THE PREPARATION AND IMMUNOLOGICAL PROPERTIES OF DETOXIFIED BACTERIAL ENDOTOXINS

by

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INTRODUCTION

The toxicity of available vaccines against Salmonella typhosa and other enterobacteriaceal infections is a problem, particularly under military conditions where the loss in personnel time due to immunization reactions may be significant. Attempts to provide protection against Salmonellae and Salmonella-like enteric diseases with polyvalent vaccines must also take into account the additive nature of the toxicities of the component organisms. Since the antigenic potential of a bacterial vaccine is influenced by its concentration of bacteria, a procedure that would minimize side reactions would be to reduce the amount of each component until the toxicity of the individual components was within tolerable limits. However, this procedure might result in using less than immunogenic concentrations of vaccine. In recent years, the attempt to chemically detoxify these bacterial components (endotoxin) without destruction of antigenicity has been under investigation (See Part V, Literature Review). This thesis describes work carried out employing chemical detoxification procedures which appear promising. However, instead of applying these detoxification procedures to a Boivin or other purer form of endotoxin, a Roschka-Edwards crude endotoxin preparation (ethanol-acetone washed cells) of a mouse virulent strain of Salmonella typhimurium was employed as the starting material. Once these preparations were made, they were

first tested for capacity to induce fever and lethality in rabbits and mice, respectively. In addition, studies concerning the immunogenic potency of these preparations by the active mouse protection test were also determined by comparing these results with the parent Roschka-Edwards preparation. The hypothesis proposed was that a nonpyrogenic, nontoxic product may still retain its immunogenicity, i.e., act as an "endotoxoid". The ultimate practical purpose of such work is to use this product as a less toxic enteric fever vaccine.

REVIEW OF LITERATURE

I. A HISTORICAL REVIEW OF IMMUNIZATION AGAINST TYPHOID FEVER IN THE ARMED FORCES

It has been well recognized that the typhoid bacillus is one of the greatest potential enemies to the soldier in the field. During the nineteenth and twentieth centuries, nearly all the armies of the civilized world have been devastated by infection with typhoid fever, but the dangers of specific epidemic communication were not realized until after the discovery of the organism by Eberth in 1880. In spite of the great losses of troops in the field, most nations were hesitant to adopt methods of immunization control for their armed forces. However, to control typhoid in its military forces during the Russo-Japanese war, the Japanese Army is credited with the first attempt by employing specifically designed sanitary procedures (Jennings and Mathieson, 1940). It is of interest that some success was probably obtained by these procedures as the incidence of typhoid fever in the Japanese Army was one-fourth of that reported by the Russian Army. It should be noted, however, that in spite of the strict sanitary disciplines which were followed in most armies, typhoid fever still prevailed at an excessive rate until active immunization became widely practiced.

Although it is claimed by some that Pfeiffer and Kolle, who in 1896 reported on experimental vaccination with killed typhoid bacilli in 13 individuals, were the first investigators to attempt vaccination

of man, the first significant attempt is usually credited to Wright (Wilson and Miles, 1964). In 1896, Wright (1896) mentioned, apparently as a mere incident concerning research on another project, the inoculation of two Indian Medical Service Officers with a killed broth culture of typhoid bacilli. Since subsequent challenge with living typhoid organisms into one of the volunteers did not produce infection, this author believed he had produced effective immunization against typhoid. The following year this same author (Wright, 1897) further reported on the blood changes in 17 individuals following typhoid vaccination with bacterial cultures by employing methods used successfully in the immunization against cholera. As a result of these data, this investigator apparently became convinced that the prevention of typhoid fever by vaccination was a definite possibility and suggested its use primarily in armies as well as in hospital personnel.

During the Boer war the British Army was the first to practice active immunization on a large scale. The case mortality rate was reduced by approximately one-half while the incidence of typhoid fever among the immunized troops was decreased by a similar amount as compared to the case reports involving non-immunized troops (Jennings and Mathieson, 1940).

Immunization against typhoid fever was also practiced in India on a large scale. Leishman (1910), in a summary covering the period 1904-1909, reported a typhoid incidence of 5.39 per 1,000 in 10,378 immunized individuals, whereas the typhoid incidence in 8,936 nonimmunized personnel was 30.4 per 1,000. In addition the mortality rates were

8.9 and 16.9%, respectively. In this same report Leishman described in detail the effects of vaccination with dead typhoid organisms on a battalion of British soldiers. In this experiment four groups of approximately six men each were immunized. Each group was immunized by a different dosage schedule prior to their departure for India in 1904. In one of the earliest carefully controlled studies of immunization against typhoid fever, Leishman took blood specimens from each man for a period of four weeks. Sera were pooled within each group and tested for the presence of antibodies. He noted that the titers of antibody response were approximately proportional to the amount of vaccine injected. It was noted that the degree of reaction, though to a lesser extent, was also proportional to the amount of vaccination. On the basis of this study, it was found that 500 million and 1,000 million dead organisms for the first and second injections respectively was established as a compromise between the degree of reaction obtained and the amount of protection established. Following these first experiments, no cases of typhoid fever resulted even though the battalion was stationed for one year in India. Further studies on the antibody levels of these men were made. Within six months, it was found that the antibody titers of these men had dropped to approximately normal levels. It is of interest to note that this immunized battalion was moved to an area of great exposure during its second and third years in India resulting in the same incidence of typhoid fever as was shown in the nonimmunized men.

In spite of this early promise in the prevention of typhoid fever;

the British authorities were reluctant to adopt the procedure of vaccination as mandatory for military personnel (Batson, 1949). However, an anti-typhoid committee evaluating the evidence favoring immunization against typhoid fever, reported in 1913 that of a total of 328 cases of typhoid which were observed in 19,314 troops, 272 cases in 8,936 nonimmunized troops while only 56 cases were noted in 10,378 immunized individuals. Greenwood and Yule (1915) analyzed these data and found that the odds against this difference in the number attacked were greater than 10,000 to 1 by chance distribution.

Typhoid vaccination was initiated in the United States Army in 1908 by Major F. F. Russell largely as a result of the experience the British were having in immunizing their troops in India and South Africa (Russell, 1913). The typhoid bacilli employed for the production of this vaccine was the Rawlings strain. This strain was originally isolated by the British from a fatal case of typhoid in 1900. Leishman (1910) selected this strain on the basis of criteria which included low toxicity for guinea pigs, active stimulation of agglutinins in rabbits, mildness of reactions elicited in man, and the ease of emulsification from solid media. This Rawlings strain was used for the production of the vaccine by the United States Army from 1908 to 1936 (Siler et al., 1941).

Typhoid fever vaccination was first adopted on a voluntary basis in the United States Army in March, 1909. During this year, under the direction of Col. F. F. Russell, less than 1,000 individuals were immunized. By 1910, approximately 15% of the Army had been vaccinated

(Siler et al., 1941). In 1911, the first instance of compulsory vaccination in the United States Army was in a division (approximately 20,000 men) of troops mobilized in Texas and elsewhere along the southern border (Russell, 1913). Compulsory immunization of the entire command, with the exception of a few individuals over 45 years of age, was actually accomplished after the troops arrived at their destination. These troops remained in the field for over 4 months, during which time only 2 cases of typhoid fever were recorded with neither terminating in death.

By September 30, 1911, immunization against typhoid fever was made compulsory for all army recruits excluding all army personnel over 45 years of age. By the end of that year approximately 85% of all army personnel had been immunized (Siler et al., 1941). It is interesting to note that Russell (1913) reported that in 1910 there were 198 cases of typhoid in all army personnel, whereas in 1911 there were 68 cases, and in 1912 there were only 27 cases out of 88,478 personnel with 19 of these latter cases being nonimmunized individuals. Russell further noted that these figures were of greater significance than those reported by the British Army where vaccination was continued on a volunteer basis. He considered it logical to assume that volunteers are predominently of a higher order of intelligence and, thus, more capable of practicing individual hygiene.

The strength and composition of the typhoid fever vaccine used in the United States Army has been changed on several occasions (Siler et al., 1941). During the years between 1909 and 1916 only a monovalent

typhoid vaccine was employed, whereas in 1917, following an outbreak of paratyphoid fever in troops stationed along the Mexican border, the paratyphoid fractions were added. This triple vaccine, later termed TAB, was used by the U. S. Army continuously from 1917 through 1927. In 1928, the paratyphoid B fraction was eliminated while in 1934 inclusion of the paratyphoid A fraction was discontinued. However, in September, 1940, both paratyphoid fractions were readded to the vaccine in view of the anticipated increase of troops in the field plus an increased likelihood of exposure of these troops to enteric organisms (Long, 1955). This vaccine contained 1,000 million typhoid bacilli and 250 million each of the paratyphoid A and B strains in each cubic centimeter. As stated earlier in this section, use of the classic Rawlings strain adopted by the United States Army for vaccine production in 1908 was terminated in 1936. At that time, investigations made by the Army Medical School (Siler et al., 1941; Longfellow and Luippold, 1943) had shown that the immunogenic strains of the typhoid bacillus were highly virulent and it was concluded that these strains should be employed for vaccine production. As assayed by mouse protection tests, the Rawlings strain was found to be inferior, and substituted in its place was the highly virulent Panama carrier strain 58. The para A strain employed was Salmonella paratyphi but this was replaced in 1942 by the English strain HA-6. The para B organism employed was Salmonella schottmuelleri (Long, 1955).

The criteria for the selection of these strains, their characteristics,

and the history of the vaccine will be discussed at length in another section. However, it is notable that these strains represented the most immunogenic strains obtainable and were selected on the basis of their virulence, their biochemical properties and productivity of agglutinins and protective substances, and their production of cross-immunity against other strains of homologous species. Throughout World War II these strains were employed in the manufacture of the TAB vaccine and are presently used today. Long (1955) reported that the effectiveness of this triple vaccine during World War II was indicated by the fact that the combined average annual rates for typhoid and paratyphoid fevers was approximately 0.05 per thousand, whereas the annual rates for the combined diseases during World War I was 0.42 per thousand. Commenting on these results, this author stated: "The occurrence of typhoid and paratyphoid fevers does not bring forth evidence against the efficacy of vaccination for the prevention of these diseases nor does it gainsay the probability of a considerably improved typhoid vaccine. These observations do, however, emphasize again that the incidence of typhoid fever and the paratyphoid fever, as well as of the other enteric infections, tends to be in proportion to the opportunities for infection existing in the environment and that specific protection through vaccination does not eliminate the necessity for proper application of sanitary measures for the control of these infections."

The active immunization against typhoid fever was made compulsory

for all personnel in the United States Navy in December, 1911 (Siler et al., 1941). Results were shown to be essentially similar to those observed in the United States Army with admission rates per 1,000 as follows: 1911, 3.62; 1912, 0.92; and 1913, 0.35. Rates continued low in the Navy and in 1925 decreased to 0.03 per 1,000. In 1924, the Navy adopted the use of the monovalent (Rawlings strain) typhoid vaccine. However, the triple vaccine remained in use for the immunization of personnel stationed in either the Caribbean or Central American areas (Siler et al., 1941).

Based on the assumption that they increased the toxicity of the vaccine, the paratyphoid A and B strains were omitted from the vaccine during the years 1911-1939 (Siler et al., 1941). In addition, paratyphoid fever infections reported in all military personnel during this time were both mild and rare. To support these assumptions, there were 382 cases of paratyphoid fever in the Army: 257 due to para A, 100 due to para B, and 25 unclassified. There were eleven deaths: 6 associated with paratyphoid A and 5 with paratyphoid B. With one exception, all of these deaths occurred in France during World War I. Within the United States, 84% of the paratyphoid infections from 1915 to 1939 were caused by paratyphoid A.

II. THE EFFECTIVENESS OF ACTIVE TYPHOID IMMUNIZATION OF MILITARY AND NON-MILITARY PERSONNEL

A significant evaluation of the effectiveness of immunization against typhoid fever is obtained by studying the reported results of such trials in military personnel. One of the earliest reports on the results of typhoid fever vaccination in American troops was given by Russell (1910). This author stated that from the introduction of vaccination in 1909 until March, 1910, there were 135 cases of typhoid among approximately 75,000 men. Russell further reported that one of these cases developed typhoid immediately following administration of the second dose of vaccine. However, since a total of approximately 1,400 men had been vaccinated during this time, it appeared reasonable to conclude that vaccination had provided real protection. In another report, Russell (1912) stated that there was a total of only 6 cases of typhoid among immunized military personnel in 1910 and 12 cases out of 80,000 individuals which had been vaccinated in 1911. This investigator suggested that if the prevailing incidence of typhoid in the nonvaccinated military personnel had occurred, about 250 cases would have been expected in this group.

Although the most extensive early trials of active immunization of military personnel were conducted in the British and American Armed Forces, military leaders in other countries followed these trials with interest and made some attempt to introduce the practice in their troops. However, in many instances, there is evidence that sufficient attention

was not given to the administrative phases of their vaccination procedures. Jude (1934) reported that prior to World War I the average morbidity of typhoid fever in the French Army was approximately 12 per 1,000 mean strength. However, after the result of some bitter experiences in the early stages of World War I, stricter attention was paid to vaccination and the morbidity rate declined sharply, reaching 0.22 per 1,000 by 1929. Similar results in the German Armed Forces were given by Thone (1938) who reported that the incidence of typhoid per 1,000 dropped from 9.3 in 1915 to 1.4 in 1917. This author indicated that this reduction was due to a rigid program of prophylactic inoculation against typhoid.

As a result of the high morbidity of typhoid encountered in the Italian Army during World War I, Castellani (1937) reported on the extra precautions taken to protect the troops prior to the Italian-Ethiopian War. During the 6 month period of the war, there were about 500,000 Italian troops committed in this campaign with a total of 458 cases of typhoid and paratyphoid and 161 deaths. These troops had received "tetra vaccine" containing typhoid, paratyphoid A and B, and cholera organisms. Castellani concluded from these results that the precautions taken showed a definite improvement over the previous experience of the Italian Army during World War I.

Malbin (1940) reported a water-borne epidemic of typhoid in a military hospital in Spain during the Spanish Civil War. In this hospital ninety per cent of the 1,700 patients and 200 staff members had been

vaccinated within the preceding 3 to 12 months. A total of 147 cases of typhoid fever were studied with the incidence among the nonimmunized being approximately 25% as compared with only 6% in the immunized. The case fatality rates for the two groups were 10.2% and 4%, respectively.

Further evidence of the efficacy of typhoid vaccination can be obtained from a study of the occurrence of the disease among the Civilian Conservation Corps. Lull (1934) stated that out of an approximate 450,000 men in the various camps during 1933 there were reported 54 cases of typhoid, 11 cases of paratyphoid B, and 2 cases of paratyphoid A. The incidence of typhoid was 0.32 per 1,000 per year, whereas, during the same period, the incidence in the regular Army was 0.04 per 1,000.

It is of interest that not all reports of the results of vaccination of military personnel against typhoid fever appear in favor of the practice. Nichols (1916) reported on 32 cases of typhoid in immunized military personnel during the period 1911-1915. Although this author did not state the total number exposed to comparable risk nor how many doses of vaccine were given to those who developed the infection, he did indicate that no toxic effect could be demonstrated in rabbits with vaccines over 8 months old. He concluded that vaccines over 1 month old were nearly inert and were responsible for the failure to protect against typhoid.

An outbreak of typhoid in vaccinated personnel at an American rest camp in England during July, 1918, was reported by Hawn et al. (1919). These authors noted that the original infections probably occurred when

members of the group went swimming in a lake at Meridian, Tennessee prior to being transported to England. During the outbreak, 248 men were exposed with 98 developing typhoid either as a result of the original exposure or from prior contact with cases. All of these 98 men had been immunized against typhoid fever. The number of doses each had received, with one exception, varied from 3 to 12. According to these authors, the resultant case fatality rate was high, (13.15%) and possibly may be explained by the lateness of admission to the hospital.

Although United States Army personnel in World War I were well vaccinated against typhoid, Burrage (1932) reported that 1,065 cases of this enteric infection occurred. He suggested that these cases were in individuals refractory to immunization or in those exposed to overwhelming doses of the infectious agent. Of the 76 vaccinated men observed by this author, 73 were in individuals who had their full course of 3 inoculations, most of them within 1 year of their injection. This series also included 9 fatal cases. Burrage did point out, however, that there was only one case in every 3,756 men in World War I as compared with 1 case in every 7 men during the Spanish-American War.

One of the most thoroughly investigated failures of vaccine to protect against typhoid was reported by Boyd (1943). This failure occurred among Axis prisoners of war captured by the British in Africa. Although the total number of prisoners observed was not given, this author noted that from January, 1941 through February, 1943, there were 305 cases of typhoid, 202 cases of paratyphoid A, and 65 cases

of paratyphoid B in these prisoners. These Axis troops supposedly had been immunized previously and during the first few days of their internment were re-immunized with a captured Italian four component vaccine. Despite this treatment a typhoid epidemic occurred among these prisoners. Subsequently, all prisoners were re-vaccinated with a British typhoid vaccine and no further epidemics occurred. Minimal laboratory tests comparing the protective potency of the British vaccine with that of the captured Italian vaccine indicated to this author that the British vaccine product was superior. It is interesting to note that Boyd contrasted the above experience with that observed among British prisoners of the Axis forces. Approximately 24,000 British troops had been captured and kept in Axis concentration camps where sanitary conditions were stated to have been deplorable. Enteric infections were common among the Axis personnel and, as evidence of the unsanitary conditions and undoubted exposure to infection, this author reported that approximately 11,000 to 12,000 cases of dysentery occurred among the British prisoners. In spite of these conditions, however, there were essentially no cases of typhoid or paratyphoid. Boyd concluded that the difference in the occurrence of enteric infections in the two groups of prisoners was due to differing degrees of immunity resulting from use of a more potent vaccine preparation by the British. He emphasized that the strains of organisms used in the preparation of the British vaccine were rich in Vi antigen.

Studies of enteric infections occurring among vaccinated American

military personnel stationed in Okinawa during World War II were reported by Syverton and his associates (1946). The outbreak occurred on Okinawa but was first noted among evacuated personnel on Guam. Infection probably resulted from eating raw vegetables including sugar cane which had been cut and allowed to lie on the ground fertilized with human excrement and, thus, heavily contaminated with enteric organisms. These authors noted that on Okinawa in the late spring and early summer of 1945 there were many cases of undiagnosed fever of unknown origin. Of the 24 cases of enteric fever reported, however, 21 were due to paratyphoid A and 3 were due to typhoid. Complete data relative to vaccination were available on 20 of the patients with nineteen of these having received the full standard course of injections, and 15 having received one or more booster injections. The interval between the last injection of vaccine and the onset of infection ranged from 5 to 15 months. No deaths were reported in these patients, but complications developed in 5 cases.

An additional account of instances of failure of vaccination to protect against enteric infections was reported by Jordan and Jones (1945). They reported that 44 cases of typhoid fever, part of an epidemic of 80 cases, were treated in a military hospital which resulted in 2 deaths. Despite the fact that all 44 of these patients had been up to date with their yearly "TAB" inoculations, all had at least 3 and the majority had 4 or 5 prior inoculations, the course of the disease was severe and unmodified. From this epidemic, these authors concluded that when artificially induced immunity to typhoid is overwhelmed,

protected men can suffer from the disease in the same way and quite as severely as though they had never been immunized. On the other hand, it was pointed out, inoculation may prevent the majority of those exposed to infection from contracting the disease.

