mole of dialkyl fluorophosphate. The plots in Fig. 1 show the relationship between CO_2 evolved and inorganic fluoride liberated after the hydrolysis of three dialkyl fluorophosphates. According to the slopes of the lines, the CO_2/HF ratios for DFP, DEFP and DBFP are 43.5, 42.5 and 46.0, respectively, as compared to a theoretical ratio of 44.8. Within the experimental error of the methods, these data indicate that 1 mole of HF is liberated for each 2 moles of CO_2 evolved. This ratio differs from that obtained by Mazur(1) with rabbit serum (1:1.49) which may be explained by the retention of CO_2 in the presence of comparatively large amounts of plasma. These results indicate that the products of hydrolysis of dialkyl fluorophosphates by enzymes of differing characteristics are the same.

Qualitative tests for inorganic phosphate and isopropyl alcohol were negative after complete hydrolysis of DFP, both by the enzyme alone and by the enzyme in the presence of cofactors.

Cohen and Warringa(10) studied the metabolism of DFP^{32} (diisopropyl fluorophosphate labelled with P^{32}) after intramuscular injection of nontoxic doses into human subjects. They observed that 50 to 60% of the injected DFP is excreted in the urine as diisopropyl phosphoric (DIP) acid during the first 10 days. The residual DFP probably remains firmly bound as a DIP-protein complex(11). It is probable that the *in vivo* and *in vitro* hydrolyses of DFP are similar.

Summary. Evidence has been presented to show that hog kidney dialkyl-fluorophosphatase (DFPase) liberates 1 mole of hydrofluoric acid and 1 mole of dialkyl phosphoric acid in the enzymatic hydrolysis of a mole of diisopropyl fluorophosphate, diethyl fluorophosphate or dibutyl fluorophosphate.

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Heat-Stability of Antibodies in Tuberculous Sera. (21279)

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The presence of hemagglutinins and hemolysins in sera of tuberculin-negative cattle has been reported from these laboratories(1). The persistence of these antibodies in low titers over a 2- to 3-year period suggested that they might be naturally occurring or "normal" antibodies with specificities for antigens similar to those present in *Mycobacterium tuberculosis*.

Preliminary results showed that antibodies in these tuberculin-negative bovine sera were eliminated by exposure to 70°C for 10 minutes. When tuberculous bovine or human sera were similarly treated, "heat-stable" antibodies could still be detected in some samples. The possibility of greater correlation of "heat-stable" antibody with tuberculous infection prompted a further study of the heat-stability of antibodies produced in response to injections of *M. tuberculosis*, B.C.G., P.P.D., and

TABLE I. Effect of Heating 6 Normal Bovine Sera on Hemagglutination and Hemolysis with O.T.-Sensitized Cells.

Unheated serum		Heated (70°C, 10 min.) serum	
Hemagglutination titer*	Hemolytic titer	Hemagglutination titer	Hemolytic titer
16	—	—	—
8	2	—	—
16	16	2	8
4	2	—	—
8	2	—	—
16	—	—	—

* All titers expressed as the reciprocal of the dilution.

Mycobacterium phlei. Accordingly, rabbits were given each agent as indicated below and titrations of fresh and heated sera were performed at frequent intervals following injection.

Materials and methods. Technics for sensitization of erythrocytes with O.T. and procedures for hemagglutination and hemolytic tests are essentially the same as originally reported by Middlebrook and Dubos(2) and Middlebrook(3), but with a modification in the quantities of serum and cells used in the tests(4). Sera were heat-inactivated for 30 minutes at 56°C, diluted 1:2 with buffered saline and adsorbed twice (each adsorption 30 minutes at 37°C) with 1/10 volume of packed, normal sheep cells to remove heterophile antibody. Samples of sera were further heated at 70° C for 10 min. All tests were incubated at 37° C for 30 min. and examined for evidence of agglutination following light centrifugation. Similar serum-red cell mixtures containing appropriate amounts of reconstituted 1:4 guinea pig complement which had been adsorbed twice at refrigerator temperature (20 minutes adsorption) with 1/10 volume of packed, normal sheep cells, were incubated and examined for hemolysis. The last dilution of serum showing complete lysis was recorded as the end-point of titration. Rabbits were infected by a single intravenous injection of 2 mg of *M. tuberculosis* var. *bovis* (Ravenel). Other animals were injected similarly with 2 mg of B.C.G. or *M. phlei.* All cultures were propagated on Petragnani's medium and harvested into physiologic saline. Uniformity of inocula was obtained by rotating bacterial suspensions in flasks containing glass beads. Two sources of P.P.D. were used. P.P.D. 49609 was kindly supplied by Dr. F. B. Seibert and P.P.D. X-0266-A by Parke-Davis Co. of Detroit, Mich. Animals were injected intravenously with 0.2 mg of these preparations contained in one ml of phosphate-buffered saline (pH 7.4).

