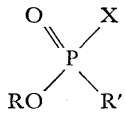
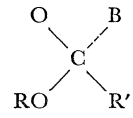
that of the natural substrates of the enzyme. It is presumably hydrogen bonding and electrostatic interactions at or close to the ester linkage which makes the enzyme combine so much more strongly with the transition state than with the substrate, which in turn causes the lowering of the activation free energy for reaction. Owing to the stability of phosphorous compounds with one more covalent bond than can occur in analogous carbon compounds, this larger interaction energy is available for binding the inhibitor molecule.

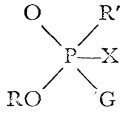
The structure of most phosphoric esters is of the kind



This imitates rather closely the presumed intermediate in the S_{N2} hydrolysis of a related ester



where *B* is a basic group. On the other hand, covalency of 5 is quite common among phosphorus compounds, and a metastable intermediate



where G is a group from the enzyme, might also imitate quite closely the transition state for a Walden inversion, as is illustrated in the Fig. 1.

A detailed discussion of the mechanism of esterase action has been given elsewhere (*3*). It should be clear that phosphorus esters are ideally suited to form stable structures corresponding both to the proposed enzyme-substrate tetrahedral complex and the pentagonal transition state for the reaction (*4*). If our speculations are correct, they may make possible the study of the geometry of the activated state by means of inhibition studies (*5*).

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References and Notes

1. A recent review of the evidence (with references) is given by G. H. Dixon, H. Neurath, and J. F. Pechere [*Ann. Rev. Biochem.* **27**, 523 (1958)].
2. T. Wagner-Jauregg and B. E. Hackley, Jr., *J. Am. Chem. Soc.* **75**, 2125 (1953).
3. S. A. Bernhard and H. Gutfreund, in International Union of Biochemistry, Symposium Series, vol. 2, *Proceedings of the International Symposium on Enzyme Chemistry, Tokyo-Kyoto, 1957* (Maruzen, Tokyo, 1958), p. 124; ———, *Progr. in Biophys. and Biophys. Chem.* **9** (1958), in press. For a more detailed discussion of the mechanism by which labile (and less specific) substrates may react with these enzymes, see Dixon *et al.* (*1*) and L. W. Cunningham [*Science* **125**, 1145 (1957)].
4. If the transition-state is tetrahedral rather than pentagonal the above arguments apply with only minor modifications.
5. We are grateful to the Study Section in Biophysics and Biophysical Chemistry of the National Institutes of Health for the opportunity jointly to participate in their program at Boulder, Colo., during the summer of 1958. One of us (S.A.B.) is indebted to the National Institute of Mental Health, Bethesda, Md., for sponsoring attendance at the program.
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Immunization of Mice against Toxic Doses of Homologous Elementary Bodies of Trachoma

Abstract. Death of mice occurs 2 to 8 hours after intravenous inoculation of concentrated viable elementary bodies of trachoma. Toxic death can be prevented by vaccinating the mice with concentrated suspensions of homologous strains inactivated by formalin or phenol. Judged by toxic challenges, at least two antigenically distinct types of elementary bodies of trachoma occur in Saudi Arabia and Egypt.

The relationship of elementary body viruses to the classical syndrome trachoma, although much disputed in the past, has been more firmly established by the recent findings of Tang *et al.* (*1*) and Collier *et al.* (*2, 3*). These reports indicate that several properties are shared by the elementary body viruses derived from conjunctival scrapings of trachoma cases in China and Gambia. Before attempts are made to prevent trachoma by immunization procedures, it is important to determine whether the strains of elementary bodies from different regions are all alike antigenically or whether there are multiple types which are immunologically distinct from one another. Toxicity for mice was reported as a property of strain SA-1, isolated from a trachoma patient from Hofuf, Saudi Arabia (*4*). Subsequently several other strains from the Middle East have been found toxic for mice (*5*) and the phenomenon thus offers a possibility for comparison of different strains. Since the elementary bodies of trachoma share the heat-stable common antigen derived from psittacosis-lymphogranuloma viruses (*2, 5*), it should be noted that certain of the latter are also toxic for mice and that several antigenic and pathologic patterns have been reported (*6, 7*).

