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Surface-Active Agents for Isolation of the Core Component of Avian Myeloblastosis Virus[1]

KURT STROMBERG

Laboratory of Biology, National Cancer Institute, Bethesda, Maryland 20014

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Sixty-one surface-active agents were evaluated in a procedure designed to assess their ability to remove the envelope from the core component of avian myeloblastosis virus (AMV). The procedure consisted of centrifugation of intact AMV through a series of sucrose gradients each containing an upper layer of agent at one of eight concentrations between 0.01 and 10%. The effectiveness of an agent in producing AMV cores was indicated by (i) the appearance of light-scattering bands in the region of core buoyant density in gradient tubes; (ii) the range of surfactant concentration over which these bands appeared; and (iii) an electron microscopy assessment by the negative-staining technique of the relative proportion of core to non-core material in each of these bands. Six nonionic surfactants were selected by this screening method for comparison in regard to recovery of core protein and endogenous ribonucleic acid (RNA)-dependent deoxyribonucleic acid (DNA) polymerase activity, as well as further morphologic evaluation by electron microscopy. The nonionic surfactants of the polyoxyethylene alcohol class (particularly, Sterox SL) were most effective. Nonionic surfactants of the polyoxyethylene alkylphenol class (particularly, Nonidet P-40) were also effective. Sterox SL and Nonidet P-40 each gave a more than fivefold increase in specific activity of endogenous RNA-dependent DNA polymerase, and each gave a low recovery of core protein. Sterox SL did not interfere to the extent that Nonidet P-40 did in procedures which involved spectrophotometric assay at 260 nm. The use of Sterox SL resulted in the least envelope contamination of core preparations by electron microscopy examination, the most recovery of protein and endogenous RNA-dependent DNA polymerase activity, and a core buoyant density in sucrose of 1.27 g/ml.

Biochemical investigation of the core of the avian and murine type C ribonucleic acid (RNA) tumor viruses has been hampered by lack of a reliable method for isolating this component in sufficient yield, purity, and integrity. Avian type C virus, as observed with the electron microscope, is enclosed in an outer membrane, or envelope, that appears to be acquired from the plasma membrane by a budding process. Within this envelope is the viral core, which consists of an inner shell, the intermediate layer, around an electron-dense spherule, the nucleoid (8). An electron-lucent space between the intermediate layer and the nucleoid is typical of mature avian type C virus; in its nonavian counterparts a separate intermediate layer is not readily distinguished, so the term nucleoid is applied to the entire core (11).

Several surface-active agents have been used to remove the envelope from the core of type C RNA tumor viruses; these include deoxycholate and Triton X-100 (8), Tween and ether (11), digitonin (10), ether (20), and nonionic surfactants of the polyoxyethylene alkylphenol class such as Nonidet P-40 and Triton X-100 (3, 9, 12) and Nonidet P-40 and ether (6). The use of different agents has been associated with discrepancies in the values obtained for buoyant density of type C cores. Values obtained for the murine leukemia virus have been 1.24 to 1.26 g/ml (11), 1.22 g/ml (10), and 1.28 g/ml (20). Values for the avian particle have been reported at 1.24 g/ml (9), 1.25 g/ml (6), and 1.27 g/ml (3). Bader et al. (3) have emphasized the structural instability and low yield of particles isolated with several of these agents. It is clear that firm characterization of envelope and core cannot be achieved without an improved technique for isolation of these components.

This report describes an evaluation of a great number of surface-active agents for their capacity to yield avian myeloblastosis virus (AMV)

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cores. Because the core component of all RNA tumor viruses is a morphologic entity and each possibly has a characteristic buoyant density, the two defining features of morphology and buoyant density served as the initial basis for screening the effectiveness of all surfactants. The screening assay consisted of centrifugation of intact AMV through a series of sucrose gradients, each of which contained an upper layer of surfactant at one of eight concentrations between 0.01 and 10.0%. The method depended upon the fact that as intact AMV was centrifuged through the layer of surfactant the envelope was removed; this released the more dense core which progressed through the gradient until its buoyant density was attained. If a distinct light-scattering band did not appear in the region of core buoyant density at any of the wide range of surfactant concentrations, the agent was judged ineffective. If bands appeared in the appropriate region, they were removed and examined with the electron microscope. If relatively large amounts of intact AMV or envelope membrane contaminated these bands, the surface-active agent was also rejected. Six of the total of sixty-one surfactants that were assessed by this screening method were considered sufficiently effective to warrant further comparative evaluation by assay for recovery of core protein and deoxyribonucleic acid (DNA) polymerase activity, in addition to morphologic examination by the negative-stain technique.

MATERIALS AND METHODS

Production and isolation of virus. An initial supply of AMV (Beard's BAI strain A) as viremic plasma was generously provided by D. W. Allen. Production of viremic plasma was carried out in the following manner. One-day-old specific pathogen-free Mt. Hope White Leghorn chicks from SPAFAS, Inc. (Gettysburg, Pa.) were injected intraperitoneally with 0.2 ml of a 1:1 solution of phosphate-buffered saline and viremic plasma [containing over 10^{12} AMV particles per ml by adenosine triphosphatase determination (7)]. Chicks were screened for development of leukemia by examination of smears of peripheral blood on the 12th and 18th day after injection. Microhematocrit tubes were then used to follow the degree of myeloblastosis. When myeloblasts comprised at least 20% of the total blood volume, chicks were bled by intracardiac exsanguination with a syringe containing 1 ml of sodium heparin (1,500 units/ml).

Virus isolated from viremic blood by differential centrifugation was used in the screening evaluation of all the surfactants. AMV was separated by centrifugation from leukemic blood drawn on the morning of use to avoid structural damage caused by freezing and thawing and to avoid exposing AMV to hypertonic gradient solutions. Cells were sedimented from viremic blood by centrifugation at 5,000 × *g* for 15 min; cell fragments and organelles were removed from the plasma by centrifugation at 10,000 × *g* for 15 min. The remaining supernatant fluid was then centrifuged at 40,000 × *g* for 60 min to pack the virus into a pellet. The standard buffer for manipulation of virus was SET [100 mM NaCl, 1.0 mM ethylenediaminetetraacetic acid (sodium salt), 50 mM tris(hydroxymethyl)aminomethene (Tris)-hydrochloride, *p*H 7.4, at 0 C, milliosmolarity between 285 and 295]. The packed virus was suspended in SET buffer by hand homogenization (25 strokes) in a Potter-Elvehjem tissue grinder [chamber clearance 0.004 to 0.006 inches (ea. 0.01 to 0.02 cm)]. The suspension was diluted with SET buffer to give a transmission of 30% at 540 nm on a DU-2 spectrophotometer. The protein content and adenosine triphosphatase activity of many different samples prepared in this manner were relatively constant, as was the low level of nonviral material seen in thin sections by electron microscopy.

For comparative biochemical assay of the six most effective surfactants in the screening evaluation, AMV was purified by rate-zonal centrifugation in Ficoll (K. Stromberg et al., *manuscript in preparation*). In brief, 8 ml of the supernatant fraction of viremic plasma after centrifugation at 10,000 × *g* for 15 min was overlaid on each of a series of linear gradients of 5 to 25% (w/w) Ficoll in SET buffer and spun in an SW-27 rotor with buckets (1.59 by 10.16 cm) on a Beckman L265B centrifuge at an $\omega^2 t$ function of 2.1×10^9 (approximately 55 min at 25,000 rev/min). The dense band of light scattering just below the Ficoll-plasma interface of each tube was withdrawn by use of the ISCO density gradient fractionation system. These bands in Ficoll were pooled, diluted 1:2 with SET buffer, and mixed, and the virus was centrifuged to pellets in an IEC model B20 centrifuge for 60 min at 40,000 × *g*. These pellets were resuspended in SET buffer with tissue grinders as described above, pooled, and diluted to a concentration of 30% transmission at 540 nm. This pure AMV was divided into 2-ml fractions, quick-frozen in dry ice-acetone, and stored in the vapor phase of liquid nitrogen.

