STUDIES ON LYMPHOGRANULOMA VENEREUM

II. The Association of Specific Toxins with Agents of the Lymphogranuloma-Psittacosis Group

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Plate 7

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The association of a toxin or toxic factor with a virus has never been demonstrated although it may have been suspected. In fact, under most circumstances it is difficult to devise experiments which would demonstrate clearly the occurrence of a toxin in association with these agents since both would be of small size and usually of labile nature. Thus, if all possible sources of confusion were controlled, a positive result would be of great value but a negative one would be without significance.

For many reasons it has been suspected in this laboratory that the agent of lymphogranuloma venereum, and the other agents which have been shown to be closely allied to it (1–5) should be separated from the true viruses and established in a group by themselves, comparable to the *Rickettisae* for example (6). It had also been suspected that the agent of lymphogranuloma venereum at least, with which we have had most experience, produces a toxin. In the acute form of generalized infection with this latter agent in man, chills, severe headache, and marked prostration occur and the whole picture suggests a toxemia (7, 8). Furthermore, in the laboratory, the convulsive deaths occuring in mice within 30 hours after intracerebral infection (9) are difficult to account for by the lesions present in the meninges, and the cause of death in the chick embryo after infection of the yolk sac has always seemed due possibly to toxemia since the agent does not invade the embryo to any marked extent (10), and careful studies have shown that no significant disturbance of nutrition results from the infection of the yolk cells (11).

The work of Gildemeister and Haagen (12) on the toxin associated with *Rickettsia mooseri* grown in the yolk sac of the chick embryo stimulated anew our interest in this problem. These authors found that suspensions of yolk sacs heavily infected with *R. mooseri*, when suspended in Ringer's solution, were lethal for mice. Following intraperitoneal inoculation of 0.5 to 1.0 ml. of such suspensions the mice died in 4 to 48 hours, often with convulsions. Smaller amounts were less active and intracerebral or subcutaneous inoculation was less effective than intraperitoneal. The toxic substance was very labile and could not be demonstrated after the suspensions had been treated in any manner which killed the *Rickettsiae*. Serum from individuals who had

suffered from endemic or epidemic typhus neutralized the toxin, and so did placental globulin. Serum from individuals immunized against typhus had some activity, but serum from normal human beings or rabbits had no neutralizing power. The authors believed they might be dealing with an endotoxin.

In our laboratory it has been possible (13) to demonstrate a toxin, or toxic factor, closely associated with the bodies of the agents of lymphogranuloma venereum, of meningopneumonitis (Francis and Magill (14)), and of mouse pneumonitis (15, 16).[1]

As will be shown in the present paper, these toxic factors behave like bacterial endotoxins and they will therefore be referred to as toxins.

Technique

All inoculations into the chick embryo, whether to obtain toxin or to titrate infective units, have been made by the yolk sac route into fertile eggs previously incubated 6 days, employing volumes of 1 ml. Suspensions of yolk sacs have been made by shaking them without beads for 30 minutes in a machine. In all but the early experiments yolk sacs to be shaken were suspended in their own yolk and fluids (with the egg albumen carefully excluded) at a volume of 1 in 2.5, 1 in 5, or 1 in 10 of their original weight after draining off excess yolk. Further 2-fold dilutions were made in a variety of diluents, discussed below, but for most purposes pooled yolk, allantoic fluid, and amniotic fluid from normal 6 day chick embryos were used. In titrating infective units 10 eggs have been used on each dilution and dilutions increased by 2-fold increments. The L_D50 was calculated by the method of Reed and Muench (17).

All intravenous inoculations into mice were made in one of the tail veins after these had been dilated by immersion of the tail in hot water. Except in early experiments a volume of 0.5 ml. was used in every case. Mice were examined at frequent intervals and deaths recorded in hours after inoculation. Deaths during the night were recorded as between so and so many hours (*e.g.* between 6 and 20 hours) and given a median value (*e.g.* 13 hours). In titrating toxic units 10 mice were used on each dilution, the dilutions were increased by 2-fold increments, and the L_D50 calculated by the method of Reed and Muench (17).

Tissues for microscopic examination were fixed in Zenker's fluid. Only tissues from mice known to have died within 30 minutes were used for pathological examinations. Eosin and methylene blue stain has been employed as routine, with special stains, such as Weigert's fibrin stain for renal capillary thrombi, used as occasion demanded. Macchiavello's stain has been employed for all smears of yolk sacs. Selection of yolk sacs for maximal toxin content has been based on the heaviness of infection indicated by smears prepared from a fragment of such sacs. This point is discussed in detail below.

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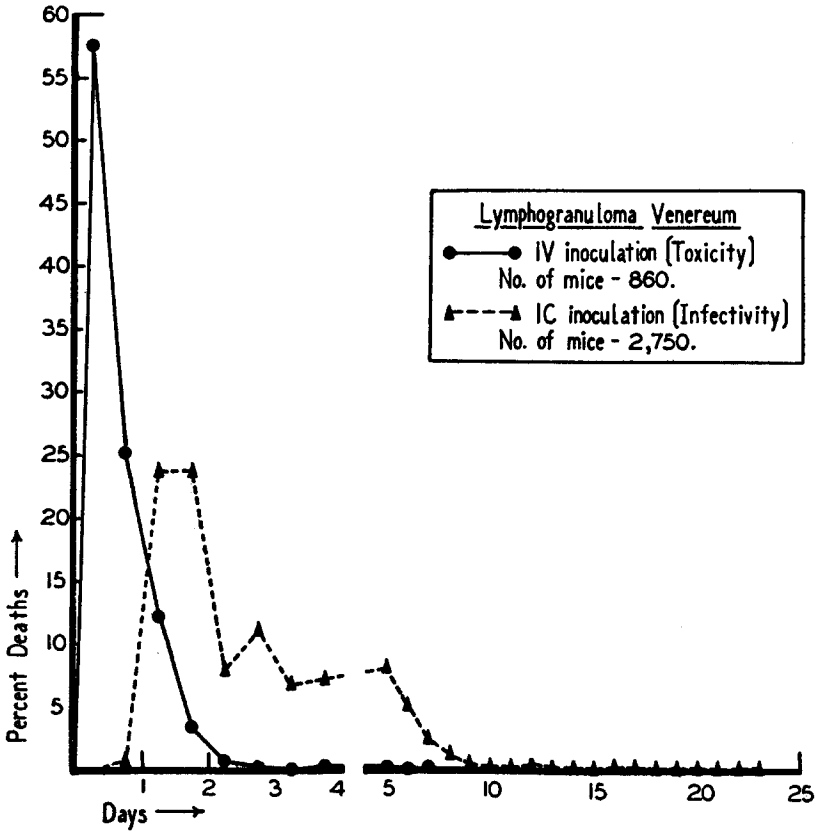
[1] The strain of mouse pneumonitis used in the present studies was the Atherton strain received through the courtesy of Dr. Clara Nigg.

The Toxin from the Agent of Lymphogranuloma Venereum

Following the inoculation of a suspension of the agent of lymphogranuloma venereum into the yolk sac of the chick, the yolk cells become heavily infected (18). By adjusting the inoculum, the time of death from specific infection can be gauged very accurately (10). It is found that when yolk



TEXT-FIG. 1

sacs are harvested shortly before or after death of the embryo, suitably diluted suspensions of those found by yolk sac smears to be heavily infected are lethal for mice following intravenous inoculation of 0.5 ml. amounts. The time of death varies, of course, with any given suspension, with the degree of dilution and type of diluent, and with the presence, or absence, of partial neutralization with serum. Deaths may occur as early as 4 hours after inoculation and are very rare after 36 hours. Text-fig. 1 shows the distribution of time of death obtained by combining all the experiments, involving 860 mice. 83

per cent of deaths occur by 24 hours and 95 per cent by 36 hours. Some of the early deaths are accompanied by convulsions but for the most part the mice become lethargic with very labored breathing and death comes quietly. In the case of both the agent of lymphogranuloma and the other agents discussed below, other tissues and fluids from the eggs were tested for the presence of the toxin, but, except in the case of the yolk, toxin is absent or present in amounts too small to be lethal. The yolk is sometimes toxic intravenously in 0.5 ml. amounts undiluted. Normal yolk is not toxic under like conditions. Even in the yolk sac itself the toxin is present in comparatively low concentration. Thus 0.5 ml. of a dilution of 1 in 40 of original weight of yolk sac was toxic in only 9 of 42 experiments and 1 in 80 was never toxic. 1 in 20 was toxic slightly less than 50 per cent of the time although, as conditions which would inactivate the toxin became understood, the percentage of tests giving some deaths at 1 in 20 could be increased.