Eight experiments were performed which involved vaccination and subsequent toxic challenges of white mice. The vaccines were prepared from yolk sacs of chick embryos which were harvested approximately 7 days after infection with the various strains. Vaccines and normal yolk sac control materials were administered intra-abdominally, and the mice were challenged at various intervals thereafter by the intravenous inoculation of viable suspensions of elementary bodies adjusted in concentration such that each mouse received one certainly fatal dose. There were two types of controls for the vaccines: (i) normal yolk sac suspensions, and (ii) 0.85 percent NaCl solution. The vaccines and control solutions were given on the basis of 0.2 ml/10 g body weight of the mice. The strains of elementary bodies were SA-1, SA-2, SA-5, and Egypt-2, derived from trachoma patients in Saudi Arabia and Egypt (*5*).

The suspensions for challenge and for vaccines were prepared as follows: chick embryos, after 6 to 8 days of incubation, were inoculated with infectious yolk sac suspensions in dosages adjusted to cause death of half the eggs about 7 days later, when the yolk sacs of surviving embryos were harvested. The inocula consisted of material derived from the 5th to 10th egg passage levels of the different strains involved. The yolk sacs were thoroughly mixed mechanically and stored at −60°C. Purification and concentration of the elementary bodies were accomplished by two cycles of centrifugation at +4°C; each cycle consisted of 1500 rev/min for 15 minutes to eliminate gross particles, followed by 5000 rev/min for 60 minutes to reduce the amounts of protein and lipids. Exposure to celite (*7*) before the second cycle was begun was also included in the procedure.

After the second high-speed run, the sediments were resuspended in one-fourth the volume of the original yolk sacs. If the material was to be used for challenge, the suspending material was sucrose PG (*8*). If the material was to be made into vaccine, the suspending medium was phosphate saline buffered to *p*H 7.2. The suspensions of elementary bodies were shown to be toxic for white mice; they were then shell frozen and stored at −60°C until ready for use. In the preparation of vaccines, the suspensions were thawed and mixed with an equal quantity of freshly prepared 0.4 percent formalin, or 0.8 percent phenol, in buffered saline. One lot of SA-2 vaccine was extracted with ether; this extraction replaced the step involving celite. After overnight storage at +4°C, each vaccine was shown to be free of bacteria by appropriate tests; the

presence of viable elementary bodies was ruled out by four successive negative transfers in chick embryos. The control materials from normal yolk sacs were prepared exactly as were those containing infectious strains. The vaccines were diluted with sterile saline just before inoculation to make the concentration of the elementary bodies equivalent to the amount in 1.0 g of yolk sac at the time of harvest; the final concentration of the inactivating reagents in the different batches was thus reduced, in the case of formalin, to 0.1 percent, and in the case of phenol, to 0.2 percent. On the basis of parallel titrations, it was estimated that each gram of infected yolk sac at the time of harvest contained approximately 12 fatal toxic doses for mice, or 10^6 infectious doses for chick embryos.

Preliminary experiments were performed with a formalin-inactivated vaccine of 10 percent yolk sac prepared by simple ether extraction; the challenge was a crude suspension of toxic yolk sac in sucrose PG. The concentration of elementary bodies in the vaccine was inadequate under such circumstances to provide immunity against toxic challenge. Furthermore, in the interval from 10 to 55 minutes after inoculation of the challenge, many of the mice died with signs of anaphylaxis. Although purification and concentration of the challenge material reduced the numbers of mice which succumbed in anaphylaxis, the phenomenon continued to be important in the experiments, since 57 vaccinated mice died of anaphylaxis, out of the total number challenged, 217. The deaths were scattered through all the different groups except the saline controls.

Among the mice which were given normal yolk sac suspensions as a control vaccine, there were 29 anaphylactic deaths in the course of the subsequent toxic challenge out of the total of 88 challenged. Just preceding anaphylactic death the mice had labored, gasping respiration, often accompanied by blood-tinged nasal discharge. At autopsy the lungs were distended and the cut surfaces showed frothy exudate. Anaphylactic death did not occur in any of the 55 mice in the saline control groups which were challenged in the same experiments. Thus the anaphylactic deaths, in the interval from 10 to 55 minutes after challenge, were easily distinguishable from the deaths due to toxic properties of the elementary bodies. The latter regularly occurred from 2 to 8 hours after challenge, without attendant signs of respiratory distress; the characteristic feature post mortem was the hemorrhagic appearance of the upper small intestine. In the report below, the anaphylactic deaths have been eliminated from the tabulations.

