1956, v76, 1.

8. Muschel, L. H., Lowe, K. M., *J. Lab. and Clin. Med.*, 1955, v46, 147.

9. Muschel, L. H., Treffers, H. P., *J. Immunol.*, 1956, v76, 20.

10. Inoue, K., Tanigawa, Y., Takubo, M., Satani, M., Amano, T., *Biken's J.*, 1959, v2, 1.

Received May 14, 1963. P.S.E.B.M., 1963, v113.

Macromolecular Properties of Hexadimethrine Bromide, an Antiheparin Agent. (28520)

GRANT H. BARLOW, LYLE J. COEN, EUGENE T. KIMURA, AND STEVEN KERESZTES-NAGY

Research Division, Abbott Laboratories, North Chicago, Ill.

Hexadimethrine bromide* has been used in recent years as a phamacological agent to neutralize the anticoagulant activity of heparin(1,2). Several publications(3-5) have reported on the relationship between molecular size of this drug and its pharmacological and toxicological properties. It appeared that a critical study of its macromolecular properties would be of interest especially if the critical molecular size for the appearance of detrimental effects could be pinpointed.

Methods. Toxicity and antiheparin studies. The same methods were employed as previously reported(5).

Light scattering. Light scattering molecular weights were determined by standard methods(6) with a Brice-Speiser-Phoenix light scattering photometer using light of 4358A. The refractive index increment at this wave length was 0.154 ml/g. Determinations of fluorescence were made by employing a yellow filter (Corning No. 3384) which transmits light above 4900A in front of the photomultiplier tube.

Sedimentation. Sedimentation data were obtained on a Spinco Model E ultracentrifuge. Sedimentation velocity runs were made at 59,780 rpm and equilibrium runs at 50,740 rpm, at or near room temperature. Calculations of sedimentation coefficients and equilibrium molecular weights were made by methods described by Schachman(7).

Diffusion. Determinations were made in a Spinco Model H Electrophoresis-Diffusion apparatus at 1°C. Diffusion coefficients were calculated from Rayleigh fringe patterns by standard methods(7). No prior dialysis of the polymer was carried out since the molecule is too small to be retained by a cellophane membrane.

Partial specific volume. This value, determined by the densitometric method, was 0.72 ml/g.

Results. All of the molecular weights reported in the literature were determined by the light scattering method. In our study we became aware that all samples fluoresced at the wave length of light used for the light scattering determinations. This means that all published figures for the molecular weight of hexadimethrine bromide are in error, being much higher than actual values.

When attempts were made to correct the results for fluorescence by the method of Brice *et al*(8) it was found that the true scattering values were too low for reliable results, being only a deflection of 10 galvanometer units at full sensitivity of the instrument. Thus, it became apparent that the true molecular weights of hexadimethrine bromide are too low to be established by the light scattering method.

On the other hand, ultracentrifuge methods are more versatile and can be used even for small molecules. Sedimentation velocity runs in a synthetic boundary cell showed very low values of the sedimentation coefficient, S_{20w}. For different samples the S_{20w} ranged from 0.23 to 0.41 S and was not dependent on concentration or ionic strength. In view of the large experimental error for such a small S_{20w} these values should be considered essen-

* Polybrene®, Abbott Laboratories, North Chicago, Ill.

TABLE I. Molecular Weight Data on Hexadimethrine Bromide.

Sample	Light scattering*	Sed. velocity	Sed. equilibrium
R-45	16,600	4,100	3,800
1561	7,500	3,800	3,700

* Not corrected for fluorescence. Presented here for comparative purposes only.

tially identical. For two samples the S_{20w} value was combined with a measured diffusion coefficient, D_{20w}, of 6.6×10^{-7}cm^2/sec, and the partial specific volume of 0.72 ml/g to obtain the molecular weights in Table I. The molecular weights were also measured by the sedimentation equilibrium method. These results are also shown in Table I. The molecular weight determined by either sedimentation method is much lower than that recorded by the light scattering method.

Since the samples were polydisperse an attempt was made to fractionate them on the basis of differences in molecular size. The gel filtration technique(9) was used. A sample (R-45) was passed through a column of Sephadex G-50 and 3 fractions were obtained. For each fraction measurements were made of the molecular weight from sedimentation equilibrium, toxicity, and antiheparin potency (Table II). There is a direct relationship between molecular weight and toxicity and an inverse relationship between molecular weight and antiheparin potency. Best overall performance is obtained with the sample of molecular weight 1800 which would correspond to a polymer of 4 monomeric units.

Discussion. Our results show that hexadimethrine bromide is a much smaller polymer than previously reported. In fact it is quite possible that all commercial samples have nearly the same average molecular weight. Differences in amount of fluorescent material among samples probably account for the reported differences in molecular size measured by the light scattering method.

TABLE II. Molecular Weight and Pharmacological Properties of Hexadimethrine Bromide Fraction.