Tribby et al. (1948) reported results gathered by the Second Medical Laboratory in French Morocco and Italy in which 19 men of the 349th Infantry regiment were hospitalized with typhoid fever within 2 to 14 months of their last dose of typhoid vaccine. These authors suggested that these soldiers were subjected to an overwhelming challenging dose of typhoid bacilli. Also, they questioned the degree of protection afforded by the various typhoid vaccines, and whether or not they have as high a protective capacity as has been claimed on the basis of statistical evidence.

Although it is generally believed that an attack of typhoid fever confers a considerable degree of immunity, recurrences occasionally take place. The second attack rate has been reported to range from 1 to 4%, but second attack rates as high as 15% are on record (Gay, 1918). Recently, Marmion et al. (1953) published an account of a second attack of typhoid fever in a British Royal Air Force unit in the Suez Canal Zone in 1950. Two large outbreaks, occurring in the same community within 5 months, produced 11 examples of second attacks. The first outbreak was caused by phage-type J bacilli and the second by phage-type E organisms, indicating that the second infections were new attacks of the disease rather than relapses of the original infection. Though

inconclusive, the evidence of these facts and figures suggested to these workers that an attack of typhoid fever does not confer more than a moderate degree of specific immunity. The possible relationship of hypo-gamma-globulinemia under these circumstances remains to be evaluated.

Understandably it is usually more difficult to evaluate the efficacy of active immunization in a civilian rather than in a military group. On the other hand, some well-controlled studies have been conducted with civilians concerning the effectiveness of immunization against typhoid. In addition, there have been occasions where recently discharged military personnel have been exposed with civilians to a common risk of infection. In these latter instances reliable estimates of the effectiveness of immunization are obtainable more readily from military personnel who usually are vaccinated under close supervision. However, evidence of protection against typhoid can be obtained from changes within the civilian population following an immunization program.

The value of immunization against typhoid was demonstrated by studies conducted by the New York City Department of Health (1916). The typhoid death rate per 1,000 decreased from 0.12 in 1910 to 0.04 in 1916. Much of this decrease coincided with an active program of vaccination where individual records revealed that, of 3,023 immunized persons directly exposed to typhoid, only 4 cases occurred. However, of 4,420 non-immunized individuals, who likewise were directly exposed to typhoid, 108 subsequently contracted the disease. In each case there was an indication that exposure to infection antedated vaccination.

Halliday and Beck (1928) reported on the protection offered by vaccination from observations made during a prolonged epidemic of typhoid in Santa Ana, California in 1924. An epidemic of gastrointestinal disorders occurred following a period of heavy rainfall during the last 2 weeks in December, 1923. It was estimated that 10,000 persons out of a population of 27,000 were attacked. During the period January to April, 1924, there were 620 cases of typhoid with the following breakdown: 226 occurred following pollution of the water supply in December, 143 followed a subsequent pollution in February, 200 cases resulted from contaminated milk, and 51 cases apparently resulted from contacts with other cases. Forty-eight of the cases terminated fatally. It is interesting to note that 9 cases occurred in persons who had had typhoid several years previously, and that only 5 cases occurred among the approximate 1,000 ex-servicemen in the city who had been vaccinated against typhoid in 1918. It appeared to these workers that vaccination had afforded a high degree of protection which was still effective after a period of 5 to 6 years.

Further evidence that individuals previously immunized during military service have retained their resistance to infection for a long period of time is evident from the report of an outbreak of typhoid at Salem, Ohio in 1920 (Pryor, 1928). In this epidemic, 882 cases of typhoid occurred in a population of 10,305. This author reported that in the age group of 20 to 30 years there were 210 ex-servicemen (World War I veterans) all of whom had received vaccination against typhoid approximately 3 years previously. The attack rate for these ex-servicemen was 1 in 70,

whereas in women of the same age group the attack rate was 1 in every 8.

A limitation in evaluating the effectiveness of immunization in civilian populations is the difficulty of determining if the immunized and non-immunized groups have been equally exposed to comparable risks of infection. By restricting observations to secondary typhoid cases in household contacts, Stebbins and Reed (1937) reported from a study conducted in New York State, 1930-1934, that the attack rate among the nonvaccinated to be approximately five times that of the vaccinated contacts.

The effects of sanitation and group immunization on the incidence of typhoid was obtained by Williams and Bishop (1936) during an intensive typhoid control program conducted in Williamson County, Tennessee (population 22,845). This program covered 13 years, ending in 1935, during which time a total of 21,157 persons received at least one full series of inoculations. As a result, these workers reported that there was a decrease in cases from an annual average of 62 for the period of 1916-1921 to only 3 for the period of 1931-1935. In addition, the typhoid rates decreased significantly in specific areas only after a major portion of the population in that area had been immunized. In other words, while typhoid practically disappeared in an area in which 87% of the population was vaccinated, there was little decrease in another area in which only 41% had been vaccinated until a specific program of sanitation was accomplished.

The efficacy of immunization in an acute water-borne outbreak of typhoid in a consolidated school in Dade County, Georgia was reported

by Bowdoin and Petrie (1940). From direct sewage pollution, twenty-seven cases of typhoid developed among a total of 323 pupils. Within the preceding 2 years, 151 of the pupils had been vaccinated and only 4 of these contacted the disease. Thus, the attack rate, according to these authors, was five times greater in the non-immunized group than in the immunized group.

Another comparison of the resistance to typhoid of immunized and non-immunized individuals was afforded in an aoubreak investigated by Duncan et al. (1946). These workers noted that infection resulted from drinking contaminated orange juice in a Cleveland residence hotel for women. Approximately 360 young women including 150 female military personnel resided in the hotel, among whom seventeen of the civilians, including one who had been vaccinated, contracted typhoid. However, as a result of immunization against typhoid, none of the female military personnel developed the disease.

The success of vaccination against typhoid in other countries has been essentially comparable to that experienced in the United States. Hogg and Latham (1926) reported the incidence of enteric fever among inmates and staff members of mental hospitals in New South Wales, Australia, during the period 1900-1924. During this time, the number of individuals upon whom the report was based increased from 4,350 to approximately 8,700 of which from 1900-1914, 241 cases of typhoid were recorded. However, vaccination was adopted in 1914 and during the period 1915-1924 there occurred a total of 12 cases, 2 of which were in vaccinated

individuals. These authors stated that their feelings were mixed in having to record in 1914-1915 that the mentally disturbed of New South Wales were better protected against typhoid than were many of the Commonwealth's soldiers in Egypt and on the Peninsula.

The failure of immunization to protect non-military groups is more difficult to evaluate than in military groups; that is, it is difficult or impossible to obtain accurate records of immunization in civilian personnel as well as to evaluate the typhoid vaccine or vaccines employed under these conditions. However, the literature does record a few isolated cases of failure to protect in typhoid vaccination of the civilian population. Seckinger (1933) reported that of 216 cases of typhoid in Georgia in 1931-1932, 21 (9.7%) of these cases were in persons immunized 2 weeks to 12 months prior to the onset of symptoms. The mortality rate in these individuals was 23.8%. The percentage of the exposed population who had been vaccinated was not known, but it appeared, according to this author, that vaccination was markedly ineffective. It is interesting to note that the Rawlings strain of organisms was used for preparation of the vaccine which apparently failed to protect. Following this investigation a study of vaccine production methods was conducted by the State Health Department and, as a result, a new strain of organisms was substituted (Mitchell strain).

Hodgson (1945), in a study of 84 cases of typhoid and paratyphoid in the British-American Hospital, Lima, Peru, reported that 71 of the cases were typhoid and that 12 of these were known to have been vaccinated

within the preceding 3 years. On the average the severity of the disease was less than in the non-vaccinated group; that is, the duration of the fever averaged only 6 days less. In addition, one of these patients died. The author concluded from this study that vaccination against typhoid fever offers some protection but it does not give absolute protection.

Allan (1943) observed 27 cases of typhoid among 114 individuals in the Devon Mental Hospital, England, in which 14 of the cases were in previously vaccinated individuals while 13 were in non-vaccinated persons. Of these 27 cases, 7 of the 17 seriously ill patients had been previously vaccinated, whereas 7 of the 10 mild cases observed had been protected. This author indicated that while inoculation did not appear to give much protection against an attack of typhoid, those who were immunized had a better chance of a mild attack. In addition, the disease in the more serious cases did not seem to be influenced by vaccination and relapses were common to both types.

III. AN EVALUATION OF THE LABORATORY AND FIELD DATA CONCERNING ACTIVE IMMUNIZATION AGAINST TYPHOID FEVER.

Although much of the information concerning active immunization to typhoid fever has been obtained by laboratory experimentation, the experimental conditions and the methods employed in different investigations have become so extensive that interpretation and comparative evaluation of the reported findings are exceedingly difficult. Making

allowances for differences in methodology by various investigators, the following is primarily a resume rather than a critical evaluation of the laboratory findings pertaining to active immunization against typhoid fever.

Although the use of bacterial suspensions grown on solid media was introduced early, the first vaccines employed by Wright were essentially broth cultures of typhoid organisms killed by heating and preserved with lysol (Leishman, 1910). Heating has been the most commonly used procedure for killing the organisms and there is little doubt that in some instances the heating has been excessive and has resulted in lessened immunogenic potency of the typhoid vaccine (Batson, 1949). The heat-killed, tricresol or phenol-preserved vaccines are the most commonly available commercial vaccines today. Slightly different temperatures are employed by different manufacturers for killing the organisms, but 56° C for 1 hour apparently does not significantly lower the antigenicity. The ability of the various typhoid vaccines to stimulate antibody production, whether measured by mouse-protection tests or by serological means, will be considered later in the text.

Following an extensive preliminary study (Felix, Rainsford and Stokes, 1941), the British Army, in 1944, discarded the heat-killed, phenol-preserved vaccine for a vaccine, which has since been abandoned as a result of field trials in Yugoslavia, in which the organisms were killed with 75% alcohol and preserved with 22.5% alcohol as recommended by Felix (1941). The criteria for this production method was the observation

by Felix and Pitt (1936) that alcohol did not destroy the O antigen, which Felix considered essential for good protection against typhoid fever, and that this concentration of alcohol was an effective preservative (Felix, 1941; Cruickshank et al., 1942), whereas both heat and phenol reportedly destroy the O antigen (Felix and Pitt, 1934; Felix and Bhatnagar, 1935). While the superior immunogenicity of the alcohol-type vaccine has been confirmed by others (Climie, 1942), Drysdale (1947) on the other hand, found no difference in the mouse-protective potency of the alcohol-type vaccine and the heat-killed, phenol-preserved vaccine.

Other killing and preserving agents that have been investigated in relation to typhoid vaccine production include formalin, acetone, chloroform, ether, and merthiolate (Rainsford, 1939; Rainsford, 1942; Sen, 1947). Although each of these agents may possess certain theoretical advantages, conclusive evidence from field trials which would provide justification for changing from the use of the present vaccine product has not as yet been presented. However, evidence may eventually be forthcoming to equal or lower the morbidity rates sufficiently from those observed during World War II by employing newer and more potent vaccines as compared to the heat-killed, phenol-preserved vaccine used at the present time against typhoid fever.

The results obtained by Grasset and his co-workers (Grasset, 1939; Grasset and Lewin, 1936) and by others (Miller, Van Blommestein and Williams, 1936; Goldsmith, Miller and Buchanan, 1938) with "endotoxoids" have been of particular interest not only because the results apparently

were highly satisfactory but also because of the immunizing product employed. This "endotoxoid" was prepared by alternately freezing and thawing fully virulent typhoid organisms followed by detoxification of the contents of the disrupted organisms with formalin at 37° C for long periods of time. The resultant product proved on extensive laboratory tests and in experimental trials in humans to be relatively nontoxic yet apparently fully immunogenic. This "endotoxoid" was subjected to an extensive study (Grasset, Lewin and Van Der Merwe, 1937) in 1936 and 1937 involving laborers in one of the gold mines in South Africa. Prior to 1936, the annual incidence of typhoid in this mine had ranged from 16 to 101 cases. Between September, 1936 and July, 1937, 5,445 of the 6,652 laborers in the mines were inoculated with a single injection of the "endotoxoid". During the following 10 months there were only 9 cases of typhoid and 8 of these were in non-immunized workers while the other case was in an individual who had been immunized only 4 days previously.

A later report (Grasset, 1939) reviewed the results of preventive immunization of native laborers of the Rand gold mines of South Africa and the statistics covered the period 1934-1936. Out of a total of 810,475 persons at risk during the above period, 152,390 laborers were immunized with the "endotoxoid" vaccine. Among these immunized workers, there were 174 cases and 35 deaths or an incidence of 1.14 per 1,000. In addition, a second group of 77,971 laborers were given oral vaccination. In this group there were 638 cases and 136 deaths, an

incidence of 8.24 per 1,000. On the other hand, in the control group of 580,514 non-immunized workers, there were 2,231 cases and 477 deaths or an incidence of 3.84 per 1,000. Since the incidence of typhoid fever in this latter non-immunized group was less than one-half that in the orally immunized group, it appears to cast some doubt on the accuracy of these results reported by this investigator. However, it is reasonable to conclude that the "endotoxoid" vaccine was apparently protective under the conditions specified since accurate statistics on the incidence of typhoid in a test population such as the native workers in these gold mines are difficult to obtain.

Rauss (1942) reported a study of the effectiveness of an immunizing agent somewhat similar to that employed by Grasset. Apparently, the main difference between the two vaccines employed by Rauss and by Grasset is that the former vaccine was an alum-precipitated detoxified extract. The field trial was conducted in 23 parishes in a district of upper Hungary where the mortality rate had been 36.5 per 10,000 population. The study was carefully controlled and no persons under 2 or over 60 years of age were included. In June 1937, 12,813 individuals were given 1 injection of the alum-precipitated vaccine; 10,962 were given 3 injections of a type unspecified vaccine; and 10,059 non-immunized persons served as a control group. This control group included several individuals who previously had received 1 or 2, but not the full 3 doses of vaccine. All persons known to have had typhoid fever within 10 years of the study were excluded. The mortality rates per 10,000

within 3 years of vaccination for the alum-precipitated immunized, vaccine immunized, and non-immunized groups were approximately 8, 7, and 23 respectively. Although there was some variation within age groups, there was no apparent difference in the protection afforded by the alum-precipitated vaccine and the conventional vaccine. However, the reduction in incidence of typhoid in the immunized as compared with the controls was highly significant.

As stated previously (See Part I, Literature Review), the strain of S. typhosa first used by the British for the production of typhoid vaccine was the Rawlings strain isolated in 1900. The basis upon which this selection was made was primarily good agglutinin production in rabbits, low toxicity for man, and ease of emulsification and suspension of the bacterial growth from solid media.

The Rawlings strain was adopted by Russell (1913) for production of typhoid vaccine for the U. S. Army by the Army Medical School in 1908 and was used until 1936. Since that time, strain 58, or Panama Carrier strain, has been employed continuously (Siler et al., 1941). However, it was not until the late twenties that the protective efficacy of the Rawlings strain was questioned seriously, although many investigators had noted its ability to dissociate (S→R) readily. For example, Grinnell (1930) noted that the bactericidal power of blood of persons immunized with a vaccine prepared from a smooth strain was increased markedly while that of individuals immunized with vaccine prepared from rough organisms was not. This author (1932) later compared the virulence

of 12 Rawlings substrains. He found that the rough strains were less virulent than were the smooth strains and that virulence and smoothness were correlated with the mouse-protective potency of vaccines made from such strains.

Other workers reported findings in general agreement with Grinnell. Perry, Findlay and Bensted (1933, 1934) observed that the original Rawlings strain and another strain given multiple passages through mice were more virulent and produced vaccines of somewhat greater protective potency. However, these authors did observe that the vaccine produced from a rough variant still was highly protective. Brown (1936) reported similar observations regarding the correlation between mouse virulence of cultures and mouse-protective potency of vaccines made from such cultures. Moreover, he concluded that variations in virulence and mouse-protective potency were dependent upon the presence or absence of the O antigen.

An extensive study of the relationship of mouse-virulence and cultural smoothness to immunogenic potency was made by Siler and his co-workers (1936, 1941). Their studies noted a definite relationship between those qualities, virulent strains being more highly immunogenic when measured either directly by active-immunity protection test in mice or indirectly by measuring the protective potency of the blood serum of humans immunized with vaccines prepared from virulent and avirulent strains. These workers felt that the mouse-protective potency of the serum of vaccinated humans was a better measure of their

immunity than was the agglutination titer. In addition, they found no consistent relationship between the agglutination and mouse-protective titers. Similar findings were reported by Grinnell (1931) who suggested further that smooth strains might possess a carbohydrate antigenic component associated with virulence and immunogenicity but which in no way entered into the agglutination reaction.

Although it is generally considered that virulence of microorganisms is associated with toxicity for man, there is disagreement as to the relationship of the degree of reactions elicited in man as well as the degree of immunity produced. Feemster (1932) found these two completely unrelated but did note that reactions following immunization were most severe in persons who previously recovered from typhoid fever. Luippold, Longfellow and Toporek (1947) observed that a reduction in dosage of typhoid vaccine in previously non-immunized individuals resulted in a decreased incidence of reactions but in an actual increase in mouse-protective antibody titers.

Unfortunately there has not been general agreement on a standard procedure for measuring the amount of active immunity produced in mice or other laboratory animals. However, in most cases, this had been approached (Siler, 1936; Siler et al., 1941; Griffitts, 1944), by vaccinating mice with a constant dose of vaccine and determining the level of their effective resistance by injecting increased multiples of lethal doses (MLD's or LD_{50}'s) to successive groups of the immunized mice. On the other hand, Batson (1948) compared the effectiveness of

graded immunizing doses as well as graded challenge doses in measuring the degree of protection afforded mice by active immunization. This investigator found that graded immunizing doses were much more effective. He also observed that at any one level of established immunity, survival or death was markedly independent of the size of challenge dose employed. Although several workers have employed various types of challenge suspensions as well as different procedures for measuring the effective resistance of immunized animals, it is possible that the reported differences in the immunizing efficacy of vaccines prepared by different methods or from different strains may be due to true differences in immunogenicity (Batson, 1949).

The development by Siler and his co-workers (1937) of the mouse-protection test as a measure of the antibody level of sera has made it possible to extend laboratory investigations. However, the information obtained from mouse-protection tests, or any other laboratory test, is not expected to approach that obtained in the field under actual conditions of exposure to infection. On the other hand, in spite of the real possibility that immunity of humans to typhoid may be primarily a cellular immunity and the humoral antibodies only a manifestation of excess immunity, the results obtained by mouse-protection tests appear to parallel the probable true resistance of the individual more closely than any other commonly employed laboratory assay procedure.

Luippold, Longfellow, and Toporek (1947) reported that, on the basis of the level of mouse-protective antibodies produced, 3 injections

of 0.5 ml (subcutaneous) each stimulated as great an immune response as did the standard course (0.5, 1.0 and 1.0 ml, subcutaneously). Several workers, including Stroebe (1929) and Taylor (1937) have noted under actual epidemic conditions that at least 2 injections of vaccine are needed to provide adequate protection. These observations appear to be in agreement with those concerning the incidence of typhoid fever in partially immunized personnel in the U. S. Army (Siler, 1941). Apparently, from these reports, multiple inoculations are of importance in establishing an effective degree of immunity.

There have been many reports on the value of oral immunization as compared with the usual parenteral (subcutaneous) injection. Those studies (Destouches, 1934), based on the actual occurrence or absence of infection, show that oral immunization affords little or no protection. However, Guerner (1927) reported a typhoid fever incidence of 0.01% in orally immunized persons in Brazil as compared with 0.17% in those vaccinated subcutaneously. In addition, Cluver (1929) reported that the oral immunization method was effective in controlling typhoid fever among laborers in the South African gold mines.