2 ml. amounts of 1 in 5 suspensions of heavily infected yolk sacs kill mice by the intraperitoneal route. Time of death and symptoms are similar to what is seen after intravenous inoculation. Higher dilutions have been ineffective by this route.

The susceptibility of mice varies with the weight. This is demonstrated more particularly with the toxin from the agent of meningopneumonitis in which higher toxicity per gram of infected yolk sac (see below) allows the demonstration of greater variation between mice at different weight levels. For practical purposes mice of 12 to 14 gm. were used in most tests; although smaller mice are killed with less toxin, there are practical difficulties in the rapid intravenous inoculation of large numbers of these very small animals.

Mice dying from toxemia show congestion of the lungs in the case of early deaths and enlarged yellow livers in those dying at 20 hours or later. Microscopically, the most constant features in deaths up to 9 hours are scattered hemorrhages in the lung or patches of edema fluid in the alveoli, and fibrin thrombi in the glomerular capillaries of the kidney. In addition, one sees occasionally necrosis of cells in some cortical tubules and exudate in the glomerular spaces and tubules of the kidney. The liver shows very occasional foci of frank necrosis and more frequent foci in which the cells appear damaged, and are loaded with fat droplets but not yet necrotic (Fig. 1). In deaths occurring at 15 to 24 hours, hemorrhage or edema in the lung is rare. No fibrin thrombi are seen in the kidney but there is occasional tubular damage. Small foci of accumulated glial cells, seen in the cerebrum of about one-third of mice examined, may represent a specific lesion. The most prominent damage, however, is in the liver. In almost every case there are numerous foci of necrosis throughout the organ (Fig. 2). Such foci are often accompanied by hemorrhage and whatever hepatic cells are not necrotic usually show marked cloudy swelling. In many cases it is difficult to determine the position of the necrosis in the liver lobule, but, in cases in which this can be established, it is always midzonal.

The toxin is labile and is inactivated by many comparatively simple treatments. Thus it disappears in eggs which die and are allowed to remain at 36°C. Inactivation is slower at room temperature (approximately 22°C.) but even here activity may drop 50 per cent in 4 or 5 hours. Small amounts of certain detergent soaps remaining in washed glassware will destroy all toxicity,—a cause of much worry in the early stages of the present investigation and one which has been overcome by using only acid-cleaned glassware. Treatment with 0.1 per cent formalin destroys all toxicity but not all antigenicity (see below). Even the choice of diluent is very important. Besides the usual mixture of yolk and egg fluids, others have been used. Of these, physiological saline and beef heart infusion broth produce some inactivation of the toxin. Normal serum, of human, bovine, or lapine origin, is even better than yolk and egg fluids in preserving full toxicity. However, many normal bovine sera, which were the most readily available, when given intravenously to mice in 0.5 ml. amounts, produce agglutination and hemolysis of red cells with rapid death and cannot be used for routine work.

Toxin can be stored at various low temperatures for future use. Most investigation of optimal conditions for storage has been carried out with the toxin of meningopneumonitis and will be discussed below. With the toxin of lymphogranuloma venereum most satisfactory storage is at 0°C.

No toxicity of the type described above can be demonstrated in freshly prepared or stored suspensions of normal yolk sac. Similarly the high-speed sediment of normal yolk sacs shows no such toxicity. It is true that normal embryo yolk and fluids, the usual diluent employed, often effect the mice particularly after storage in the cold. This is especially true when care has not been taken to exclude albumen from the fluid mixture, and the reaction can be avoided if the fluid diluent is centrifuged for 20 minutes at 1800 R.P.M. However, the nature of this reaction differs from the action of the toxins under consideration in that the mice show immediate symptoms with convulsions or extreme prostration, and death or recovery supervenes in a few minutes.

The reason for considering the material producing death in the mice receiving infected yolk sac suspensions as a toxin is discussed below. At this point attention should be drawn to those observations which indicate that we are not dealing with an infection. Foremost of these is the rapidity of death. Included in Text-fig. 1 is a distribution curve of time of death of mice infected intracerebrally with the agent of lymphogranuloma venereum—the most rapid method of infection known—obtained by combining many experiments totaling 2750 mice. This should be compared with the curve of toxic deaths. From infection no deaths occur before 21 hours have elapsed and only 0.7 per cent in the first 24 hours (83 per cent in the case of the toxin). 24 per cent of deaths take place in the first 36 hours (toxin 95 per cent), and deaths continue to occur for 3 weeks, accompanied by all degrees of chronic infection.

Similar delayed deaths do not follow intravenous or intraperitoneal inoculation. The mice either die rapidly or soon become well. In fact limited experiments indicate that these mice, having received intravenously high concentrations of yolk sac material, which not merely is toxic but contains large numbers of viral bodies potentially infectious by other routes, do not become carriers, despite the relative ease with which the carrier state is set up in mice surviving infection (9, 19). The agent is not demonstrable by the most sensitive methods of detection in the brain or spleen of mice surviving sublethal doses of toxin. The changes found in the tissues also speak against death from infection. All of those described above are such as are usually associated with administra-

TABLE I

Ratio of Toxic to Infective Units under Various Conditions

Agent	Treatment	L_D50 Infectivity for chick embryos	L_D50 Toxicity for mice	Toxicity/ infecticity ratio
Lymphogranuloma venereum	Freshly harvested	1/370 million	1/13	1/28 million
" "	" "	1/260 "	1/7.7	1/34 "
" "	" "	1/265 "	1/6.2	1/43 "
Meningopneumonitis	" "	1/1180 "	1/33	1/36 "
"	" "	1/1560 "	1/43	1/36 "
"	−72°C. 11 days	1/495 "	1/43	1/11.5 "
"	−72°C. 35 days	1/210 "	1/45	1/4.7 "
"	−72°C. 85 days	1/210 "	1/24	1/8.8 "
"	Frozen and thawed six times	1/125 "	1/17	1/7.4 "

tion of a toxic material. No evidence of any inflammatory change, or other alteration usually associated with viral or bacterial infection, is present.

Attempts have been made by three principal methods to determine the nature of the toxin. Everything done so far indicates that it is closely associated with the actively infectious bodies of the agent. Thus filtration of suspensions of infected yolk sacs through Seitz pads, which hold back the infective agent (20), results in atoxic filtrates. Moreover, when infected yolk sacs suspended in infected yolk are first centrifuged at 1500 R.P.M., and the supernate from this recentrifuged at 18,000 R.P.M. for 1 hour in the cold, the toxin remains with the final sediment of elementary bodies and is not found in the supernate from which 99 per cent of the agent has been removed (18). It has, in fact, proved possible to wash and resuspend the sediment repeatedly with full retention of toxicity. Finally, simultaneous titration of the number of

toxic units (for mice) and infective units (for eggs) in a given freshly harvested yolk sac suspension has revealed that these bear a relationship to each other which is as constant from test to test as could be expected in biological tests of this nature (Table I). As will be pointed out below, no complete separation of toxic and infective factors has yet been accomplished, but in the case of the agent of meningopneumonitis certain manipulations result in decrease of infectivity with retention of toxicity.

The Toxin from the Agent of Meningopneumonitis

Following the same technique as that described for the agent of lymphogranuloma venereum, it has proved possible to demonstrate a toxin associated with the agent of meningopneumonitis, first isolated and described by Francis and Magill (14). The toxin in this case can be demonstrated in higher dilution by intravenous inoculation than can the toxin of lymphogranuloma, although routine smears of yolk sacs always suggest a lighter infection in meningopneumonitis. Thus a dilution of 1 in 40 of yolk sacs by weight was toxic in 77 of 98 tests killing 233 of 442 mice, and a dilution of 1 in 80 was toxic in 41 of 84 tests killing 97 of 374 mice. Dilutions of 1 in 20 or less were almost always fatal, killing all of the mice. Even a dilution of 1 in 160 was toxic in 5 of 41 tests and such high titers can probably be obtained more frequently by the use of normal lapine or human serum.