Instrumentation and biochemical assays. Gradients were preformed with an ISCO density gradient former, or poured manually with a dual-chamber lucite block. ISCO fractionation equipment included the UA-2 ultraviolet (UV) monitor, model 610 recorder, model 566 fraction collector, and model D density gradient fractionator. Buoyant density was determined by refractometry (Bausch and Lomb) or by weighing 0.5 ml of material in micropipettes on an analytical balance. Density gradient band photography was carried out with the ENI density gradient-band camera (Electro-Nucleonics, Inc., Fairfield, N.J.). Sonic oscillation was performed on a Raytheon external sonic oscillator, model DF 101, at 0.8 amp, for 2 min at 4 C. Adenosine triphosphatase determinations were done by the method of Mommaerts et al. (17). DNA polymerase activity, with use of the endogenous RNA template, was performed by the method of Spiegelman et al. (22), which involved pretreatment of each sample with 0.2% Nonidet

P-40 for 10 min. Protein concentration was determined by the procedure of Lowry et al. (14).

Electron microscopy. Samples were prepared for electron microscopy either by negative staining of the unfixed suspension of material in a light-scattering band or pellet, or by thin-sectioning of fixed and embedded pellets.

For negative staining, 400-mesh copper grids were covered with a substrate of 2% parlodion in isoamyl acetate and then with a layer of evaporated carbon. A drop of specimen was placed on the grids and after 1 min was withdrawn by filter paper. In rapid succession, a series of 2 drops of SET buffer was applied and removed with filter paper; in a similar manner, 2% potassium phosphatungstate (PTA) at *p*H 6.5 was applied twice. The grids were finally allowed to dry in air.

For fixation and embedding, pellets were prepared by centrifuging samples into SVC conical BEEM capsules; capsules were arranged in a polylucite adapter (21) and centrifuged for 90 min in an SW50 rotor at 30,000 rev/min. The pellets were fixed for 30 min in 2% glutaraldehyde buffered at *p*H 7.4 with 0.05 M cacodylate. After postfixation for 1 hr in 1% osmium tetraoxide in cacodylate buffer, pellets were stained in 0.5% uranyl acetate for 1 hr, dehydrated in a graded series of ethanol, and embedded in Epon Araldite (16). Subsequently, the conical pellets were re-embedded in a flat mold in an orientation that permitted sectioning of the entire length of the pellet. Thin sections were cut on an LKB ultratome and stained with uranyl acetate and lead citrate (23).

All specimens were examined with an Hitachi HU-11E electron microscope with a 50-nm objective aperature and an accelerating voltage of 75 kv. Counts of viral material on negatively stained grids were performed at a magnification of 31,000 × after random selection of well stained grid squares at the scanning magnification of 250 ×. Counts of structures were made on all negatively stained regions in at least five grid spaces from each of at least three grids. This was done to obtain a representative sample of the distribution of morphologic entities that was produced by disruption of intact AMV with a particular surface-active agent.

Surface-active agents. Four cationic, 10 anionic, and 47 nonionic surfactants were tested (Table 1). Most of the nonionic surfactants belonged to the class of "polyoxyethylene" surfactants because polyoxyethylene units were a part of their structures (for complete classification see references 18 and 19). The predominant types of nonionic surfactants used in this investigation were polyoxyethylene alkylphenols (Nonidet P-40, Renex 690, and most of the triton series), polyoxyethylene primary or tertiary aliphatic alcohols (Sterox, Neodol, Trycol, and Brij series), and polyoxyethylene esters (G-2162, G-2151). Each of the surface-active agents was prepared in 5% sucrose in SET buffer at eight concentrations: 0.01, 0.05, 0.1, 0.5, 1.0, 2.0, 5.0, and 10.0% (v/v or w/v depending on the nature of the agent). When necessary, nonionic surfactants which were solid at room temperature were melted in a 56 C water bath to permit preparation on a volume to volume basis. Samples of the Alfol and Sterox series were ethoxylated to precise levels under controlled conditions on a small scale in the laboratory of Reilly N. Weston (Monsanto Chemical Co.) who also calculated their hydrophile-lipophile balance (HLB) values. The HLB value is a designation (13) which indicates the per cent weight of the hydrophilic portion (the polyoxyethylene chain) of a nonionic emulsifier divided by an arbitrary factor of five. The HLB values of all other nonionic surfactants were supplied by their commercial manufacturers.

Method of surfactant assay. Surface-active agents were evaluated for effectiveness by a method which required the removal of envelope as virions were centrifuged through a layer of surfactant on top of a linear gradient. If the agent was effective, intact AMV released their denser cores, which then migrated to their buoyant density during equilibrium centrifugation to form a light-scattering band. The design of the gradient is illustrated in Fig. 1. Each step was carried out in the cold (0–4 C). Preformed 10 to 70% (w/v) sucrose gradients in SET buffer were poured to a level of 3.4 ml in polyallomer SW56 centrifuge tubes. Surfactant (0.4 ml) at each of eight concentrations between 0.01 and 10% in 5% (w/v) sucrose in SET buffer was laid over the top of the linear gradients and allowed to equilibrate for 90 min. A 0.2-ml amount of a 30% transmission concentration of AMV in SET was then layered over the series of tubes containing the various concentrations of a given surfactant. As quickly as possible (about 10 min) the SW56 rotor was loaded, and centrifugation was begun in the Beckman L265B. Centrifugation for 2 hr at 53,000 rev/min (approximately 400,000 × g) was sufficient to establish conditions of equilibrium; further centrifugation did not change the location of light-scattering bands which indicated the position of virus cores and other material in the gradient. The tubes were photographed in the ENI centrifuge-band camera. Light-scattering bands that were evident in the polaroid print were removed by puncturing the side of the tube with a 25-gauge needle on a 1-ml syringe. The buoyant density of each band was determined by refractometry.

The buoyant density between 1.20 and 1.28 g/ml was designated as the region of core buoyant density. Negatively stained grids were prepared from each light-scattering band in this region for electron microscopy examination as previously described. An assessment was then made of the relative proportion of core to non-core material in all those light-scattering bands in the core region of buoyant density. The structures observed were grouped into several categories: (i) AMV virions, identified by their size, peripheral spikes, and their customary lack of interior penetration of PTA stain; (ii) cores, which were smaller in size and usually had interior stain penetration; and (iii) envelopes, curvilinear shapes of varying sizes which often surrounded core structures. Virions and envelopes constituted non-core material. Each of these light-scattering bands was then scored according to the relative proportion of core to non-core material observed; less than 50%

TABLE 1. *Surface-active agents tested*[a]

