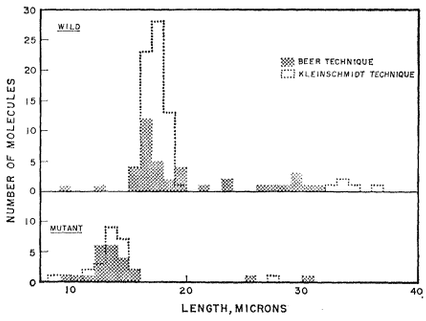

Fig. 2. Histogram showing molecular lengths. (Top) A single batch of DNA molecules from $\lambda cb_2^+b_5^+$, containing both linear and circular forms, measured after electron microscopic preparation by the two different techniques. Both yield the same value for monomer length (both linear and circular), while the more stressful technique of Beer results in more scattered lengths being seen. Dimers occur in both preparations. (Bottom) DNA molecules from the density mutant λcb_2b_5. Those prepared by the technique of Beer had been heat-treated to favor the formation of circles, while those prepared by the technique of Kleinschmidt had received no heat treatment.

was touched with a grid covered by a carbon film, dried, and shadowed with platinum at an angle of 7° in two or three different directions. Some results typical of over 260 molecules that were measured are shown in Fig. 1.

The lengths of these molecules were measured by tracing enlarged projections with ink and determining their contour length with a map-distance measuring device. The total magnification was determined by micrographs of a grating replica (*10*). The calculated molecular lengths of 163 molecules are assembled in histograms shown in Fig. 2. Two things can be seen. (i) The wild-type molecules are 17.2 ± 0.9 μ long (96 measurements) while the mutant molecules are 13.2 ± 1.5 μ long (48 measurements) or 23 percent shorter. This is in agreement with other estimates of the fraction of DNA missing from the mutant molecules, namely: 23 percent, calculated from the densities of the phage particles (*3*) and 20 percent, calculated from the relative sedimentation rates of the DNA molecules in sucrose gradients (*6*). (ii) The mean length measured on carbon grids, 17.1 ± 1.1 μ, is the same as that measured in cytochrome *c* films, 17.2 ± 0.8 μ. Since it is unlikely that both procedures would lead to exactly the same perturbation in length, we shall assume that neither has been significantly disturbed. At the time of deposition, the molecules were in solution and thus in their most hydrated form. If this were equivalent to the B crystallographic form (*11*), the sodium salt of the molecule should have a linear density of 192 daltons per angstrom. Multiplying this number by the measured length gives a molecular weight of 33 million for the DNA molecule of wild-type λ. This value for the molecular weight is in good agreement with the value of 31 million obtained by Burgi and Hershey (*12*) from the sedimentation rate of this DNA in sucrose gradients. This agreement encourages the belief that our assumption of the B configuration was justified.

About half of the molecules whose lengths are shown in Fig. 2 were in the circular form. The lengths of circular and linear monomers were the same within the limits of experimental error. Thus the point of union must be at or near the ends of the molecule. To examine this point of union more carefully, we have inspected the central regions of eight linear dimers. Three of these are shown in Fig. 1 (*D*, *E*, and *F*). We can see no obvious joining point.

Finally, we turn to the frequencies of different types of molecules seen in grids prepared from DNA solutions which had received the two different heat treatments. Samples were prepared with cytochrome film and every molecule seen was photographed. Table 1 shows the composition of the total group comprising 97 molecules and fragments. The results leave very little doubt that slow cooling from 75°C or incubation at 60°C does cause the DNA molecule from λ bacteriophage to assume a circular form.

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Density Gradient Centrifugation of a Murine Leukemia Virus

Abstract. *The Rauscher leukemia virus separated as a single band upon density gradient centrifugation in cesium chloride, rubidium chloride, sucrose, potassium citrate, or potassium tartrate. Prolonged exposure to concentrated potassium citrate or potassium tartrate solutions caused lysis of the virus; the resulting products, having different densities, separated on the density gradients.*

