TSH, ACTH) this suppression becomes balanced according to the push and pull principle, which applies readily to the tropins producing secondary peripheral hormones (balanced tropins). With regard to those pituitary hormones which are not held in abeyance by the feed-back mechanism of their secondary peripheral hormones (LTH, ADH and MSH), an overshoot is to be expected. This would classify LTH, ADH and MSH as unbalanced tropins.

Our hypothesis has been confirmed recently by Scott and Nading(16). They reported that phenothiazine tranquilizers caused release of MSH by counteracting hypothalamic inhibition.

With regard to our experiments it is relevant that the melanophore effect was obtained only in Rana frogs and Bufo. Hyla frogs did not react to reserpine with darkening of the skin. This would indicate that the tree frog has a more stable regulation of MSH secretion than Rana. Hyla melanophores have indeed been considered to react more specifically to MSH and ACTH assays than those of Rana or Bufo(17,18).

Summary. Reserpine injected into the dorsal lymph sac of Rana frogs at dose levels of 0.05-0.25 mg was shown to provoke melanophore-dispersion in the skin. The melanophore index, M.I., in the hind webs of the frog rose from 1 to 5 within 24-72 hours after reserpine injection. Reserpine did not cause any change of skin color *in vitro*. It is suggested that this effect is due to intensified endogenous MSH secretion.

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Distribution and Particle Size of Type A Botulinum Toxin in Body Fluids of Intravenously Injected Rabbits.* (26604)

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The present study reports distribution of type A botulinum toxin in various body fluids after intravenous administration of the toxin to rabbits. The sedimentation coefficient of the toxin in plasma and lymph was determined by partition cell ultracentrifuga-

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tion. These observations were undertaken to investigate whether or not exposure of the toxin to the *in vivo* environment might result in the appearance of toxophoric particles smaller in size than can be attributed to a protein molecule. Ingested toxin which appears in the lymph draining the intestinal tract has the dimensions of a protein(1).

Materials and methods. Concentration of toxin in specimens was expressed in LD_{50} values calculated by the method of Reed and Muench, employing white mice for the assay as previously described(1,2). Sedimentation coefficients (S_{20w}) of toxin in fluid specimens were calculated from measurements of the distribution of toxin between upper and lower portions of partition cells after ultracentrifugation as outlined by Heckly *et al.* (1). Crystalline type A toxin dissolved in 0.05M phosphate-0.2% gelatin buffer was injected into marginal ear veins of New Zealand white male and female rabbits weighing between 2 to 5 kg. This toxin had S_{20w} values of 12-21 when measured by the partition cell centrifugation method.

Sampling of body fluids. Pentobarbital –Na (30-60 mg) was injected intravenously when an anaesthetic was required.

Blood was taken from the right ventricle at termination of an experiment. Consecutive samples of blood were taken from the cannulated jugular or femoral vein through a polyethylene tube flushed with saline both before and after each sample was taken. Heparin was added to the collected blood. Lymph was taken by continuous sampling from cannulated left thoracic duct, and collected under ice to avoid clotting without addition of anticoagulant. Fetal blood was taken from 3- to 4-week pregnant rabbits at the time of maternal respiratory failure. The fetus was exposed, the thorax opened and blood aspirated from the right ventricle. Blood from fetuses of a litter was pooled. Spinal fluid was obtained by suboccipital puncture of the cisterna cerebello-medullaris. Ocular fluid was taken through a No. 26 needle inserted in the lateral rim of the cornea and directed towards the middle of the anterior eye chamber. For continuous sampling, the needle was left in place and the ocular fluid allowed to drip into a vial through a small polyethylene tube attached to the needle. Bile was taken from the gall bladder at termination of an experiment. In some cases the common bile duct was cannulated and the bile collected continuously under ice. Urine was taken from the urinary bladder at termination of an experiment or the bladder was catheterized and the urine sampled continuously under ice. Specimens of body fluids showing macroscopic evidence of blood contamination were discarded.

Results. The particle size of botulinum toxin is a function of its degree of association with a separable hemagglutinating protein(2). Since dissociation of the hemagglutinin from the toxic entity might occur *in vivo,* it was important to establish the sedimentation coefficient of toxin freed from the hemagglutinin. Consequently, hemagglutinin-free toxin was prepared by exposing crystalline toxin dissolved in an isotonic sodium chloride-phosphate buffer, pH 7.3, at a maximum temperature of 10°C to successive batches of chicken erythrocytes. Under these conditions selective adsorption to the red cells reduced the hemagglutinin titer of the toxin solution to zero. While the method results in losses of toxin, the toxin remaining in solution is free of hemagglutinin after centrifuging out the red cells. This hemagglutinin-free toxin was found to have S_{20w} values in the range of 3.7 to 8.4.