The inadequacy of oral immunization based on serological or mouse-protection tests has been reported by various workers (Tuft, Yagle and Rogers, 1932; Valentine et al., 1935; Downs and Bond, 1937; Elledge, Kennedy and Cumming, 1943). Moreover, Hoffstadt and Thompson (1929a, 1929b) and Hoffstadt, Martin and Thompson (1929) made a careful study of the antibody response to oral immunization and found that, although there

was a definite response produced by this method, titers were definitely lower than resulted from subcutaneous injection of vaccine.

The degree of antibody response to intracutaneous vaccination has also been studied by many investigators. Most workers (Tuft, 1931; Valentine, et al., 1935; Perry, 1937; Siler and Dunham, 1939; Naumer and Nerb, 1943) have reported that the antibody response is approximately equal to or greater than that obtained by the subcutaneous route even when the dosage is reduced markedly. Longfellow and Luippold (1940) and Luippold (1944) reported extensive studies of the response to intracutaneous immunization based on mouse-protection tests. These authors suggested the method was adequate for restimulation of previously vaccinated individuals but did not recommend the procedure for initial immunization. Presumably the major value of the intracutaneous injection procedure is that fewer systemic reactions occur than are obtained following subcutaneous injections of the larger amounts of vaccine. However, it is interesting to note that Luippold (1944), though admitting local and systemic reactions are more frequent and pronounced following administration of the standard subcutaneous doses of TAB vaccine than following the intracutaneous injection of one-tenth or one-fifth of these doses, felt that the risk of any lowering of protection was not worth the questionable reward of milder reactions. This author further reported that under the best conditions of immunological response to artificial immunization it was not expected that the standard method of vaccination would be adequate at all times in the field to the challenge

of infecting organisms. Adoption of the intracutaneous injection of reduced doses of TAB vaccine, according to this author, would seem to increase this inadequacy.

Information concerning the duration of effective immunity following vaccination is probably best obtained from data on the incidence of typhoid fever in former military or similar personnel exposed to a common risk with non-immunized individuals. Employing this basis it appears that the duration of effective immunity is at least 2 or 3 years (Pryor, 1928; Bowdoin and Petrie, 1940; Siler et al., 1941) and may be even 5 or more years (Halliday and Beck, 1928). For further discussion concerning the duration of effective immunity, please see Part B of the Literature Review.

Several workers (Davison, 1918; MacLean and Holt, 1940; Kolmer, Bond and Rule, 1942; Hitch, Ashcroft and Green, 1943) have reported on the use of polyvalent immunizing agents of which typhoid vaccine was a component part. In general, it appeared to these investigators that the inoculation of such mixtures does not subtract from the immunizing potency of any one component; in fact, immunogenicity may even be enhanced. On the other hand, the relative protection against typhoid effected by monovalent typhoid vaccine as well as polyvalent typhoid-paratyphoid vaccine apparently has not been established with certainty. Some indication that response to the polyvalent vaccine was less than the monovalent vaccine was suggested by Longfellow and Luippold (1943). However, it is interesting to note that their reported difference was slight

and was not observed in a controlled comparison. Therefore, their findings should be considered inconclusive.

With regard to the immunogenically important components of S. typhosa, both the somatic O and the Vi antigens are considered responsible for the production of protective antibodies in animals and man.

Much effort has been made to extract specific chemical fractions from the typhoid bacillus in hope of obtaining a fraction suitable for use in active immunization. To be of practical value such a fraction should be stable, nontoxic, readily soluble, and of such nature as to permit its preparation in large quantities. At least partial attainment of the above objective has been reported by several investigators (See Part IV, Literature Review). The products obtained by these workers were more or less similar lipopolysaccharides of low nitrogen content even though the methods employed in extracting the antigenic fractions were different. In general, these products reacted with specific antisera and appeared to be strongly antigenic. In addition, some of these extracts have been shown to produce active immunity against typhoid fever in mice and to stimulate production of mouse-protective antibodies in man. Unfortunately, many of these antigenic fractions are highly toxic and efforts to detoxify these products will be presented in Part V of the Literature Review.

The discovery by Felix and Pitt (1934) of the Vi antigen resulted from their study that strains freshly isolated from typhoid patients often were inagglutinable and that such strains were highly virulent

for mice; thus the name Vi. The mechanism of the virulence-enhancing action of the Vi antigen has not been completely established but it has been suggested by some investigators (Bhatnagar, 1935; Felix and Bhatnagar, 1935; Felix and Pitt, 1951a) to be the result of a protective action of the antigen against phagocytosis. The Vi antigen is different from and independent of the somatic O antigen (Felix and Pitt, 1935). Following injection of living Vi strains into rabbits, a specific Vi antibody is produced in a relatively low titer. The microorganisms rich in Vi antigen are agglutinated by sera containing Vi antibodies but not by sera containing only specific anti-O agglutinins. Gladstone (1937) found that retention of the Vi antigen during propagation in culture media is dependent upon the utilization of glucose or similarly complex carbohydrates while the O antigens apparently can be synthesized from simpler carbon compounds.

Although the Vi and O antigens are chemically distinct, they are difficult to separate chemically (Topley et al., 1937; Henderson, 1939a; Henderson, 1939b). In addition, the Vi antigen is more sensitive to heat than is the O antigen. That is, unlike the O antigen, the Vi antigen is rapidly destroyed when heated at 100° C, and heating at 60° C for 30 minutes may also destroy it. Moreover, the immunizing quality of the Vi antigen is so altered by formalin that the resultant antibody produced against it is deficient in protective potency while retaining its agglutinating properties (Felix and Bhatnagar, 1935; Felix and Petrie, 1938). Felix and Bhatnagar (1935) found that the immunogenicity

of the Vi antigen is essentially destroyed by 0.5% phenol. However, Bensted (1940) failed to confirm their findings and concluded further that both the O and Vi antigens are necessary for full protective immunity.

Felix and Pitt (1936) observed that treatment with alcohol destroyed the specific Vi agglutinability of Vi strains but did not impair their ability to stimulate production of Vi antibodies in rabbits. It was on this basis that Felix and his coworkers (1951b, 1951c) developed the alcohol-killed and alcohol-preserved typhoid vaccine for immunization of humans. There is little doubt that this product can stimulate the production of specific Vi antibodies in a greater proportion of persons than does the heat-killed, phenol-preserved vaccine such as used today by the United States Armed Forces. Although the antibody response to the latter type of vaccine is predominantly of the anti-O type (Siler, 1941), there is no denying the fact that the most successful experiences to date in mass immunization per se (see below) have been accomplished with the heat-killed, phenol-preserved vaccine (Siler, 1941; Holt, 1948). However, as was suggested by Luippold (1946), it may well be that future research will indicate the possibility of modifying production procedures so as to retain the Vi antigen in an unaltered state or that it may be advisable to fortify the presently used vaccine with extracts containing Vi antigen.

In recent years, Webster, Landy and Freeman (1952) and Landy and Webster (1952) have reported on the purification and immunological properties of a purified Vi antigen derived from Escherichia coli

5396/38. Their method for extracting this antigen from acetone dried organisms was an ethenol-salt fractionation procedure followed by acetic acid hydrolysis. The final product was a fraction free of contamination with E. coli somatic antigen and showed a high degree of serological activity and specificity especially when absorbed on either colloidion particles or on erythrocytes. Furthermore, this purified Vi antigen was markedly effective in the protection of mice against challenge with virulent strains of S. typhosa. Later observations by Landy (1954) on the immunological response of human beings to this purified Vi antigen showed that, of a total of 432 subjects given a single subcutaneous injection of 0.04 mg of antigen, 95% developed Vi antibody which persisted for periods up to 2 years. This was in contrast to the rapid disappearance of Vi antibody following recovery from typhoid fever.

In 1953 Landy (1953) described the preparation and standardization of a dehydrated typhoid vaccine from acetone-killed and dried S. typhosa and paratyphoid components. From estimates of absolute and relative mouse protective potencies of dehydrated and of heat-killed, phenol-preserved vaccines it was shown that the dehydrated vaccine was markedly superior in this respect. Moreover, the dehydrated vaccine stimulated the production of Vi antibody in rabbits. Passive mouse protection tests on the sera of rabbits immunized with the two products respectively revealed the immunogenic superiority of the dehydrated over the heat-phenol vaccine. It was further reported that the stability of the

dehydrated vaccine was observed following storage in the dry state at 50° C for 1 year with no significant loss in Vi immunogenic activity. Evidence that the dehydrated vaccine stimulated the production of Vi antibody in man was later reported by Landy et al. (1954). They found that of the vaccines tested in man all gave good O agglutinin titers, whereas the Vi antibody levels were limited with the heat-phenol-vaccine but considerably higher for the acetone-dehydrated product.

More recently, a series of papers from the Walter Reed Army Institute of Research (Edsall et al., 1954; Spring et al., 1956; Edsall et al., 1960) have reported on the studies of infection and immunity in experimental typhoid fever employing the chimpanzee as the experimental animal. Detailed clinical observations, stool and blood cultures, and serological studies confirmed the impression that the disease produced in these animals closely resembled the mild form of human typhoid fever frequently seen in childhood. Furthermore, gross and histologic examination of intestines, mesenteric lymph nodes, liver, spleen, and other organs of orally infected chimpanzees demonstrated pathological findings that are essentially indistinguishable from those seen in mild typhoid fever in man. Moreover, the clinical symptoms observed in these animals ranged from moderately severe illness, through transitory illness, to afebrile infection with or without bacteriemia, occasionally leading to the development of persisting biliary infection and the carrier state. Thus the range of illness observed in

chimpanzees resembled that seen in man except that the severe and complicated forms of typhoid fever were not observed in the chimpanzee. This production of the spectrum of typhoid fever in the chimpanzee, according to these authors, had made it possible to study the basic problems in this disease which are not easily defined through the use of prevailing laboratory techniques.

Further studies were made by Gaines and his coworkers (1956, 1961) on the efficacy of various antityphoid immunizing agents in immunizing chimpanzees against typhoid fever produced by feeding viable S. typhosa. These workers found that both acetone-killed (Landy) and heat-killed, phenol-preserved typhoid vaccines were effective in protecting against infection produced by either homologous or heterologous strains of typhoid bacilli. On the other hand, purified O antigen (phosphorylated lipopolysaccharide) induced no discernible protection, whereas some immunity was afforded in the chimpanzee by the administration of purified Vi antigen (Webster).

No doubt the most thorough investigations to date of the immunizing efficacy of the various typhoid fever vaccine preparations in man have been the series of field trials conducted under the auspices of the World Health Organization. The first of these field studies was organized in 1954 in the town and district of Osijek, Yugoslavia (Yugoslav Typhoid Commission, 1962). One-half of a single large pool of bacterial harvest of S. typhosa, strain Ty 2, was processed by the alcohol method of Felix (1941), and the other half was inactivated by

heating at 56° C for one hour and preserved with 0.5% phenol. These vaccines were given, together with control vaccine made with Shigella flexneri, on a double-blind, strictly randomized basis. The three groups, each consisting of approximately 12,000 persons, were injected subcutaneously with two doses of vaccine at a three-week interval. The first dose was equivalent to 750 million and the second to 1500 million organisms. A booster dose equal to the first dose was given after one year to 75% of the volunteers. During the first two years, 31 proven cases of typhoid occurred in the control group, 23 in the group given alcoholized vaccine, and only 9 in the phenolized vaccine group. The latter preparation evoked significant protection (approximately 70%) particularly in children 5 to 14 years old. In addition, no increase in protection was observed in those who received the booster injection one year later, and comparable protection was still afforded in 1958. Moreover, preliminary serological results suggested a significantly higher titer of Vi antibodies in those who had received the alcoholized vaccine.

Reactions to inoculation in the primary course of immunization, in which local and systemic reactions were recorded in 640 persons chosen at random, were studied in 1954. Each person was visited 24, 48 and 72 hours after each of the two injections. Although it was not possible to observe much difference in the reactions between the vaccines, it was generally observed that reactions were not severe. However, the reactions to the second injection were generally somewhat

greater and more frequent than those to the first one. Five persons developed sterile cysts, as no bacteria were found in their contents, at the inoculation site.

In 1956, an additional study was carried out among military recruits in Zagreb, Yugoslavia where typhoid fever was not active. The immunization schedule, employing another lot of these same vaccines, consisted of three doses of 750 million, 1500 million, and 750 million organisms each at three-week intervals. Serial bleedings were obtained from 377 men representing the three vaccine groups. Specimens were obtained before vaccination, three weeks after the first dose of vaccine, three weeks after the second dose, immediately prior to revaccination which was done three months after completion of initial immunization, and three weeks after the revaccination. The results of these studies revealed that the H-agglutinin titers were consistently and significantly higher in the group given the phenolized vaccine. In addition, the O-agglutinin titer was higher soon after phenolized vaccine was given, whereas after five months the difference in O antibodies was not significant in the Osijek group. In both the Osijek and the Zagreb studies, the mean Vi antibody level was slightly higher after primary immunization with phenolized vaccine, but after several months the Vi-antibody titers were higher in those vaccinated with the alcoholized vaccine. It is of interest that Edsall et al. (1959) confirmed the superiority of this alcoholized vaccine used over the phenol-preserved product in employing the usual mouse protection tests (active and

passive) and in the production of Vi hemagglutinins in rabbits. However, as the studies show above, in man, the protection afforded by the alcoholized vaccine was inferior to that observed with the phenolized vaccine.

Further evidence suggesting that high Vi antibody levels appear not to have any effectiveness in protecting man against typhoid was seen in the 1960 field trials in British Guiana and in Yugoslavia. In British Guiana (Ashcroft, 1963, 1964; Ashcroft, Ritchie and Nicholson, 1964), a total of about 72,000 children, 5 to 14 years of age were immunized on a random basis with two doses of vaccine, given three to five weeks apart. Each dose consisted of 0.5 ml (500 million organisms) of either an acetone-inactivated and dried typhoid vaccine (Landy, 1953), a heat-killed, phenol-preserved vaccine, or tetanus toxoid control. The effectiveness of these preparations in protecting against clinical disease revealed that five cases of typhoid fever occurred over the next three years in the acetone-inactivated vaccine group, and 23 in the heat-phenol-inactivated vaccine group, as compared with 83 in the control group, indicating an effectiveness of 93% and 75% respectively. In groups of over 3300 each, the protection given by a single dose was of the same order as that given by two; that is, the disease incidence was 0.3 and 14 cases, respectively.

The 1960 field study in Yugoslavia (Yugoslav Typhoid Commission, 1964) was carried out simultaneously with the study in British Guiana and tested the same vaccines. Two doses of vaccine were given four

weeks apart. Groups of approximately 6500 children each, ages 2 to 14, received 0.5 ml doses of either the acetone-inactivated and dried vaccine or the heat-killed, phenol-preserved vaccine or the tetanus toxoid control. In addition, three groups of approximately 8700 each within the age range 15 to 60 years were given two 1 ml doses totaling 2000 million organisms. Two years after immunization an outbreak of typhoid fever occurred in Pristina and was the consequence of a three-week contamination of the city water supply. Among the 5000 volunteers in each group in this community, 16 cases of blood-culture-positive typhoid fever occurred in the acetone-killed vaccine treated group, 37 in the heat-phenol vaccine treated group, and 75 in the control group, indicating 79% and 51% effectiveness, respectively, in the prevention of typhoid fever.

The relative antigenic potency of the above vaccine preparations was estimated by the usual serological tests. These tests involved approximately 100 children who were bled two or more weeks before inoculation and again two weeks after the second dose of vaccine had been given. The typhoid vaccines had been administered at a normal dose level of 0.5 ml followed by a second dose three weeks later. Each dose contained approximately 500 million typhoid bacilli. The results of these serological tests revealed that the acetone treated vaccine elicited a significantly greater anti-H response, while the anti-O response was better after immunization with the heat-killed, phenolized vaccine. Moreover, no difference was found in Vi response

whether measured by direct bacterial agglutination or by hemagglutination of red blood cells sensitized with the Vi antigen.

Reactions to inoculation were studied in 1,265 vaccinated persons in Bitola in which examinations were made on the first, second, third and fourth days at about 10, 24, 48, and 72 hours after inoculation. No real difference was seen between the reactions to the acetone-dried and the heat-phenol vaccines. Moreover, no differences in reactions were apparent between these vaccines and the alcoholized and heat-phenol vaccines used in the first Yugoslav field trial. In addition, the reactions to the acetone-dried and to the heat-phenol vaccines during the later trials were very similar. While there were more reactions to the first injection of heat-phenol vaccine than to the first acetone-dried vaccine injection, it seemed that the second dose of acetone-dried vaccine gave rise to more reactions than the second dose of heat-phenol vaccine. However, reactions to the second dose were significantly milder than to the first dose of either of the vaccines.

Preliminary results of the field trials carried out during 1961 in Poland in which over 800,000 individuals were vaccinated with several different preparations were reported by Benenson (1964). The typhoid vaccines employed for this study were prepared by the following techniques: acetone-inactivation and drying (vaccine P), formal-phenol (vaccine N), Westphal (vaccine S), and Grasset-Slopek (vaccine T). Tetanus toxoid was again used as the control preparation. Early results suggested that the acetone-inactivated and formal-phenol vaccines were

both highly effective in protecting against typhoid. Furthermore, serological tests showed that the acetone-inactivation technique provoked the highest H-agglutinin response, despite the fact that only one-half as many bacteria were injected as compared with the other preparations. The O-agglutinin response to the formal-phenol preparation, when compared to the other preparations, was significantly higher, while the Vi antibody responses of all the vaccines were not significantly different. The antibody responses to the other two preparations were worthy of note also according to this author. The alum-absorbed extract prepared by the Grasset-Slopek technique did not differ from the acetone-inactivated vaccine in Vi and O responses, but the H antigenicity was suppressed. On the other hand, the Westphal vaccine, which is essentially a pure somatic substance, yielded an insignificant rise in O antibodies with no change in H- and Vi-antibody titers.

As previously mentioned, the alcohol method had been developed by Felix and his coworkers (1941, 1951b, 1951c) because it stabilized Vi antigen and was superior to other vaccines in protecting mice against intraperitoneal challenge with S. typhosa. However, as the field trials in Yugoslavia show in which the hypothesis was tested that antibodies against the Vi antigen were the protective substance for man, the protection afforded by the alcoholized vaccine was inferior to that provided by the phenol vaccine (Cvjetanović, 1957). Although the 1960 field trials in Yugoslavia showed no apparent difference between the two vaccines in the Vi-antibody levels, the protective difference in

the two preparations in man was significant. Thus, at least as far as these two field trials are concerned, the results (Benenson, 1964) argue against the concept that the titer of Vi antibodies measures the immunity of man against infection with the typhoid bacillus.

The level of O-agglutinins, as a measure of protection in man against typhoid fever, has apparently been abandoned by the World Health Organization as a result of these field trials (Benenson, 1964). This decision follows from the elevated O-agglutinin response in only those individuals receiving the phenol vaccine during the 1954 field trial, whereas, in 1960, the O-agglutinin response was superior only in those who had received the alcoholized vaccine.

Benenson (1964) further reported that, in the 1954 Yugoslav trials, the phenolized vaccine was superior to the alcoholized product only in its ability to evoke in man and rabbits a higher H-agglutinin level. In the 1960 field trials, both the acetone and the heat-phenol preparations were equally effective in protecting man against typhoid in British Guiana. In the Yugoslav studies, the phenol vaccine was effective in children, but only the acetone-inactivated vaccine was equally protective to individuals of all ages. According to this investigator, the outstanding characteristic of the acetone-inactivated vaccine is its H antigenicity. That is, in two conclusive studies, the H antigenicity appeared to be the attribute of the vaccine which correlates best with protection of man against infection with S. typhosa. Moreover, in the Polish studies, the acetone-inactivated vaccine was

again predominant in H antigenicity, and preliminary data indicated that this and the formal-phenol vaccines are the most protective.

