been found previously to be optimum when chlortetracycline was fed. This may not have been true for other antibiotics. The mode of action of certain antibiotics in the economy of Vit. A and beta carotene metabolism is being studied.

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A Study with Low and High Molecular Weights of Hexadimethrine Bromide—an Antiheparin Agent. (27698)

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Chemicals available for neutralization of heparin include toluidine blue, an organic dye; protamine sulfate, a polypeptide of marine origin; and, more recently, hexadimethrine bromide,* a synthetic polymer. Certain pharmacologic and toxicologic properties of hexadimethrine have been described(1-5). Experimentally, a basic question still remains as to the possible relationship between polymer size, toxicity, *in vitro* mast cell disruption and antiheparin potency.

This report is concerned with certain toxicologic and pharmacologic aspects of various polymer sizes of hexadimethrine. Our initial studies were carried out with polymers whose molecular weights were expressed as average values; subsequent studies were carried out with dialysate fractions of several of these polymers.

Methods and materials. Toxicity Studies. Acute toxicities were determined for each lot of hexadimethrine bromide in female Scientific strain mice weighing between 17-24 g, using 10 to 20 animals per dose level. Each lot was diluted in saline to 1 and 2 mg/ml concentration and injected at a rate of 1.2 to 2 ml/minute. Survivors were observed for 1 to 2 weeks. The LD_{50} and associated confidence limits were determined by the method of Litchfield and Wilcoxon(6).

Mast Cell Studies. Studies on the effect of hexadimethrine bromide on tissue mast cells were carried out *in vitro,* using mast cells from rat mesentery as test objects. This method, described previously by Norton(7), is an adaptation of the technic of Mota *et al.* (8). Female Holtzman rats, weighing between 150-250 g, were used in our study. Approximately 10 pieces of mesentery were obtained from each rat and immersed in various concentrations of hexadimethrine bromide for 30 ± 1 minutes. Pieces of mesentery from at least 3 different rats were used for each concentration. After fixing and mounting, 4 separate fields were selected under 125 × magnification. Each of these fields was re-observed under 250 × magnification for mast cell disruption, using the first 10 cells, reading clockwise from the upper left-hand corner. For each drug concentration, approximately 560-600 cells were examined.

Antiheparin Studies. The antiheparin potency of each lot of hexadimethrine was determined by using a modification of the U.S.P. XV assay method for heparin. Briefly, graded amounts of the polymer are added to a series of test tubes each containing fixed amounts of heparin and citrated sheep plasma. That tube forming a 50% clot is taken as the end point. Calculations with results obtained with a hexadimethrine standard run concurrently yielded antiheparin potencies expressed as per cent of the standard.

Molecular Weight Determinations. Light Scattering. Light scattering molecular weights were determined on a Brice-Speiser-Phoenix light scattering photometer, using light of 4358 A. The refractive index increment, dn/dc, at this wave length was 0.154 ml/g.

Sedimentation. Sedimentation data were obtained on a Spinco Model E Ultracentri-

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

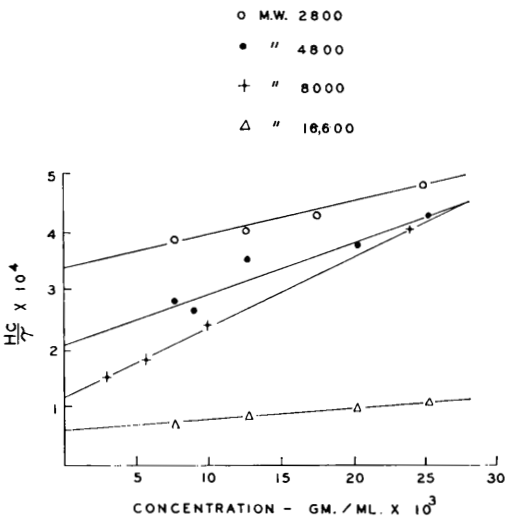

FIG. 1. Molecular weight determination by light scattering. All solutions in isotonic saline. Solutions filtered through millipore filter until dissymmetry was 1.05 or less. The extrapolated intercept on the ordinate is the reciprocal of molecular wt.

fuge. All runs were at 59,780 r.p.m. and near room temperature.