Table 1. Immunization of mice against toxic challenge. Ratios, survivors to total (see text regarding anaphylactic deaths). Summary of eight experiments.

Vaccination schedule	Two doses, 0* and 7 days	Three doses, 0*, 7, and 21 days					
Day of challenge Challenge strain	17th, 20th SA-2	28th, 28th, 29th SA-2	29th SA-5	30th Eg-2	30th SA-1	Total Homologous	Total Heterologous
Vaccine	†	‡					
SA-2 formalin	6/11	18/21		0/10	0/9	18/21	0/19
SA-2 phenol	4/7	5/5				5/5	
SA-2 ether and formalin	5/10	3/3				3/3	
Total	15/28	26/29				26/29	0/19
SA-1 formalin		0/15		4/5	0/3	0/3	4/20
SA-5 formalin		1/7	7/7	0/10	0/8	7/7	1/25
Eg-2 formalin		0/3	7/10	7/7	3/5	7/7	10/18
Total						14/17	15/63
Control							
Normal yolk sac, formalin	0/11	0/14		0/6	0/4	0/24	
Normal yolk sac, phenol	0/10	0/6				0/6	
Saline only	0/11	1/18	1/8	0/9	0/9	2/44	
Totals	0/32					2/74	

* Mice were weaned approximately 3 weeks after birth. "Zero day" indicates the beginning of the tests, which occurred within 1 day of weaning.
† Summary of results of experiments 1 and 2.
‡ Summary of results of experiments 3, 4, and 5.

The data from eight different experiments are assembled in Table 1. Approximately half of the mice (15 of 28) which received two doses of the SA-2 vaccines were immune to the lethal toxic dose of SA-2 elementary bodies; after a third dose of vaccine at 21 days the survival rate was considerably higher (26 of 29). The data further suggest that there was no important difference between the SA-2 vaccines prepared with formalin, phenol, or ether extraction plus formalin. Formalin-inactivated vaccines of SA-1, SA-5, and Egypt-2 did not protect against SA-2 challenge.

When the other strains were used for challenge, none the the SA-2 vaccinated mice was protected (no survivors of 19 challenged). Vaccine prepared from Egypt-2 gave partial protection against toxic challenges of SA-5 and SA-1, and complete protection against Egypt-2. The results suggest that there may be an antigenic overlap among the strains SA-1, SA-5, and Egypt-2. The controls were satisfactory, there being only 2 survivors out of 106 challenged.

These experiments indicate that it is possible to protect mice against toxic death by three doses of concentrated suspensions of homologous trachoma elementary bodies, inactivated by formalin or phenol. Although the tests were complicated by anaphylactic phenomena, the method can be applied to one of the major problems in research on trachoma, namely, to determine how many different antigenic types or groups of elementary body viruses are involved in the etiology of clinical trachoma. Our findings suggest that there are at least two types currently prevalent in Saudi Arabia and Egypt (*10*).

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References and Notes

1. F. F. Tang, H. L. Chang, Y. T. Huang, K. C. Wang, *Chinese Med. J.* 75, 429 (1957).
2. L. H. Collier and J. Sowa, *Lancet* 1, 993 (1958).
3. L. H. Collier, S. Duke-Elder, B. R. Jones, *Brit. J. Ophthalmol.* 42, 705 (1958).
4. E. S. Murray, J. C. Snyder, S. D. Bell, Jr., paper presented at the 6th International Congress of Tropical Medicine and Malaria, Lisbon, Portugal, 12 Sept. 1958.
5. E. S. Murray, S. D. Bell, Jr., R. Nichols, A. Hanna, J. C. Snyder, in preparation.
6. G. Rake and H. P. Jones, *J. Exptl. Med.* 79, 463 (1944).
7. G. P. Manire and K. F. Meyer, *J. Infectious Diseases* 86, 226 (1950).
8. M. Bovarnick and J. Miller, *J. Biol. Chem.* 184, 661 (1950).
9. M. Bovarnick, J. Miller, J. C. Snyder, *J. Bacteriol.* 59, 509 (1950).
10. These experiments were supported in part by the Aramco Trachoma Research Program, Medical Department, Arabian American Oil Company, Dhahran, Saudi Arabia.

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