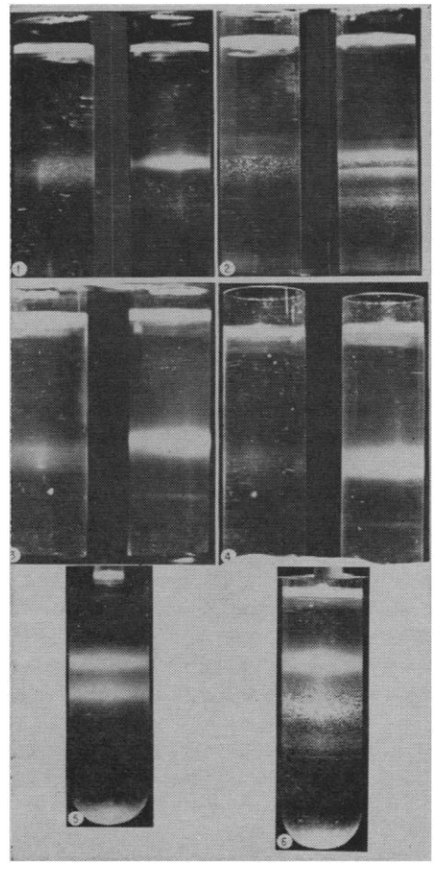
Over a dozen viruses (*1*) have been isolated and identified as causative agents of murine leukemias. Several of these have been prepared in large amounts by a procedure involving differential centrifugation (*2*). Electron microscopy (*3*) of the murine leukemia viruses indicates that they are typical "membrane viruses," because, like myxoviruses and viruses of the avian leukosis group, they are assembled at the plasma membrane of the infected cell, from which they are released by a budding process. The virus particles measure approximately 100 mμ and contain denser-staining cores (presumably nucleoprotein) surrounded by lesser-staining double outer membranes (presumably derived from the plasma membrane).

Myxoviruses can be lysed by chemical treatment; the internal structures of various members of this group are serologically interrelated and contain nucleoprotein filaments which show individual differences in morphological fine structure (*4*). Similar filaments have been reported as internal constituents of the avian myeloblastosis virus (*5*) and of virus-like particles associated with two cases of human leukemia (*6*).

Thus a controlled splitting of murine leukemia viruses should result in a better physical and serological characterization of viruses of this group and should also provide a guideline for studies on suspected human-leukemia viruses. We are reporting the results of centrifugation of a murine leukemia virus (Rauscher) in a new medium—potassium citrate—as well as in a variety of conventional media. Upon centrifugation in all the media, the virus separated as a single band, and the recovery of infectious virus was particularly high on short centrifugations in potassium citrate. However, the virus lysed into several components of different densities after prolonged centrifugations in either potassium citrate or potassium tartrate.

McCrea and colleagues (*7*) have used gradients of potassium tartrate in centrifugations of influenza A, influenza B, and Newcastle disease viruses, and obtained high recoveries of infectivities, in short (0.66 to 3 hours) centrifugations. We chose potassium citrate as a density gradient material because this salt has a relatively high molecular weight, is readily soluble in water, can readily be buffered, in solution, by addition of small quantities of citric acid, and is inexpensive in reagent grade, and because the citrate ion has been shown to stabilize the infectivity of the viruses of both Rous sarcoma (*8*) and several murine leukemias (*2*, *9*). Density gradients of either potassium citrate or potassium tartrate have to be preformed, conveniently with the aid of a layering device (*10*), but once formed are quite stable and provide a linear density range (1.0 to 1.45 g/cm^3) which should be adequate for the centrifugation of most of the known animal viruses.

The Rauscher leukemia virus (R-NCI-2) was prepared and purified by the differential centrifugation of the pooled plasma of viremic BALB/C mice that had been infected with the virus 20 to 30 days previously (*9*); the final virus concentrate was then sedimented at 30,000*g* for 60 minutes, after which time the viral pellets were resuspended in a volume of 0.05 molar sodium citrate (*p*H 6.75) such that 1 ml of the final virus stock derived from an initial 10 ml of mouse plasma. Pooled plasma of normal, noninoculated BALB/C mice of the same age was treated according to an identical protocol to provide a control sample. Stock samples of the virus and of the control were frozen and stored at −70°C and thawed just prior to use. Portions (0.5 ml) of the virus stock or control sample were layered onto the surface of preformed density gradients (density: 1.05 to 1.36 g/cm^3) of the appropriate salts in lusteroid tubes (1.3 by 5 cm) and the tubes were then placed in the SW39L Spinco rotor and centrifuged at 36,000 rev/min at 0° to 4°C in a Spinco model L preparative centrifuge. At various times the tubes were removed, illuminated from the top, and photographed. The tubes were then punctured at the bottom, to allow collection of fractions by the drop, or were subjected to further centrifugation. The densities of the fractions were determined (*11*) or else the fractions were dialyzed successively at 4°C against increasing dilutions of the density gradient solutions and finally against 0.05 molar sodium citrate (*p*H 6.7), and they were then assayed for infectivity (*9*) or were examined by electron microscopy.