Since heparin was employed at times *in vivo* and *in vitro,* it was necessary to learn whether or not this might affect the assay for toxin. Titrating the blood and plasma of 2 heparinized rabbits given toxin resulted in 100% recovery of the expected toxicity values. Thus, *in vivo* use of heparin does not appear to affect the assay for toxicity.

In vitro heparin can precipitate toxin dissolved in salt solutions. The precipitation, however, can be prevented or reversed by addition of serum albumin. Ultracentrifugation revealed that the sedimentation coefficient of toxin in plasma and serum under the conditions of the present investigation was unaffected by the presence of heparin or citrate. On the other hand, when these anticoagulants were added to a salt solution of

TABLE I. Distribution of Toxin between Plasma and Whole Blood in Intravenously Injected Rabbits.

Dose (mouse LD_{50}/kg)	Sample time after inj., min.	Mouse LD_{50}/ml* Whole blood	Plasma
200,000	120	3,200	6,000
400,000	120	6,400	12,000
1,000,000	120	14,000	28,000

* Toxic blood removed from rabbit, and blood titrated. A sample specimen was centrifuged at 10°C for recovery and titration of plasma. Since red cells occupy about 40% volume of blood, the approximate 2-fold greater toxicity of plasma than whole blood argues in favor of the complete presence of the toxin in the liquid phase of blood.

toxin, increased values for the sedimentation coefficient of toxin were found. It is therefore felt that the sedimentation coefficients of toxin in plasma and lymph reported here are independent of the presence of added anticoagulant.

Since toxin was injected directly into the blood stream, the possibility was explored that toxin might be distributed between erythrocytes and plasma. It can be concluded from the data in Table I that all of the toxin remains in the liquid phase.

Toxin in plasma and lymph. The toxicities of plasma and lymph were determined at various time intervals after injection of 4 rabbits with 2×10^6 mouse LD_{50}/kg. Toxin could be demonstrated in lymph within 25 minutes, though the highest concentrations required longer periods for their appearance (Table II). The toxicity of lymph did not exceed between 1/6 to 1/12th part the toxicity of plasma. Thus toxin appears to escape slowly from blood to the lymph collectable from the thoracic duct. The disproportion between the toxicity of plasma and lymph could be solely a reflection of a lack of time to reach a true plateau value. To answer this question, it would be necessary to repeat this experiment employing lower levels of toxin, to permit survival of groups of animals for significantly longer periods of time.

Determinations of sedimentation coefficients of the toxin are recorded in Table II. The size of the toxic material in lymph and plasma cannot be said to differ. The variations in sedimentation coefficients are within the limits expected from the large experimental errors inherent in the procedures employed. What is worth noting is that all the values are in a range expected for proteins. The size of the toxin after intravenous injection is significantly less than that of crystal-

TABLE II. Toxicity and Sedimentation Values of Toxin in Plasma and Lymph of Rabbits Intravenously Injected with 2,000,000 Intraperitoneal Mouse LD_{50} per kg of Subject Rabbit.

Rabbit	Sampling time post-inj., min.	Plasma Toxicity, mouse LD_{50}	Plasma $*S_{20w}$	Lymph Toxicity, mouse LD_{50}	Lymph $*S_{20w}$	Death (D) or termination (T) time, min.
A	5	113,000	13.9	—	—	
	24	113,000	9.1	700	—	
	40	74,800	12.2	800	—	
	55	64,000	12.5	5000	—	55 D
B	4	50,000*	4.2	—	—	
	20	61,000*	10.0	236*	7.7	
	40	43,000*	5.9	2300*	10.3	
	90	43,000*	6.4	3100*	11.4	
	100	—	—	2600*	6.6	100 T
C	5	80,000	4.0	—	—	
	20	—	—	57	—	
	40	77,000*	7.3	7800	14.7	
	70	57,000	9.6	9100	14.2	
	77	—	—	9600	7.4	77 D
D	1	80,000	11.2	—	—	
	25	57,000	8.2	<16	—	
	110	64,000	12.6	4000	9.0	
	130	—	—	3800	7.0	130 T

* Toxicity values are based on a calculation from toxicities of top and bottom specimens of the ultracentrifuge partition cell by the method recorded in Heckly *et al.*(1).

TABLE III. Test for Presence of Toxin in Pools of Fetal Blood of Litters from Rabbits at Time of Maternal Respiratory Failure after Intravenous Injection with Type A Crystalline Botulinum Toxin.

Rabbit	Dose (mouse LD_{50}/kg)	Toxicity of blood*: Maternal	Toxicity of blood*: Fetal	Respiratory failure time, min.
1	400,000	6,000	0	70
2	1,000,000	16,000	0	87
3	1,000,000	12,000	0	114

* Expressed as mouse LD_{50}/ml of blood. With fetal blood the method employed would permit detection of 2 or more mouse LD_{50}/ml.

line toxin. This could be due to partial dissociation of hemagglutinin units from the toxic entity. The S_{20w} values obtained are too large to permit the conclusion that the toxin becomes completely free of hemagglutinating material. The measurements are similar to those found for toxin appearing in lymph taken from rats given crystalline toxin intraduodenally (1).