Category	Name	Source
Anionic	Digitonin	Fisher Scientific Co.
	Pyronate-L	Witco Chemical Co.
	Sarkosyl	Giegy Co.
	Sodium cholate	Schwarz/Mann
	Sodium deoxycholate	Schwarz/Mann
	Sodium dodecyl sulfate	Schwarz/Mann
	Sodium glycocholate	Schwarz/Mann
	Sodium taurocholate	Schwarz/Mann
	Sulframin OBS	Witco Chemical Co.
	Sulframin 85	Witco Chemical Co.
Cationic	Cetab (cetyltrimethyl ammonium bromide)	Eastman Chemical Co.
	Ethofat 60/20	Armour Industrial Chemical Co.
	Ethomeen 18/60	Armour Industrial Chemical Co.
	Hyamine 1620	Rohm and Haas Co.
Nonionic	Alfol 10	Monsanto Co.
	Alfol 16-18	Monsanto Co.
	Brij series (30, 35, 56, 58, 76, 78)	Atlas Chemical Industries, Inc.
	G-2151, G-2162	Atlas Chemical Industries, Inc.
	Isodecyl alcohol NB 20–39	Trylon Chemicals, Inc.
	Neodol series (23-6.5, 25-7, 25-9, 25-12)	Shell Chemical Co.
	Nonidet P-40	Shell Chemical Co.
	PEG-600 monolaurate	Trylon Chemicals, Inc.
	Renex 690	Atlas Chemical Industries, Inc.
	Sterox series (DE, DL, SD, SL, SM, SN, SO, SP, 66L, 67K)	Monsanto Co.
	Tween series (20, 40, 60, 80)	Schwarz/Mann
	Triton series (N-101, X-45, X-67, X-102, X-114, X-165, X-205, X-305, X-405)	Rohm and Haas Co.
	Triton X-100	Packard Instrument Co.
	Tricol series (TDA-9, TDA-12, TDA-15)	Trylon Chemicals, Inc.
	Tryfac 610-K	Trylon Chemicals, Inc.
	Trymeen CAM-10	Trylon Chemicals, Inc.

[a] Alphabetical compilation by ionic category of the name and source of each of the 61 surface-active agents that were evaluated in the screening assay.

cores (+), 50 to 75% cores (++), 75 to 90% cores (+++), and more than 90% cores (++++).

The effectiveness of all surface-active agents in core production was arbitrarily divided into classes to permit a comparison among them. This classification was based on assay of light-scattering bands in the region of core buoyant density for the range of surfactant concentration over which bands appeared, and on the relative proportion of envelope and core material in these bands by electron microscopy. A surface-active agent was classified as ineffective if no bands were observed in the region of core buoyant density in any of the series of eight gradient tubes. If between one and four different concentrations yielded bands each with 50 to 90% cores (i.e., ++ or +++), or up to seven concentrations each with less than 50% cores (i.e., +), an agent was classified as partially effective. When five or six different concentrations resulted in core bands, each containing over 75% cores (i.e., +++ or ++++), the agent was classified as effective. When seven different concentrations of an agent gave bands in the region of core buoyant density, each with over 75% cores (i.e., +++ or ++++), the agent was classified as most effective.

The six surfactants that were most efficacious in core isolation in the screening assay described above were selected for quantitative comparison with AMV which had been purified by rate-zonal centrifugation in Ficoll. Each sample was assayed for recovery of core protein, endogenous RNA-dependent DNA polymerase activity, and evaluation by electron microscopy with the negative stain technique.

To obtain samples for these assays, 2 ml of intact AMV in SET buffer (at a protein concentration of 0.8 mg/ml) was mixed with 0.5 ml of each of the six surfactants at a concentration of 7.5% (v/v) in SET buffer (final concentration 1.5%) for 10 min in an ice-water bath. Untreated virus served as the control. The seven samples were placed over discontinuous gradients in SW56 tubes which consisted of a layer of 15% (w/w) sucrose on top of an equal volume of 48% (w/w) sucrose. Centrifugation was for 2 hr at 53,000 rev/min. The resulting pellets

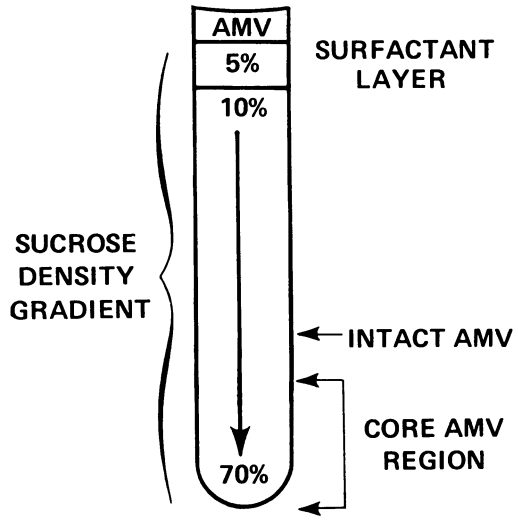

FIG. 1. *Design of gradient used to screen all surfact-active agents. For each agent, eight gradient tubes of this type, each with a different concentration between 0.01 and 10.0%, were prepared in 5% sucrose to form the surfactant layer illustrated.*

were suspended to a final volume of 200 μliters in 0.1 M Tris-hydrochloride, pH 7.4. Samples were sonically treated and quick-frozen in samples for subsequent assay for DNA polymerase activity and protein content. At the same time, a similar set of seven samples was obtained in an identical manner as specimens for electron microscopy.

RESULTS

The sensitivity of the screening assay used to evaluate all surfactants (Table 1) is shown in Fig. 2A–E, in which density-gradient-band photographs illustrate the effect of five nonionic surfactants at eight concentrations between 0.01 and 10%. Beneath each tube with a light-scattering band in the gradient region of core buoyant density, + marks represent the results of electron microscopy assessment of these bands by the negative stain technique (see Fig. 5–8). Notice that differences in chemical structure of nonionic surfactants as minor as one ethoxylation (EO) level can be detected in this assay in terms of the least concentration of surfactant which resulted in a band in the core region of the gradient. For example, an EO level of 14 (Sterox SO, Fig. 2E) required a concentration of 2% before this band was evident. If the EO level was 13 (Sterox SM, Fig. 2D), a concentration of 0.5% was necessary. An EO level of 12 (Sterox SL, Fig. 2C) required a concentration of only 0.05%. With an EO level of 11 (Sterox 67K, Fig. 2B), the bands that were present at concentrations between 0.05 and 10% were faint and diffuse. Notice also that as the appearance and intensity of bands in the core region became evident with increasing concentration of surfactant, there was a corresponding diminution of the light-scattering bands at the level of the untreated intact AMV (single arrows, far left side of each series of tubes).

Electron micrographs of the morphologic appearance by the negative stain technique of several of these light-scattering bands is shown in Fig. 3–8. They are representative illustrations of untreated control AMV (Fig. 3), of untreated AMV when PTA stain penetrates the envelope to reveal the appearance of the interior of intact virions (Fig. 4), and of the four categories which were designated from + (Fig. 5) to ++++ (Fig. 8) according to the percentage of cores seen in each light-scattering band in the core region of the gradient tubes.

The range of surfactant concentrations over which light-scattering bands appeared in the core region of the gradient, and which had the highest proportion of cores morphologically, reflected the specificity of a surface-active agent for AMV core isolation. On this basis, Fig. 2A–2E suggested that Sterox SL with an EO level of 12 was most appropriate. The use of Nonidet P-40 resulted in bands in the core region of the gradients at a range of concentrations between 0.1 and 10%. However, in comparison to Sterox SL, the bands after Nonidet P-40 treatment had a larger percentage of envelope contamination by electron microscopy (compare Fig. 6 and 7).

The results of the screening assay are summarized in Table 2. From the total of 61 surfactants that were examined, 18 were selected either as instructive or as most efficacious in their ionic category. The remaining 43 surfactants (Table 1) were all classified as ineffective or partially effective. Among the anionic surface-active agents evaluated, Digitonin gave a band in the region of core buoyant density at concentrations between 0.05 and 10%; however, the dominant morphologic component of each of these bands was envelope structures (see Fig. 5). Sodium cholate at a concentration of 1% resulted in a band in the region of core buoyant density with between 50 and 75% cores. Very faint light-scattering bands were observed in tubes that contained Hyamine 1622 at concentrations between 0.05 and 1.0%. Although these bands contained little envelope material or intact virions by negative-stain evaluation, very few cores were seen in comparison to the nonionic surfactants. At concentrations of Hyamine 1620 over 1%, no light-scattering bands were observed in the core region of gradients.