Figs. 1–6. Density gradient tubes containing virus or control samples after centrifugation under various conditions. Fig. 1. Tubes containing control (left tube) and virus (right tube) after 4 hours' centrifugation in potassium citrate gradient. Fig. 2. Same tubes as Fig. 1 after 22 hours' centrifugation. Fig. 3. Tubes containing control (left tube) and virus (right tube) after 3 hours' centrifugation in preformed cesium chloride gradient. Fig. 4. Same tubes as Fig. 3 after 22 hours' centrifugation. Fig. 5. Tube containing virus, which had been pre-incubated for 40 hours in 1.15 g/ml density potassium citrate, after 2 hours' centrifugation in potassium citrate gradient. Fig. 6. Same tube as Fig. 5 after 22 hours' centrifugation.

Centrifugation of the virus for 0.75 to 6 hours in potassium citrate gradients resulted in the formation of a single intense opalescent band (density: 1.16 g/cm^3) whereas identical treatment of the control sample gave only a very weak and diffuse opalescence at a slightly higher density position (Fig. 1). Electron microscopy showed a high concentrate of the characteristic leukemia virus particles in the 1.16 g/cm^3 fraction, whereas other fractions showed no such particles except for the lightest fraction, which possibly desorbed particles from the tube wall during draining of the tube. High titers of infectious virus were associated with the opalescent zone (Table 1); the recovery of infectious virus was particularly high in a short centrifugation when extra calcium and magnesium salts were incorporated in the gradient (Table 1, experiment 1), although in this case the sharpness of the opalescent band and associated infectivity were somewhat reduced.

Quite different results were obtained when the centrifugation of the virus in potassium citrate gradients was continued for more prolonged periods. After 22 hours' centrifugation several opalescent bands had formed, and while an opalescent band remained at the 1.16 g/cm^3 density, it was substantially reduced in intensity (Fig. 2). The new bands did not contain infectious virus and only a small titer of infectious virus was found in the remains of the 1.16 g/cm^3 band after 48 hours centrifugation (Table 1, experiment 3).

The behavior of the Rauscher virus on centrifugation in potassium tartrate gradients was generally similar to that observed in the citrate gradients in that a single opalescent band (density: 1.16 g/cm^3) containing infectious virus (Table 1, experiment 4) was obtained on short centrifugations, whereas prolonged centrifugation gave multiple opalescent bands with densities similar

to those obtained in the potassium citrate system.

On the other hand, when the Rauscher virus was centrifuged in preformed cesium chloride gradients for either 3 hours (Fig. 3 and Table 1, experiment 5) or 22 hours (Fig. 4) a single but somewhat diffuse opalescent band was obtained. A similar result was obtained if the virus was premixed with solutions of either cesium chloride or rubidium chloride and then centrifuged for 22 hours (Table 1, experiments 6 and 7) to spontaneously establish density gradients, as previously described by Crawford (*12*) in studies on the Rous sarcoma virus. The infectivity titers of the virus after centrifugation in cesium chloride or rubidium chloride gradients were considerably lower than those obtained after short centrifugations in potassium citrate or potassium tartrate gradients but were not significantly altered by the duration of the centrifugation.

Preliminary experiments indicate that the Rauscher virus separates as a single band (density: 1.15 g/cm^3) after centrifugation for 22 hours in sucrose-saline density gradients, but infectivity assays on recovered virus are still pending.

The multiple bands obtained on prolonged centrifugation of the Rauscher virus in potassium citrate and tartrate gradients could arise from a final equilibration of various density components originally present as a mixture in the virus preparation. In addition to the separation of the virus as a single band in cesium chloride, rubidium chloride, and sucrose-saline gradients, as noted above, several pieces of evidence do not support such an explanation. No splitting of the viral opalescent band was observed during sedimentation through a shallow potassium citrate gradient (density: 1.0 to 1.20 g/cm^3) to the final band position during centrifugation at various speeds for periods up to 5 hours. Although multiple bands in the potassium citrate and tartrate gradients were not clearly observable until the virus had been centrifuged for approximately 16 hours, the material isolated from the new bands could be recentrifuged within 2 hours in identical gradients to their equilibrium positions (materials isolated from the bands were dialyzed against 0.05 molar sodium citrate prior to recentrifugation). Incubation of virus in 1.15 g/cm^3 density potassium citrate for 40 hours at 4°C altered the virus so that after a 2-hour centrifugation of the incubated mixture in a potassium

Table 1. Separation and infectivity of Rauscher virus centrifuged in various density gradient media.