Hexadimethrine Bromide. Polymer sizes of hexadimethrine available for clinical usage have been restricted to those with average molecular weights between 5000 and 10,000. For our current studies, the following polymer sizes were used: 2800 (R-81), 4800 (R-84), 6000 (R-54), 7500 (1561-156-1), 8000 (R-67-1), 10,600 (R-62-1) and 16,600 (R-45). Analyses to ascertain molecular weights based on their weights included light scattering and ultra-centrifugation. Average molecular weights for the above samples were determined by light scattering.

Several of the specimens were dialyzed for 7 days against either isotonic saline or water with changes outside every 24 hours at a ratio of 1 to 3. Comparative intravenous LD_{50}s were determined in mice with the control specimens, their non-dialyzable fractions and their dialysates.

Results. Fig. 1 shows typical curves obtained by light scattering for polymers of high, intermediate and low average molecular weights. On the abscissa is plotted the concentration in grams/milliliter, on the ordinate, Hc/τ where H is a constant, c is concentration and τ is absolute turbidity of the sample. The extrapolated intercept on the ordinate is the reciprocal of the molecular weight. These data show that polymers of different molecular size had been produced.

Table I shows rate of dialysis of polymer size 16,600. Results are expressed as total per cent dialyzed at 24-hour intervals over a 96-hour period. Concentrations of the fractions were determined by differential refractometry. In all samples treated, at least 65% of the weight was dialyzed.

Sedimentation analysis on the samples in a synthetic boundary cell showed in every case a single sedimenting boundary with S_{20w} ranging from 0.23S to 0.41S. Analysis of the peak by boundary spreading technics showed obvious polydispersity.

The acute intravenous toxicities for 5 polymer sizes of hexadimethrine are presented in Table II, column 2. Results show that the toxicity of hexadimethrine in mice increases with an increase in polymer size. Toxicities for the intermediate polymer sizes increased in direct proportion to an increase in molecular weight.

Symptoms were moderate to marked depression with some respiratory involvement and were as described previously. Deaths occurred within one minute or were delayed up to 10 days after injection. The majority of fatalities occurred between 2 to 120 hours.

Results on the effect of polymer size on mast cell disruption are presented in Table II, column 3. These data show that an increase in polymer size from 2800 through 16,600 is accompanied by a concomitant increase in disruptive action on the mast cells. When per cent of mast cell disruption was plotted against drug concentration for each polymer size of hexadimethrine, there was a linear relationship both with respect to dosage and to polymer size.

TABLE I. Rate of Dialysis of Hexadimethrine Bromide of Average Molecular Weight 16,600.

Dialysate No.	Time (hr)	Total % dialyzed
1	24	35
2	48	56
3	72	68
4	96	70

TABLE II. Relationship between Molecular Weight, Toxicity, Mast Cell Disruption and Antiheparin Potency of Various Polymer Sizes of Hexadimethrine.

Hexadimethrine bromide, avg mol wt	Mouse I.V. LD_{50}, mg/kg (95% C.L.)	Tissue mast cell disruption, ED_{50} in mg/ml	Antiheparin potency (% of standard)
2800	26.9 (25.3-28.6)	2.18	111.7
6000	19.6 (18.7-20.6)	1.47	86.9
8000	17.5 (16.7-18.4)	.81	103.3
10,600	16.4 (15.6-17.2)	.75	100.2
16,600	12.0 (10.5-13.7)	.67	99.6

TABLE III. Comparative Toxicities of Dialysates and Non-Dialyzable Fractions of Hexadimethrine Bromide of High, Intermediate and Low Molecular Weights.