The nonionic surfactants were far more useful than the anionic and cationic surfactants in core

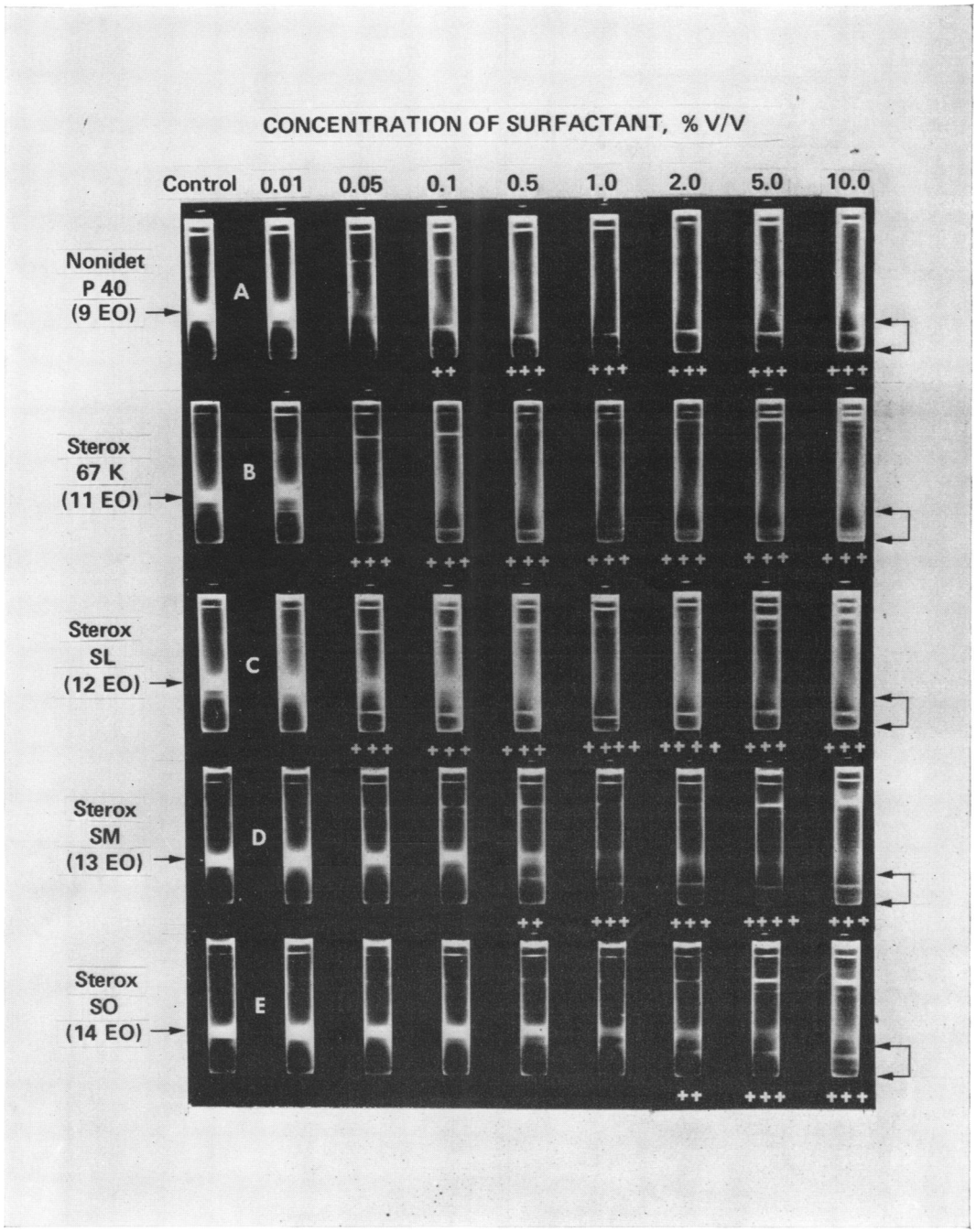

FIG. 2. *Density gradient band photographs of the effects of five surfactants selected from the surfactant screening data in Table 2. The control tube of intact AMV without surfactant treatment (single arrow, far left of each series) had a buoyant density of 1.16 g/ml. The + marks beneath tubes with bands in the core region of the gradient (1.20 g/ml to 1.28 g/ml shown in brackets, far right side of each series) indicate the proportion of cores in these bands by morphologic examination with the negative stain technique (see Fig. 3–8 for representative electron micrographs). Notice that Sterox SL had the widest range of surfactant concentration which gave light-scattering bands in the core region of the gradient, the most light-scattering in the bands, and the highest proportion of cores on negative stain examination of these bands.*

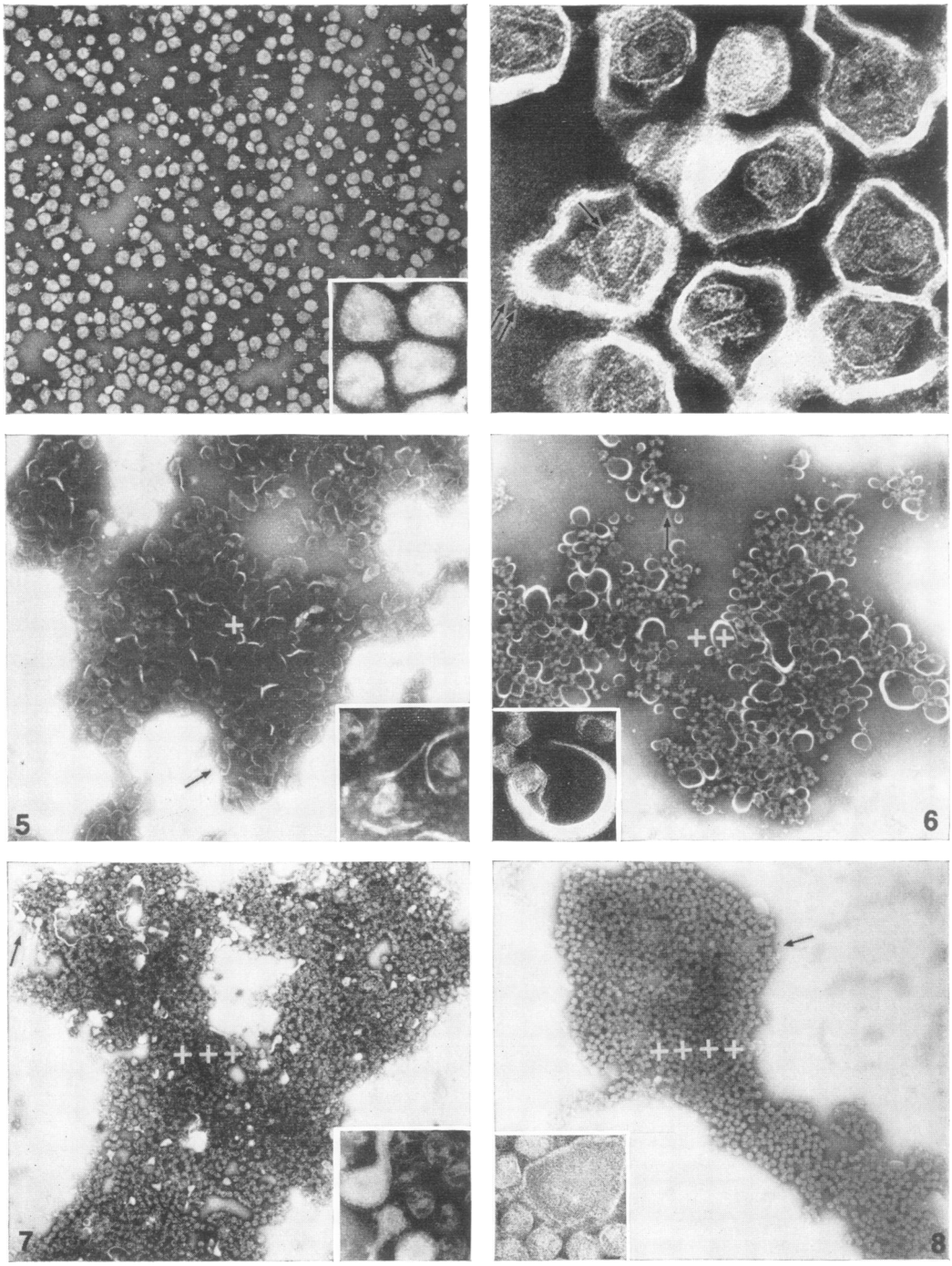

FIG. 3–8.