Expt. No.	Separation characteristics			Infectivity assay*				
	Time of centrifugation (hr)	Densities of opalescent bands (g/cm^3)	Character of opalescent band	Density gradient region assayed (g/cm^3)	Animals with palpable splenomegaly	Time (days) to 50% incidence of splenomegaly	No. of deaths	Time to 50% incidence of deaths
	Potassium citrate in phosphate-buffered saline containing 0.5 g of magnesium chloride and 0.5 g of calcium chloride per liter†							
1	0.75	1.16	Moderately diffuse	1.18	10/16	47	3/16	
				1.16	23/23	12	15/23	30
				1.12	21/22	41	9/22	
				Control	(12/12)	(12)	(10/12)	(27)
	Potassium citrate–citric acid (p*H 7.1*) *in water*†							
2	4	1.16	Sharp	5-drop fraction at 1.16. Entire duplicate gradient (100 drops)	16/16	26	12/16	46
					17/17	20	15/17	38
	Potassium citrate in phosphate-buffered saline†							
3	48	1.16	Moderately diffuse	1.16	4/23		0/23	
		1.19	Diffuse	1.19	0		0	
		1.25	Moderately diffuse	1.25	0		0	
				Control	(16/17)	(12)	(10/17)	(30)
	Potassium tartrate-tartaric acid in water (p*H 7.5*)†							
4	2	1.16	Sharp	1.25	0/25		0/25	
				1.18	0/25		0/25	
				1.16	17/25	28	8/25	
				1.13	16/19	58	1/19	
	*Cesium chloride in 0.05*M *sodium citrate* (p*H 6.75*)†							
5	3	1.16	Moderately diffuse	1.18	2/28		0/28	
				1.16	14/26	62	0/26	
				1.14	3/27		0/27	
				Control	(24/24)	(12)	(19/24)	(28)
	Virus layered on cesium chloride solution (density: 1.20 g/ml) in phosphate-buffered (p*H 7.0*) *saline containing 0.04% bovine serum albumin*							
6	22	1.175–1.21	Diffuse	1.21	0/21		0/21	
				1.20	0/24		0/24	
				1.19	22/23	61	10/23	
	Virus admixed with rubidium chloride solution in phosphate-buffered saline (p*H 6.9*) *containing 0.03% bovine serum albumin. Virus input was three times the concentration of experiments 1–6.*							
7	22	1.17–1.20	Diffuse	1.198	0/13		0/13	
				1.192	3/18		0/18	
				1.188	10/15	111	0/15	
				1.186	5/10	80	4/10	
				1.181	12/14	57	0/14	
				1.173 (top of tube)	12/15	63	0/15	

* Based on palpable splenomegaly and deaths produced in inoculated BALB/C mice; 0.5-ml virus stock was used in each density gradient experiment, except for experiment 7. Dialyzed fractions from the gradient were diluted to 5 ml with 0.05*M* sodium citrate. Young (3- to 14-days-old) mice of the same age were then inoculated with 0.1-ml portions of either the fraction dilutions or 10^{-1} dilutions of the virus stock in sodium citrate as controls.
† Denotes gradients preformed with the aid of a gradient-forming device (*10*).

citrate gradient (*13*) two opalescent bands separated (Fig. 5); continued centrifugation for 20 hours did not affect the appearance of the upper band but the lower band showed splitting into multiple diffuse bands (Fig. 6). All of the above observations indicate that prolonged exposure of the Rauscher virus to concentrated potassium citrate or tartrate causes a lysis of the virus, which is aggravated by the centrifugation conditions, and a simultaneous release of subviral components which then separate in the density gradient.