Results. As seen in Table I, the exposure of cattle sera to 70°C resulted in elimination of all antibody from 5 of 6 normal sera while hemagglutinin in the sixth serum was reduced 8-fold. Similarly, agglutinins were completely eliminated from 3 of 8 tuberculous bovine sera (Table II), and from 10 of 12 samples of tuberculous human sera (Table III). Titers of the other sera in both groups were also reduced. There is no apparent correlation between unheated serum titers and amount of reduction produced by heat.

TABLE II. Effect of Heat on Hemagglutinins in 8 Tuberculous Bovine Sera.

Hemagglutination titer before heat	Hemagglutination titer after heat (70°C, 10 min.)
128	8
64	2
128	16
512	8
4	—
64	—
64	—
64	—

Serologic tests were performed on unheated and heated sera from 4 rabbits injected with

TABLE III. Effect of Heat on 12 Tuberculous Human Sera.

Unheated serum		Heated (70°C, 10 min.) serum	
Hemagglutination titer	Hemolytic titer	Hemagglutination titer	Hemolytic titer
32	4	—	—
64	—	—	—
64	—	—	—
64	64	8	32
16	—	—	—
16	—	—	—
64	—	—	—
2	—	—	—
64	8	4	2
32	—	—	—
64	4	—	—
256	8	—	—

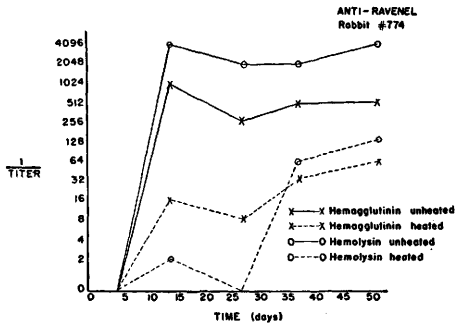

FIG. 1. Comparative antibody titers in unheated and heated tuberculous rabbit serum.

M. tuberculosis var. *bovis* (Ravenel), with samples obtained before injection and from bleedings at intervals to 50 days. Representative data are presented in Fig. 1.

Both hemagglutinins and hemolysins were present in maximal titers in unheated sera by 14th day, the hemolysin titer being higher. Hemagglutinins and hemolysins also were present in heated sera. In contrast to unheated sera, the highest titers of both heat-stable antibodies appeared later in the course of infection, occurring in maximal titers on 50th day, which was the last blood sample taken prior to death of the rabbit.

Four rabbits were injected with B.C.G. as described previously. The animals were bled periodically during the ensuing 65 days at which time they received a second injection of antigen. The results presented in Fig. 2 from a single animal are characteristic of the group. Agglutinins were demonstrable early in heated sera, but titers did not increase after 2 weeks as with tuberculous rabbit sera. Again, the hemolysin in heated sera appeared later than other antibodies and in one rabbit did not

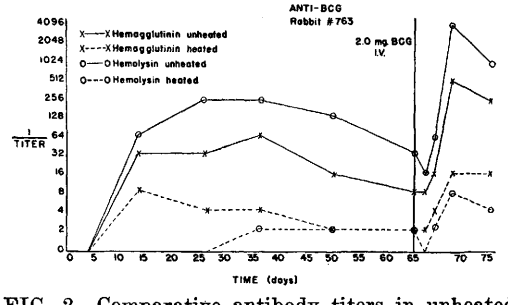

FIG. 2. Comparative antibody titers in unheated and heated sera from rabbits injected with B.C.G.

appear at all until after second injection. Following the second injection of antigen, the titers of all antibodies increased beyond the original titers.