The concept that the H antibody is synonymous with resistance to infection by S. typhosa is still difficult to evaluate at the present time. Tully and Gaines (1961) confirmed the work of many others in that they were unable to implicate H antigen or antibody in typhoid infection of the mouse. Furthermore, Tully, Gaines and Tigertt (1963) were unable to protect chimpanzees against a typhoid challenge by exposure to either a typhoid strain containing H but no Vi or O antigen (Mrs. S. Rough strain) or an acetone-inactivated and dried Mrs. S. Rough vaccine preparation, despite high anti-H titers. And, finally, the frequency of human relapses in the presence of high H-agglutinin titers suggests that a correlation between immunity to typhoid fever and H-antibody levels is highly unlikely.

IV. A HISTORIAL REVIEW OF METHODS EMPLOYED IN THE EXTRACTION OF ENDOTOXIN

The major connotation of the term "endotoxin" is a substance found in most all gram-negative bacteria which appears to be a structural component of the organism. Indeed, a body of information exists suggesting that the cell wall material of smooth strains of gram-negative bacteria are the major sources of endotoxin. Endotoxin is found in culture filtrates of gram-negative bacteria as a consequence of bacterial lysis. Van Heyningen (1950) suggested that this endotoxin acts

on animals in the same way without regard to the bacterial source. In marked contrast to the exotoxins, the endotoxins are heat stable and not destroyed by alcohol at room temperatures. The chemical characteristics of endotoxin, according to the pioneer work of Boivin (1933), are largely those of a phospholipid-polysaccharide complex which has been identified serologically as the O or somatic antigen. These O antigens have been extracted from dysentery organisms (Boivin and Mesrobeanu, 1937; Morgan, 1937; Morgan and Partridge, 1941; Goebel et al., 1945; Smolens et al., 1946; Barnes et al., 1947) as well as the salmonella group (Boivin et al., 1933; Walker, 1940; Palmer and Gerlough, 1940; Freeman, 1942; Roberts, 1949; Webster et al., 1955; Westphal, 1957; Ribi et al., 1961; Haskins et al., 1961; Haskins et al., 1963). Also, antigens of this same general type have been found in brucella (Miles and Pirie, 1939), meningococcus and gonococcus organisms (Boor and Miller, 1944; Mergenhagen et al., 1963). In animals and man O antigen or endotoxin has been found to produce a wide variety of effects including the production of fever, leukopenia, nonspecific enhancement of resistance and the Shwartzman reaction (Boivin et al., 1933; Boor and Miller, 1944; Hook and Wagner, 1959; Atkins, 1960). Repeated injections (Beeson, 1947a; Tennent and Ott, 1953) of endotoxin in rabbits induced tolerance to the fever effect, whereas in tumor bearing mice (Shear and Turner, 1943) endotoxin was shown to produce hemorrhogic necrosis within the tumors.

Several O antigen isolation procedures have been described since

that first reported by Boivin et al., (1933). Their trichloroacetic acid extraction procedure enabled them to isolate toxic and immunizing fractions from many gram-negative organisms. Their method of isolating endotoxin from enterobacteriaceal organisms was followed by many other extraction procedures. Using ethanol extraction after trypsin digestion, Raistrick and Topley (1934) were able to isolate antigenic material from a strain of Salmonella typhimurium. Their chemical findings suggested a substance very similar to that of Boivin and his colleagues using trichloroacetic acid on the same organism. Later, Morgan (1937) employed an extraction procedure which could be carried out at a neutral reaction and at low temperatures. This investigator was able to isolate a specific antigen which was associated with the "smooth" form of Shigella dysenteriae (Shiga). Depending upon the solubility of antigenic complexes in organic solvents, he was able to extract at 37° C an antigenic material with diethylene glycol followed by fractional precipitation of the active material from the aqueous solution by acetone or ethanol. This antigenic material was capable of inducing an anti-bacterial immunity response in mice which was qualitatively identical with that produced by the specific antigen of the intact microorganisms.

Walker (1940), on the other hand, reported a method for the isolation of toxic and immunizing fractions from bacteria of the salmonella group employing aqueous solutions of urea. He found these substances to contain a large amount of carbohydrate with traces of protein. Also, these substances were highly toxic and, in addition,

could confer active immunity in mice.

By employing an alcoholic precipitation procedure (sodium acetate and 95% ethanol), Morgan (1941) reported that an antigenic material had been prepared from cultures of S. typhosa grown on a synthetic medium. This investigator further reported that, in sub-lethal amounts, this purified antigen was able to induce leukopenia and fever in rabbits as well as leukopenia in guinea pigs, rats and mice. Moreover, this antigen was able to induce the Shwartzman reaction in rabbits when employed either as the preparatory or eliciting agent. Later, Morgan (1945) found that this purified antigen as well as those prepared from cultures of Salmonella paratyphi and Salmonella schottmuelleri produced less (local and constitutional) reactions than those produced by the TAB bacterial vaccine when injected subcutaneously in man. These data and others suggested to this author, that these purified antigens possessed the advantages of potency, stability and compactness.

A few of the early investigators developed methods for extracting antigenic polysaccharide substances from bacteria other than the entero-bacteriaceal organisms. Fuller (1938) employed the formamide procedure for the extraction of polysaccharides from hemolytic streptococci. It was indicated by this author that this method had the advantage over the existing methods of obtaining antigenic substances from organisms by completely dissolving the bacteria thereby producing potent extracts as well as removing protein substances which might give cross-reactions. In addition, he suggested that this method might be applicable to all

species of bacteria. Using suspensions of "smooth" Brucella melitensis, Miles and Pirie (1939) were able to obtain with both chloroform-water and phenol a substance which appeared to contain the antigenic components of the whole organism. They designated this substance as a phospholipid (PL), amino-polyhydroxy (AP), protein-like (S) complex or PLAPS. Extracting with organic solvents to remove the lipids and acetic acid to remove the protein-like material respectively, they obtained the resulting material called amino-polyhydroxy (AP). They considered AP analogous to the SSS that had been isolated from S. typhimurium, S. typhosa, and Sh. shigae as well as the somatic antigen isolated by Boivin et al. from a number of gram-negative bacteria. In a later paper, Boor and Miller (1944) reported obtaining a carbohydrate-lipid fraction from both the gonococcus and meningococcus organisms. Extracting with trichloroacetic acid, they were able to show an extractable, toxic antigenic product which varied in amount with species, strain, and physical and chemical pre-treatment of cells. However, no difference was found by these authors in the toxicity of like quantities of glucolipid from various strains of gonococci and meningococci irrespective of virulence. Recently, Mergenhagen et al. (1963) described some of the properties of an endotoxin isolated by phenol-water extraction and further purified by differential ultracentrifugation from a Group C Neisseria meningitidis strain. Their lipopolysaccharide contained 20% lipid with polysaccharide constituents identified as glucose, galactose, glucosamine and sialic acid. In addition, their product contained

less than 1% protein, and less than 1% nucleic acid. Analytical ultracentrifugation and the separation of lipopolysaccharide and of capsular polysaccharide by gel filtration provided evidence that sialic acid formed an integral part of the biologically active lipopolysaccharide.

Different ratios of phenol and water have been utilized by many investigators for the extraction of antigenic substances from enterobacteriaceal organisms. Palmer and Gerlough (1940) used repeated extractions of acetone dried typhoid bacilli (Panama carrier strain) with 95% phenol. They obtained a final product which gave strong precipitin reactions with antityphoid rabbit serum. In addition, they reported that a single intraperitoneal injection of 0.4 mg killed 50% of 40 mice within 24 hours, whereas intravenous injections of 0.1 mg in rabbits produced an average maximum elevation of temperature of 2.2° F (2 of the 10 rabbits died within 2 hours after injection). The first use of a 90% phenol and water solution for the removal of a water-soluble antigenic complex isolated from a "smooth" culture of Sh. dysenteriae (Shiga) was reported by Morgan and Partridge (1941). In addition, by this procedure they obtained an approximately equal amount of a non-specific, water-insoluble simple protein. However, similar extraction of the corresponding "rough" variant yielded no antigenic material but only a considerable quantity of the simple protein. When tested in rabbits, these investigators found the material extracted from the "smooth" strain to be a powerful "Shiga" antigen. Goebel and Barry (1958), in a paper reporting on a substance having colicine K

activity, found that 90% phenol could be used to dissociate the lipopolysaccharide-protein complex (O antigen) of E. coli K_{235} to yield a protein-like constituent bearing the colicine K activity. The lipopolysaccharide portion of the complex comprised 80% of the original complex. In an earlier paper Goebel et al. (1945) used an aqueous pyridine solution (50%) for the extraction of the Type V specific antigens in the Flexnor group of dysentery bacilli. They found that their yield of antigen varied considerably depending upon the type of organism as well as the strain employed.

Using the same group of organisms, Smolens et al. (1945) employed an HCl acid extraction procedure (56° C for 20 hours) which also proved successful in obtaining the somatic antigen. Their experiments with this apparent somatic antigen indicated an extremely potent antigen as well as being the principle carrier of the toxicity of the whole organism. However, they concluded from their immunization studies using both man and animals that their isolated somatic antigen did not appear to be superior to the killed whole bacterial cell as a vaccine against bacillary dysentery. In describing a simple and effective method, Roberts (1949), reported that water suspensions of E. coli when heated at 80° C for 60 minutes could also yield a toxic and pyrogenic material apparently identical with the O antigen.

The separation, characterization, and biological significance of a common antigen found in enterobacteriaceal organisms has been recently described by Kunin (1963). By employing DEAE cellulose chromatography,

this investigator has been able to isolate this antigen from closely associated endotoxin fractions. Unlike endotoxin, however, this purified common antigen failed to coat erythrocytes, was non-antigenic, and was composed primarily of polysaccharide and polypeptide. The author indicated that the most important property of this antigen thus far determined was its interference with the specificity of the hemagglutination test commonly employed to measure antibody to O antigen of Enterobacteriaceae. Moreover, this purified common antigen may have some significance in classification of this family of bacteria.

Classically, the products obtained by these procedures are usually complexes consisting of varying amounts of proteins, lipids, and polysaccharides. They all apparently evoke the characteristic endotoxin reactions, but differ widely in their potency on a weight basis especially their capacity to produce lethal effects. Since the pioneer work of Boivin, little progress has been made in determining the composition and structure which elicits the characteristic reaction of endotoxins in mammals. In recent years, however, Landy and his associates (Webster et al., 1955; Landy et al., 1955; Landy and Johnson, 1955) described a method for the further purification of the O antigen from a strain of S. typhosa employing a series of fractionations using ethanol in high salt concentrations, ammonium sulfate, and ethanol respectively for the isolation of a phosphorylated lipopolysaccharide component essentially free of protein. They were able to show by immunological studies that when a somatic antigen is freed of protein, the resultant lipopolysaccharide

has an increased antigenic activity in mice, rabbits and men. All evidence, including quantitative immunochemical procedures revealed that the immunologically active form of the somatic O antigen was the lipopolysaccharide component. These investigators further reported that microgram amounts of this protein-free lipopolysaccharide were capable of producing the various biological phenomena generally attributed to endotoxins.

Employing heated phenol-water mixtures (approximately 1:1 by weight), Westphal and his colleagues (1957) were able to obtain a lipopolysaccharide fraction from a strain of E. coli which contained neither protein nor nucleic acid. Moreover, they reported that the yield of their material was in the range of 2 to 3% of the whole dry weight of the bacteria. In addition, their product was electrophoretically homogeneous as well as being highly pyrogenic.

Because of the association of lipid and polysaccharide in endotoxin, and the generally accepted idea that the protein moiety is not essential, there exists a current tacit assumption that these two components are responsible for the toxic and antigenic properties respectively. From such lipopolysaccharide complexes, Westphal and Luderitz (1954) showed that chloroform-soluble material obtained by hydrolysis of bacterial lipopolysaccharides with dilute hydrochloric acid retained some of the endotoxic properties of the original material. Therefore, they postulated that the lipid component (lipid A) was responsible for the endotoxic activities of lipopolysaccharides. Moreover, Westphal and his associates

(1954, 1957) further suggested that for lipid A to exert endotoxic effects, it must be suitably dispersed in an aqueous medium, and they further predicted that were it possible to disperse lipid A in water comparable to its original state when bound to the polysaccharide carrier, it would display activity similar to that of endotoxin. Experimentally, they found that lipid A exerted one-fifth the potency of the original endotoxin when it was dispersed by detergents or coupled to an inert protein. However, Landy and Ribi and their associates (Ribi et al., 1961; Haskins et al., 1961) have accumulated evidence against the concept that lipid A is responsible for the biological effects of endotoxin. They obtained endotoxins prepared by the cold aqueous-ether procedure from Salmonella enteritidis which contained a much reduced content of lipid without any lowering of its pharmacologic or antigenic activity. In addition, parallel quantitative bioassays of both lipid A and the endotoxins from which they had been derived revealed that these lipids contained less than 1% of the potency of intact endotoxin.

In support of the idea that lipids are not responsible for the biological effects of endotoxin, Tauber et al. (1961) presented data that the lipid moiety of endotoxin could be removed by hydroxylaminolysis. They noted marked differences between endotoxins prepared by the phenol-water method from 3 different species of gram-negative bacteria. After hydroxylaminolysis, however, the differences between these 3 species were retained in spite of the loss of the lipid component from the endotoxins. In later studies, Creech, Breuninger and Adams (1964)

made determinations of the chemical composition and antitumor properties of several polysaccharide fractions obtained by high speed centrifugation of aqueous extracts of the cells of Serratia marcescens following phenol extraction and trypsin digestion. According to these investigators, the use of methods that insured complete removal of lipid gave polysaccharides containing firmly bound amino acid residues. These polysaccharides were highly effective in causing complete regression of tumors in mice. They further reported that the products derived primarily from the cell wall were more potent than the polysaccharides of extracellular and cytoplasmic origins. In addition, extensive differences were noted in the chemical composition of the polysaccharide complexes isolated from the two sources.

On the other hand, Nowotny (1963b) described several methods for the extraction of lipids from their endotoxic O antigen from a strain of Serr. marcescens. Best results were obtained with dilute formic acid. He found that this crude lipid mixture, when analyzed by paper chromotography, consisted of at least 16 different components. These and other data comparing the chemical structure of the liberated lipid with that of the lipid intact in the cell wall suggested to this investigator that there is still no method that will isolate all the lipid components intact and free from other constituents of the endotoxic O antigen.

Recently, Haskins et al. (1963) reported on simplifying the procedure for purifying the S. enteritidis endotoxin complex. By

treating the aqueous ether extract of S. enteritidis with phenol-water, they were able to reduce the nitrogen content from as much as 6% to approximately 0.5%, while most of the lipoidal material (initially as much as 6-8%) was removed by treatment with $LiAlH_4$ (lithium aluminum hydride). Their end product was a high molecular weight polysaccharide which contained only small amounts of fatty acids, nitrogenous material and phosphorous.

That an aqueous ether extract of S. enteritidis endotoxin undergoes extensive changes when subjected to treatment with 0.1 N acetic acid was reported also by Haskins et al. (1961). They were able to show a marked reduction in the biological potency which occurred within minutes after such treatment. This reduction in biological potency continued progressively and after 60-90 minutes almost all of the original endotoxic activity had been abolished. These investigators hypothesized that a complex of critically large size was essential and that the destructive effect of 0.1 N acetic acid represented a splitting of bonds by which particles of the size of haptene are held together. On the other hand, they hypothesized that the major endotoxin properties were due to some small moiety whose attachment to a macro-molecular carrier was essential for activity. To substantiate these hypotheses, Ribi et al. (1962) extensively investigated the immunochemical and physiochemical changes of the S. enteritidis endotoxin complex when subjected to hydrolysis with 0.1 N acetic acid. Samples of the product of hydrolysis, removed at various intervals, were examined in gel diffusion tests, in

the optical ultracentrifuge, and were also assayed for biological activity. Their gel diffusion tests revealed evidence of a progressive increase in the concentration of haptene during the exposure of endotoxin to acid. In the ultracentrifuge, the starting endotoxin showed as a single entity. However, as hydrolysis progressed, a second much slower moving boundary appeared. In addition, they were able to show that as the area under the fast moving endotoxin boundary decreased, the area under the slow moving boundary increased correspondingly. Further, their bioassays (lethality for BCG treated mice, resistance to infection, tumor damage, and pyrogenicity) showed that the degree of hydrolysis of the parent endotoxin and the per cent destruction of biological activity were approximately paralleled. The results obtained by these investigators were consistent and revealed that the rate at which host reactive properties were abolished paralleled the rate of dissociation of the endotoxin complex into haptenic units, particles whose size was of the order of 1/100 that of the original endotoxin.

The separation and purification of Vi and O antigens of S. typhi employing a column precipitation technique has been reported recently by Griffiths, Diena and Greenberg (1963). These authors reported that both Vi and O antigen fractions have been obtained in purified as well as separated forms employing alcohol fractionation as well as salt fractionation of the crude extract on an inorganic column bed. Alcohol fractionations alone neither separated nor purified the antigens significantly. However, when cetyl pyridinium chloride was maintained

in constant concentration in the elution solvents, the Vi and O serologically active materials could not be eluted by the usual alcohol-water solvents, but could be removed in separated and purified forms by the addition of $MgCl_2$ to the above alcohol-water solvent.

In other recent studies, Nowotny and coworkers (1963a), in trying to relate structure to function in somatic antigens involving the use of six different methods for the extraction of endotoxin from three different enterobacteria, reported that not one of the methods can be applied generally for all gram-negative O antigen isolations. The different tribes of Enterobacteriaceae, as investigated by these workers, each required a different isolation procedure. Studies of the chemical composition of their preparations revealed many differences with no correlation between the chemical components and lethal toxicity or their serological reactivity. They concluded that the structural components or properties responsible for biological activity cannot be detected by gross chemical analysis.

The data and results reported above by the various investigators and especially the results reported by Nowotny and coworkers apparently suggest that some of the differences of opinion between various scientific groups may be due largely to the individual properties of the particular bacterial strain under study. That this observation might prove to be true is seen from the elegant studies of Osborn *et al.* (1964). According to these investigators, the use of mutants of *S. typhimurium* in which biosynthesis of specific lipopolysaccharide precursors is blocked has

made possible both biosynthetic studies and structural analyses which provide the basis for the structure of the core polysaccharide. These workers found that the simplest mutant, which is unable to synthesize UDP-glucose, forms only the backbone structure, containing heptose, phosphate, and keto-deoxy-octonate. To this backbone are attached side chains containing glucose, galactose, and N-acetylglucosamine. The resulting core structure is found in the lipopolysaccharide of the rough strain, as well as in that of the GDP-mannose-deficient mutant. On the other hand, long O-antigenic chains composed of repeating units containing galactose, mannose, rhamnose, and abequose are linked to the core in the wild type organism. These authors further found that no mutants have thus far been detected which lack the backbone or lipid portions of the lipopolysaccharide. Furthermore, it was thought that these parts of the lipopolysaccharide might play an essential role in the physiology of the organism.