Molecular wt (avg)	mg/kg I.V. LD_{50} (95% C.L.) in mice				
	Control	Non-Dialyzable	Dialysate #1	#2	#3
16,600	12.0 (10.5-13.7)	8.3 (7.0- 9.8)	30.5 (26.3-35.4)	11.5 (10.5-12.6)	11.9 (10.7-13.2)
8000	17.5 (16.7-18.4)	8.8 (7.6-10.2)	19.9 (18.1-21.9)	12.7 (11.0-14.6)	10.5 (8.9-12.3)
7500*	13.1 (12.1-14.2)	9.9 (8.9-11.0)	14.3 (12.9-15.9)	12.4 (11.4-13.5)	10.5 (9.8-11.3)
4800	20.8 (19.1-22.6)	16.5 (14.7-18.5)	11.4 (10.3-12.7)	10.9 (10.0-11.8)	14.1 (12.9-15.4)

* A production batch rejected because of its high toxicity.

Table III shows the intravenous toxicity of various dialysates and nondialyzable fractions of hexadimethrine. Polymers with high, low and intermediate average molecular weights were used in this study to determine its poly-dispersity pattern. Fractionation of the 16,600 molecular weight polymer showed its first dialysate to be the least toxic, having an LD_{50} value of 30.5 mg/kg. This was more than twice the control value of 12.0 mg/kg. By contrast, succeeding dialysate fractions as well as the non-dialyzable portions were about 2½ to 4 times as toxic as this first dialysate fraction.

Dialysate fractions from polymer size 8000 showed a similar toxicity pattern, the first dialysate fraction being the least toxic with succeeding fractions and the non-dialyzable portions being more toxic than control values.

When the ratios between toxicity values of the first dialysates and their corresponding controls were expressed as per cent, dialysis enabled the lethal components of polymer sizes 16,600, 8000 and 7500 to be reduced by 154%, 14% and 9%, respectively. This stepwise decrease, concomitant with a decrease in weight average molecular weight may be explained on the basis of correspondingly smaller amounts of high molecular weight (*i.e.*, toxic) polymers being present in the original whole undialyzed specimen. At a certain average molecular weight range, a cut-off point is reached wherein dialysis no longer materially alters the toxicity pattern. Such a concentration appears to be below 7500 in these studies. This perhaps represents the area below which the high molecular weight components may not be present in such comparatively significant amounts that its removal by dialysis would markedly alter the toxicity.

The LD_{50}s of the non-dialyzable fractions of these polymer samples (column 3) were fairly constant, ranging from 8.3 to 9.9 mg/kg.

Toxicity data in column 4 show that an increase in polymer size was accompanied by a concomitant step-wise decrease in the toxicity of the first dialysate. This may be explained on the basis that the molecular weights of these polymers are only average values, representing the average of 2 or more fractions whose molecular weights may spread from low through high values. Thus, a sample of hexadimethrine with a relatively high average molecular weight (*i.e.*, toxic) may have as one or more of its components, fractions which are relatively less toxic to offset and balance the toxic components. Such may be the case since those original whole samples of hexadimethrine with higher average molecular weights have as one of their components a

fraction with correspondingly lower toxicity. The molecular weight of the first dialysate from polymer size 16,600 was now found to be 3600. Further, a 4800 average molecular weight specimen, with an LD_{50} value of 20.8 mg/kg apparently consisted of much more of the low toxicity material. Its non-dialyzable fraction was only half as toxic as those from the other polymers tested (column 3).

Antiheparin potency assays showed that potency was not materially affected, *i.e.*, a decrease in toxicity was achieved without sacrificing antiheparin potency. Further, antiheparin potency and toxicity tests on the dialysates from 5 to 6 additional hexadimethrine specimens, previously rejected because of their high toxicity, now showed toxicity to be within the clinically acceptable range while still retaining potent antiheparin activity.

Discussion. Conclusions relating polymer size to pharmacologic activity (Table II) must be tempered with the knowledge that the molecular weights of these polymers are only average values. Thus, a sample of hexadimethrine with a weight average molecular weight of 6000 may represent an average of 2 or more polymers whose individual molecular weights may spread from a low of 2000 to a high of 16,000; or may be a mixture of 2 or more molecular weight samples whose spread is only from 5500 to 6500.