The behavior of the Rauscher virus on centrifugation for short periods in potassium citrate or tartrate gradients, or on centrifugation for prolonged periods in cesium chloride, rubidium chloride or sucrose gradients, is characteristic of that expected of a "membrane virus" as shown by previous studies on influenza (*7*), Newcastle disease (*7, 14*) and Rous sarcoma (*12*) viruses. In view of the reported chemical composition of the influenza (*15*) and the avian myeloblastosis (*16*) viruses, it appears probable that "membrane" viruses in general have outer lipoprotein membranes, which comprise significant fractions of the total viruses and which enclose more dense nucleoprotein cores. Electron microscopy of the materials recovered from the prolonged centrifugation of the Rauscher virus in potassium citrate gradients suggests that dissolution of the viral membrane is concomitant with the viral lysis. Accordingly it was of interest to determine whether the behavior of the Rauscher virus on these gradients was characteristic of "membrane viruses" or a peculiarity of this etherlabile virus. The Moloney leukemia virus separated as a single band (density: 1.16 g/cm^3) in potassium citrate gradients after 3 hours centrifugation but gave multiple bands on centrifugation for 22 hours. The Rous sarcoma virus (Bryan strain), on the other hand, separated as a single band (density: 1.16 g/cm^3) in the potassium citrate gradient after centrifugation for 18 hours (*17*). Thus the susceptibility to lysis in potassium citrate gradients may reflect constitutive differences between viruses which show similar density behavior.

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N-Ethylmaleimide Inhibition of Horseshoe Crab Hemocyte Agglutination

Abstract. *The sulfhydryl inhibitor* N-*ethylmaleimide* (*NEM*) *inhibits the agglutination of horseshoe crab hemocytes. The inhibitory action is neutralized by adding cysteine to the NEM before it is mixed with the hemolymph. This new method for arresting horseshoe crab hemocyte agglutination suggests the participation of sulfhydryl groups in the process.*

Loeb (*1*) and Copley (*2*) have described two phases in the clotting of hemolymph (blood) of the horseshoe crab (*Limulus polyphemus*): hemocyte agglutination and subsequent gelation of the hemolymph. The study reported here concerns the first phase, the rapid and apparently irreversible clumping (agglutination) of the cellular elements of the shed hemolymph. In agreement with a previous report (*2*), low temperature, nonwettable surfaces, and heparin were largely ineffective in preventing hemocyte agglutination. The cation binding agents (oxalate, citrate, and ethylenediamine tetraacetate) were also ineffective. Recently, certain sulfhydryl inhibitors were shown to prevent the agglutination of mammalian platelets (*3*). We now report that under certain conditions, agglutination of *Limulus* hemocytes is inhibited by *N*-ethylmaleimide (NEM). This material alkylates sulfhydryl groups in a specific manner (*4*); the reaction is difficult to reverse (*5*).

Hemolymph was collected by inserting an 18-gauge needle into a clean leg joint and allowing the hemolymph to flow into a Krogh-Keys syringe pipet. Hemolymph appears to be an appropriate term for the fluid thus collected since there is an open circulation in this area of the *Limulus* (*6*). In these studies the hemolymph was always diluted ten times in the collection step by having in the syringe pipet a sufficient volume of one of the following test solutions: buffered NaCl, NEM, cysteine, and NEM-cysteine solutions. The diluent for *Limulus*

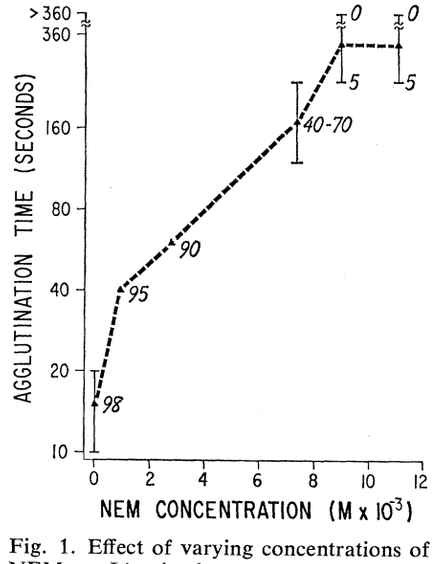

Fig. 1. Effect of varying concentrations of NEM on *Limulus* hemocyte agglutination. The points on the curve represent the time interval between the puncture of the joint and macroscopic agglutination. Included beside the points are the microscopically estimated percentages of agglutinated cells after 5 minutes of observation. Agglutination was absent for points greater than 360 seconds.