The presence of substantial amounts of nucleic acid in certain endotoxic extracts was shown by Ribi (1964a, 1964b) and Milner (1963) and their associates as evidence that some cellular mechanism facilitates the transfer of protoplasmic constituents into the cell wall extracts. They noted that the integrity of the cell wall was maintained upon extraction with either TCA or with aqueous ether, whereas extraction with phenol-water resulted in rupture of this structure with the consequent mixture of protoplasm and cell wall components. They further noted that, although neither TCA nor aqueous ether appear to rupture

cell walls, nucleic acids, under some circumstances, can pass into the endotoxic extracts. With these experimental observations in mind, the possibility was recognized that proteins, peptides, lipids, and/or carbohydrates from the protoplasm might, during the process of extraction, form stable complexes with endotoxic material from the cell wall at the moment they become solubilized, thus giving misleading information regarding the chemical composition of bacterial endotoxins. To gain further insight into this problem, two strains of E. coli, one strain of S. enteritidis, and a strain of Serr. marcescens were disrupted mechanically (Ribi Refrigerated Cell Fractionator - Servall) and separated into cell walls and protoplasm (Fukushi et al., 1964b). These fractions plus extracts from each were then compared as to gross chemical composition and biological properties. They reported that a sharp separation had been effected, with biological properties largely restricted to the cell wall fraction. In addition, the protoplasm was rich in nitrogen as well as appreciable amounts of hexosamine, esterified and amidic linked fatty acids, and carbohydrates. Of special interest, however, was the very substantial amount of nondialyzable carbohydrate in protoplasm, which approximated that in the cell wall. By exploring this latter experimental observation further, these investigators (Milner et al., 1963; Anacker et al., 1964; Ribi et al., 1964a, 1964b) noted that when protoplasmic material from E. coli was extracted with TCA, as much as 6% of this protoplasm was extracted in this reagent. Following dialysis, the product which they obtained had

a gross chemical composition similar to that of endotoxin prepared from intact cells, but differed from endotoxin in that it failed to stimulate the production of antibodies and to elicit the various host reactions. Apparently, this protoplasmic extract possessed the same serologic specificity and mobility in gel as their previously mentioned acid haptene produced by 0.1 N acetic acid hydrolysis for 90 minutes. Furthermore, qualitative sugar analysis of endotoxin and protoplasmic haptene by paper strip electrophoresis indicated similar composition. The haptene contained fatty acids (determined by the hydroxamic acid method and expressed as palmitic acid) in the same proportion as endotoxin from the same strain; however, it was not yet certain whether or not these are qualitatively the same. In addition, no significant differences were noted in the infrared spectra of the protoplasmic haptene, acid haptene and endotoxin. Chemically, this hapten differed from endotoxin and from acid hapten in that it lacked phosphorus, heptose, long-chain fatty acids, and 2-Keto-3-deoxyoctonate. Ultracentrifugation revealed that the acid haptene was polydisperse, whereas the protoplasmic product, which had a similar sedimentation constant, was much more uniform, and possibly even monodisperse.

Although Ribi and his associates (1961, 1962) have previously shown that haptene is the product of acid hydrolysis of endotoxin or of degradation by host enzymes, they do not believe the haptene extracted from protoplasm is a cleavage product of cell wall antigen. However, they do feel it is an anabolic product and it remains to be elucidated

whether this haptene has the necessary attributes for organization into a larger structure solely by colloidal-physical means or whether an enzymatic process will be required to construct the endotoxin molecule which will possess the characteristic antigenic and endotoxic properties.

New types of acid polysaccharides, apart from the typical O-antigen lipopolysaccharide, which are capable of evoking some of the endotoxic reactions in animals has been described by Orskov et al. (1963) and Westphal et al. (1964). Employing extensive studies with the genus of E. coli; that is, more than a hundred serologically classified strains were extracted with phenol-water, and the water extracts were analyzed, Westphal and his associates found that many of the Escherichia strains produced an acid polysaccharide which can be separated from the lipopolysaccharide and nucleic acid fractions by ultracentrifugation followed by cetavlon fractionation of the supernatant.

The acid polysaccharide derived from a representative E. coli strain was obtained in a yield of 5.5% of the dried bacteria. Analysis revealed no O-acyl other than O-acetyl linkages of carbohydrates and no amino acids were found. However, small amounts of N-acetyl and N-acyl carbohydrates amide bound to B-OH long-chain fatty acids were present. The ratio of glucosamine:rhamnose:glucuronic acid:mannose was close to 1:1:1:2.

This acid polysaccharide formed rather viscous solutions in water, and the data obtained from the sedimentation and diffusion constants

permitted the calculation of the molecular weight as being close to 100,000. Periodate oxidation of the original and of the alkali-treated substance gave exactly the same results suggesting a large proportion of the O-acetyl groups being linked to the C_6 position of mannose units.

On the other hand, these investigators reported that the lipopolysaccharide (endotoxin) from the same strain was obtained in a yield of 2.5 to 3.0% with the sugar constituents being glucosamine, heptose, galactose, glucose and mannose. It was noted also that heptose, and especially galactose and glucose occur only in the lipopolysaccharide and not in the acid polysaccharide. In addition, they further noted that this lipopolysaccharide contained 12% of long-chain fatty acid ester, whereas the acid polysaccharide material contained no detectable amount of long-chain fatty acid ester. On the basis of these chemical differences for this particular strain of E. coli, these workers felt that the acid polysaccharide contained less than 2% of lipopolysaccharide.

From another strain of E. coli, these authors reported the isolation of a similar acid polysaccharide which was obtained in a yield of 7 to 8%. The main constituents were galactose, fucose, and galacturonic acid (1:3:2) which accounted for 85% of the substance. The corresponding lipopolysaccharide was composed of the same constituents as the lipopolysaccharide from the previous E. coli strain: glucosamine, heptose, galactose, glucose, and mannose, with additional N- and O-acetyl carbohydrates and long-chain fatty acids.

The purest preparations of these acid polysaccharides plus the

homologous lipopolysaccharides were tested for pyrogenicity in rabbits and horses (iv) and for toxicity in mice (ip). The minimal pyrogenic dose (MPD) of the acid polysaccharide obtained from the first strain of E. coli was found to be 0.003 ug/kg, which was of the same order as for the homologous lipopolysaccharide - also 0.003 ug/kg. In addition, the same results were found for both preparations when tested in horses. However, lethality tests in mice indicated that the acid polysaccharide was about 10 times less toxic than the lipopolysaccharide. These workers also noted that short treatment with 0.1 N NaOH at 56° C or incubation of the acid polysaccharide in normal horse serum at 37° C for 6 to 24 hours led to complete inactivation of the pyrogenicity. The rather high pyrogenicity, but rather low toxicity, of these preparations of acid polysaccharides, according to these investigators, is as yet unexplained. In addition, traces of lipopolysaccharide could not be excluded from all these preparations. However, they recalled that all acid polysaccharides analyzed so far have had three constituents in common with endotoxins, namely glucosamine, an amide-like bound Beta-hydroxy long-chain fatty acid, and O-acetyl groups. Since these constituents could not be fully excluded with certainty, the possibility remained that they may be an integral part of a structure, common to endotoxically active bacterial products.

V. A REVIEW OF METHODS EMPLOYED IN THE DETOXIFICATION OF ENDOTOXIN.

Many attempts have been made to alter either selected antigenic fractions or the whole organisms, but the apparent loss in toxicity has almost always been accompanied by a corresponding decrease in antigenicity. In 1936, Morgan (1936) briefly mentioned the preparation of a specific polysaccharide isolated from Sh. dysenteriae (Shiga) by direct treatment of the micro-organisms with hot acetic acid obtaining a degraded polysaccharide with little or no serological activity. In a later paper, Morgan and Partridge (1940) found that repeated formamide treatment of the soluble antigen, extracted from the same organism with diethylene glycol with subsequent ethanol fractionation, yielded a polysaccharide which was very similar to the earlier reported degraded polysaccharide. It is of interest, however, that neither of these preparations were active antigenically. On the other hand, there was an apparent loss in mouse toxicities reported with the latter preparation.

Freeman (1942), in reporting on the chemical characterization of an acetylated derivative, employed acetic anhydride to a "crude" polysaccharide prepared by tryptic digestion followed by 0.1 N acetic acid hydrolysis from S. typhosa Ty_1. He obtained a colorless amorphous solid which was insoluble in cold ethanol, ether, and water, but was soluble in chloroform, pyridine and acetone. He showed that pyridine and chloroform solutions of this acetyl polysaccharide did not precipitate with S. typhosa O-antiserum. He concluded from these serological studies that the polysaccharide haptene loses its property of specifically

reacting with the homologous antibody on conversion into the triacetyl compound.

Freeman (1943) also reported that a degraded polysaccharide can be obtained by direct extraction of the antigenic complex of S. typhosa with mild acetic acid hydrolysis. He found the liberated polysaccharide to be non-antigenic and relatively non-toxic with a minimum molecular weight of 10,000. However, it was apparently responsible for the specific properties of the intact antigenic complex. In addition, this author reported that similar procedures were applied to the antigenic complex of S. typhimurium. The polysaccharide obtained from this organism yielded very similar results from those already obtained with S. typhosa, but quantitative differences in the nature of the specific polysaccharides were found which apparently accounted for the immunological dissimilarity of the two species.

In attempts to detoxify the Type V antigen extracted by aqueous pyridine (50%) from Shigella paradysenteriae, Goebel et al. (1945) met with disappointment. Among other procedures, acetylation with ketene gas as well as deamination with nitrous acid had no effect. Their attempts to detoxify this antigen revealed the remarkable stability of the toxic component to ordinary chemical manipulation.

Neter et al. (1956), studying the effects of heat and chemicals on erythrocyte-modifying, antigenic, toxic and pyrogenic properties of enterobacteriaceal lipopolysaccharides, reported that periodate-treated lipopolysaccharides lose their antigenic specificity and toxicity

but retain their pyrogenicity. However, prolonged treatment (60 min) of enterobacteriaceal lipopolysaccharides with diluted NaOH caused a decrease in both pyrogenicity and toxicity. This apparent reduction of biological effects induced by chemical modification of the endotoxin molecule suggested to these investigators that there may be specific reactive sites responsible for various toxic properties.

Although quite complete chemical characterizations have been made by these early investigators, little or no information on the biological properties of their fractions is available with the exception that acetylated antigens apparently did not react serologically with antisera to the whole bacteria. However, Treffers (1946), and Treffers, Rubin and Bell (1946a) reported on having prepared a series of acetylated derivatives which were antigenic and comparatively non-toxic from the somatic lipopolysaccharide complex of Sh. dysenteriae (Shiga) and of S. typhosa (Army strain 58). They dissolved the soluble toxic antigens in a small amount of water, diluted with several volumes of pyridine, and then allowed it to react at room temperature with an excess of acetic anhydride. Generally, these workers followed the technique of Freeman (1942) with the exception that his samples were removed at shorter intervals, and the reactions stopped by pouring aliquots into water. In most cases, the water soluble fractions showed an appreciable decrease in toxicity over that of the original material, but the water-insoluble fractions after acetylation were the least toxic. Toxicity tests in mice with doses as high as 3.0 mg produced no lethality upon

ip injection with the acetylated soluble fractions obtained from the Sh. dysenteria organism, whereas the mice injected with the undetoxified Shiga soluble antigen all died at doses of 0.4 mg. These investigators further reported on the temperature response in rabbits and found that approximately a 60 fold increase in dose (0.3 mg) of the Shiga acetylated fraction produced responses which were approximately equal to those given by the original toxic material (0.005 mg). In addition, they were able to show that, by prolonged immunization of rabbits, the Shiga acetylated fractions gave rise to antibody levels which would form precipitates with the acetylated fractions but not agglutinate the intact organisms. More important, however, they were able to show some active protection against intracerebral challenge with live Sh. dysenteriae organisms by subcutaneous or intraperitoneal injection (0.05 mg) of these same acetylated fractions. Preliminary experiments in animals employing the acetylated derivatives of S. typhosa indicated to these investigators that a comparatively non-toxic substance was also produced which was apparently antigenic.

In recent years the detoxification of endotoxin without the apparent loss of antigenicity by various chemical methods has been reported by several investigators. In a series of papers, Freedman et al. (1961), Freedman and Sultzer (1962), and Sultzer and Freedman (1962) described methods by which they were able to dissociate many of the biological properties from endotoxin by acetylation of a Boivin-type lipopolysaccharide extracted from S. typhosa. They obtained

three fractions (L, E, and P) employing acetic anhydride in the presence of anhydrous sodium acetate. Whereas fraction E retained full endotoxin potency as compared to complete inactivity for fraction L, fraction P showed dissociation of some of the biological effects of the parent endotoxin. They reported that pyrogenicity and acute toxicity were markedly reduced, and the ability to confer tolerance to the lethality and pyrogenicity of homologous or heterologous endotoxins was diminished. In contrast to these characteristics, they were able to show that this acetylated (P) fraction retained its ability to stimulate phagocytosis and to enhance nonspecific host resistance to infection to a variety of bacterial infections (E. coli, S. typhimurium, Staphylococcus aureus, and Pseudomonas aeruginosa). Furthermore, they reported that their detoxification procedure was reversible. The original toxicity and pyrogenicity was recovered followed by mild saponification. It was suggested by their data that the observed dissociation of biological properties could not be explained either by deacetylation of the derivative in vivo or by the observable alteration of the gross physical state.

Noll and Braude (1961) reported the detoxification of endotoxin by reductive cleavage of ester bonds employing $LiAlH_4$ yielding a preparation (RE fraction) of high immunogenic potency from a Boivin antigen of E. coli. Their chemically modified product showed virtually no toxicity or pyrogenicity when tested in mice and rabbits respectively. This reduction of pyrogenicity and toxicity was suggested by these

investigators from their infrared studies of the removal of fatty acid esters. Further, their product failed to cause leukopenia in rabbits as well as intradermal inflammation in man. Unlike Freedman and Sultzer's preparation, however, these investigators reported that their product conferred tolerance to both the pyrogenicity and lethality of the parent endotoxin. In addition, comparative studies showed that reduction with $LiAlH_4$ and controlled alkaline hydrolysis with LiOH gave products of different chemical and biological properties. Mild alkaline hydrolysis caused only moderate reduction of the pyrogenicity for rabbits as well as lethal toxicity for mice.

Haskins et al. (1963) noted that if endotoxin, when prepared from S. enteritidis by either the aqueous ether or the trichloroacetic acid method, was treated with $LiAlH_4$ and the reaction mixture was decomposed by the use of 1.0 N HCl by employing the method of Noll and Braude (1961), approximately one-half of the endotoxin was precipitated with the aluminum hydroxide. That is, the material they recovered from the supernatant fluid retained pyrogenicity, toxicity and the ability to stimulate nonspecific resistance of mice to challenge with S. typhosa. Further, the endotoxin they separated from the aluminum hydroxide precipitate by extraction with citric acid solution also retained these biological properties. However, when the reaction mixture was decomposed with water alone by the method of Noll and Braude (1961), the products obtained had a substantial loss of activity. This loss of activity was thought to be due to the high alkalinity

of the reaction mixture after addition of water. In a more recent paper, Fukushi et al. (1964a) reported on the results of studies with the same culture and procedures as employed above by Noll and Braude (1961). They sought to confirm their findings and to obtain additional chemical data on the nature of the changes in bacterial endotoxins produced by treatment with $LiAlH_4$. These investigators found that treatment of Boivin-type endotoxin from E. coli with $LiAlH_4$ according to the procedures of Noll and Braude (1961) yielded a small amount (about 10%) of an essentially pure nonphosphorylated polysaccharide which was nontoxic and nonpyrogenic. In contrast to Noll and Braude's RE fraction, this product did not stimulate the production of antibodies in rabbits, precipitate antibody nor produce tolerance to endotoxin. Further, the bulk of the product resulting from treatment with $LiAlH_4$ consisted of biologically unaltered endotoxin. This fraction was isolated as a rapidly soluble, lipid-containing aluminum citrate-endotoxin complex, the infrared spectrum of which was nearly identical to that of Noll and Braude's RE fraction with 100-fold reduced pyrogenicity. Moreover, no correlation was found between the fatty acid ester content, as estimated from infrared spectra, and the toxic and pyrogenic properties of the endotoxin.

The use of a variety of chemical procedures for the purpose of detoxifying endotoxin preparations has been recently reported by Nowotny and his coworkers (1962, 1963) and by Johnson and Nowotny (1964). Employing endotoxin isolated by the trichloroacetic acid method of

Boivin from Serr. marcescens, these investigators found that toxicity could be decreased or completely abolished by chemical methods such as treatment with borontrifluoride, potassium methylate, pyridinium formate, or anhydrous liquid ammonia gas. Moreover, the ability to induce the second fever peak of the biphasic fever response characteristic of endotoxin was abolished, and, in addition, the capacity to prepare for the local Shwartzman reaction was reduced after such treatments. On the other hand, the resulting preparations produced in all cases a specific antiserum. However, the borontrifluoride procedure apparently reduced the antigenic character of the endotoxin. Further, they found that these endotoxoids greatly enhanced the non-specific resistance of mice and rabbits against experimental infections. Nowotny (1964) suggested that the structural changes brought about by the use of these detoxification procedures produced one common specificity: cleavage of O-acyl linkages of carbohydrates. He arrived at this conclusion by employing the above chemical detoxification procedures on several compounds which had identical or very similar linkages with those present in the endotoxic O antigen (among others, D-glucose pentacetate, Tween 20, N-acetyl D-glucosamine, methyl-D-glucoside, D-glucosamine 6-phosphate, and bis-p-nitrophenyl-phosphate).

Methods other than chemical have been suggested for the detoxification of somatic antigens. Goebel and coworkers (1945) reported that after 3 hours of ultraviolet irradiation, antigens prepared from the Flexnor group of dysentery bacilli either by aqueous pyridine or

diethylene glycol lost 90% of their toxicity. However, they concluded that this reduction in toxicity was closely paralleled by the loss of antigenicity, thus not favoring this procedure as a sound approach for the detoxification of somatic antigens. On the other hand, Barnes et al. (1947) have been more optimistic about the use of ultraviolet irradiation. Using a somatic antigen extracted from Sh. paradysenteriae (Flexnor) by acetone fractionation, they have been able to report the separation of the toxic property from the protective property if the irradiation procedure is carried out slowly and with a low degree of uv light intensity. They have shown that 90% or more net reduction in toxicity can be achieved when the antigen is irradiated for 12 hours with little loss of antigenicity.

Mergenhagen, Martin and Schiffmann (1963a) have recently isolated and characterized the endotoxin from a strain of a group C N. meningitidis. They reported that the lipoid isolated from this endotoxin appeared to be nontoxic and nonpyrogenic when tested for endotoxin activity. However, this same lipoid material in microgram quantities stimulated resistance in mice against a lethal E. coli infection. In additional studies, Mergenhagen et al. (1963b) reported that, contrary to observations with similar doses of lipopolysaccharide, lipid-treated animals developed no phase of negative resistance to a lethal dose of E. coli. They noted that the livers and spleens from the lipid-treated mice took up large numbers of E. coli after intravenous injection in comparison to those from untreated animals. In contrast to the protection afforded

mice against E. coli endotoxin pretreatment with meningococcal lipopolysaccharide, meningococcal lipid-treated mice appeared to be more susceptible to graded doses of endotoxin. Further, these investigators reported that a particulate fraction from mouse liver released chloroform-soluble lipid from meningococcal lipopolysaccharide. as shown by the release of C^{14}-labeled lipid from C^{14}-labeled meningococcal lipopolysaccharide. This lipid was also able to increase host resistance in mice. From these data, the investigators proposed that lipid, released from lipopolysaccharide in vivo by tissue enzymes, may be one of the mediators of increased host resistance induced by lipopolysaccharides.

