tility and decrease in respiration of the sperm, followed by death of the cells. Apparently, with a more concentrated suspension of cells, sensitization is prolonged. The reason for this is not known at the present time. The protection afforded by the addition of catalase to the medium indicates that a photochemical oxidation producing H_2O_2 is occurring as a result of the radiation.

In order that this phenomenon may be defined as photodynamic action, it is necessary that there be some fluorescent substance present which can absorb radiant energy (7). The activated sensitizer transfers its energy to an acceptor, presumably a cellular protein, which then undergoes oxidation. To determine the source of the photosensitive agent, the effect of light on spermatozoa suspended in various media was tested. Since these cells will not survive at room temperature for a prolonged period of time, except in coconut-milk extender, it was necessary to modify slightly the experimental design and expose the samples to a higher light intensity (1400 ft-ca) for a shorter time. The results are shown in Table 2. The data clearly reveal that the photosensitive agent is not a constituent of any of the media tested or of the antibiotics which supplement them, although the latter may enhance the photochemical reaction. Comparison of Tables 1 and 2 indicates that the effect of radiation—presumably an oxidative process—is in accord with the Bunsen-Roscoe reciprocity law (7). Further, the effect is independent of temperature, as demonstrated by the results with the skim-milk diluent at 5°C. These are characteristics usually identified with a photosensitized oxidation (7).

On this basis, then, it can be concluded that there is a photosensitizer present within the sperm itself and that the radiation affects the cell directly. Calcutt (8), working with *Paramecium*, suggests that light has a direct action upon the test material, inducing a cellular change which facilities the photodynamic response. This phenomenon apparently involves cytoplasmic damage, as compared with the nuclear effects of ultraviolet radiation (9).

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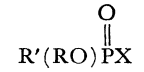
9. Preliminary attempts to delineate the light source into narrower spectral bands indicates that the photosensitive agent within the sperm has a maximal absorption in the blue. We thank I. D. Porterfield of the dairy husbandry department for furnishing us with bovine semen, the College of Agriculture, West Virginia University, for financial support, and C. E. Johnson for his technical assistance.

28 April 1959

Mechanism of Enzyme Inhibition by Phosphate Esters

Abstract. A theory for the rapid specific reaction of certain phosphorous-containing esters with many proteolytic enzymes based on the ability of phosphorous to form one additional bond relative to carbon is presented. A stable tetrahedral phosphate ester is compared with a labile tetrahedral orthocarbonyl ester and a relatively stable pentagonal enzyme-phosphate ester complex is compared with a pentagonal enzyme-carbonyl substrate complex. The latter complex is assumed to be the transition state in the enzyme-catalyzed reaction. If the theory is correct, it opens up the possibility of studying intermediates and transition states from the known structures of chemical inhibitors.

The specific reaction of a group of organic phosphate esters of the type



where R is an alkyl group and R′ an alkyl or alkoxy group, with a class of esterases and phosphoglucomutases has long been known. Some pertinent chemical facts concerning the inhibition reaction are: (i) The reaction products are the degraded fragment (X) and the enzyme, phosphorylated on the hydroxyl group of a serine residue (1). (ii) The reaction is stoichiometric with the number of enzymatic sites and not with the total number of serine residues. (iii) The reaction proceeds with a high velocity with all the enzymes for which the stoichiometric relationship holds. (iv) The phosphorylated enzyme is catalytically inactive.

The extreme velocity of the inhibition reaction contrasts with the rates of homogeneous solution reactions of the phosphate esters. Tetraethylpyrophosphate, for example, is remarkably stable in neutral aqueous solutions. Even di-isopropyl-phosphofluoridate is much more stable than acetic anhydride (2). Nonetheless, it reacts much more rapidly and specifically with suitable esterases.

In this report we wish to suggest that the action of the phosphate esters differs from that of most other enzyme inhibitors in that it is the unstable transition state of the enzyme-substrate complex which is imitated by the inhibitor in one of its stable combinations with the enzyme. More frequently, it is assumed that inhibitor action is effected by imitation of either the substrate itself or a *stable* combination of substrate with enzyme.

If our postulate is correct, one can understand why the side group specificity of the inhibitors is so different from

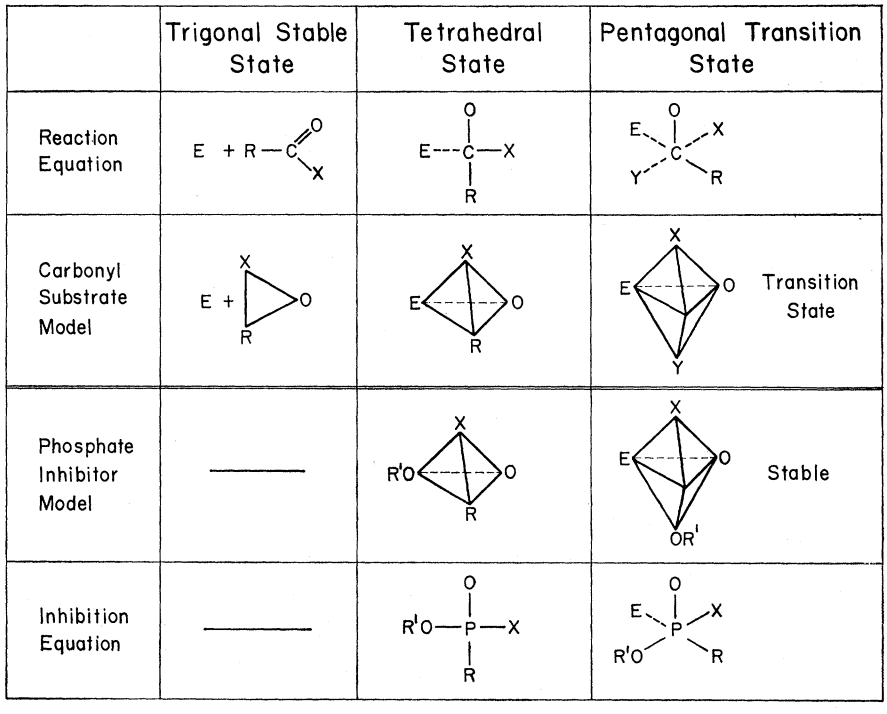
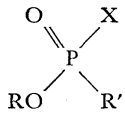


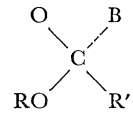
Fig. 1. Stereochemical representation of enzyme action.

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.

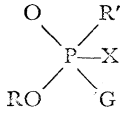
The structure of most phosphoric esters is of the kind



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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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