The distribution of the toxin in the tissues and fluids of the embryo is similar to that of the toxin of lymphogranuloma. As might be expected, higher titers than with lymphogranuloma venereum can be obtained with the yolk from meningopneumonitis, which is occasionally toxic at a dilution of 1 in 10.

2 ml. amounts of freshly harvested yolk sacs are toxic for mice intraperitoneally in far higher dilution than is the case with yolk sacs infected with lymphogranuloma. The endpoint tends to be less sharp by this route than by the intravenous (Table II).

As stated above, the susceptibility of mice to those toxins of agents of the lymphogranuloma-psittacosis group which have been tested differs with weight. Table III shows the result of a titration with the toxin of meningopneumonitis.

Two explanations exist for the higher titers of toxin found in meningopneumonitis. Either the agent of meningopneumonitis is more toxic than is that of lymphogranuloma, infective unit for infective unit, or it is not more toxic but its infective units are present in greater amount in infected yolk sacs. That the latter explanation is correct is shown by the data in Table I. As will be seen, the number of toxic units per gram of freshly harvested infected yolk sacs bears a constant relationship to the number of infective units, and the toxicity-infectivity ratio for both agents is as close as could be expected from such biological experiments. The agent of meningopneumonitis is present in greater amounts in such yolk sacs than is the agent of lymphogranuloma.

The time of death of mice inoculated intravenously with the yolk sac sus-

pension of meningopneumonitis differs markedly in one respect from the time with the yolk sac suspension of lymphogranuloma. It will be seen from Text-fig. 2 that there is no sharp end to the mortality but that deaths continue for as long as 3 weeks. As might have been predicted, these deaths, unlike those with the suspension of lymphogranuloma, are due to infection as well as toxemia. At first it seemed impossible to separate deaths due to the one cause

TABLE II

Comparison of Toxicity of Meningopneumonitis Material by the Intravenous and Intraperitoneal Routes

Date of test	Intravenous					Intraperitoneal					
	1/10	1/20	1/40	1/80	1/160	1/5	1/10	1/20	1/40	1/80	1/160
6/14/43	5/5*	5/5	1/5	0/5		3/5	3/5	1/5			
6/21/43	3/3	3/3	3/3	4/4		5/5	2/5	3/4			
7/ 6/43		4/4	4/4	2/4		4/4	3/5				
8/ 3/43			5/5	4/5	1/5		10/10	5/9	0/4	0/4	
8/16/43	5/5	5/5	5/5	4/5		10/10	3/5				
8/30/43	5/5	5/5	5/5			5/5	5/5	5/5	3/4		
9/ 2/43	5/5	5/5	5/5	3/5	0/4		3/5	3/5	3/5	2/5	0/5

* 5/5 = 5 mice of 5 inoculated died within 60 hours.

TABLE III

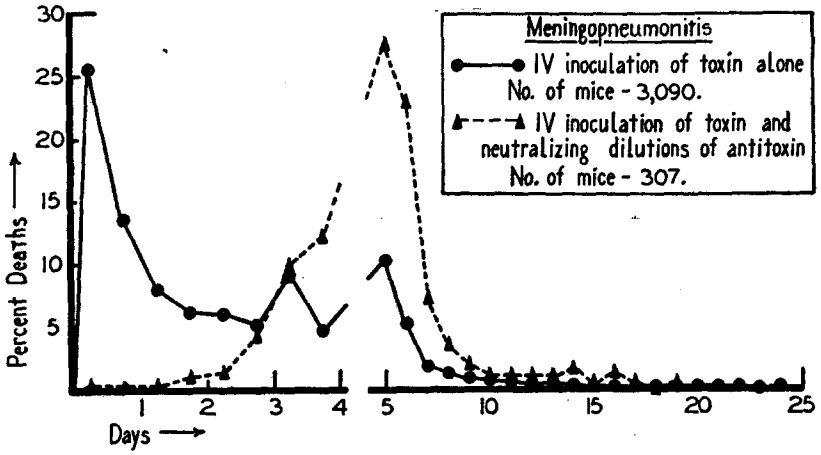
Toxicity of Meningopneumonitis Material in Mice of Different Weights

Weight of mice	Dilution of toxin				
	1/10	1/20	1/40	1/80	1/160
gm.					
10–12	6/6	5/6	5/6	2/6	0/6
12–14		4/6	2/6	0/6	0/6
15–17	4/4	5/6	2/6	0/6	0/6
20–22	6/6	4/6	0/6	0/6	0/6

from those due to the other, but it has not proved difficult. As Text-fig. 2 shows, the first part of the curve follows closely that of lymphogranuloma venereum but the curve rises again at 72 hours. The appearance of the mice suggests that this latter rise is due to infection and such an assumption is borne out by the microscopical findings and by the neutralization tests which will be discussed later. Even sera of high titer, which completely prevent deaths in the first 60 hours, *i.e.*, those deaths comprising the first part of the curve, fail in most instances to prevent the later deaths. As the curve of distribution of time of death following the use of antitoxic sera shows (Text-fig. 2), the distribution in this case covers the same range as the second, or

infectivity, rise described above. For practical purposes, therefore, all deaths occurring up to 60 hours have been considered as toxic deaths and none later have been so considered. This obviously introduces the error of including some infective deaths and excluding a few toxic deaths around the 60 hour period but such errors probably counterbalance each other.

Mice dying from the toxin of meningopneumonitis show symptoms very similar to those described for that of lymphogranuloma venereum. The later infectious deaths are preceded by characteristic symptoms of infection with ruffled fur, hunched back, and marked loss of weight.



TEXT-FIG. 2

The lesions found in mice receiving yolk sac suspensions of meningopneumonitis intravenously are striking. In the early deaths, both before 12 hours and up to 60 hours, the macroscopic appearances resemble those seen following inoculation of the agent of lymphogranuloma. The bright yellow livers suggest extensive necrosis. In later deaths the macroscopic changes in the liver may become even more striking because of regeneration of new liver lobules; in addition, pin-point, plum-colored areas appear in the lungs, and the spleen is often large and soft, or may show yellowish grey areas. In deaths as late as 7 days the surface of the brain appears dull and the brain itself is swollen and softer than usual.

Microscopically, changes in the organs in deaths up to 21 hours are similar to those seen following inoculation of the agent of lymphogranuloma venereum. Thus, in deaths within 6 hours, fibrin thrombi are found in the renal glomerular capillaries and there is fluid in some pulmonary alveoli. Damaged fat-holding cells are found in the midzonal region of the hepatic lobule at 4 hours and by 6 hours a few foci of frank necrosis are present. During the next 12 hours the areas of hepatic necrosis become more extensive and are definitely midzonal in distribution (Fig. 3). In the kidney there is damage to some cortical tubules with death of some cells. At 25 hours the

hepatic lesions are even more extensive but a new lesion, necrosis of the Malpighian corpuscles, has appeared in half of the spleens examined. At 39 to 44 hours hepatic changes are the same as at 25 hours. In the lung not only is fluid present in the alveoli, as often occurs in early deaths, but in many areas there is frank hemorrhage as well. The splenic changes are similar to those seen at 25 hours. By 48 hours a new feature has appeared, namely, resolution of the areas of hepatic necrosis. Other lesions are the same as before except that the areas of necrosis in the spleen are definitely larger (Fig. 4). By 66 to 71 hours the resolution of areas of hepatic necrosis is being followed by regeneration of lobules. In the lung an early interalveolar monocytic infiltration is present in most cases and there is an early monocytic infiltration of the cerebral pia mater (Fig. 5.). Thus the first signs of infection are evident at this stage. At 90 to 94 hours some of the spleens examined show areas of necrosis often taking up whole Malpighian corpuscles, while other spleens show an appearance resembling acute splenic tumor, the whole pulp being filled with monocytes. In the lung definite though small foci of monocytic consolidation are found and foci of monocytic infiltration are present in the cardiac muscle and pericardium (Fig. 6). In some cases the Kupffer cells of the liver are distended with viral vesicles (10) (*i.e.*, little colonies of the agent in the cytoplasm). Other changes are as before. By 7 days the areas of necrosis in the liver have disappeared for the most part and there is marked regeneration of lobules. The meningitis is much more extensive and the monocytic infiltration includes the choroid plexus and the linings of the ventricles. Some perivascular cuffing is apparent. Foci of monocytic infiltration are present in the cortex of the kidney.