Since sera from many normal cattle contain antibodies which will react with O.T.-sensitized cells (as indicated in Table I), the suggestion was made that non-infectious mycobacteria might be responsible for the sensitization(5). The antibody response of rabbits injected with *M. phlei* was similar to results observed with sera from infected animals (Fig. 1).

Scott and Smith(6) reported stimulation of agglutinins in humans by tuberculin testing; tuberculin-positive individuals responded more actively. In a further effort to characterize the antigenic stimuli responsible for antibodies in heated sera, 8 normal rabbits were injected with P.P.D. and antibody determinations made as indicated in Fig. 3. The data

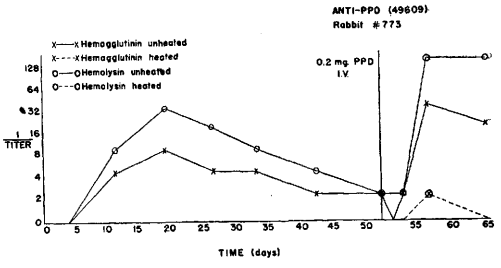

FIG. 3. Comparative antibody titers in unheated and heated sera from rabbits injected with P.P.D.

are representative of the results observed with 6 of the 8 animals. No heated-serum antibodies were demonstrated until 4 days after the second P.P.D. injection. Titers were very low and disappeared 10 days later. During the same period of time titers of hemagglutinin and hemolysin in unheated sera rose to maximal levels but did not approach those observed in animals injected with Mycobacteria.

Agglutinins were present consistently in heated sera of the other 2 animals, although agglutinins in unheated sera were only demonstrated erratically. Both the heat-labile and heat-stable hemolysins appeared about the same time, but varied somewhat as to their appearance in sera from subsequent bleedings. Titers of antibodies in unheated serum rose to maximal values after the second injection

of P.P.D., but differences in antibody titers in heated sera were minimal.

Discussion. The common response of rabbits to all 4 agents was primarily heat-labile antibodies. Since the earliest heat-stable antibodies were agglutinins, apparently some of the agglutinin molecules, in addition to being heat-stable, also are incapable of fixing complement to result in lysis. The heat-stable hemolysin usually appeared later, the titers of both antibodies in heated tuberculous and anti-phlei sera continued to rise.

It is evident that heat-stable antibodies are produced maximally in response to antigens present in *M. tuberculosis* (Ravenel) and *M. phlei,* less readily following injection of B.C.G., and least following P.P.D. However, both heat-stable antibodies may appear in some rabbits at the same time and quite soon after injection of P.P.D.

Follensby and Hooker(7) reported that heated equine antihemocyanin combined with antigen but lacked the capacity to precipitate optimally the same concentration of antigen demonstrated with unheated serum. They also stated that heated serum inhibited precipitation, and concluded that heating resulted in the formation of complexes of antibody globulin and serologically inert protein. Similar observations of inhibition have been made by others after studying the sera from other species or different antigen-antibody systems (8-13).

Other reasons have been presented by some of the above authors(11-13) for the effect of heat on reactivity of immune sera. The principal characteristics of heated rabbit antisera seem to be antibody which specifically combines with antigen, does not result in precipitation or agglutination and inhibits the demonstration of antibody present in unheated serum. In the present work, no inhibition could be demonstrated.

The specificity of the heat-stable agglutinins must be directed toward antigenic sites different from those required by labile hemolysin since its demonstration was not inhibited by heated serum. Further proof of the independence of the heat-stable agglutinins is the fact that their titers in tuberculous and anti-phlei sera eventually approximated their labile counterpart. The non-lytic nature of heat stable agglutinin and its distinct specificity are evidenced by the fact that an excess added to tuberculous serum failed to affect the hemolytic titer, but markedly reduced the hemolytic titer in unheated serum from normal cows.