In recent years, the method of detoxifying endotoxin by soluble tissue extracts has been described. Although the main body of this line of research is primarily interested in the degree in which most animals are able to destroy or detoxify endotoxins by natural resistance mechanisms, it is worthwhile noting the possible significance that might be achieved in vivo without the use of an outside stimulus such as specific immunization. That is, if the natural body defense mechanisms responsible for the inactivation of endotoxin could be stimulated at the time of an enterobacteriaceal infection, the clinician could have at his disposal another weapon for combating diseases caused by the gram-negative bacteria.

Trapani and his associates (1962) have described the in vitro inactivation of endotoxin extracted from both Serr. marcescens and

S. enteritidis by animal tissues. Their experiments indicated that cell free extracts of perfused rabbit liver inactivated these endotoxins, whereas rabbit liver slices yielded negative results. Of the other organs of the perfused rabbit which they examined, brain, heart and spleen produced negative results while the adrenal, kidney, and lymph nodes yielded products of significant activity. They suggested that detoxification by the agent from rabbit liver was consistent with the requirements of an enzyme-catalyzed reaction. Keene (1962), in describing the detoxification of bacterial endotoxin by soluble tissue extracts, also suggested that detoxification of endotoxin by tissue extracts is enzymatic in nature. He found that in vitro incubation of water-soluble extracts of rabbit liver, kidney, spleen and heart with endotoxin derived from Serr. marcescens resulted in inactivation of the pyrogenic and tumor-necrotizing properties of endotoxin and was found to be dependent upon time and temperature of incubation. However, Smith et al. (1963) have described an endotoxin-detoxifying factor in tissue which differed from those reported by Trapani et al. and by Keene. They demonstrated their endotoxin-detoxification factor in dog spleen, and in low concentration in the liver, but not in other tissues of this animal. Further, they reported this factor to be a non-particulate fraction of dog spleen as well as being a protein with an esterase type of enzymatic behavior. Corwin and Farrar (1964), in reporting on the nature of the endotoxin-inactivating principle in guinea pig liver, found that these liver preparations inactivated Serr. marcescens

endotoxin as assayed by the sensitive chick embryo test. The activity was optimal at pH 6.5 to 7.0 and 8.5 to 9.0. In addition, it was found that the mitochrondia and the supernatant fraction containing microsomes possessed activity with the mitochrondia being active only at the acid pH optimum. These workers further reported that the activity of acetone powder extracts of mitochrondia was increased by adenosine triphosphate and nicotinamide adenine dinucleotide, whereas the mitochrondia themselves were also activated by malate. It was concluded from these studies that the enzymes which inactivate endotoxin involve fatty acid activation and oxidation thus suggesting to these authors that the lipid moiety of endotoxin is required for toxicity.

In addition to these methods of detoxifying endotoxin by soluble tissue extracts, the incubation of bacterial endotoxins in either human serum or defibrinated plasma has been shown to result in repression of many of the biological properties of endotoxin (Atkins, 1960). Recently, Rudbach and Johnson (1962) and Yoshioka and Johnson (1962) reported on the characteristics of endotoxin altering fractions obtained from normal human serum. Employing the alcoholic fractionation of normal human defibrinated plasma by Cohn's method 10, these workers obtained two fractions capable of altering bacterial endotoxins. The incubation of endotoxin in Fraction IV_I, resulted in a marked reduction in the ability of the specific antiserum to precipitate endotoxin. In addition the ability of this endotoxin to elicit a pyrogenic response in rabbits was also abolished. They further reported that

Fraction III_O was generally incapable of producing such effects unless it was activated by several procedures. All other fractions resulted in no effect on these properties of endotoxins. It is of interest that the alteration of the lethal activity of endotoxin was not evident after incubation in Fraction IV_I, but was reduced after exposure to activated Fraction III_O. They suggested that their data were consistent with the hypothesis that a possible enzyme system in normal human serum was responsible for destruction of the pyrogenic and serologic properties of endotoxin. Their results indicate this enzyme activity to be confined to Fractions III_O and IV_I.

The in vitro inactivation of gram-negative bacterial endotoxins by papain has been recently reported by Kim and Watson (1964). They found that reduced crystalline papain inactivated the pyrogenic and lethal activities of purified gram-negative bacterial endotoxins. The possible presence of peptide, ester and amide linkages within the macromolecular toxin and the known ability of the enzyme to split linkages of all 3 types indicated that it was not possible to implicate a specific linkage or group in endotoxin activity. However, they suggested that this purified enzyme may prove useful for further studies on the chemical nature of the active groups within the endotoxin molecule.

Mergenhagen and Martin (1964) have recently described a phenol-water preparation of C^{14}-labeled E. coli endotoxin of low solubility by treatment with lysozyme at pH 5.0 or 8.0 which effected a dissociation of this preparation. The products of dissociation were equally

distributed in the chloroform and water phases after extraction. It was further noted by these authors that concomitant with dissociation, recoverable endotoxin after lysozyme treatment had a reduced content of bound lipid, and dissolved easily in aqueous media to yield a clear solution. Moreover, ultracentrifugation of the lysozyme-treated endotoxin revealed that it sedimented as a single major boundary with a sedimentation coefficient of 13.3. In addition, lysozyme-treated endotoxin was more potent than was the conventional endotoxin as shown by lethal activity in rabbits and pertussis-sensitized mice. Gel diffusion analysis also indicated to these investigators that the higher molecular weight component associated with conventional endotoxin was separated by lysozyme treatment, whereas lysozyme-treated endotoxin, as shown by immunoelectrophoresis, was observed as a single sharp band of precipitation which migrated toward the cathode.

MATERIALS AND METHODS

I. EXPERIMENTAL ANIMALS

A. Rabbits

Testing for pyrogenicity was done in rabbits (Oryctoloagus cuniculus) that had been properly "screened" by methods previously reported (Martin and Marcus, 1964b). To summarize, previously sham and pyrogen-free (PF) saline tested male and female rabbits obtained from a local source were used for testing. All rabbits weighed between 3 and 5 kg, and were caged individually or in pairs in a constant temperature room. Pyrogen tests were carried out according to techniques described in the United States Pharmacopeia (USP), XVI, (1960) with the following exceptions: all glassware was boiled in detergent solution, then rinsed with nonpyrogenic distilled water and heated in an oven at 250° C for 2 hours; solutions to be tested were at room temperature rather than 37° C; temperature recordings were taken prior to injection and at 15 minutes, 1, 2, and 3 hours after injection. The results were interpreted as per the USP.

In each experiment one group of rabbits injected with PF saline was included. This group served as a control for technique and other external factors such as temperature, humidity and noise. Rectal temperatures were measured with an electric-type thermometer operating on the thermocouple principle (model TE3; Ellab Instruments, Copenhagen, Denmark). Rabbits which yielded positive fever results, and were used

in more than one experiment involving pyrogenicity, were "rested" for two or more weeks as outlined by the United States Pharmacopeia (1960).

B. Mice

Tests for immunogenicity and lethality were performed in white Swiss mice (Mus musculus) weighing approximately 20-25 grams; deaths were recorded for a period of one week.

II. CULTURES

A. Bacterial Strains

One strain each of Salmonella tennessee and S. typhimurium was employed. These organisms were originally obtained from the Utah State Department of Health. The organisms are maintained by transfer culture at monthly intervals on tryptose-phosphate agar slants, incubated at 37° C for 24 hours and thereafter kept at room temperature.

In order to determine the comparative virulence of these two strains, the following mouse challenge experiment was performed. Eighteen hour cultures of both S. tennessee and S. typhimurium grown on tryptose-phosphate agar slants were harvested with PF water. The turbidity of each suspension was adjusted approximately to a McFarland 3 employing the turbidity reference standard of the Division of Biological Standards (DBS), National Institutes of Health (NIH). Both live and mucin cell suspensions were prepared for challenge inoculum by employing serial 10-fold dilutions using PF water. The mucin suspension was prepared by suspending 5 grams of dehydrated hog gastric mucin (Wilson,

type 1701-W) in 100 ml of distilled water. The pH was adjusted to 7.2-7.4 with sodium hydroxide and the mucin suspension stirred with a magnetic stirrer at about 80° C for 15 minutes. The suspension was then strained through gauze and autoclaved for 30 minutes. Five-tenths ml of each of the 10^{-1} and 10^{-7} dilutions was added to 4.5 ml of the 5% mucin preparation thereby obtaining 10^{-2} and 10^{-8} dilutions of the organisms in the mucin suspension. In addition 10^{-2} and 10^{-8} dilutions containing live cell suspensions (without mucin) were employed. Each set of six mice was inoculated intraperitoneally (ip) with 0.5 ml of the appropriate challenge inoculum. Moreover, one set of mice served as a control, being injected with PF water. All mice were observed over a three-day period which is the minimal observation time suggested by the Division of Biological Standards (DBS) of the National Institutes of Health (1953).

The results of the virulence tests employing both organisms in mice are recorded in Table 1. It is apparent that the approximate MLD of the ip administered challenge was the dilution containing 10^{-2} org./0.5 ml for both organisms regardless of whether mucin was present or not. However, the strain of S. typhimurium appeared to be slightly more virulent under these conditions. This strain was employed exclusively in all of the experimental procedures to follow.

III. PREPARATIONS

A. Roschka-Edwards (RE) Procedure: Crude pyrogen substance was prepared

TABLE 1

VIRULENCE TESTS IN MICE EMPLOYING THE FOLLOWING SUSPENSIONS OF *SALMONELLA TENNESSEE* AND *SALMONELLA TYPHIMURIUM*

Organism	Dilution 0.5 ml ip	Deaths 72 hours after Challenge * Live	Mucin
S. tennessee	10^{-2}	3/6	6/6
	10^{-8}	2/6	2/6
S. typhimurium	10^{-2}	6/6	6/6
	10^{-8}	2/6	0/6
Control (PF water)		0/6	---

* mortality ratio (deaths/total)

according to the following procedure (Edwards, 1951):

Roux bottles containing approximately 100 ml of tryptose-phosphate broth (Difco) plus 2% agar were incubated with S. typhimurium for 18 hours at 37° C and the growth gently washed off with saline. The pooled washings were then heated in a boiling water bath for 2 hours. Sterility tests performed at this time and after 4 days were found to be negative. The sterile cell suspension was centrifuged at 4° C for 1/2 hour and the sediment was resuspended in 50 ml of absolute ethanol, incubated for 24 hours at 37° C, and recentrifuged at 4° C. The sediment was resuspended in 50 ml of absolute ethanol and incubated for another 24 hours at 37° C. The cells were again centrifuged at 4° C, the alcohol was replaced with acetone, and then taken through two further changes of acetone. The sediment was dried at 37° C and then ground to a fine powder. This powder, consisting primarily of the "hulls" of these Salmonella organisms, was further disintegrated by the use of an ultrasonic disintegrating device. Fifteen ml of PF water was added to 1500 mg of this crude endotoxin and disintegrated (Sonifier, Branson Instruments, Stanford, Connecticut) for 15 minutes under sterile conditions. The suspension was then filtered (Millipore filter) and the precipitate dried overnight at 37° C. Employing this procedure of sonification produced no visible changes in yield or in alteration of this crude endotoxin. Hereafter, this product will be referred to as the parent RE preparation.

B. Acetylation Procedure (Freedman and Sultzer, 1962):

To 500 mg of sonified parent RE preparation were added 115 ml of acetic anhydride and 75 mg of anhydrous powdered sodium acetate as a basic catalyst. The reaction mixture was heated in a capped centrifuged bottle in a boiling water bath for 4 hours with occasional mixing. After cooling, the acetic anhydride was removed as a clear solution by decantation. The remaining insoluble material was freed of anhydride by drying at room temperature under a stream of nitrogen. Once dried, it was ground to a powder by mortar and pestle. This was washed 3 times with 100 ml portions of saline and once with water. The washes were removed by centrifugation at low speed yielding a clear supernatant solution which was discarded due to its apparent inactivity in the test animal. However, Freedman and Sultzer (1962) obtained an opalescent solution at this point. By dialyzing their combined washes, they were able to obtain a preparation (fraction E) which amounted to 20-25% of their starting material which retained full endotoxic potency. However, unlike our starting material, they started with a Boivin preparation. The washed precipitate was dried under nitrogen and ground to a powder. This powder, amounting to approximately 65% of the starting material, was granular in appearance, light tan in color and strongly lyophobic. The yield was 324 mg (64.8%) with some loss due to personal technique. Chemical analysis of the acetyl content (Australian Microanalytical Service, University of Melbourne) as $COCH_3$ (methanolic NaOH used as the saponification agent) was 6.8%. A stock solution of 50 mg/10 ml was readily

prepared by adding a small amount (0.5-1 ml) of saline and triturating thoroughly with a mortar and pestle in order to wet and disperse the solids. Saline was added in small aliquots as the lyophobic powder was worked into suspension. It should be noted that, as with the parent RE preparation, this stock solution should be mixed thoroughly before testing since the suspension is rather dense and separates readily. Hereafter, this product will be referred to as the acetylated Roschka-Edwards (Acet-RE) preparation.

Attempts to recover an anhydride-soluble, water-insoluble material by pouring the anhydride solution into excess cold water and filtering it yielded a small amount of solids which accounted for less than 10% of our starting material. Since the yield was small, no attempt was made to test its biological effects in animals. Freedman and Sultzer (1962), however, obtained a rather large amount (30-35%) of this anhydride-soluble, water-insoluble material from their Boivin preparation of endotoxin (fraction L) which they reported as having complete inactivity for pyrogenicity and lethality in their test animals.

C. Periodate Procedure (Neter et al., 1956):

The periodate-treated RE crude endotoxin was prepared as follows: 200 mg of the parent RE preparation was suspended in 160 ml of PF distilled water; 20 ml of 1.0 N sodium acetate buffer (pH 5.0) as well as 20 ml of 0.1 N potassium periodate solution were added. This material was kept in the dark for 24 hours at room temperature and then dialyzed in cellophane (Visking)casing against distilled

water for 24 hours. Finally, it was concentrated by pervaporation at room temperature and lyophilized. The resulting product was a light, white, fluffy powder with a yield of 112 mg (56%). Although Neter and his associates employed 0.1 N sodium periodate solution in their procedure, it is of interest to note that the potassium periodate-treated RE material in identical concentrations reacted in a similar manner; i.e., our lyophilized oxidation product was also insoluble in distilled water, but was soluble upon the addition of small amounts of 1.0 M NaOH and subsequent neutralization with dilute acetic acid. The soluble stock suspension of 50 mg/10 ml was readily prepared and had a characteristic milky color.

D. Potassium Methylate Procedure (Nowotny, 1963):

The parent RE preparation was "purified" by extraction with anhydrous methanol, a procedure which removes inert impurities but does not alter the biological properties of endotoxins (Nowotny et al., 1963). To 1 gram of dry RE preparation, 250 ml of methanol were added, and the resulting suspension refluxed on a hot-plate with continuous magnet-stirring for 60 minutes, cooled and finally centrifuged in the cold at 2,500 rpm for 10 minutes. The clear supernatant was drawn off and the precipitate dried in a vacuum desicator for 48 hours. This "purified" RE preparation was subjected to the following chemical procedures. To 200 mg of the foregoing RE preparation, 40 ml of 0.02 M potassium methylate in anhydrous methanol were added and the mixture refluxed for 60 minutes. The insoluble residue was

centrifuged in the cold at 2,500 rpm for 10 minutes, washed with methanol and dried in a vacuum desicator. This preparation, unlike the Acet-RE preparation was a very fine, light powder which was ivory in color, and sparingly soluble in saline in small amounts. The yield was 169.1 mg (84.6%).

E. Boron Trifluoride (BF_3) Procedure (Nowotny, 1963):

In this procedure 500 mg of previously "purified" (methanol refluxed) RE preparation was refluxed for 1 hour with 50 ml of 2% boron trifluoride in anhydrous methanol (ca. 80° C). The reaction mixture was diluted with 4 volumes of water and dialysed against distilled water for 96 hours. The dialysed material was then centrifuged in the cold at 2,500 rpm for 10 minutes and the resulting residue dried in a vacuum desicator. This preparation resembled the Acet-RE preparation in that it was tan in color, granular in appearance with a consistency of fine sand and mildly lyophobic. The yield was 233.3 mg (46.7%).

Stock solutions of the potassium methylate and BF_3 preparations were prepared in the following manner. A stock solution of 50 mg/10 ml was readily prepared by adding 0.1 ml of saline and triturating with mortar and pestle in order to thoroughly wet and disperse the solids. Saline was added in small aliquots as the powder was worked into suspension. It should be noted that, as with the Acet-RE and RE preparations, these stock solutions should be thoroughly mixed before testing since both suspensions are dense and separate readily.

In all procedures, precautions to avoid contamination by extraneous pyrogens were rigorously observed. All glassware and other apparatus were heated at 250° C for 2 hours before use and saline and distilled water were proved to be pyrogen-free (PF).

F. Boivin Preparation:

Lipopolysaccharide extracted by trichloroacetic acid (TCA) from S. typhimurium was used as the source of "pure" endotoxin (Difco Laboratories, Detroit).

G. Heat-Killed, Phenol-Preserved (HP) Vaccine:

A freshly prepared heat-killed, phenol-preserved (HP) vaccine of S. typhimurium was prepared in the following manner. Eighteen-hour cultures of S. typhimurium, grown on tryptose-phosphate (Difco) agar, were harvested in saline. After filtering the suspension through sterile glass wool, the organisms were heated in a 56° C waterbath for 2 hours. Following heating, the suspension was cooled to room temperature and standardized turbidimetrically by the McFarland Method (Kolmer and Boerner, 1945). Dilutions were prepared in PF saline to give a final concentration of 1.8×10^8 organisms per ml (McFarland #6) of saline containing 0.5% phenol. The vaccine was then put up in sterile vials, fitted with the appropriate sterile rubber stoppers, and stored in the refrigerator until ready for use. All sterility tests made on each lot of vaccine were negative for growth.

IV. METHOD OF CHALLENGE

A. Active-Immunized Mouse Protection Test:

The potency of the vaccines was, for the most part, determined by active-immunized mouse protection tests as outlined by the Public Health Service, Division of Biologic Standards (1953). In each test, groups of 20 mice were immunized with 2 weekly subcutaneous (sc) injections in a volume of 0.5 ml. The number of bacteria in 0.5 ml of the HP vaccine was approximately 900 x 10^6 organisms. A group of 20 mice given 0.5 ml of saline served as a control for each challenge. All mice were challenged with the same S. typhimurium strain 7 days after immunization with approximately 10 LD_{50} (LD_{84}) doses by the ip route. This challenge dose was sufficient to kill about 80-90% of the mice in the saline control group within 24-48 hours and was prepared from the harvest of an 18 hour tryptose-phosphate agar slant suspended in PF water. This harvest was standardized turbidimetrically by employing the turbidity reference standard (DBS, NIH), and confirming the challenge numbers by subsequent viability counts. The mouse virulence of each challenge culture was ascertained by ip inoculation of 0.5 ml of the culture suspended in PF water. LD50 values of approximately 2.66 x 10^6 bacilli, as calculated by both the methods of Reed and Muench (1938) and Miller and Tainter (1944) were obtained. For further discussion concerning S. typhimurium as a challenge inoculum reference please see Experimental Results, Part III.

V. SEROLOGICAL TESTS

A. Preparation of Anti-O Serum (Edwards and Ewing, 1962):

Since the procedure for preparing the parent RE crude endotoxin inactivates the H antigen while retaining the O antigen, this preparation was used to immunize rabbits for the production of anti-O serum. Each rabbit received four intravenous (iv) injections administered at 4 day intervals, the first of which consisted of 0.5 ml of a suspension comparable in density to a 24-hour broth culture. The doses were increased in volume (twofold increments) at each injection and the suspensions rapidly increased in density so that very large numbers of the organisms are administered at the third and fourth injections. Employing standard agglutination procedures, high titered sera (1:2560) were obtained by this method. The immune sera were obtained from these animals by cardiac puncture six days after the last injection. The serum was separated from the clot, heated at 56° C for 30 minutes, and stored in a refrigerator at -20° C.