It will be noted that the early lesions found following inoculation of the agent of meningopneumonitis resemble those found following inoculation of the agent of lymphogranuloma and are undoubtedly due to the toxin. Starting at about 60 hours there are found lesions which are due to infection, namely, the meningitis, the interalveolar infiltration and foci of consolidation in the lungs, the monocytic infiltration of the cardiac muscle and the cortex of the kidney, and the acute splenic tumor. It is interesting to note that the lesions in the lung and the central nervous system resemble closely those described by Francis and Magill (14) as beng produced by the agent of meningopneumonitis. They do not note having seen the lesions which we believe to be due entirely to the toxin, namely, those in the liver and kidney.

Following administration of the toxin by the intraperitoneal route, lesions are found which resemble those seen after intravenous inoculation, namely, necrosis in the liver and fluid in the pulmonary alveoli. Particularly marked is the destruction of the cells of the renal cortical tubules.

It has not been possible to demonstrate lesions in the organs of chick embryos moribund or recently dead after yolk sac inoculation with the agent. If, therefore, the death of the embryo is due to toxicity, it must occur too rapidly for lesions to be produced (as in the most rapid deaths in mice) or the tissues of the embryo must fail to respond in a manner similar to those of the mouse.

The lability of the toxin of meningopneumonitis resembles that of the toxin of lymphogranuloma. For the most satisfactory tests yolk sacs from moribund embryos should be used, but appreciable amounts of toxin can be demonstrated in yolk sacs of embryos known to have been dead for not more than 3 or 4 hours. Table IV gives the data of representative tests for the

TABLE IV

Stability of the Meningopneumonitis Toxin under Various Conditions of Storage

Date of preparation	Date of testing	Condition of storage	Dilution of toxin				
			1/10	1/20	1/40	1/80	1/160
		°C.					
5/ 4/43	5/ 4/43	—		2/3*	3/3		
"	5/11/43	−32	0/4	0/4	0/4	0/4	
"	"	−72	4/4	3/4	0/4	0/4	
"	7/28/43	"	9/9	6/9	0/10	0/9	
6/14/43	6/14/43	—	5/5	5/5	1/5	0/5	
"	6/15/43	0	5/5	5/5	5/5	2/5	
"	6/17/43	"	5/5	4/4	4/5	1/5	
"	6/21/43	"	5/5	5/5	5/5	1/5	
"	7/22/43	"	5/5	1/5	2/5	0/5	0/5
"	7/24/43	"	3/5	0/5	0/5	0/5	0/5
"	6/15/43	−32	5/5	3/5	0/4		
"	6/22/43	"	5/5	1/5			
"	7/22/43	"	5/5	2/5	1/5		
"	6/15/43	−72	5/5	3/5	1/5	0/5	
"	6/22/43	"	5/5	3/5	2/5		
"	7/22/43	"	4/4	5/5	1/5	0/5	
"	7/23/43	"	4/4	5/5	1/5	0/5	
"	8/ 6/43	"	5/5	4/5	0/4		
"	9/15/43	"	9/9	3/10	0/10		
7/26/43	7/26/43	—	4/4	0/4	0/4	0/4	
"	8/30/43	0	4/4	5/5	1/5		
"	9/27/43	"	4/4	2/5	0/5		

* 2/3 = 2 mice of 3 inoculated died within 60 hours.

retention of toxicity after storage at various low temperatures. It will be seen that when material is stored in rubber-stoppered, dope-sealed[2] tubes at −72°C. in dry ice there may be an initial loss in potency, but thereafter the potency remains approximately unaltered over a period of 3 months. It is essential that the tubes stored in this manner be carefully sealed with rubber stoppers and airplane dope since otherwise rapid inactivation will occur, owing prob-

[2] Cellulose acetate dope, No. 5332, Du Pont.

ably to a pH alteration. At −32°C. there is an even greater initial loss of potency and for this reason this storage temperature has not been employed. On storage at 0°C. the toxicity usually shows an initial increase which appears within 24 to 48 hours. This may be due to slow liberation of elementary bodies from previously intact yolk cells. Full activity is then usually maintained over periods as long as 2 months, the longest period of test, although occasionally there may be an abrupt and as yet unexplained loss in potency. For practical purposes storage either at −72°C. or 0°C. has been adopted.

As has been pointed out above, the constant ratio between toxic and infective units has held only with freshly harvested yolk sacs. Attempts have been made to separate completely toxic action and infectivity, without any success. The toxin is so labile that any manipulation which removes all infectivity also removes all toxicity. However, with the agent of meningopneumonitis partial separation has been achieved. Thus, as is shown in Table I, the simple process of storing yolk sac suspensions at −72°C. decreases the infectivity in contrast to the toxicity so that the toxicity-infectivity ratio decreases from about 1 to 37 million to about 1 to 8 million, a decrease which is consistent and significant. Moreover, repeated freezing and thawing of freshly harvested material has the same effect (Table I).

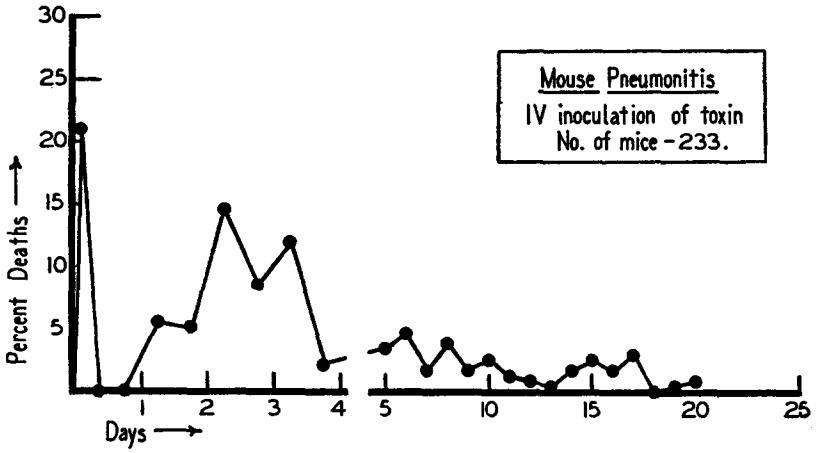
The Toxin from the Agent of Mouse Pneumonitis

Studies have also been made on the agent of mouse pneumonitis (15, 16). The distribution of time of death following intravenous injection with suspensions of this agent (Text-fig. 3) is quite unlike that of the two discussed above. The greater number of deaths (21 per cent of the 233 mice included in Text-fig. 3 and 29 per cent of the 309 mice studied up to the present) occurs in the first 4 hours and then no more deaths occur before 30 hours have elapsed. It seems clear that we are dealing with a toxin more intensely lethal for the animals than are either of the others since it kills the mice so rapidly. These mice recover from the injection and remain apparently well for about 30 minutes. They then become sick and are rapidly prostrated and die. The deaths occurring at 30 to 48 hours also follow only a short period of obvious illness and the mice behave as though they were suffering from a toxemia. Later deaths are characteristic of infection with this agent. The animals show ruffled fur, extreme loss of weight, and they have a hunchbacked high gait.

A scrutiny of the lesions found in these mice offers some explanation of the signs observed. Mice dying in the early period, *i.e.* up to 4 hours, show none macroscopically or microscopically. In those dying between 30 and 60 hours the liver is bright yellow and friable while occasional pin-point, plum-colored lesions may be seen in the lungs. In such mice microscopic examination reveals areas of early interstitial pneumonia with patches of edema fluid in the alveoli. There may be a few necrotic tubules in the renal cortex and in one case this process was very extensive. As in the case of

the other agents, however, the most intense damage is that in the liver. This follows the usual pattern of necrosis spreading out from the midzone of the lobule. There is one striking and characteristic feature, namely, distension of the Kupffer cells with viral vesicles. It would appear that this represents removal of elementary and initial bodies of the agent from the blood stream by the Kupffer cells, with subsequent multiplication of the agent until the cytoplasm of these cells is filled with vesicles. The necrosis of the liver cells, appearing as late as it does, would seem to be due to the toxic action of these accumulations of the agent in the Kupffer cells.