production; and, within the nonionic class, the polyoxyethylene alcohol type were more useful than the polyoxyethelene alkylphenol type (Table 2). Among this latter type with an EO of 9 or 10, Nonidet P-40 at concentrations between 0.1 and 10% resulted in bands which had a higher percentage of cores than Triton X-100 or Triton N-101, surfactants that differ slightly in chemical structure from Nonidet P-40. The use of Triton X-114 and Triton X-102, similar surfactants with EO levels of 7 to 8 and 12 to 13, respectively, gave few light-scattering bands in the core region of the gradients. It was characteristic of polyoxyethylene alkylphenol surfactants that envelope structures were seen among cores during negative stain evaluation of the bands (Fig. 6).

Polyoxyethylene alcohols were the most efficacious type of nonionic surfactant. In this group Sterox 67-K and Sterox SL, straight chain C_{14-15} primary alcohols ethoxylated in the laboratory to a level of 11 and 12, respectively, were classified as most effective (Table 2). The use of Sterox 67-K, with an EO level of 11, resulted in more diffuse light-scattering bands in the region of core buoyant density (compare Fig. 2B and C). In addition to having no surfactant concentrations which gave bands with over 90% cores, Sterox 67-K yielded cores that had a fragmented morphology in comparison to Sterox SL. Variations in surfactant structure from Sterox SL and Sterox 67-K were less useful in core formation. For example, a similar surfactant prepared for industrial application (Neodol 25-12, a C_{12-15} primary alcohol with 12 EO) could only be classified as effective (Table 2). Use of a C_{14-15} tertiary alcohol with an EO level of 12 (Trycol TDA-12) was also only effective. If the chain length of the primary alcohol was decreased to C_{10} (Alfol 10) or increased to C_{16-18} (Alfol 16–18) with the same level of ethoxylation of 12, no light-scattering bands appeared in the core region of the gradient. Further extension of the EO level to 15 (Trycol TDA-15) or reduction to 9 (Neodol 25-9) with the polyoxyethylene alcohol class of surfactants was also ineffective. Table 2 also illustrates that the HLB value correlated with the effectiveness of the polyoxyethylene alcohol surfactants. For example, those surfactants of this type with an HLB value between 13.8 and 14.5 were most useful in core isolation. All other polyoxyethylene alcohol surfactants in Table 1 had HLB values above or below this range; each was classified as ineffective after evaluation in the screening assay.

A quantitative basis for selection of the optimal surfactant for AMV core isolation is presented in Table 3, which compares the efficacy of six nonionic surfactants from Table 2. Using AMV purified by rate-zonal centrifugation, the core material that resulted from treatment with these surfactants was assayed for endogenous RNA-dependent DNA polymerase activity, protein content, and negative stain morphology. The recovery of protein in the core samples with these six surfactants was low, and five of six were essentially equivalent at between 11 and 13%. The recovery of specific and total endogenous RNA-dependent DNA polymerase activity with these five surfactants was similar (about 42 to 64%). The highest recovery was obtained with Sterox SL and Nonidet P-40; both gave an approximately five-fold increase in specific activity above untreated intact AMV. By negative stain evaluation the

FIG. 3–8. *Electron micrographs after negative staining with 2% potassium phosphotungstate (PTA) which shows intact AMV (Fig. 3–4) and avian myeloblastosis virus (AMV) after treatment with four surfactants (Fig. 5–8) from Table 2. The + marks in the center of Fig. 5–8 illustrate the different categories of percentages of AMV cores present in light-scattering bands in the surfactant screening assays. In these figures, the arrow in the low magnification view indicates the location of the high magnification inset.*

FIG. 3. *A representative field of AMV, clarified by differential centrifugation, from the light-scattering bands at 1.16 g/ml buoyant density from one of the far left control tubes of figures 2A–E. × 12,000; inset × 60,000.*

FIG. 4. *The structural components of AMV were evident when PTA penetrated through the envelope into the interior of the virion: envelope with peripheral spikes (double arrows), and core which consists of an intermediate layer (single arrow) that surrounds a central density. Compare with Fig. 10B inset for the appearance in thin section* at the same magnification. *× 144,000.*

FIG. 5. *The light-scattering band at 1.22 g/ml after Digitonin treatment at 1.0% was comprised of less than 50% core structures and so was designated as +. × 12,000; inset × 60,000.*

FIG. 6. *A representative field after treatment of AMV with Nonidet P-40 at 0.1% (see Fig. 2A) which had a percentage of cores between 50 and 75% cores, or ++. × 12,000; inset × 60,000.*

FIG. 7. *A (+++) field of cores (between 75 and 90%) from the light-scattering band which resulted from treatment with Sterox SL at 0.1% (see Fig. 2C). Notice in the inset the intact AMV and the penetration of PTA into the interior of the core. × 12,000; inset × 60,000.*

FIG. 8. *A view of the (++++) category, in which over 90% of all structures seen were cores. The sample was from the band which resulted from treatment with Sterox SM at 5.0% (see Fig. 2D). Even at this high concentration, this surfactant yielded cores with little PTA penetration into the core interior. However, envelope-like structures remain evident in the inset. × 12,000; inset × 60,000.*

TABLE 2. *AMV core isolation with selected surfactants*[a]

Category	Chemical formula	EO avg moles	HLB value	Classification
Anionic				
Sodium cholate	$C_{24}H_{39}O_5Na$			Partially effective
Digitonin	$C_{56}H_{92}O_{29}$			Partially effective
Cationic				
Hyamine 1622	Diisobutylphenoxyethoxyethyl dimethyl benzyl ammoniun chloride			Effective
Nonionic				
Triton X-114[b]	$C_8H_{17}C_6H_4O(CH_2CH_2O)_{7-8}H$	7–8	12.4	Partially effective
Nonidet P-40*[b]	$C_8H_{17}C_6H_4O(CH_2CH_2O)_9H$	9	13.1	Effective
Triton N-101[b]	$C_9H_{19}C_6H_4O(CH_2CH_2O)_{9-10}H$	9–10	13.4	Effective
Triton X-100[b]	$C_8H_{17}C_6H_4O(CH_2CH_2O)_{9-10}H$	9–10	13.5	Effective
Triton X-102[b]	$C_8H_{17}C_6H_4O(CH_2CH_2O)_{12-13}H$	12–13	14.6	Ineffective
Neodol 25-9[c]	$C_{12-15}O(CH_2CH_2O)_9H$	9	13	Ineffective
Sterox 67-K*[c]	$C_{14-15}O(CH_2CH_2O)_{11}H$	11	13.79	Most effective
Sterox SL*[c]	$C_{14-15}O(CH_2CH_2O)_{12}H$	12	14.16	Most effective
Neodol 25-12[c]	$C_{12-15}O(CH_2CH_2O)_{12}H$	12	14.2	Effective
Trycol TDA-12[c]	$C_{13}H_{27}O(CH_2CH_2O)_{12}H$	12	14.5	Effective
Sterox SM*[c]	$C_{14-15}O(CH_2CH_2O)_{13}H$	13	14.48	Effective
Sterox SO*[c]	$C_{14-15}O(CH_2CH_2O)_{14}H$	14	14.77	Partially effective
Trycol TDA-15[c]	$C_{13}H_{27}O(CH_2CH_2O)_{15}H$	15	15.4	Ineffective
Alfol 10[c]	$C_{10}O(CH_2CH_2O)_{12}H$	12	15.39	Ineffective
Alfol 16-18[c]	$C_{16-18}O(CH_2CH_2O)_{12}H$	12	13.52	Ineffective