Fraction	M.W. sed. equilibrium	LD_{50} (mg/kg)	Antiheparin potency (% of std)
Control (R-45)	3,800	12.0	99.6
1	3,600	18.0	144.6
2	1,800	40.0	86.7
3	800	145.0	37.0

Although earlier observations of the relation of molecular weight to biological properties were based upon unreliable light scattering data, by chance the general conclusions may be approximately correct. They are in fact supported by our present studies of preparations of different molecular size as separated by gel filtration and by our earlier work on dialyzed preparations. If the fluorescent material were toxic it could account for the fortuitous correctness of the earlier conclusions, *i.e.*, the more fluorescent material the greater the toxicity and the higher the apparent, (erroneous) molecular weights determined by light scattering.

Another interesting point is the limited size of the available samples of polymer. Previously it appeared that a wide range of polymer sizes had been synthesized, but our present findings rule this out. Thus it seems now that there is a size-limiting reaction occurring in the production of the polymer. Two possible reactions which would terminate the polymerization are cyclization and dehydrobromination. No experiments have been performed to establish either of these possibilities.

It seems apparent that the best preparation of hexadimethrine bromide for use as a pharmacological agent will require some fractionation following the polymerization reaction. We feel that either dialysis or gel filtration would serve as a convenient fractionation method and that a final material with a molecular weight approximately 1800 would be desired.

Summary. 1. Previously reported light scattering molecular weights of hexadimethrine bromide are in error due to the presence of fluorescent material. The true molecular weights are much lower and may in fact be nearly identical for all commercial samples. 2. Fractionation by gel filtration followed by pharmacological and toxicological studies indicate that a polymer of 4 monomer units would be the preferred one for clinical use.

1. Preston, F. W., *Proc. Central Soc. Clin. Re-*

search, 1962, v25, 63.
2. Weiss, W. A., Gilman, J. S., Catenacci, H. J., Osterberg, A. E., *J.A.M.A.*, 1958, v166, 603.
3. Lalezeri, P., Spaet, T. H., *J. Lab. and Clin. Med.*, 1961, v57, 868.
4. Lalezeri, P., *Blood*, 1962, v19, 109.
5. Kimura, E. T., Young, P. R., Barlow, G. H., PROC. SOC. EXP. BIOL. AND MED., 1962, v111, 37.
6. Bender, M., *J. Chem. Ed.*, 1952, v29, 15.
7. Schachman, H. K., *Methods in Enzymology*, Academic Press, Inc., N. Y., 1957, vIV.
8. Brice, B. A., Nutting, C. G., Halwer, M., *J. Am. Chem. Soc.*, 1953, v75, 824.
9. Porath, J., Flodin, P., *Nature*, 1959, v183, 1657.

Received May 15, 1963. P.S.E.B.M., 1963, v113.

Effect of Actinomycin D on HeLa Cell Nuclear RNA Metabolism. (28521)

HILTON B. LEVY
U. S. Department of H.E.W., Public Health Service, Nat. Inst. Health, Nat. Inst. of Allergy and Infectious Diseases, Bethesda, Md.

Actinomycin D inhibits DNA-dependent RNA snythesis, both in intact cells(1) and in cell-free systems(2). The mechanism suggested for its action is a combination with DNA, so that the DNA is not capable of functioning as a template(1,3). Actinomycin D can stop all or virtually all RNA synthesis, and this has been interpreted as meaning that all cell RNA synthesis is DNA mediated.

It has been shown by autoradiography that tritiated uridine is first incorporated into the nucleus of cells, and then appears in the cytoplasm. It was thought desirable to test the effect of actinomycin D on this migration of RNA from the nucleus to the cytoplasm.

Experimental. Two types of experiments were performed. In the first HeLa cell monolayers on coverslips in Leighton tubes, grown in Eagle's medium, were exposed at 37°C to actinomycin D, 1 μg/ml for 20 minutes, then to tritiated uridine 1 μc/ml for 10 minutes. Controls were not treated with the antibiotic. Radioautograms on the washed, acid extracted cells were made with Kodak Ltd. AR 10 film, by standard methods. Previous exposure to actinomycin D virtually abolished the incorporation of uridine into acid insoluble polynucleotide (Fig. 1, 2). The tendency to nucleolar concentration in the untreated cells can be seen. This is in agreement with previously published reports(4).

In the second type of experiment, HeLa cells were exposed to tritiated uridine, 1 μc/ml for 15 minutes to allow incorporation into nuclear and nucleolar RNA. Further incorporation of the labelled compound was then stopped by diluting the radioactivity by addition of 50 μg/ml unlabelled uridine. Actinomycin D was then added to some of the cultures at a final concentration of 1 μg/ml, equivalent volume of diluent was added to control cultures, and both types of cultures were incubated for an additional 2 hours. After extracting acid soluble components, radioautograms were made and are shown in Fig. 3 and 4.

In the cells which were not treated with Actinomycin D there was the normal expected migration of radioactivity from the nucleus to the cytoplasm. In the actinomycin treated cultures there was virtually no migration to the cytoplasm of the radioactivity that was present in acid insoluble form in the nucleus prior to addition of the actinomycin. The nucleolar concentration of radioactivity that had been present prior to addition of actinomycin was no longer present. The total amount of acid insoluble radioactivity was about the same in both treated and untreated cultures.

Discussion. The data presented here indicate that actinomycin inhibits the transfer from nucleus to cytoplasm of radioactivity that appears associated with ribosomes, a process not previously reported to be DNA dependent. Three hypothetical explanations for this observation are: 1) continued synthesis of DNA-dependent RNA is needed for