In mice dying on the 3rd or 4th days after inoculation the macroscopic appearance of the liver is unaltered. The spleen is very large and consolidation in the lung is more obvious. Microscopically, the changes in the liver are of the type described above but are more advanced. Those liver cells which are not necrotic show marked



TEXT-FIG. 3

cloudy swelling. There are occasional foci of monocytes. In the spleen there is commencing necrosis in the Malpighian corpuscles, and viral vesicles are present in some of the endothelial cells of the sinuses. In the lung, besides focal interstitial infiltration of monocytes, there are fluid and fibrin in the alveoli.

In still later deaths, *i.e.* 7 days or later, a characteristic finding is generalized subcutaneous edema, ascites, and pleural effusion. The liver is very large and as much as 50 per cent of the lung may show consolidation. Microscopically, the liver shows early cirrhosis with infiltration by fibroblasts and commencing regeneration of liver lobules. In focal areas the alveoli of the lung contain monocytes and a few neutrophils —a typical lobular pneumonia. Other lesions are inconstant and their significance of some doubt. Thus in one case there were foci of necrosis in the cardiac muscle, and in another two small areas of complete destruction of hippocampal cells in the cerebrum.

The rapid deaths, *i.e.*, those up to 4 hours have occurred only with dilutions of yolk sac of 1 in 2.5 or 1 in 5. As will be pointed out below, routine smears of

yolk sacs have shown a lighter infestation with this agent than occurs with either lymphogranuloma venereum or meningopneumonitis. Titration of infected yolk sacs by the yolk sac route in chick embryos also indicates that infestation of the yolk cells is less with this agent than with the other members of the group thus far studied. This apparently accounts for the low titer of toxin. Infectivity occurs with dilutions of 1 in 40 or even occasionally 1 in 80.

As with the other agents, toxin can be obtained from eggs with embryos moribund or recently dead. The optimal storage conditions for the toxin are the same as for meningopneumonitis.

Neutralization Tests with Serum

Following immediately upon the demonstration of the toxins described above, antisera were prepared. Rabbits were injected intravenously with fresh untreated suspensions of infected yolk sacs, similar to those used for demonstrating toxicity in mice except that normal saline was used as diluent. In addition, rabbits were injected intravenously and chickens intravenously or intraperitoneally with formalin-treated toxoid. Tests showed that such formalin-treated material was non-toxic for mice and non-infective for eggs.

The schedule for both rabbits and chickens consisted of three consecutive daily injections of increasing amounts of toxin or toxoid. Injections of toxin or toxoid began at 0.1 ml. of a 10^{-2} dilution made up to a volume of 1 ml. in physiological saline and increased to 0.2 ml. and 0.3 ml. of a 10^{-2} dilution for the first 3 day period. This was followed by a 4 day rest and injections were then resumed on the 5th day at 0.1 ml. of a 10^{-1} dilution. Increments from then on were as before (*i.e.* 0.2 ml. and 0.3 ml. of a 10^{-1} dilution) and after the 4 day rest period injections were resumed at the dose given in the last inoculation before the rest period (*i.e.* 0.3 ml. followed by 0.4 ml. and 0.5 ml. of a 10^{-1} dilution). Three such series were given and the animal then rested for 3 weeks. A new series of injections was then begun. Some animals received as many as five 3 week series of inoculations. Trial bleedings were taken on the day that a new series of injections was begun and before such injections were made. During the 3 week rest period trial bleedings were taken on the 5th day after the last inoculation and again on the day that a new series of injections was started. Toxoid was prepared by mixing equal amounts of highly toxic yolk sac suspensions, diluted 1 to 5 in their own yolk and fluids, with 0.2 per cent formalin in physiological saline. This mixture was allowed to stand at 37°C. for at least 24 hours before use and was stored at 0°C.

Two methods of neutralization have been used. In the first the mice (weighing between 12 and 14 gm.) have been given intravenously 0.25 ml. of a given dilution of antitoxic serum in physiological saline followed within 5 minutes by 0.25 ml. of a suspension of toxin at a concentration presumed sufficient to give a dose of 2 M.L.D. As a control, mice received 0.25 ml. of physiological saline or normal rabbit serum followed by toxin. The number of M.L.D. in the toxin is titrated at the same time. In the second method equal amounts of a given dilution of antitoxic serum in normal

yolk and fluids, and suspension of toxin diluted with normal yolk and fluids to contain approximately 4 M.L.D. per 0.5 ml., are thoroughly mixed and left for 2 hours at room temperature. Mice (12 to 14 gm.) are then inoculated intravenously with 0.5 ml. amounts of these mixtures. As controls, and to determine the exact number of toxic units used, mice are inoculated with mixtures of equal parts of the same toxic suspensions and of normal human or rabbit serum in the same dilution as the test sera. These controls, after standing at room temperature for 2 hours, are further diluted 2-fold, 4-fold, and 8-fold in normal yolk and fluids and then inoculated in 0.5 ml. amounts. None of the normal human or lapine sera tested so far have shown any neutralizing power against any of the toxins. In the second method an occasional apparent inconsistency appears with the higher dilutions of inactive sera. These will seem to protect mice while lower dilutions show no protection. It can, however, be demonstrated that this phenomenon is due to diluting out the serum and thus losing some of the protective power which normal or inactive serum has on the toxin (see above). If the toxin is present in quantities just sufficient to produce death in the presence of higher serum concentrations, too low a concentration of serum will result in survival of some mice.

Since both of the methods outlined above give good and comparable neutralization (Table V), the second method of neutralization *in vitro* has been adopted as a routine for the sake of simplicity. It is clear, however, that passive transfer of protection is possible and, in fact, in the case of the toxin of meningopneumonitis, which kills in quite high dilution after intraperitoneal inoculation (Table II), one can give the toxin intraperitoneally and protect the mice by intravenous injection of antiserum given immediately before the administration of toxin.

The toxin of lymphogranuloma venereum has never proved as good an antigen as has that of meningopneumonitis. Thus in rabbits the highest titer of antiserum, produced with fresh toxin of the former agent, which gives complete protection has been 1 in 10 against 2 lethal doses of toxin, and no response was obtained with toxoid in one rabbit. Only toxoid has been used in chickens and here a titer of 1 in 10 after 3 series of injections was the maximum obtained. On the other hand, inoculation with fresh toxin of meningopneumonitis has given titers in rabbits as high as 1 in 200 against 2 or 3 lethal doses after as few as 2 or 3 series of injections. Toxoid in rabbits has given titers as high as 1 in 80 after 2 series of injections and in a chicken a titer of 1 in 250 against 2 lethal doses after 4 series of injections. These titers, however, are only against the toxin. These antitoxic sera show little if any protection against the infection which follows intravenous inoculation of yolk sacs infected with the agent of meningopneumonitis.

The striking thing has been the absence of any cross-neutralization with these antitoxic sera. Table VI shows representative cross-neutralization tests with rabbit and chicken sera. In only one of all sera examined, namely, the one bleeding from rabbit 60 shown in Table VI, was there any evidence of cross-

TABLE V

Neutralization in Vivo and in Vitro of the Toxin of Lymphogranuloma Venereum

	0.25 ml. R 64 serum diluted 1/2 in saline	0.25 ml. saline	Control 0.5 ml. toxin in normal yolk and fluids			
			Diluted 2-fold	Diluted 4-fold	Diluted 8-fold	Diluted 16-fold
	Followed by 0.25 ml. toxin					
In vivo	S,* S, S. S, S	<17‡, <17, <17, <17, 42	<18, <18, <18	<18, 42, <42	S, S, S	S, S, S
	Toxin + R 64 serum diluted 1/2	Toxin + R 64 serum diluted 1/8	Control Normal rabbit serum + toxin After incubation, further diluted in yolk and fluids			
			Undiluted	Diluted 2-fold	Diluted 4-fold	
In vitro	S, S, S, S, S	<39, S, S, S, S	<14, <14, <14, <14, <14	<14, 18, 18, 20, S	S, S, S, S, S	

The toxin used throughout was a 1/5 yolk sac suspension. In this table and elsewhere in the paper primary, not final, serum dilutions are given.

*S = survived.

‡< 17 = died in less than 17 hours.