[a] Results of the screening assay for avian myeloblastosis virus (AMV) core isolation with various surfactants. The classification of effectiveness after surfactant treatment was based on the range of concentration which resulted in light-scattering bands in the region of core buoyant density, and the relative proportion of core to non-core material in these bands by electron microscopy. A detailed description of the system of classification is found in the Materials and Methods section. Each surfactant with an asterisk is shown in Fig. 2. Ethoxylation level, EO; hydrophile-lipophile balance, HLB.

[b] Polyethoxylene alkylphenol type.

[c] Polyethoxylene alcohol type.

pelleted material which resulted from Sterox SL treatment had the least proportion of non-core structures (3%). Treatment with polyoxyethlene alkylphenol surfactants (Nonidet P-40 or Triton X-100) left core material with a larger amount of envelope contamination (10 and 12%, respectively).

An optical density scan of sucrose density gradient tubes that compares untreated AMV with Sterox SL-treated AMV after isopycnic centrifugation is shown in Fig. 9. Intact AMV banded at the characteristic buoyant density of 1.16 g/ml; AMV treated with Sterox-SL had a buoyant density of 1.27 g/ml, and left an upper band between the aqueous layer and the top of the sucrose gradient. (This upper light-scattering band had no consistent ultrastructural characteristics by direct negative stain of the band, and no pellet resulted from prolonged centrifugation at 65,000 rev/min of many pooled fractions of this upper band material.) Unlike Nonidet P-40 and Triton X-100 (which both contain phenyl rings), a useful characteristic of Sterox SL was the absence of significant UV absorbance at 260 nm. Morphologically, the untreated AMV from the peak in Fig. 9 consisted of a homogeneous population of AMV particles (Fig. 10A and B), and treatment with Sterox SL resulted in a homogeneous preparation of AMV cores by negative stain (Fig. 11A) and thin section (Fig. 11B) electron microscopy. However, even after isopycnic centrifugation in sucrose, envelope constituents are at times seen near the edge of negative stain fields of cores (Fig. 11A, arrows) isolated with the use of Sterox SL. The coarse border to the intermediate layer of the cores on thin section examination is a consistent observation (Fig. 11B), even though on the same preparation of cores the appearance of this intermediate layer by negative stain is sharply defined (Fig. 11A).

The morphology of cores by the negative stain technique received extensive examination because electron microscopy was the principal method of assay for core formation in this study. In general, the type of surfactant influenced the ultrastructural appearance of cores only in regard to the

TABLE 3. *Comparative quantitation of AMV core isolation with selected surfactants*[a]

Surfactant	Protein		Endogenous RNA-dependent DNA polymerase assay			Negative stain morphology
	Micrograms recovered in 200 μliters	Per cent recovered in core sample	Net counts (15 min incubation at 37 C)	Specific activity (pmoles ^{3}H-TTP[b] incorporated per min per mg at 37 C)	Per cent recovered (total activity of core sample)	Proportion of non-core material (%)
Control (untreated)	1,610	100 (intact AMV)	718	2.25	100 (intact AMV)	100 (intact AMV)
Nonidet P-40	190	11.7	2,000	10.9	57.5	10
Triton X-100	178	11.1	1,600	9.3	46	12
Sterox 67-K	205	12.8	1,530	7.4	42.2	5
Sterox SL	210	13	2,200	10.9	63.7	3
Neodol 25-12	192	12	1,900	10.3	55	8
Sterox SM	96	6	550	6.0	16	8

[a] Rate-zonal purified avian myeloblastosis virus (AMV) was divided into seven equal samples of 2 ml each. To each of six samples, 0.5 ml of one of the above surfactants was added at 7.5% (v/v) (1.5% v/v final concentration), intermittently mixed in an ice-water bath for 10 min, and placed over SW56 tubes which contained 15% sucrose layered over an equal volume of 48% sucrose in SET buffer. Untreated virus served as control. Centrifugation was for 2 hr at 53,000 rev/min. The supernatant fluid was removed, and the pellets were suspended in 200 μliters of 0.01 M Tris-hydrochloride, *p*H 7.4. After sonic treatment each sample, including the control, was assayed for protein concentration (14) and endogenous RNA-dependent DNA polymerase activity (22). An equivalent set of seven samples was prepared for negative stain morphology. Quantitation of the percentage of non-core material (envelopes and virions) was then determined as detailed in the Materials and Methods section.

[b] ^{3}H-TTP = ^{3}H-thymidine triphosphate.

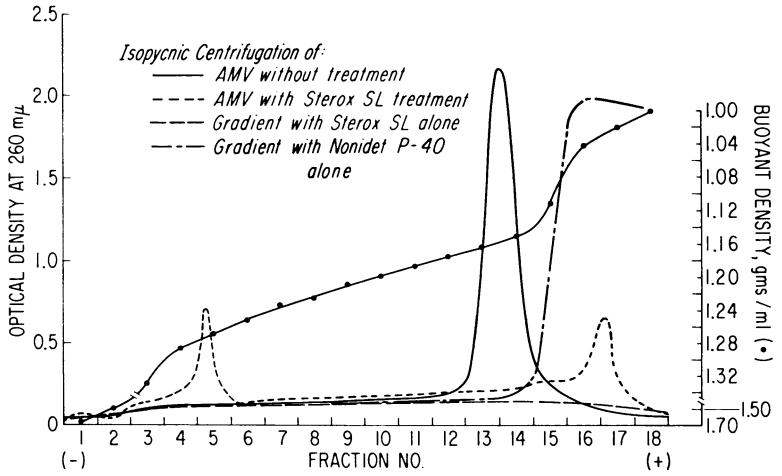

FIG. 9. *Isopycnic centrifugation in sucrose gradients of untreated avian myeloblastosis virus (AMV) and AMV after treatment with Sterox SL. One-half of a preparation of 4 ml of rate-zonal purified AMV, at a protein concentration of 0.8 mg/ml, was added to 0.5 ml of 7.5% (v/v) Sterox SL in SET buffer in an ice-water bath for 10 min. As a control, 0.5 ml of SET buffer alone was added to the remaining 2 ml of intact AMV. Each was overlaid on preformed 25 to 65% (w/w) sucrose density gradients on top of a 2-ml cushion of 2.5 M Cs_2SO_4 in tubes [0.63 by 4 inch (ca. 1.6 by 10.2 cm)]. Centrifugation was for 18 hr at 25,000 rev/min at 0 C in an SW27 rotor. The untreated AMV banded at a buoyant density of 1.16 g/ml and was morphologically a uniform population of intact AMV (see Fig. 10). The Sterox SL-treated AMV banded at 1.27 g/ml and consisted of a uniform population of core components (see Fig. 11). The absence of A_{260} at the sucrose-Cs_2SO_4 interface indicated that there is no substantial pellet of degraded core material during core isolation with Sterox SL. Control tubes overlaid with 2.5 ml of Sterox SL or Nonidet P-40 in SET buffer (final concentration 1.5% v/v) indicated that Sterox SL had minimal A_{260} alone, unlike Nonidet P-40.*