Toxin in fetal blood. Toxin was absent in 3 pools of fetal blood taken at the time of respiratory failure of the poisoned parents. The toxicity of maternal blood was about 10,000 mouse LD_{50}/ml (Table III). There appears to be no rapid transfer of toxin from maternal to fetal blood.

Toxin in ocular fluid. Ocular fluid was taken from rabbits at periods from 30 to 120 minutes after intravenous doses of toxin were given in the range from 200,000 to 2,000,000 mouse LD_{50}/kg. Upon initial puncture of the anterior eye chamber ocular fluid was collected with no detectable toxicity for the mouse in 20 out of 33 experiments. The technic of titration employed would give such a negative result if less than 10 mouse LD_{50} doses were present per ml of ocular fluid, and would represent values of toxicity for the ocular fluid of less than 1/400 to 1/3600 part of the toxicity present per ml of plasma of the rabbits. In the remaining 13 experiments detectable toxicity was present, the highest value recorded being about 5% of the toxin in one ml of plasma. Contamination of the ocular fluid with blood can be excluded as a cause of the presence of toxin in these positive instances. The toxicity values obtained could be correlated neither with amount of the injected toxin nor interval of time between injection of the toxin and sampling of ocular fluid. It would appear that normally a barrier exists to the passage of toxin from blood to ocular fluid but that this barrier varies with individual animals. Inducing inflammation of the eye can cause the appearance of some toxin in the intraocular fluid (Table IV).

In 14 experiments, after the initial withdrawal of ocular fluid, sampling was permitted to go on continuously. In these instances the ocular fluid was drained as fast as it was formed, which would permit an increase in the difference between intracapillary and intraocular pressures. Under these circumstances of reduced intraocular pressure the toxicity of the ocular fluid rose substantially, in some cases to values approaching that for plasma. Since rupture of blood vessels could be excluded as the cause of this rise in toxicity, the presence of toxin in the ocular fluid can be attributed to filtration processes. Table IV gives data in support of this conclusion. That filtration is the prob-

TABLE IV. Accumulation of Toxin in Ocular Fluid under Conditions of Normal and Low Intraocular Pressure.

Rabbit	Time of sampling postinjection, min.	Toxicity (mouse LD_{50}/ml): Blood	Ocular fluid: Normal	Ocular fluid: Low pressure*
A	30	16,000	0 (one eye) 20 (other eye) †	
B	60	6,000	0 (one eye) ‡	8,000§
	90	6,000	0 (other eye)	
C	60	8,000	0 (one eye)	6,000
	90	8,000	0 (other eye)	
D	30		0 (one eye)	8,000
	120	6,000		

* Low pressure achieved by continuous tapping of ocular fluid for time interval indicated.

† This eye was artificially inflamed by instilling allyl-isothiocyanate into conjunctival sac.

‡ One eye sampled and ocular fluid found to be non-toxic, then allowed to drain continuously to prevent intraocular pressure from building up to normal, and ocular fluid retested for toxicity. The other intact eye was then sampled at the same time.

§ The higher toxicity in ocular fluid than in blood is due to the fact that toxin is limited to plasma. Blood toxin values should be approximately doubled for plasma toxin values (Table I).

able mechanism for the appearance of toxin in ocular fluid is also supported by an experiment in which, after removal of ocular fluid, saline was used to replace the fluid and thus prevent anything other than a transient reduction in intraocular pressure. In this situation the ocular fluid remained non-toxic after retapping. Maintaining the intraocular pressure acts against escape of toxin from the capillaries of the eye, as would be expected if toxin enters the intraocular fluid by a filtration mechanism.

Toxin in urine and bile. Toxin mixed *in vitro* with urine and bile was inactivated at body temperature, particularly in the case of bile. The quantitative aspects of these inactivations have not been worked out, but the phenomenon shows that finding an absence of botulinum toxin in urine or bile which has remained for a time in the fluids' reservoirs might reflect toxin inactivation and not a true inability of the toxin to make its way into these body fluids. This might explain Legroux and Levaditi's(3) findings of toxin in blood of injected rabbits with the occasional failure to find toxin in urine taken directly from the bladder. Therefore, a series of experiments were performed by direct cannulization of the urinary bladder and the common bile ducts. The specimens were collected under ice for one hour and then immediately injected intraperitoneally into mice. The specificity of deaths was authenticated by including among the injected mice individuals which had been passively immunized with specific antitoxin.