B. Double Diffusion Test Procedure:

Agar gel precipitin tests were carried out by the method of Ouchterlony (1948). Different concentrations of endotoxoids as well as immune and normal rabbit sera were placed in the wells appropriately arranged in agar plates. In other experiments different endotoxoid preparations but at the same concentrations were similarly placed in the wells and tested against either immune or normal rabbit sera. The diameter of the antigen and antibody wells was 7 mm and 9 mm respectively,

and the distance between centers of the wells was 18 mm. In order to inhibit nonspecific band formation in these tests, two per cent agar (Difco) in pH 7.2 glycine buffer (14%) was employed. The bands of precipitation were observed after incubation for 1 to 3 weeks at room temperature.

EXPERIMENTAL RESULTS

I. EXPERIMENTS DESIGNED TO STUDY THE PYROGENIC PROPERTIES OF SALMONELLA TYPHIMURIUM ENDOTOXOID PREPARATIONS

A. Effect of injecting rabbits with the parent RE preparation.

Results reported previously (Martin and Marcus, 1964a) indicated that the minimum pyrogenic dose (MPD) of dry material from either an RE or a Boivin preparation from the 055:B5 strain of *E. coli* was approximately 1.0 ug. Table 2 shows similar results in rabbits when an RE preparation from *S. typhimurium* was employed. Both the 1 and 10 ug amounts of this preparation produced comparable fevers in rabbits as interpreted by USP procedure.

B. Effect of injecting rabbits with a Boivin preparation.

In this experiment, rabbits were injected with a comparatively "pure" endotoxin. A TCA extract of the working strain of *S. typhimurium* yielded the data shown in Table 3. As was noted previously concerning the *E. coli* preparations, these results in Table 3 as well as those in Table 2 show excellent agreement with the results obtained with the same 2 preparations from a strain of *S. typhimurium*. That is, a 1 ug amount of either an RE or a Boivin preparation from a strain of *S. typhimurium* also represents the MPD. Moreover, these results indicate that the RE preparation is pyrogenic in rabbits in concentrations similar to the relatively "pure" endotoxin (Tables 2 and 3).

C. Effect of injecting rabbits with a potassium periodate RE preparation.

Tables 4 and 5 show the pyrogen test results obtained with the potassium periodate RE preparation as interpreted by USP definition. It can be seen from Table 4 (Maximum Temperature Change column) that all of the rabbits tested with this preparation gave negative results to the 1, 10, and 50 ug test amounts. On the other hand, Table 5 shows a MPD response in rabbits with 200 ug of the potassium periodate RE preparation as compared to the negative pyrogen test results recorded for the control animals shown in both Tables 4 and 5.

D. Effect of injecting rabbits with an Acet-RE preparation.

The pyrogen test results obtained with the Acet-RE preparation are shown in Tables 6 and 7. It can be seen from the Maximum Temperature Change column in Table 6 that all of the doses tested for pyrogenicity in rabbits gave negative results. However, definite febrile responses in rabbits are shown in Table 7 tested with 200, 500, and 1,000 ug amounts, respectively. Negative results recorded for the control animals are shown in both Table 6 and 7.

E. Effect of injecting rabbits with a potassium methylate RE preparation.

Tables 8 and 9 show the pyrogen test results obtained with the potassium methylate, and BF_3 preparations as interpreted by USP definition. It can be seen from the Maximum Temperature Change column in Table 8 that only the 1 ug amount of the potassium methylate preparation tested for pyrogenicity in rabbits gave a negative result, whereas the 10 and 100 ug amounts both yielded positive febrile responses in rabbits respectively.

F. Effect of injecting rabbits with a boron trifluoride RE preparation.

Table 9 shows the fever responses in rabbits when injected with 1, 10 and 100 ug amounts of the BF_3 preparation. With the exception of the saline injected control animals, all 3 of these preparations were pyrogenic in rabbits.

G. A summary of the pyrogen test responses in rabbits employing the endotoxoid preparations.

The pronounced reduction in pyrogenicity exhibited by the Acet-RE and the potassium periodate RE preparations are shown in summary form in Table 10. The data indicate a $>100<200$ fold reduction in rabbit pyrogenicity for both preparations when compared with comparable doses of parent RE and Boivin preparations. Although these experiments approximate the upper and lower limits of decrease in pyrogenicity for the preparations concerned in this dissertation only, it is interesting to note that the pyrogen test results with the Acet-RE preparation compare favorably with those reported by Freedman and Sultzer (1962). On the other hand, the results obtained with the potassium periodate RE preparation indicate an approximate 25,000 fold reduction in pyrogenicity when compared to the periodate-treated E. coli lipopolysaccharide (MPD 0.0013 ug/kg) as reported by Neter et al. (1956). Furthermore, under similar conditions, the potassium methylate and BF_3 procedures yielded a 10 fold loss and no loss of pyrogenicity respectively when compared to the parent RE preparation. As elaborated upon in the Discussion these results are comparable with those recently reported by Johnson and Nowotny (1964).

TABLE 2

EFFECT OF INJECTING RABBITS INTRAVENOUSLY WITH A

ROSCHKA-EDWARDS (RE) SALMONELLA TYPHIMURIUM PREPARATION +

Rabbit Number	Amounts Tested	Temperature, °C					Max. Temp. Change
		Control Temp.	15'	1 hr.	2 hr.	3 hr.	
19	PFPSS *	39.4	39.7	39.7	39.7	39.7	+0.3
25	"	39.8	40.1	40.0	39.8	39.9	+0.3
20	"	39.5	39.9	39.7	39.5	39.7	+0.4
5	0.1 ug/33 ml PSS	39.6	39.5	39.6	39.7	39.8	+0.2
9	"	39.6	39.4	39.7	39.8	39.8	+0.2
1	"	39.1	39.0	39.4	39.5	39.4	+0.4
21	1 ug/33 ml PSS	39.4	39.6	39.4	39.6	39.9	+0.5
24	"	40.1	40.7	40.6	41.4	41.5	+1.4
27	"	40.4	40.6	40.6	40.7	41.0	+0.6
31	10 ug/33 ml PSS	40.1	40.8	41.1	41.0	41.4	+1.3
29	"	39.6	39.9	40.3	40.6	40.2	+1.0
30	"	39.6	39.8	40.1	40.2	40.3	+0.7

+ interpreted as per USP XVI procedure

* pyrogen-free saline, 10 ml/kg; negative control group

TABLE 3

EFFECT OF INJECTING RABBITS INTRAVENOUSLY WITH A

BOIVIN PREPARATION EXTRACTED FROM SALMONELLA TYPHIMURIUM +

Rabbit Number	Amounts Tested	Temperature, °C					Max. Temp. Change
		Control Temp.	15'	1 hr.	2 hr.	3 hr.	
1	PFPSS *	39.9	40.3	40.0	39.7	40.0	+0.4
5	"	40.6	40.7	40.3	39.9	40.2	+0.1
10	"	40.2	40.4	40.1	39.9	40.3	+0.2
11	0.1 ug/33 ml PSS	40.4	40.6	40.3	40.2	40.5	+0.2
7	"	40.5	40.4	40.2	39.7	40.0	-0.8
15	"	40.4	40.2	40.2	40.2	40.5	+0.1
14	1.0 ug/33 ml PSS	39.9	39.7	39.9	40.4	40.6	+0.7
13	"	39.6	39.4	39.8	39.8	40.3	+0.7
16	"	40.0	39.7	39.7	40.3	40.5	+0.5

+ interpreted as per USP XVI procedure

* pyrogen-free saline, 10 ml/kg; negative control group

TABLE 4

EFFECT OF INJECTING RABBITS INTRAVENOUSLY WITH A POTASSIUM PERIODATE RE SALMONELLA TYPHIMURIUM PREPARATION +

Rabbit Number	Amounts Tested	Temperature, °C					Max. Temp. Change
		Control Temp.	15'	1 hr.	2 hr.	3 hr.	
25	PFPSS *	40.3	38.7	38.7	39.1	40.0	-1.6
24	"	40.4	38.8	38.9	39.4	40.8	+0.4
23	"	39.9	38.7	38.6	38.9	39.1	-1.3
22	1 ug/33 ml PSS	40.2	38.4	38.5	38.8	39.1	-1.8
20	"	40.2	38.4	38.5	39.0	39.5	-1.8
18	"	40.2	38.7	38.8	39.1	39.5	-1.6
17	10 ug/33 ml PSS	40.4	38.6	38.7	39.2	39.8	-1.8
15	"	40.2	38.7	38.9	39.2	39.7	-1.5
4	"	40.3	38.8	39.0	39.3	39.7	-1.5
3	50 ug/33 ml PSS	40.3	40.7	40.6	39.9	40.6	+0.4
5	"	40.2	40.5	40.4	40.2	40.5	+0.3
6	"	40.1	40.3	40.4	39.8	40.6	+0.5

+ interpreted as per USP XVI procedure

* pyrogen-free saline, 10 ml/kg; negative control group.

TABLE 5

EFFECT OF INJECTING RABBITS INTRAVENOUSLY WITH A POTASSIUM PERIODATE RE SALMONELLA TYPHIMURIUM PREPARATION +

Rabbit Number	Amounts Tested	Temperature, °C					Max. Temp. Change
		Control Temp.	15'	1 hr.	2 hr.	3 hr.	
1	PFPSS *	40.4	40.6	40.6	40.6	40.1	+0.2
26	"	40.2	40.5	40.4	40.4	40.2	+0.3
3	"	39.6	39.9	39.9	40.0	39.6	+0.4
23	100 ug/33 ml PSS	39.9	38.2	38.6	39.3	39.8	-1.7
24	"	40.5	38.9	39.4	40.6	41.0	+0.5
25	"	40.5	39.1	39.9	40.4	40.7	+0.2
7	200 ug/33 ml PSS	40.3	40.3	40.6	40.3	41.3	+1.0
8	"	39.8	40.1	39.4	39.8	40.9	+1.1
10	"	40.1	40.3	40.6	40.4	41.5	+1.4
15	500 ug/33 ml PSS	39.8	40.2	40.8	40.0	40.7	+1.0
17	"	40.1	40.6	41.2	41.1	41.1	+1.1
18	"	40.2	40.6	41.4	40.3	42.1	+1.9

+ interpreted as per USP XVI procedure

* pyrogen-free saline, 10 ml/kg; negative control group

TABLE 6

EFFECT OF INJECTING RABBITS INTRAVENOUSLY WITH AN ACET-RE *SALMONELLA TYPHIMURIUM* PREPARATION +

Rabbit Number	Amounts Tested	Control Temp	Temperature, °C 15'	1 hr.	2 hr.	3 hr.	Max. Temp. Change
29	PFPSS *	40.1	40.4	40.1	40.0	39.8	+0.3
30	"	40.1	40.1	40.2	39.8	39.7	+0.1
31	"	40.5	40.5	40.5	40.0	40.1	-0.5
35	1 ug/33 ml PSS	40.6	40.6	40.6	40.3	40.4	-0.3
19	"	40.3	39.9	40.1	39.9	39.9	-0.4
21	"	40.4	39.9	40.1	40.0	40.3	-0.5
24	10 ug/33 ml PSS	40.9	40.7	40.3	40.4	41.0	+0.1
25	"	40.6	40.4	39.9	40.5	41.0	+0.4
20	"	40.1	39.6	39.3	39.3	39.6	-0.8
1	100 ug/33 ml PSS	40.8	40.4	40.6	40.7	41.0	+0.2
7	"	40.9	40.3	40.4	40.6	40.9	-0.6
9	"	40.7	40.3	40.4	40.6	41.2	+0.5

+ interpreted as per USP XVI procedure

* pyrogen-free saline, 10 ml/kg; negative control group

TABLE 7

EFFECT OF INJECTING RABBITS INTRAVENOUSLY WITH AN ACET-RE *SALMONELLA TYPHIMURIUM* PREPARATION +

Rabbit Number	Amounts Tested	Temperature, °C					Max. Temp. Change
		Control Temp.	15'	1 hr.	2 hr.	3 hr.	
2	PFPSS *	40.3	40.2	40.0	39.4	39.5	-0.9
3	"	39.7	39.8	39.7	39.5	39.7	+0.1
4	"	40.0	40.1	39.9	39.8	39.7	+0.1
1	200 ug/33 ml PSS	40.4	40.3	40.8	41.1	41.0	+0.7
6	"	40.5	40.5	40.7	41.2	41.3	+0.8
7	"	40.3	40.4	40.6	40.8	40.7	+0.5
5	500 ug/33 ml PSS	39.5	39.7	39.9	39.9	40.3	+0.8
8	"	40.3	40.3	40.8	40.8	41.3	+1.0
10	"	40.4	40.2	40.7	40.7	40.9	+0.5
12	1000 ug/33 ml PSS	40.1	40.1	40.4	40.4	41.1	+1.0
14	"	40.6	40.2	40.4	40.5	41.3	+0.7
15	"	40.5	40.4	40.9	40.7	41.2	+0.7

+ interpreted as per USP XVI procedure

* pyrogen-free saline, 10 ml/kg; negative control group

TABLE 8

EFFECT OF INJECTING RABBITS INTRAVENOUSLY WITH A

POTASSIUM METHYLATE RE SALMONELLA TYPHIMURIUM PREPARATION +

Rabbit Number	Amounts Tested	Temperature, °C					Max. Temp. Change
		Control Temp.	15'	1 hr.	2 hr.	3 hr.	
15	PFPSS *	39.5	39.1	39.5	39.5	39.7	+0.2
14	"	40.0	39.7	40.2	40.2	40.4	+0.4
18	"	39.9	39.6	39.8	39.6	39.8	-0.3
30	1 ug/33 ml PSS	39.3	38.9	39.2	39.2	39.6	+0.3
27	"	40.3	40.1	40.1	40.2	40.5	+0.2
20	"	39.4	39.0	39.2	39.2	39.4	-0.4
25	10 ug/33 ml PSS	39.8	39.7	40.3	40.2	40.5	+0.7
24	"	39.9	39.8	41.0	40.9	41.2	+1.3
29	"	39.9	39.7	40.2	40.8	40.9	+1.0
31	100 ug/33 ml PSS	40.0	40.3	41.0	40.9	41.6	+1.6
21	"	39.3	38.8	39.2	39.6	40.1	+0.8
19	"	39.2	39.5	39.9	40.2	40.5	+1.3

+ interpreted as per USP XVI procedure

* pyrogen-free saline, 10 ml/kg; negative control group

TABLE 9

EFFECT OF INJECTING RABBITS INTRAVENOUSLY WITH A BORON TRIFLUORIDE RE SALMONELLA TYPHIMURIUM PREPARATION +

Rabbit Number	Amounts Tested	Temperature, °C					Max. Temp. Change
		Control Temp.	15'	1 hr.	2 hr.	3 hr.	
1	PFPSS *	39.7	39.6	39.6	39.6	39.7	-0.1
5	"	39.5	39.5	39.5	39.6	39.8	+0.3
9	"	39.3	39.4	39.4	39.5	39.7	+0.4
10	1 ug/33 ml PSS	39.6	39.8	40.0	40.2	40.5	+0.9
11	"	39.9	40.0	40.4	40.6	40.9	+1.0
15	"	39.4	39.5	39.6	39.7	39.8	+0.4
13	10 ug/33 ml PSS	39.0	39.0	39.2	39.5	39.7	+0.7
14	"	40.0	39.8	40.0	40.3	40.4	+0.4
35	"	39.4	39.4	39.7	40.4	40.5	+1.1
18	100 ug/33 ml PSS	39.6	39.7	40.5	40.5	40.5	+0.8
17	"	40.2	40.3	40.5	40.9	41.2	+1.0
16	"	39.3	39.5	40.1	40.5	40.5	+1.2

+ interpreted as per USP XVI procedure

* pyrogen-free saline, 10 ml/kg; negative control group

TABLE 10

A SUMMARY OF THE PYROGEN TEST RESPONSES IN RABBITS EMPLOYING

THE ENDOTOXOID PREPARATIONS OF SALMONELLA TYPHIMURIUM

Amounts Tested *	Fever Responses with					
	RE	Boivin	Acet-RE	BF_3	Potassium Periodate	Potassium Methylate
0.1	-	-				
1.0	+	+	-	+	-	-
10	+		-	+	-	+
50					-	
100			-	+	-	+
200			+		+	
500			+		+	
1000			+			

* ug/33 ml PF saline

II. EXPERIMENTS DESIGNED TO STUDY THE LETHAL EFFECTS OF SALMONELLA TYPHIMURIUM ENDOTOXOID PREPARATIONS

A. Lethality of potassium periodate RE, RE, and Boivin preparations injected intraperitoneally into mice.

Table 11 presents the data from experiments comparing the lethality in mice of ip injections of 5 individual potassium periodate RE concentrations with that of the parent RE and Boivin preparations, respectively. Both the periodate-treated RE and the parent RE preparations, employing similar conditions, indicate no lethality in mice at the concentrations shown ($>$2,500 ug). These data suggest that the oxidation of the parent RE preparations with potassium periodate had little effect on mouse lethality. Moreover, these results correlate well with the findings of Neter et al. (1956) in that the periodate procedure yields a compound of low toxicity. The results shown for the Boivin (TCA) preparation indicate that a "pure" endotoxin is much more lethal at higher concentrations than either the potassium periodate RE or parent RE preparations, respectively. That is, the Boivin preparation shows an LD_{50} response in mice with approximately 2 mg, whereas under similar conditions, neither the periodate-treated nor the parent RE preparations approach an LD_{50} response in mice 7 days after ip injection of 2.5 mg.

B. Lethality of Acet-RE, RE, and Boivin preparations injected intraperitoneally into mice.

The data from experiments comparing mouse lethality by ip injection

of 5 individual acetylated concentrations with that of the parent RE and Boivin preparations respectively are shown in Table 12. Comparing the parent RE preparation, which again is apparently non-lethal for mice at the doses shown, with similar amounts of Acet-RE preparations suggests that acetylation may induce lethality in mice at high concentrations ($>$500 ug). However, as was shown in the previous table (Table 11), the Boivin preparation shows an LD_{50} response between 500 ug and 2000 ug, whereas neither the Acet-RE nor the RE preparations approach an LD_{50} response throughout the observation period with any of the doses shown. As elaborated upon in the Discussion, these data are in line with those of Freedman and Sultzer (1962).

C. Lethality of potassium methylate RE, boron trifluoride RE, and RE preparations injected intraperitoneally into mice.

Table 13 shows the data from experiments comparing the lethality in mice by ip injection of 5 individual potassium methylate and BF_3 preparations with that of the parent RE preparation. A comparison of the parent RE preparation with similar amounts of potassium methylate and BF_3 preparations suggests that neither of these procedures altered the lethality of the parent endotoxin molecule. That is, all of the preparations are apparently non-toxic in mice in amounts of at least 2.5 mg. Furthermore, the mouse lethality data shown for the potassium methylate and BF_3 preparations in Table 13 agree with those of Johnson and Nowotny (1964) who reported similar mouse LD_{50} values with their preparations.