TABLE VI

Cross-Neutralization with L.V. and Men. Pn. Antitoxic Sera

Serum	Prepared against	Serum dilution	Tested against	
			L.V. toxin	Men. Pn. toxin
Rabbit 64 bled 4/15/43	L.V. toxin	1/2	S,* S, S, S, S	<16, <16, <16, <16, <16, <16
		1/8	<39,‡ S, S, S, S	<16, <16, <16, <16, <16
Rabbit 60 bled 8/9/43	Men. Pn. toxin	1/2	S, S, S, S, S	
		1/10	<17, <17, <17, <17, <41	
		1/50	<17, <17, <17, 22, <41	
		1/100		S, S, S, S, S
		1/500		<42, <42, <42, <42, <42, 48, <66
		1/2500		<17, <17, 21, 25, 25, <42
Chicken 1 bled 6/15/43	L.V. toxoid	1/2	S, S, S, S	<17, <17, <41, <65, <65
		1/10	23, S, S, S	<17, <17, <41, <65
Chicken 2 bled 7/26/43	Men. Pn. toxoid	1/2	<19, <19, <19, <43, S	
		1/10	<19, <19, <19, <19, <19	
		1/50		S, S, S, S, S
		1/250		<16, S, S, S, S
		1/1250		<17, <17, <17, <17
L.V. pool 2	L.V. toxin	1/2	<19, <43, S, S, S	<16, <16, <16, <16, <16
		1/10	<19, <19, S, S, S	
Men. Pn. pool 2	Men. Pn. toxin	1/2	<18, <18, <18, <18, <18	
		1/10	<18, <18, <18, <18, <18	
		1/50	<18, <18, <18, <18, <18	
		1/100		<41, S, S, S, S
		1/200		S,S, S, S, S
		1/400		<18, 26, <41, <41, S
		1/800		<18, <18, 22, <41

Approximately 2 lethal doses of toxin, as shown in control mice inoculated at the same time, were used in all instances.

* S = survived the effects of the toxin. (In the case of L.V. this means survival for over 21 days. In the case of Men. Pn. it means survival for 60 hours: most of the mice died later, *i.e.*, usually at 5 days or later, from infection.)

‡ <39 = died in under 39 hours.

neutralization and, as is apparent, this is very slight (in dilution of only 1 in 2 with the heterologous toxin compared to 1 in 100 with the homologous).

In the above cases hyperimmune sera from animals apparently not infected were utilized. Some studies have been made with human sera from individuals infected with, or recovered from, lymphogranuloma venereum, psittacosis, and trachoma. All of these sera gave good fixation of complement with yolk sac antigen prepared against lymphogranuloma venereum.[3]

Sera have been tested from 11 cases of lymphogranuloma venereum.[4] Ten of these gave neutralization against homologous toxin in dilutions of 1 in 2 or 1 in 10 and none neutralized the meningopneumonitis toxin. One (Table VII) showed no significant neutralization of either toxin. It was taken from a case of lymphogranuloma venereum of less than 1 month's duration which still showed the primary lesion. A later specimen from this case gave homologous neutralization. Eleven sera from cases of atypical pneumonia not due to any one of this group of agents[5] have been tested and none gave any neutralization. Two sera from cases of trachoma[6] failed to give any neutralization. Six sera from individuals recovered from psittacosis[7] have been tested and none of these gave any significant neutralization against the toxins of either lymphogranuloma venereum or meningopneumonitis.

The fact that none of the sera from individuals recovered from psittacosis gave significant neutralization is interesting. It is true that 3 of these sera had been stored for between 1 and 2 years before testing and there might have been some loss of antibody in this time. However, as Table VII shows, the lymphogranuloma serum S.A. had been stored for 3 years and still gave complete homologous neutralization at 1 in 10, and others not shown had been stored almost as long. Moreover, in the case of hyperimmune sera, very high titers are obtained with meningopneumonitis antisera as compared to antisera from lymphogranuloma venereum. The fact, therefore, that the sera from human cases of psittacosis fails to give any neutralization with toxin from the agent of meningopneumonitis would suggest a distinct difference between these

[3] Some of these tests were carried out by Dr. M. F. Shaffer to whom we wish to express our thanks.

[4] Our thanks are due to Dr. A. W. Grace, Long Island College Hospital, Brooklyn; Dr. H. C. de Sousa-Araujo, Institut Oswaldo Cruz, Rio de Janeiro, Brazil; Colonel W. B. Martin, Percy Jones General Hospital, Battle Creek, Michigan; and Dr. S. E. Sulkin, St. Louis, Missouri, for these specimens of sera.

[5] Our thanks are due to Dr. T. P. Magill of the New York Hospital for these specimens of sera.

[6] Our thanks are due to Dr. P. Thygeson, Presbyterian Hospital, New York, for these specimens of sera.

[7] Our thanks are due to Dr. H. A. Schorsh of Cooks County Hospital, Chicago, and Dr. A. M. Williams, Pennsylvania Department of Health, Pittsburgh, for some of these sera.

two agents, wider than has been supposed by other investigators. It would seem that we are dealing with a highly specific test, and this assumption is borne out by the cross-neutralization studies with hyperimmune sera already described. Proof must await studies on a larger series of sera.

As has been pointed out in a preliminary communication (13), mice recovered from sublethal doses of the toxin of lymphogranuloma, while not completely protected against reinoculation with homologous toxin at a later date, are significantly more resistant than are normal mice. The same has proven true with mice recovered from sublethal doses of the toxin of meningopneumonitis. However, no extensive data has been obtained on this point. One of the chief difficulties lies in the circumstance that the first inoculation of toxic material sensitizes the mice so that a majority of them, usually about 60 per cent, die

TABLE VII

Neutralization of L.V. and Men. Pn. Toxin with Human Sera

Serum	Remarks	Serum dilution	Tested against	
			L.V. toxin	Men. Pn. toxin
W I	Early L.V. Less than 1 mo. duration	1/2	<15, 22, S, S	
		1/10	<15, <15, <15, <15	
W II	Later specimen of above case	1/2	S, S, S, S, S	<14, <14, <14, <14, 20
		1/10	<17, <41, S, S, S	
S.A.	Chronic L.V. (Stored 3 yrs. at 0° before testing)	1/2	S, S, S, S, S	<15, <15, 23, 23
		1/10	S, S, S, S, S	<15, <15, <15, 19
Pe	Recovered psittacosis. (Stored 2 yrs. at 0° before testing)	1/2	<14, <14, <14, <14	<17, <43, <43, <43, 46
		1/10	<14, <14, <14, <14	<17, <17, <43, <43, <43
F406	Recovered psittacosis	1/2	<18, <18, <18, <18	<15, 21, 21, <63, S
		1/10		<15, <15, 21, 23, 23

Approximately 2 lethal doses of toxin, as shown in control mice inoculated at the same time, were used in every case.

from anaphylactic shock within a few minutes of receiving the second intravenous inoculation. This immediate anaphylactic death can be produced in these recovered mice with suspensions of normal yolk which never affect normal mice. Since there have never been large numbers of survivors from the original inoculation and since approximately 60 per cent of such survivors die of shock on challenge inoculation, the number of mice available on which to assess the degree of this acquired immunity has always been small. No evidence of acquired heterologous immunity has been obtained.

DISCUSSION

It has been possible to demonstrate for three members of the lymphogranuloma-psittacosis group of agents the presence, in infected chick embryo yolk sacs, of a factor very rapidly fatal to mice. That these rapid deaths are not due to infection is shown by six facts. Foremost is the fact that the deaths

occur within 4 hours in the case of one agent (mouse pneumonitis) and within 12 to 24 hours in the case of the other two. Infections do not act thus rapidly. Moreover, inoculation of two of the agents (meningopneumonitis and mouse pneumonitis) produces a biphasic curve of distribution of deaths and the appearance of the mice dying in the second part of the curve suggests infection. Third, microscopic examination of tissues from mice recently dead from inoculation of any of the agents gives evidence of infection in the mice dying late (during the secondary part of the curves) and only lesions characteristic of toxemia in the earlier deaths. Fourth, meningopneumonitis antitoxic sera, which as will be pointed out below, have only a limited degree of power to neutralize and prevent the infection no matter how high their antitoxic titers may be, blot out the first part of the curve but leave the second unchanged, (Text-fig. 2). Fifth, in the case of lymphogranuloma venereum in which no secondary curve of deaths occurs, it has not been possible to demonstrate the presence of the agent in a latent form in either the brain or spleen despite the ease with which the carrier state is induced following infections with this agent. The final evidence lies in experiments described elsewhere (21). As pointed out above, the agent of mouse pneumonitis produces a biphasic curve of distribution of deaths. Moreover, infections with this agent are susceptible to sulfonamide therapy (22). If our assumptions are correct it should be possible to eliminate the second part of the biphasic curve and not affect the first part by sulfonamide therapy; and such has proved to be the case.