Further, the proportionate amounts of each of the various molecular weights contained in any given sample of hexadimethrine may influence the final toxicity. For example, the above sample of hexadimethrine with an average molecular weight of 6000 may have quite a significant amount of higher molecular weight fraction (with its concomitant higher toxicity) while another specimen of hexadimethrine with the same average molecular weight of 6000 may have a proportionately larger amount of lower molecular weight fraction (with its concomitant lower toxicity).

Again, citations of average molecular weight values must be identified as to whether they are expressions of number average, weight average or of zeta average.

Results shown in Table II represent initial studies carried out with average molecular weight polymers with no attention being given to the precise identity of each of its components. However, our choice of the 5 polymers having an average molecular weight spread from a low of 2800 to a high of 16,600 was based on an attempt to cancel out any fringe-area overlapping of responses.

Based on these findings, we have concluded that the intravenous toxicity of hexadimethrine bromide in mice increases concomitantly with an increase in average polymer size. Thus, it appears that hexadimethrine bromide also follows the general toxicity pattern seen for other macromolecular substances. For example, Grönwall *et al.*(9) showed previously that a decrease in molecular weight of the dextran sulfates was accompanied by a decrease in toxicity in experimental animals.

The effect of hexadimethrine on mast cells was studied, since these cells are known to contain such agents as histamine, serotonin, heparin and hyaluronic acid. Certain toxic side effects from this polymer when given in large doses in experimental animals, as well as in therapeutic doses in certain susceptible clinical cases suggest reactions to one or more of the pharmaco-active agents. Factors other than these no doubt are concerned in the over-all pharmacologic and toxicologic response to hexadimethrine.

Our comparative findings obtained in the second phase of our studies (Table III dialysates and non-dialyzable fractions) tend to support the theory suggested by one of us (G.H.B.) that differences in average molecular weights of hexadimethrine are due, essentially, to presence of a comparatively small yet toxicologically significant amount of high molecular weight fractions of this polymer; and, secondarily, that removal of these fractions from specimens of hexadimethrine should, theoretically, result in samples having lower molecular weights with concomitant lowered toxicity. Such appears to be the case. Further, this decrease in toxicity was accomplished without appreciable loss of antiheparin potency. Thus, a way may be clear now to obtain specimens of hexadimethrine of high antiheparin potency with greatly diminished toxicity.

Summary. The above findings represent studies on the effect of hexadimethrine bro-

mide on intravenous toxicity in mice, on *in vitro* mast cell disruption, and on antiheparin potency. Polymers of average molecular weight as well as dialysates of several such polymers were used in these studies. Results showed (a) that there is a definite trend for the toxicity of hexadimethrine to increase concomitantly with an increase in average polymer size, (b) that both intravenous toxicity and effects on mast cells are interrelated through the average molecular size of the samples used, and (c) that dialysis can fractionate the average molecular weight polymers into their components of varying toxicity without appreciable loss of antiheparin potency.

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Suppression of Antibody Forming Capacity with Thymectomy in the Mouse.* (27699)

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Previous reports have indicated that surgical ablation of the thymus gland has a variable effect on the immune response of the operated animal. While the immunologic-depressive effect of thymectomy appeared to be a function of the age at which the thymus was removed(1-5), further investigation has also revealed wide difference in immunologic maturation at birth or hatching in various species(6-9). Thymectomy within 24 hours after birth in the rabbit appeared to lower the response to antigenic stimulation when antibody was measured by quantitative technics(10). However, thymectomy, even within the immediate neonatal period, did not completely abolish the immune response. We report here that complete thymectomy performed within 24 hours after birth in the DBA/2 strain of mice almost completely abolishes the ability of survivors to respond to stimulation when bacteriophage T_2 (*E. coli* B) is used as antigen.

Material and methods. Newborn mice of the DBA/2 strain were thymectomized by a procedure described previously(5). Sham operated controls were subjected to a similar operative procedure, but the thymus was left intact. At 2 months of age, controls and thymectomized survivors were given a single dose of T_2 bacteriophage (2×10^{10} particles) intraperitoneally and bled 7 days later from the retro-orbital plexus into heparinized capillary tubes. The tubes were centrifuged at 4°C at $1500 \times g$ and the serum removed. Virus

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