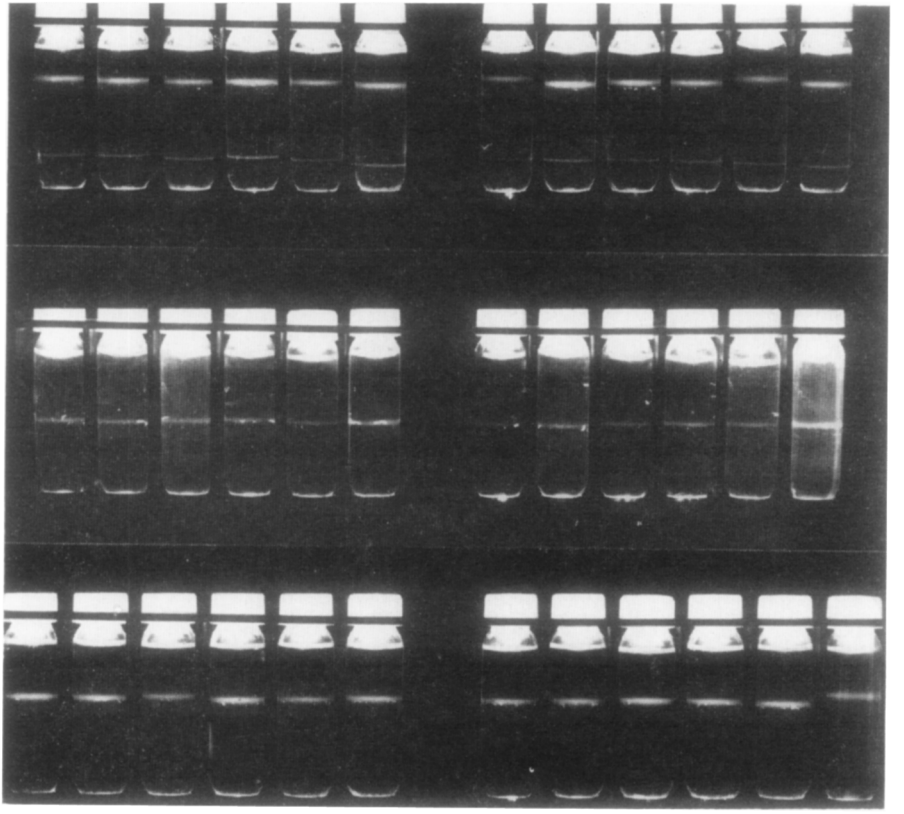

FIG. 3. Rat liver banded in gradients prepared with the gradient-distributing device. The bands were photographed by scattered light. The even alignment of the bands in each set of tubes serves to indicate the reproducibility of the gradients. (Upper) Rat liver banded in a cesium chloride gradient. (Middle) Rat liver banded in a potassium citrate gradient. (Lower) Rat liver banded in a sucrose gradient.

which ranged in density from 1.10 to 1.51 gm/ml. Figure 3 (lower) shows the alignment of the particulate bands in a sucrose gradient. The tubes were centrifuged for 1 hr, and the density of the gradient in this instance extended from 1.13 to 1.26 gm/gl. The potassium citrate gradient and the sucrose gradient were not sufficiently dense to allow glycogen to band.

DISCUSSION

The even alignment of the rat liver bands in the three sets of tubes demonstrates that the gradient distributor rotor is capable of distributing

a gradient solution equally among 12 tubes. The banding pattern of the rat liver in these tubes is not the same pattern that would be observed had the tubes been centrifuged longer. The centrifugation time was purposely kept short to show that the gradients were produced by even distribution of the gradient material and not by simple diffusion.

The greatest advantage of the gradient-distributing rotor is that gradients can be prepared in a very short time. Gradients of the three different solutions used for this study were prepared in less than 30 min, or about 10 min for each set of 12 tubes.

If the gradient solution is fed to the distributor rotor properly, there should be less than 1 ml variation in the volumes of the gradient in any two tubes in the rotor. Good distribution of the gradient solution can be achieved only if the gradient solution is fed to the rotor in an uninterrupted stream which has a constant flow rate. The flow rate of the gradient must be regulated so that it is not great enough to flood the gradient loading cavity or small enough to enter the loading cavity as discrete drops. In this study the sucrose gradient was loaded at a rate of 60 ml/min, the potassium citrate was loaded at 120 ml/min, and the cesium chloride was loaded at 260 ml/min. Slower or faster rates of gradient addition may be used provided a steady, uninterrupted flow of the gradient solution can be maintained during the loading operation. Any sudden change in the flow rate of the gradient solution during loading will cause a few tubes to be overloaded. The imbalance produced by overloading a few tubes in the spinning rotor causes the rotor to precess (wobble). When the loading process is carried out at 2000 rpm, this precession is very small and is not great enough to represent a hazard to either the operator or the centrifuge. Tubes have been purposely overloaded by increasing the flow rate of the gradient as much as ten times during loading. The precession caused by this sudden change in flow rate was noticeable and would produce a nonuniform series of gradients. The loading process should, of course, be terminated if precession of the rotor is observed. The rotational speed of the rotor during loading is also very important for equal distribution.

Loading speeds of 500, 1000, 2000, and 3000 rpm were tested (the ultracentrifuge drive cannot be started when the lid is open and the rpm selection switch is set at greater than or equal to 4000 rpm). Gradient solutions loaded at 500 rpm were distributed very poorly. The gradient volumes varied as much as 5 ml between some tubes. At 1000 rpm the No. 30 rotor gyrated so badly that it was impossible to keep the nozzle of the gradient inflow line in the gradient loading cavity. Loading at 2000 rpm was selected at the B-XXI loading speed because it was the lowest speed tested that yielded good gradient distribution. As mentioned

above, there was less than 1 ml variation of the gradient volumes in any two tubes of the set of 12 tubes when the loading is done at 2000 rpm. Good gradient distribution can be achieved by loading at 3000 rpm, but this increase in speed does not appear to be necessary.

It should be mentioned that good gradient distribution can be achieved only if the B-XXI has been carefully machined, properly balanced, and centered on the No. 30 rotor.

In the s-ρ technique, subcellular particles are separated first on the basis of sedimentation rate in a zonal centrifuge. Each of the recovered fractions is then banded isopycnically in angle-head rotors. In previous studies, relatively steep diffusion-formed gradients were used. The B-XXI distributor head now allows shallow gradients to be made in which a much higher resolution separation of endoplasmic reticulum fragments, cell membranes, and viruses is possible. Experimental studies with this system will be described elsewhere.

SUMMARY

Identical liquid density gradients required for s-ρ separations have been prepared in 12 angle-head rotor tubes in less than 10 min by a spinning distributor rotor. The technique is especially useful for the preparation of gradients with materials that would take too long to establish gradients by simple diffusion, such as sucrose or potassium citrate.

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