To exclude the possibility that circulatory failure after large intravenous injections of toxin would interfere with excretory functions of the liver and the kidney, mean arterial pressure was recorded in the femoral artery. During the first hour after intravenous injection of 10,000 to 1,000,000 mouse LD_{50}/kg of toxin, the blood pressure of the rabbits did not fall below 100 mm Hg. Only during the terminal stages of asphyxia following respiratory failure did arterial pressure change significantly.

The urine collected within an hour from 6 rabbits receiving intravenous injections of 1,000,000 mouse LD_{50}/kg contained 30 to 230 mouse LD_{50}/ml. In 3 experiments bile was toxic to the extent of 100 to 140 mouse LD_{50}/ml. It is evident that toxin can appear in urine and bile. In relation to the dose received, the amount of toxin eliminated by these routes is too small to signify a therapeutically significant loss of toxin. Since it is well known that small quantities of protein occur in the excretions of the livers and kidneys of normal animals, the toxin appearing in urine and bile cannot be considered unique examples of passage of protein through these organs.

Cerebrospinal fluid. From 10 rabbits receiving 200,000 to 1,000,000 mouse LD_{50}/kg, specimens of cerebrospinal fluid were negative for toxicity in 9 cases. Four of the negative specimens were drawn 30 minutes postinjection, 4 were drawn 90 minutes postinjection, and 1 drawn 120 minutes postinjection. The one specimen showing toxicity (10 mouse LD_{50}/ml) was taken at time of death (100 minutes) from a rabbit having received 200,000 mouse LD_{50}/kg with a blood level of 3,000 mouse LD_{50}/ml. The experience cited does not point to capacity of toxin to pass readily into the cerebrospinal fluid, at least during the time intervals of our experiments, and does not exclude the possibility for appearance of small quantities of toxin under conditions other than those employed. One might infer that in natural cases of food poisoning, where much lower levels of toxin will appear in blood than in these experiments, the presence of measurable amounts of toxin in cerebrospinal fluid will be quite doubtful. No toxin could be found in cerebrospinal fluid of a rabbit 10 hours after 11,000,000 mouse LD_{50} were instilled intraduodenally and a toxic blood level of 160 mouse LD_{50}/ml had been achieved.

Discussion. The distribution of intravenously injected type A toxin seems to parallel the distribution of protein in body fluids. Apart from plasma, only lymph appears to show a consistently high toxicity. The finding that increase of filtration pressure will lead to a greatly increased toxicity of the ocular fluid is compatible with the assumption that a toxic protein is passing by filtration from the blood stream into other body

fluids. Our data are in accord with the behavior of proteins in plasma and the knowledge that the glomerular membrane is relatively impermeable to protein in spite of a high filtration rate in the kidney.

The data suggest that in diagnosis of poisoning by botulism it is advisable to collect blood for toxicity tests. It should be pointed out, however, that in human cases brought to autopsy toxin has been detected in extracts of liver when blood specimens have been negative(4).

Summary. In rabbits which were given type A botulinum toxin intravenously, and which died rapidly of acute poisoning the toxin was found in lymph and plasma with no tendency to diffuse into erythrocytes. Ratio of toxin in lymph to plasma ranged between 1:6 to 1:12. Sedimentation coefficients of the toxin in these body fluids were similar and had the dimensions of a protein. The particle size was decreased in relation to crystalline toxin, was similar to toxin in lymph from rats given toxin intraduodenally, but was not sufficiently smaller to warrant the assumption of complete dissociation of toxin from the hemagglutinin present in crystalline toxin.

Toxin was absent from fetal blood, but appeared in small quantities in urine and bile. Cerebrospinal fluid was free of toxin except for a small amount at death. In the normal eye none or only a small quantity of toxin appears in the intraocular fluid. By experimental reduction of intraocular pressure toxin in intraocular fluid tends to approach concentrations present in plasma. Inflammation can lead to the occurrence of toxin in intraocular fluid. The findings are compatible with the hypothesis that the toxin *in vivo* has the dimensions of protein and passes tissue barriers by filtration mechanisms normally operative.

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Amino Acid Composition of Mycobacterial Cell Wall.* (26605)

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Determinations of mycobacterial cell wall composition have been made semi-quantitatively for taxonomic purposes by Cummins and Harris(1), and quantitatively by Kotani, *et al.*(2), who also studied particulate and soluble fractions from the same mycobacterial cells. Glutamic acid, alanine, and α,a'-diaminopimelic acid were not determined quantitatively by the latter authors but were identified as cell wall components. Antigenic properties of mycobacterial cell wall have been examined by Ribi, *et al.*(3). The present report concerns the quantitative determination of amino acids in cell walls from 4 mycobacteria.

Materials and methods. Cells of *Mycobacterium tuberculosis,* strains† $H37R_a$, and

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