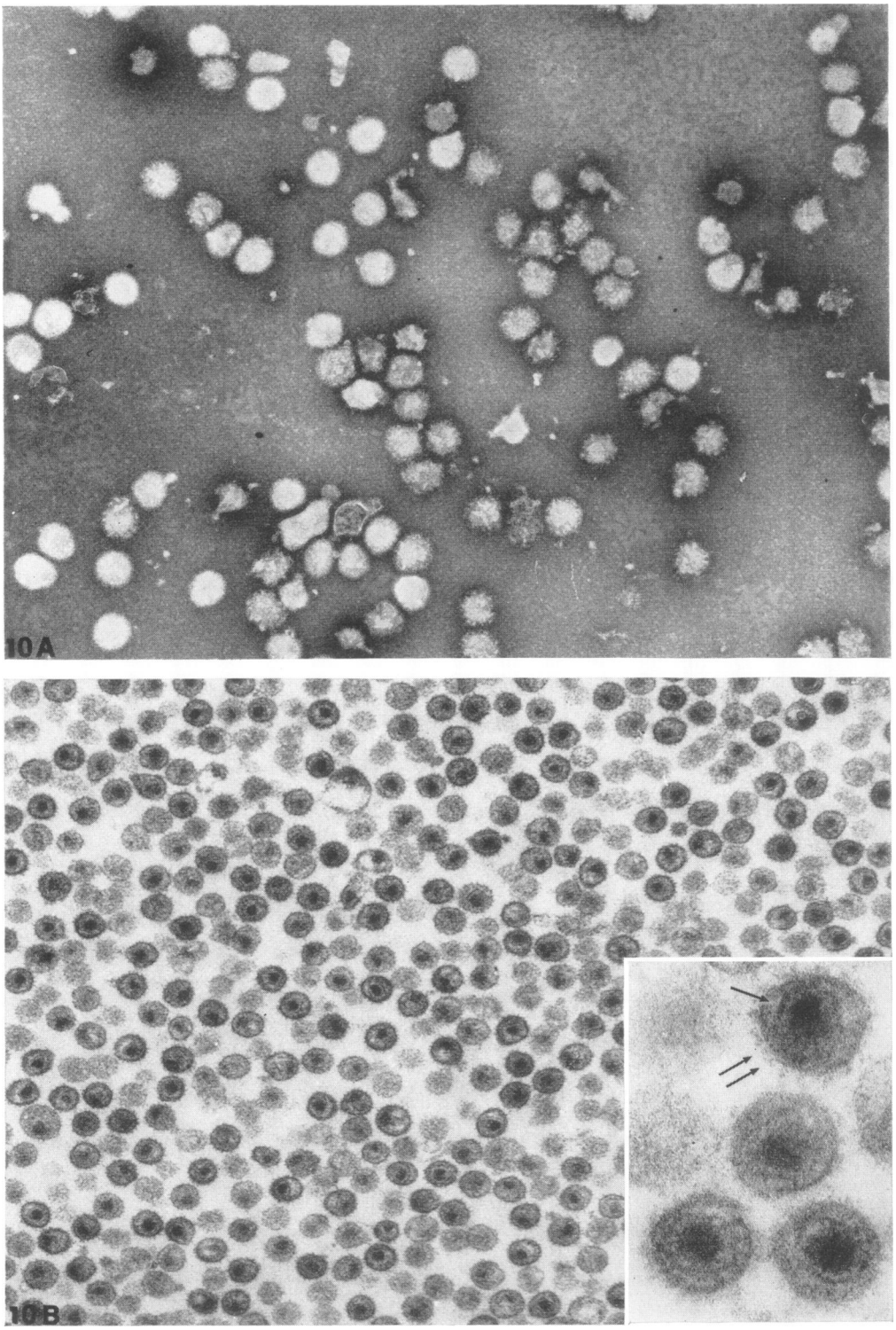

FIG. 10. *Appearance by negative stain (10A) and thin section (10B) of the intact avian myeloblastosis virus at buoyant density 1.16 g/ml from fractions 13 and 14 in Fig. 9. A: a negative stain preparation with potassium phosphotungstate made directly from the intact material in sucrose; × 40,000. B: the sample in sucrose was diluted with SET buffer, mixed, and placed in SW50 tubes that were equipped with Lucite adaptors to situate conical SVC BEEM capsules. Centrifugation at 30,000 rev/min for 90 min pelleted the structural material which was then processed for thin-section electron microscopy. The high magnification inset illustrates the peripheral spikes on the envelope (double arrows), and the core which consists of an intermediate layer (single arrow) that surrounds an electron-dense central nucleoid. × 40,000; inset × 144,000.*