These rapid deaths, therefore, are not due to infection and must be due to some toxic factor specific for each agent. The next question to be decided is whether these toxic factors are a product of the agents themselves or are produced by the action of the agent on the infected yolk cells and are actually a product of these cells. It would seem that the high degree of specificity of these toxic factors argues in favor of their being a product of the agents themselves, but more definite evidence than this is required. This would seem to be given by two observations. Both bear on the fact that, in the case of lymphogranuloma and meningopneumonitis, in which the point has been investigated, the toxic factors are closely associated with the bodies of the agent. Thus parallel titrations of toxic units for mice and infective units for chick embryos indicate that, in suspensions of freshly harvested yolk sacs, the two bear a constant ratio one to the other. It would seem improbable that, for example, 1 million infective units of a given agent would always produce the same degree of damage in yolk cells and induce the formation of the same amount of toxic material from such cells, whereas if the toxic factor is associated with the bodies of the agents (as an endotoxin) such a constant ratio would be expected. Furthermore, differential centrifugations of a character known to separate out in the final sediment all the bodies of the agent also separate out in the same final sediment all of the toxic factor. This would be possible only in the un-

likely event that the hypothetical toxic factor from the yolk cells has a sedimentation constant similar to the bodies of the agent.

All of the evidence at present available points to the conclusion that these toxic factors are products of the agents themselves. In this case it would seem that they should be known as toxins just as are similar products from other microorganisms. *i.e.*, bacteria. Bacterial toxins may be defined as substances elaborated by bacteria which are poisonous for certain cells of susceptible animals. It has been convenient and conventional to divide these toxins into the exotoxins which diffuse readily from the living bacteria producing them, and endotoxins which remain within the bodies of the living bacteria and are liberated only after death and dissolution of the organisms. The toxins of the lymphogranuloma-psittacosis group of agents would appear to fit into the general class of endotoxins. They are not liberated from the bodies of the agents under natural conditions and so cannot be termed exotoxins. In fact it has been difficult to show any separation of the toxins from the intact (and infectious) bodies of the agents because of their lability, and partial separation has been achieved only by the more delicate of those methods usually employed for liberating endotoxins from bacterial cells. The minimal lethal dose of these toxins is large, and they are not very active antigens in the production of antitoxic sera, both of which are resemblances to bacterial endotoxins. Thus, as has been pointed out above, while high serum titers can be obtained in the case of the meningopneumonitis antitoxin, the neutralization is against only relatively few lethal doses, *i.e.* between 1 and 4, and the law of neutralization in multiple proportions (which applies to exotoxin-antiexotoxin reactions) has not been found to apply. If the dose of toxin from these agents is too large, no amount of serum will protect.

In two ways the toxins of these three agents differ from the more characteristic bacterial endotoxins. Thus they are labile to heat and other conditions; and they produce characteristic lesions in mice, particularly in the liver. On the whole, however, it would appear that there is much to relate the toxins to bacterial endotoxins and little ground on which to separate them.

Certain features as regards the neutralization of these toxins with antisera are of interest. As has been pointed out above, although for reasons of convenience neutralizations are carried out in the test tube at room temperature and the mixtures later tested for toxicity by inoculation into mice, it is readily possible to immunize the animal passively (and in this way carry out the neutralization tests) either by inoculation of both toxin and antitoxin at different times but by the same route (*i.e.* intravenous) or by inoculation of the antitoxin intravenously at the time the mouse receives the toxin intraperitoneally. Thus the possibility that such neutralization as has been demonstrated is due to a purely artificial reaction *in vitro*, analogous for example to the precipitin-precipitinogen reaction, is excluded.

It has been pointed out that the antitoxins we have been using have little power to prevent infection in, for example, meningopneumonitis, since the mice surviving sublethal doses of toxin die later from infection. In most cases sera have been prepared in rabbits. However, following upon the report of Phair, Smith, and Root (23) on the high specificity obtained with chicken sera prepared against the meningococcus, a few sera were prepared in chickens. Because of the susceptibility of chickens to ornithosis (24) and because of the demonstrated close relationship between members of the lymphogranuloma-psittacosis group of agents (1–5), it was decided to use only toxoid in these chickens. Following the use of toxoid, antitoxins have been obtained from chickens which have titers as high as those induced by inoculation of toxins, and far higher than those induced by inoculation of toxoids, in rabbits. It is possible that the use of untreated toxin in chickens would allow the production of still more active sera which would have the power of preventing infection as do the chicken sera described by Hilleman and Gordon in a recent article (25), (which appeared after the present work had been completed). It should be pointed out that the neutralization tests in the present paper have been carried out by varying the dilutions of sera, whether chicken or rabbit, against constant amounts of toxin, and never with undiluted sera against different dilutions of toxin.

The specificity of the hyperimmune sera against these toxins is particularly striking in the light of the marked serological cross-reactions between members of the group as seen for example in complement fixation tests. This specificity seems also to be a feature of sera obtained from persons suffering from infection with members of the group. In fact this marked specificity may be the reason why sera from persons recovered from psittacosis fail to neutralize the toxin of meningopneumonitis, and may serve to place this latter agent in a category separate from psittacosis—a point on which there has been some controversy. It seems possible that the toxin-antitoxin reaction here described may offer a better method of distinguishing clearly between the different members of the lymphogranuloma-psittacosis group than any hitherto available.[8]

In choosing yolk sacs to use in the preparation of toxin it has been found advisable to select them on the basis of smears of the sacs prepared in a routine manner and stained with Macchiavello's stain. Experience has taught that only yolk sacs giving heavy smears should be used since these are indicative of

[8] While the present work was in press work was carried out with the agent of feline pneumonitis (26, 27), recently shown to belong to the lymphogranuloma-psittacosis group of agents (28). A toxin similar in many regards to the three described in this paper has been found associated with this agent. As in the case of the other toxins, the toxin-antitoxin reaction with the agent of feline pneumonitis has been highly specific. The pathological picture in mice has differed in some respects from that produced by the other toxins (29).

the heaviest infection. In this connection it should be reiterated that the smears from eggs infected with lymphogranuloma will appear more heavily infected than those infected with meningopneumonitis which in turn give heavier smears than those infected with mouse pneumonitis. This is true despite the fact that the infective unit of the agent of meningopneumonitis, for example, is present in higher concentration in the cells of the yolk sac than that of lymphogranuloma (some 3- to 6-fold higher—see Table I). The fewer bodies of the agent seen in the smears may be due to differences in physical properties preventing the bodies of the agent of meningopneumonitis from being dispersed as readily from ruptured cells. A suggestion of this is also apparent in the morphology of the latter agent as seen in smears. Thus initial bodies seem to be more plentiful and tend to appear in clusters more frequently than is the case in lymphogranuloma venereum. In the case of mouse pneumonitis all forms suggestive of a developmental cycle similar to that described for lymphogranuloma (10) can be seen in the smears but it has been observed that the elementary bodies of this agent are characteristically smaller than are those of the other two and approach more closely the limit of clear resolution.

SUMMARY

Toxins, which resemble in most respects bacterial endotoxins, are associated with the agents of lymphogranuloma venereum, meningopneumonitis, and mouse pneumonitis as they grow in the yolk sac of the chick embryo. They are labile and are not readily separated from the bodies of the agent. They kill mice rapidly after intravenous, and in some cases after intraperitoneal, injection but the minimal lethal dose is relatively large and, in those freshly harvested yolk sacs which have been tested, has corresponded to about 36 million infective units. Characteristic lesions are produced in all mice except those which die very rapidly, and are found especially in the liver where necrosis of the midzone of the lobule occurs.

Antitoxic sera which are effective against a few lethal doses of the toxin can be produced in rabbits or chickens by using either toxin or toxoid as antigen. Such sera behave in a manner analogous to antiendotoxins against bacterial endotoxins and they do not neutralize by the law of multiple proportions. Antitoxic sera can also be obtained from human beings convalescent from at least one of these diseases. The toxins and antitoxins appear to be highly specific and they may offer a useful tool in distinguishing between different members of this closely interrelated group.