Although practically all antibody in sera of tuberculin-negative cattle was eliminated by heat, the presence of heat-stable antibodies in the sera of only 16% of tuberculous humans and in 62.5% of the infected cattle, as well as their appearance in sera of rabbits injected with *M. phlei* seems to contradict the original premise of a greater correlation between these antibodies and tuberculosis. However, the occurrence of heat-stable antibodies in progressive tuberculosis in rabbits has been established. If the comparison with rabbits is valid, the absence of such antibodies in sera from some cases of human and bovine tuberculosis is due to lack of proper antigenic stimulus, either in quantity or kind. Thus, further study of heat-stable antibodies in experimentally infected animals and in cases of known tuberculosis is warranted to determine whether or not their continued presence may indicate a poor prognosis, or if their elimination would serve as a criterion for the effectiveness of therapy.

Summary. 1. Antibodies in the sera from a group of tuberculin-negative cattle were destroyed by heating at 70°C for 10 minutes, while heat-stable antibodies were detected in some tuberculous sera. 2. Experimental tuberculosis in rabbits was accompanied by the appearance of heat-stable agglutinins early in the course of infection, and later by heat-stable hemolysins. 3. Similar findings were observed in rabbits injected with B.C.G. and *M. phlei.* 4. Several injections of P.P.D. were required to stimulate the production of heat-stable antibodies.

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Serum Accelerator Factors and Antihemophilic Factor (AHF) in Early Phases of Clotting.* (21280)

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In recent years a number of plasma factors have been described which are required for the optimal conversion of prothrombin to thrombin. A simple, yet effective, partial separation of these substances can be achieved with $BaSO_4$ and other alkaline earth salts. At least two plasma factors, AHF and Ac-globulin, are adsorbed hardly at all with $BaSO_4$; others, particularly PTC and convertin (SPCA) are adsorbed almost completely. Prepared from plasma, the citrate eluate of the $BaSO_4$ adsorbent contains prothrombin; however, made from serum, it is free of prothrombic activity when the parent blood has been clotted with thromboplastin. The $BaSO_4$ eluate from such serum will be designated hereafter as serum accelerator factor (SA) with the understanding that it consists of at least 2, and possibly other, clotting components. A preliminary report of our findings has been published previously(1).

Materials and methods. Blood and *native plasma* were obtained from normal and hemophilic dogs, as described previously(2). *Platelet-poor hemophilic plasma* was prepared similarly by centrifugation at 15,000 *g* for 2 hours at 4°C in siliconed tubes. The source of the *antihemophilic factor* (*AHF*) was prothrombin-free plasma, prepared from normal oxalated dog plasma by $BaSO_4$ adsorption (40 mg/ml) and dialysis against .032% sodium citrate in 0.9% NaCl (2 hr, 4°C). Plasma from both normal and hemophilic dogs was adsorbed in a similar manner and used as the diluent in the experiments of Fig. 2. *Serum accelerator factor* (*SA*) was prepared from normal canine blood to which thromboplastin had been added. The resulting serum was adsorbed with $BaSO_4$, and the adsorbent eluted with 3.2% sodium citrate. This eluate contained no prothrombin, AHF, or Ac-globulin activity, and did not clot citrated plasma in 6 hours or fibrinogen in 10 minutes. The eluate, after dialysis against normal saline, was stored at −20°C at a concentration of 20-25 SPCA units per ml.(3). *Plasma and serum prothrombic activity* were determined by the one-stage method of Langdell, Graham and Brinkhous(4), after the samples had been diluted with prothrombin-free normal or hemophilic plasma. *Plasma and serum prothrombin concentration* were determined by the Iowa 2-stage method(5), modified by the addition of Factor V(6) to the diluting fluid.

Results. The effect of the addition of SA was determined in several types of clotting systems.

The reaction of *hemophilic whole blood* is shown in Table I, Tests 2-5. The addition of SA caused the clotting time to be reduced to the normal range of 5-8 minutes, but did not alter the defective prothrombin utilization (Test 3). When SA and AHF were added together, both clotting time and prothrombin utilization were more rapid than in normal blood (cp. Tests 1 and 5). The prothrombin

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