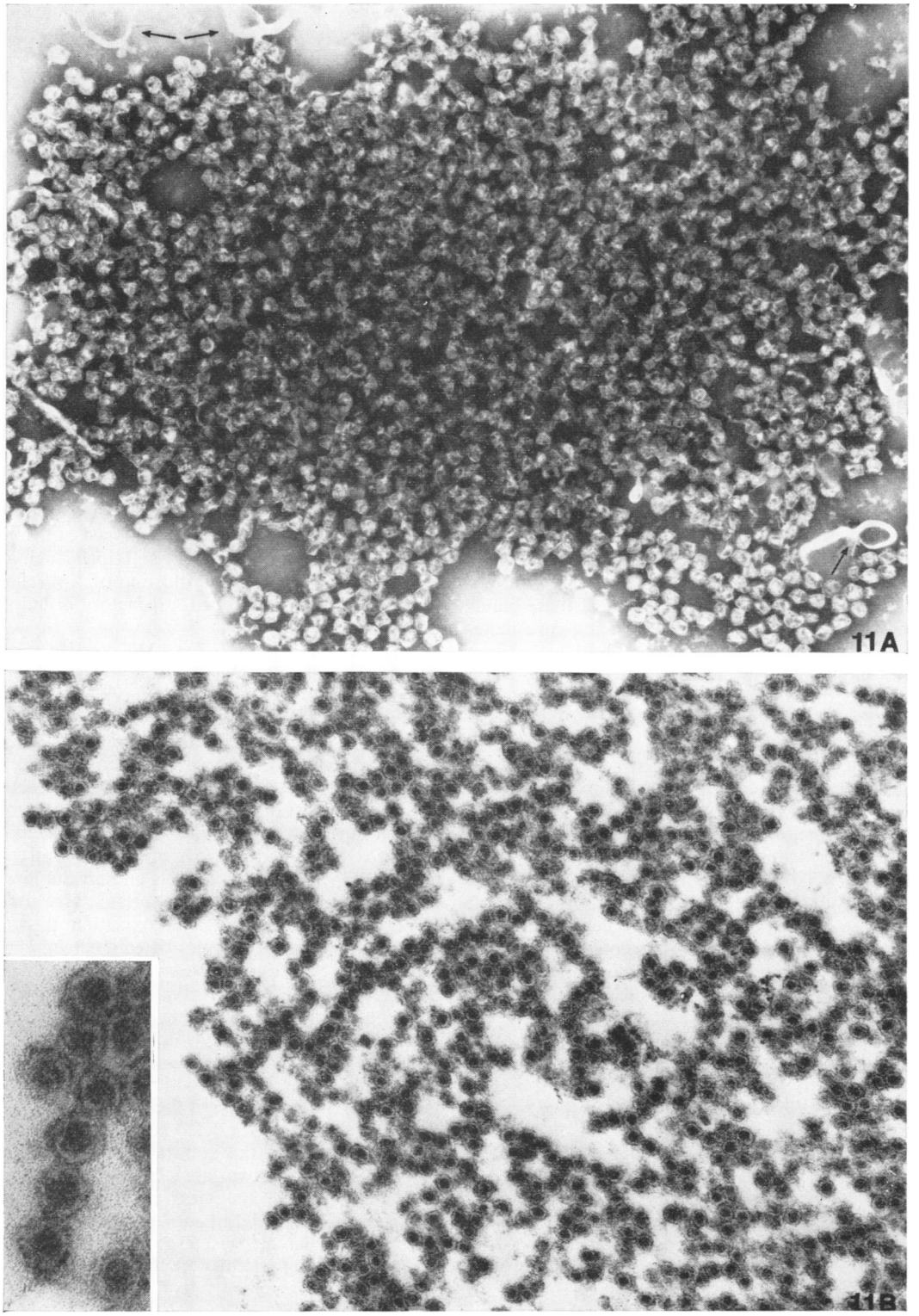

FIG. 11. *Negative stain and thin-section micrographs, respectively, of the material in the core peak at buoyant density 1.27 g/ml from fractions 4 and 5 in Fig. 9. A: a negative stain preparation with potassium phosphotungstate made directly from the core material in sucrose shows the high degree of core homogeneity and integrity upon isopycnic centrifugation of intact avian myeloblastosis virus (AMV) treated with Sterox SL. However, infrequent envelope structures, devoid of spikes, remain present (single arrows). × 40,000. B: thin section of this same material after pelleting into a conical SVC BEEM capsule. This view of the upper surface of the pellet, where envelope structures if present would collect, consists of a uniform field of cores. Notice the absence of envelope in comparison to intact AMV in Fig. 10B inset at the same magnification. × 40,000; inset × 144,000.*

ease with which phosphotungstate stain penetrated through the intermediate layer to reveal the interior of the core. For example, Sterox SM produced cores with less interior stain penetration (Fig. 8 inset) than with Sterox SL (Fig. 7 inset), and much less than the fragmented appearance of cores after treatment with Sterox 67-K. The envelope structures which are present by negative stain in many core preparations (Fig. 5–8 and their insets and Fig. 11A) invariably lacked the peripheral spikes characteristic of untreated intact AMV (Fig. 3 inset, 4, and 10A).

DISCUSSION

Nonionic surfactants were superior to anionic and cationic surfactants in stripping the envelope from AMV to release the core component. Use of polyoxyethylene alcohol and polyoxyethylene alkylphenol types of nonionic surfactants, within a narrow range of suitable ethoxylation, resulted in light-scattering bands in the region of core buoyant density at a wide range of surfactant concentrations. Morphologic evaluation of these bands revealed a higher proportion of non-core material, usually envelope structures, in the most useful alkylphenol surfactant (Nonidet P-40, 10%) than with the most useful alcohol surfactant (Sterox SL, 3%). The finding of envelope structures with core material following Nonidet P-40 treatment of intact AMV agrees with a recent observation that use of this surfactant resulted in core material becoming entrapped in large membranous inclusions; extraction of this material with ether was employed to release the trapped cores (6). The use of subsequent ether treatment makes quantitative studies of recovery difficult because of loss of material at the gelatinous interface between the ether and aqueous phases.

The biochemical data with selected surfactants were in line with the morphological evaluation. Use of Sterox SL to produce cores resulted in material with a slightly higher recovery of protein and endogenous RNA-dependent DNA polymerase activity than use of several other nonionic surfactants. The amount of protein that was recovered in the core was low, but the specific activity of endogenous RNA-dependent DNA polymerase in core preparations was enriched approximately fivefold over untreated intact AMV. This latter result was not due to differential enzyme activation between core and intact AMV with preincubation in Nonidet P-40 because increase in concentration of Nonidet P-40 up to 5% (v/v) in the preincubation stage of the polymerase assay with intact AMV did not significantly alter the specific activity (R. R. Gantt and K. Stromberg, *unpublished data*). Thus, the endogenous RNA substrate and the DNA polymerase enzyme(s) are core constituents in agreement with earlier reports for avian tumor viruses (9, 6, 15). An association of similar enzyme(s) with virus cores has been reported in a murine leukemia virus (12).

Sterox SL was markedly more effective than Nonidet P-40 in AMV core isolation when AMV was obtained from viremic plasma which by adenosine triphosphatase activity had only about 5×10^{11} particles per ml. Under these conditions, approximately one-half of the material seen after Nonidet P-40 treatment as done in Table 2 was envelope structures. The use of satisfactory methods of AMV isolation and purification was critical for core isolation. Only viremic plasma of high titer was used in this study (over 10^{12} particles/ml by adenosine triphosphatase determination). Moreover, the use of rate-zonal purification in Ficoll permitted rapid purification of AMV under nearly isoosmotic conditions. Prolonged exposure to hypertonic density gradient solutions, such as sucrose or salts, during purification of AMV has in our experience precluded satisfactory isolation of AMV cores.

The optimal HLB value for AMV core isolation among the polyoxyethylene alcohol class of surfactants was 14.16 from a $C_{14\text{-}15}$ primary alcohol ethoxylated to a precise level of 12 under controlled laboratory conditions (Sterox SL). Use of a $C_{14\text{-}15}$ primary alcohol with a precise EO level of 11 or 13, or a C_{10} or $C_{16\text{-}18}$ primary alcohol with an EO level of 12 was less effective. A commercial surfactant of similar chemical structure to Sterox SL, Neodol 25-12 which has a mixed range of $C_{12\text{-}15}$ primary alcohols and an EO level of 12, seemed to be less appropriate. This may illustrate the variability of ethoxylation that is present in industrial samples of commercial surfactants (R. N. Weston, *personal communication*). Similarly, only those surfactants of the polyoxyethylene alkylphenol class that were ethoxylated to a level of 9 or 10 were effective. These results indicated that the effectiveness of nonionic surfactants in AMV core production depended upon three interrelated factors: (i) the level of ethoxylation (EO level), (ii) the HLB value, and (iii) the chemical nature of the lipophilic constituent of the surfactant. The variable behavior of these surfactants toward the viral envelope suggests that this response might be a tool for analysis of membrane structure. Selection of an appropriate surfactant for a particular biological application from those commercially available (1) can be facilitated by HLB guidelines (2).

Remarkably little is known about the mechanism of action of nonionic surfactants in biological systems. The nonionic surfactants evaluated in this study probably lead to AMV core

production by virtue of their surface active properties on the envelope. Bean (4) indicated that a solution exhibiting maximal activity should be at the critical concentration for micelle formation. The critical micelle concentration is influenced by additives (such as electrolytes or urea) and temperature (5). That several surfactants were effective over a 1,000-fold range in concentration (0.1 to 10%, v/v) suggests that once the critical micelle concentration was achieved, an excess did not adversely affect AMV core production.

In summary, use of Sterox SL resulted in core preparations with the highest recovery of protein and endogenous RNA-dependent DNA polymerase activity. Sterox SL did not significantly interfere with biochemical assays based on UV absorbance. Because Sterox SL was synthesized under controlled conditions in the laboratory to a precise level of ethoxylation, it avoids the possible variation in EO level that may occur in similar nonionic surfactants prepared for industrial use. Most importantly, morphologic examination by electron microscopy indicated that treatment with Sterox SL resulted in a population of cores with the greatest structural integrity and uniformity and with the least envelope contamination.

Note. Inquiries regarding samples of Sterox surfactants should be addressed to R. N. Weston, Monsanto Co., St. Louis, Mo. 63166.

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