BIBLIOGRAPHY

1. Rake, G., Eaton, M. D., and Shaffer, M. F., *Proc. Soc. Exp. Biol. and Med.*, 1941, **48**, 528.
2. Eaton, M. D., Martin, W. P., and Beck, M. D., *J. Exp. Med.*, 1942, **75**, 21.

3. Pinkerton, H., and Moragues, V., *J. Exp. Med.*, 1942, **75,** 575.
4. Rake, G., Shaffer, M. F., and Thygeson, P., *Proc. Soc. Exp. Biol. and Med.*, 1942, **49,** 545.
5. Levine, S., Holder, E. C., and Bullowa, J. G. M., *J. Immunol.*, 1943, **46,** 183.
6. Rake, G., Shaffer, M. F., Jones, H., McKee, C. M., and Hamre, D. M., *J. Bact.*, in press.
7. Harrop, G. A., Rake, G., and Shaffer, M. F., *Tr. Am. Clin. and Climat. Assn.*, 1941, **56,** 154.
8. Harrop, G. A., Rake, G., and Shaffer, M. F., *Tr. Assn. Am. Physn.*, 1941, **56,** 101.
9. Jones, H. P., Rake, G., and Stearns, B., data to be published.
10. Rake, G., and Jones, H. P., *J. Exp. Med.*, 1942, **75,** 323.
11. Rake, G., and Jones, H. P., unpublished data.
12. Gildemeister, E., and Haagen, E., *Deutsch. med. Woch.*, 1940, **66,** 878.
13. Rake, G., and Jones, H., *Proc. Soc. Exp. Biol. and Med.*, 1943, **53,** 86.
14. Francis, T., Jr., and Magill, T. P., *J. Exp. Med.*, 1938, **68,** 147.
15. Gönnert, R., *Zentr. Bakt., 1. Abt., Orig.*, 1941, **147,** 11.
16. Nigg, C., *Science*, 1942, **95,** 49.
17. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27,** 493.
18. Rake, G., McKee, C. M., and Shaffer, M. F., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43,** 332.
19. Jones, H. P., Rake, G., and McKee, C. M., *Proc. Soc. Exp. Biol. and Med.*, **1941, 48,** 318.
20. Rake, G., Shaffer, M. F., Jones, H. P., and McKee, C. M., *Proc. Soc. Exp. Biol. and Med.*, 1941, **46,** 300.
21. Rake, G., and Hamre, D. M., *Proc. Soc. Exp. Biol. and Med.*, 1944, in press.
22. Rake, G., Jones, H., and Nigg, C., *Proc. Soc. Exp. Biol. and Med.*, 1942, **49,** 449.
23. Phair, J. J., Smith, D. G., and Root, C. M., *Proc. Soc. Exp. Biol. and Med.*, 1943, **52,** 72.
24. Meyer, K. F., and Eddie, B., *Proc. Soc. Exp. Biol. and Med.*, 1942, **49,** 522.
25. Hilleman, M. R., and Gordon, F. B., *Science*, 1943, **98,** 347.
26. Baker, J. A., *Science*, 1942, **96,** 475.
27. Baker, J. A., *J. Exp. Med.*, 1944, **79,** 159.
28. Thomas, L., and Kolb, E. M., *Proc. Soc. Exp. Biol. and Med.*, 1943, **54,** 172.
29. Hamre, D. M., and Rake, G., *J. Bact.*, in press.

EXPLANATION OF PLATE 7

All of the sections were stained with eosin and methylene blue.

FIG. 1. Lymphogranuloma venereum toxin. Earliest lesion in liver showing fat globules in the cells. × 100.

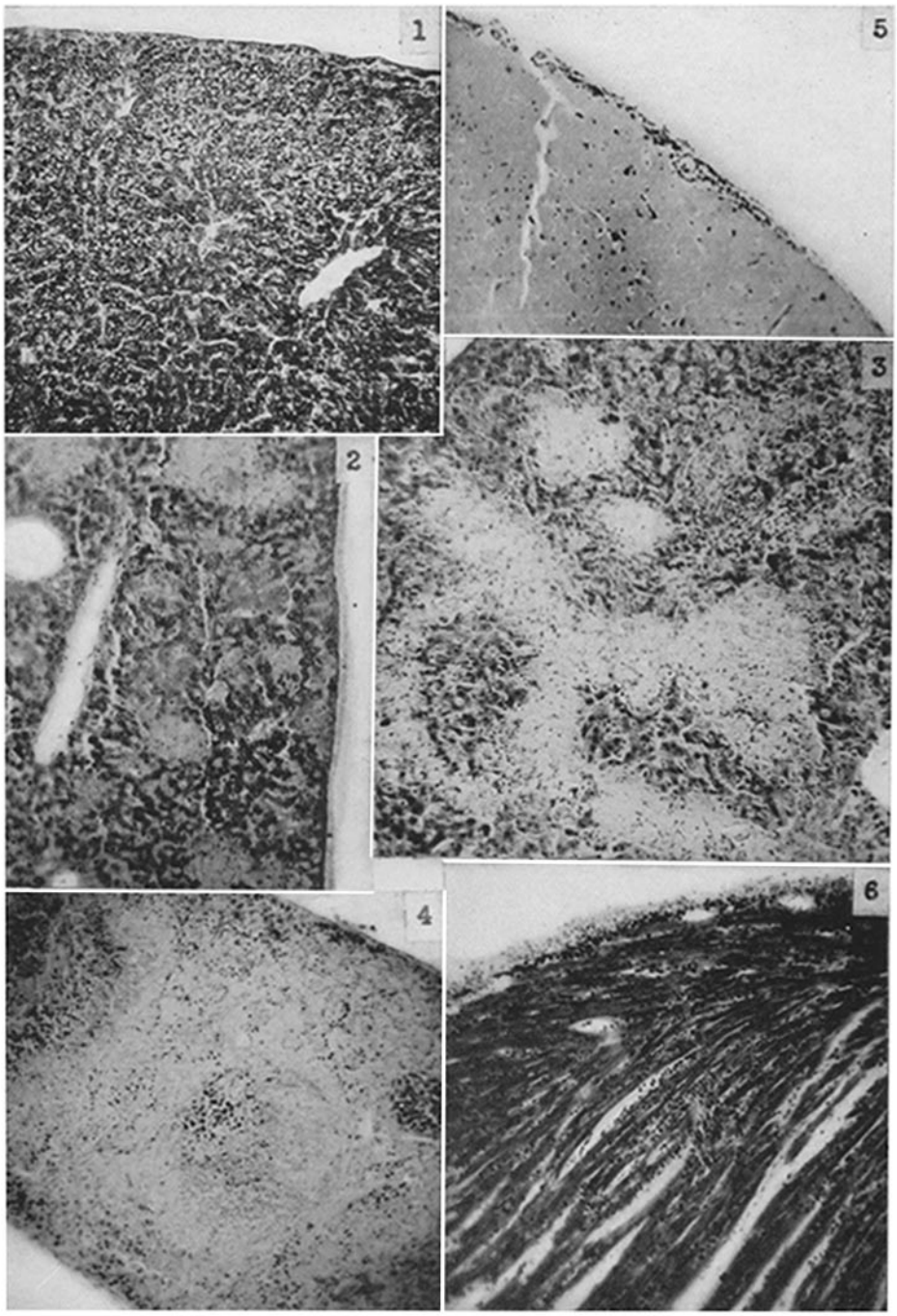
FIG. 2. Lymphogranuloma venereum toxin. Liver showing commencing necrosis involving small groups of cells. × 100.

FIG. 3. Meningopneumonitis toxin. Liver showing extensive midzonal necrosis. × 100.

FIG. 4. Meningopneumonitis toxin. Spleen showing almost complete necrosis of a Malpighian corpuscle. × 100.

FIG. 5. Meningopneumonitis toxin. Brain showing earliest evidence of meningitis at 60 to 72 hours. × 100.

FIG. 6. Meningopneumonitis toxin. Areas of monocytic infiltration of the cardiac muscle and pericarditis. × 100.



(Rake and Jones: Lymphogranuloma venereum. II)