TABLE 11

LETHALITY OF POTASSIUM PERIODATE RE, RE, AND BOIVIN PREPARATIONS OF SALMONELLA TYPHIMURIUM INJECTED INTRAPERITONEALLY INTO MICE

Preparation	Dose * (ug)	No. of Mice	Cumulative Deaths, in Days 1	2	3	7
Potassium Periodate RE	2.5	8	0	0	0	0
	25	8	0	0	0	0
	250	8	0	0	0	0
	500	8	0	0	0	0
	2500	8	0	0	0	0
RE	2.5	8	0	0	0	0
	25	8	0	0	0	0
	250	8	0	0	0	0
	500	8	0	0	0	1
	2500	8	0	0	1	1
Boivin	2.5	8	0	0	0	0
	25	8	0	0	0	0
	250	8	0	0	0	1
	500	8	2	2	3	3
	2500	8	5	7	7	8
Saline Controls	---	8	0	0	0	0

* all preparations given in 0.5 ml PF saline

TABLE 12

LETHALITY OF ACET-RE, RE, AND BOIVIN PREPARATIONS OF

SALMONELLA TYPHIMURIUM INJECTED INTRAPERITONEALLY INTO MICE

Preparation	Dose * (ug)	No. of Mice	Cumulative Deaths, in Days			
			1	2	3	7
Acet-RE	2.5	8	0	0	0	0
	25	8	0	0	0	0
	250	8	0	0	0	0
	500	8	1	1	1	1
	2500	8	2	2	2	2
RE	2.5	8	0	0	0	0
	25	8	0	0	0	0
	250	8	0	1	1	1
	500	8	0	0	0	0
	2500	8	0	0	0	0
Boivin	2.5	8	0	0	0	0
	25	8	0	0	0	0
	250	8	1	1	1	1
	500	8	1	2	4	4
	2500	8	6	7	7	7
Saline Controls	---	8	0	0	0	0

* all preparations given in 0.5 ml saline

TABLE 13

LETHALITY OF POTASSIUM METHYLATE RE, BORON TRIFLUORIDE RE, AND RE PREPARATIONS OF SALMONELLA TYPHIMURIUM INJECTED INTRAPERITONEALLY INTO MICE

Preparation	Dose * (ug)	No. of Mice	Cumulative Deaths, in Days 1	2	3	7
Potassium Methylate RE	2.5	8	0	0	0	0
	25	8	0	0	0	0
	250	8	0	0	0	0
	500	8	0	0	0	0
	2500	8	0	0	0	0
Boron Trifluoride RE	2.5	8	0	0	0	0
	25	8	0	0	0	0
	250	8	0	0	0	0
	500	8	1	1	1	1
	2500	8	0	0	0	0
RE	2.5	8	0	0	0	0
	25	8	0	0	0	0
	250	8	1	1	1	1
	500	8	1	1	1	1
	2500	8	0	0	0	0
Saline Controls	---	8	0	0	0	0

* all preparations given in 0.5 ml saline

III. EXPERIMENTS DESIGNED TO STUDY THE IMMUNOGENIC PROPERTIES OF THE SALMONELLA TYPHIMURIUM ENDOTOXOID PREPARATIONS

A. Preparation of a challenge inoculum reference standard from a strain of S. typhimurium.

The expected efficacy of the various vaccine preparations in humans depends upon an experimental design consisting of a prescribed method of immunization of mice followed by parenteral challenge. In order to standardize as nearly as possible the challenge inoculum of S. typhimurium to be used against the various vaccine preparations, the following experiment was performed. The challenge dose was prepared by harvesting an 18 hour tryptose-phosphate agar slant in PF water, standardizing the suspension turbidimetrically by employing the turbidity reference standard (DBS, NIH) and confirming the challenge numbers by subsequent viability counts employing one ml portions diluted 10^{-6} through 10^{-9}. The data shown in Table 14 suggest that the proper dilution permitting a plate count is in the range of 10^{-7}. Thus, each ml contained approximately 133×10^7 organisms. The mouse virulence of the 10^{-1} through 10^{-4} dilutions of S. typhimurium, as shown in Table 15, was ascertained by ip injection of 0.5 ml employing PF water suspensions of the organisms. Employing the protocol of the U. S. Department of Health, Education, and Welfare for typhoid fever vaccine indicated that the 10^{-1} dilution will kill all of the mice injected within a 3 day period. Moreover, it can be seen from Table 15 that an approximate MLD was obtained only for the 10^{-1} and 10^{-2} dilutions

TABLE 14

AVERAGE VIABLE PLATE COUNTS AFTER 24 HOURS

INCUBATION AT 37° C

Dilution	Averages *
10^{-6}	TNC
10^{-7}	133
10^{-8}	14.3
10^{-9}	2.3

* calculated from 3 plates per dilution

TABLE 15

VIRULENCE TESTS IN MICE EMPLOYING THE FOLLOWING DILUTIONS OF SALMONELLA TYPHIMURIUM

Dilution	No. of bacteria per mouse ip*	No. of Mice	Cumulative Deaths, in Days						
			1	2	3	4	5	6	7
10^{-1}	6.65×10^{7}/0.5 ml	10	9	10	10	10	10	10	10
10^{-2}	6.65×10^{6}/0.5 ml	10	2	5	6	8	9	9	9
10^{-3}	6.65×10^{5}/0.5 ml	10	1	1	2	5	6	7	7
10^{-4}	6.65×10^{4}/0.5 ml	10	1	1	1	1	4	6	6
Control	PF water/0.5 ml	10	0	0	0	0	0	0	0

* from Table 14 where undiluted suspension contained approximately 133×10^{7} org./ml

in a 7 day period. The LD_{50} determination employing the 72 hour end-point for mortality was calculated by the method of Reed and Muench (1938) as shown in Table 16. It can readily be seen that the LD_{50} dilution for this particular experiment was equal to the $10^{-2.4}$ dilution. That is, the ip injection of 2.66×10^6 S. typhimurium organisms per 0.5 ml into 10 mice should kill 50% of them within 72 hours.

In order to further evaluate the above LD_{50} calculation, the same data (Table 15) were subjected to the probit (LD_{50}) method of Miller and Tainter (1944). Table 17 shows the probit values corresponding to the respective mouse deaths for each dilution. These data are depicted graphically in Figure 1 showing both the LD_{50} (probit 5) and the $10LD_{50}$ (probit 6) doses. Thus, it can be seen from this graph that there is good agreement with both methods in determining the LD_{50} as well as the $10LD_{50}$ dose response.

B. The immunogenic effect of HP vaccine preparation in mice against an LD_{50} and a $10LD_{50}$ challenge of S. typhimurium.

The following experiment was carried out in order to evaluate the protective capacity of the HP vaccine, as well as the challenge assay procedure, against both an LD_{50} and a $10LD_{50}$ challenge of S. typhimurium. Two groups of mice were immunized with the HP vaccine by sc injection with two 0.5 ml doses (900×10^6 org./0.5 ml) at 7 day intervals. In addition, one group of mice served as controls receiving only sc injections of saline. Seven days after immunization one group of

TABLE 16

DETERMINATION OF THE LD_{50} WITHIN A 3 DAY PERIOD

BY THE METHOD OF REED AND MUENCH

Dilution	No. of bacteria per mouse ip*	Died	Survived	Cumulative Values Died	Survived	Ratio	%
10^{-1}	6.65×10^7/0.5 ml	10	0	19	0	19/19	100
10^{-2}	6.65×10^6/0.5 ml	6	4	9	4	9/13	69
10^{-3}	6.65×10^5/0.5 ml	2	8	3	12	3/15	20
10^{-4}	6.65×10^4/0.5 ml	1	9	1	21	1/22	4.5

* from Table 14 where undiluted suspensions contained approximately 133×10^7 org/ml

Calculations:

a) $$\frac{\text{\% mortality above 50\% minus 50\%}}{\text{\% mortality above 50\% minus \% mortality below 50\%}} = \frac{69-50}{69-20} = \frac{19}{49} = 0.4$$

b) Negative log of LD_{50} titer = negative log of dilution plus proportionate distance above 50% mortality. Therefore, the negative log of the LD_{50} titer = 2.0 + 0.4 = 2.4 or the LD_{50} titer = $10^{-2.4}$.

c) LD_{50} titer of $10^{-2.4}$ is also equal to the following number of S. typhimurium organisms: 2.66×10^6 (2,660,000) org./0.5 ml.

TABLE 17

DETERMINATION OF THE LD_{50} AND $10LD_{50}$ CHALLENGE WITHIN A 3 DAY PERIOD BY THE METHOD OF MILLER AND TAINTER

Dilution	Mortality Ratio *	%	Probit Values
10^{-1}	10/10	100	6.96
10^{-2}	6/10	60	5.25
10^{-3}	2/10	20	4.16
10^{-4}	1/10	10	3.72

* $\frac{\text{number dead}}{\text{total}}$

Calculation of the $S.E.LD_{50}$:

Probit 5 (LD_{50}) $\cong 10^{-2.4} = 2.66 \times 10^6/0.5$ ml

Probit 6 $\cong 10^{-1.25} = 3.73 \times 10^7/0.5$ ml

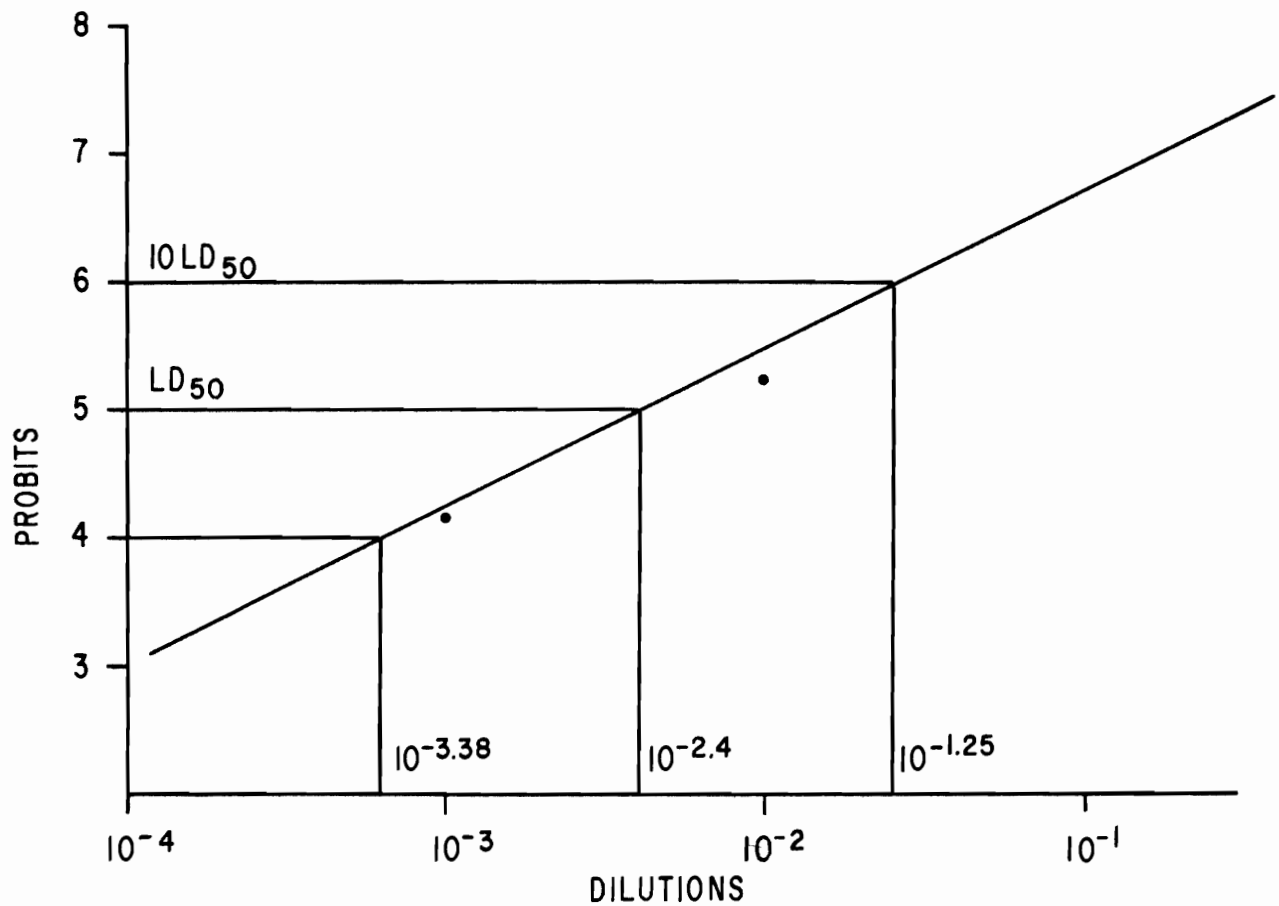
Probit 4 $\cong 10^{-3.38} = 2.77 \times 10^5/0.5$ ml

$2S = P6-P4 = 367.2 \times 10^5$

$$S.E.LD_{50} = \frac{2S}{\sqrt{2N'}} = \frac{367.2}{\sqrt{40}} = \frac{367.2}{6.325} = 5.81 \times 10^6$$

Fig. 1

GRAPHIC ESTIMATION OF THE LD_{50} AND $10\,LD_{50}$ CHALLENGE DOSE.



immunized mice as well as the group of saline control mice were challenged ip with approximately $10LD_{50}$ doses, whereas the second group of immunized mice received an LD_{50} challenge dose. From Table 18, it can be seen that by employing the DBS criteria for recording mortalities (3 days), both groups of immunized mice were protected against their respective challenges. However, the saline control mice show mortalities of greater than 90% in the same 3 day period thus indicating good agreement with the estimate of a $10LD_{50}$ response. It is of interest to note that after 3 days, the cumulative deaths of the immunized mice challenged with the $10LD_{50}$ dose showed a marked increase in mortality on the fourth day, whereas the immunized mice challenged with an LD_{50} dose were protected through the sixth day.

C. The immunogenic effect of potassium periodate RE, RE, and HP vaccine perparations in mice against a $10LD_{50}$ challenge of S. typhimurium.

Since it was shown in the previous experiment that a $10LD_{50}$ challenge dose gave a reproducible endpoint within the 3 day observation period, it was decided to employ this dose for all challenge experiments involving the various vaccine preparations. Table 19 shows results obtained with four groups of mice immunized with potassium periodate RE, RE, saline, and HP vaccine preparations, respectively. Each animal received 2 sc injections, 7 days apart, containing an arbitrarily arrived at dose of 100 ug/0.5 ml since <200 ug of the potassium periodate RE preparation was found to be the approximate MPD in rabbits (see Part I of the Experimental Results). All groups of mice, including

TABLE 18

EFFECT OF IMMUNIZATION OF HP VACCINE PREPARATION IN MICE AGAINST AN LD_{50} AND A $10LD_{50}$ CHALLENGE OF SALMONELLA TYPHIMURIUM

Treatment 0.5 ml, sc +	No. of bacteria per mouse ip*	No. of Mice	Cumulative Deaths, in Days 1	2	3	4	5	6	7
Immunized (HP vaccine)	3.73×10^7/0.5 ml ($10LD_{50}$)	20	0	0	1	14	16	18	19
Immunized (HP vaccine)	2.66×10^6/0.5 ml (LD_{50})	17	0	0	0	0	0	8	13
Non-immunized Saline Controls	3.73×10^7/0.5 ml ($10LD_{50}$)	25	19	22	23	24	25	25	25

+ 2 injections 7 days apart

* challenged 7 days from last injection

TABLE 19

EFFECT OF IMMUNIZATION OF POTASSIUM PERIODATE RE, RE, AND HP VACCINE PREPARATIONS IN MICE AGAINST A $10LD_{50}$ CHALLENGE OF SALMONELLA TYPHIMURIUM *

Treatment 0.5 ml, sc+	Immun. Dose	No. of Mice	Cumulative Deaths, in Days						
			1	2	3	4	5	6	7
Potassium Periodate RE	100 ug	20	12	14	17 (S:>.05)# (HP:<.01)	19	20	20	20 (S:>.05) (HP:<.01)
RE	100 ug	20	6	6	11 (S:.01)	14	15	17	18 (S:>.05)
HP	--	19	0	0	0 (S:<.01)	5	6	9	11 (S:<.01)
Saline	--	20	16	19	19	20	20	20	20

* challenged ip 7 days from last injection with approximately 3.32×10^7 org./0.5 ml

\+ 2 injections 7 days apart

\# P value

the saline control group, were challenged one week after the last immunizing injection with an approximate $10LD_{50}$ of S. typhimurium. By employing the DBS criteria of recording lethalities over a 3 day period, it can be seen in Table 19 that the HP vaccine immunized mice show the best protection followed by the parent RE and potassium periodate RE preparations, respectively. These data show that for all practical purposes the potassium periodate (oxidation) procedure destroys vaccine immunogenicity. It can be seen that at the 100 ug immunizing dose level there is an 85% mouse mortality within the 72 hour observation period which correlates well with the saline control mice mortality of greater than 90%. That is, both groups are responding to the $10LD_{50}$ challenge within the 3 day observation period. Further evidence that the periodate procedure destroys mouse immunogenicity is seen also with the parent RE preparation in Table 19. When compared to this vaccine preparation under similar conditions, it is obvious that the procedure of periodation has a deleterious effect on the immunogenicity of the parent RE preparation.

Statistical treatment of the above data, as well as all subsequent challenge data, by chi-square analysis (Mainland, Herrera, and Sutcliffe, 1956) suggest with confidence that the potassium periodate procedure destroys immunogenicity. The difference in protective capacity between the periodate vaccine preparation and the saline (S:) control mice at both the third and seventh day of the observation period, as shown in Table 19, indicates no level of protection. However, when the periodate preparation was compared with the HP vaccine (HP:) preparation on the

same days of observation the results were significant at the one per cent level. That is, the mouse protection afforded by the HP vaccine compared to the potassium periodate RE vaccine preparation was significantly different.

D. The immunogenic effect of Acet-RE, RE, Boivin, and HP vaccine preparations in mice against a $10LD_{50}$ challenge of S. typhimurium.

The data in the next two tables (Tables 20 and 21) show results of groups of mice immunized with the Acet-RE, RE, Boivin, saline, and HP vaccine preparations, respectively. Each animal received 2 sc injections, 7 days apart, with the amounts shown under the column headed Immunizing Dose. The immunizing doses of 100 and 200 ug/0.5 ml for the various preparations shown in both tables were arbitrarily arrived at since, as mentioned earlier in Part I of the Experimental Results, $<$200 ug of the Acet-RE preparation was found to be nonpyrogenic in rabbits. All groups of mice, including the saline control group, were challenged one week after the last immunizing injection with an approximate $10LD_{50}$ dose.

By employing again the DBS criteria of recording deaths over a 3 day period, it can be seen in both of these tables that the HP vaccine provided the best protection when compared to the respective saline control mice as well as the mice immunized with 100 ug dose amounts. Of the latter, as shown in Table 20, the Boivin preparation immunized mice show the greatest protection followed by the RE and Acet-RE vaccine preparations, respectively. However, as shown in Table 21, the deaths

TABLE 20

EFFECT OF IMMUNIZATION OF ACET-RE, RE, BOIVIN, AND HP VACCINE PREPARATIONS IN MICE AGAINST A $10LD_{50}$ CHALLENGE OF SALMONELLA TYPHIMURIUM *

Treatment 0.5 ml, sc+	Immun. Dose	No. of Mice	Cumulative Deaths, in Days						
			1	2	3	4	5	6	7
Acet-RE	100 ug	20	10	13	13 (S:<.01)# (HP:<.01)	15	18	18	18 (S:>.05)
RE	100 ug	20	2	11	11 (S:<.01)	15	17	18	18 (S:>.05)
Boivin	100 ug	19	3	5	7 (S:<.01)	11	15	16	17 (S:>.05)
HP	--	20	0	0	1 (S:<.01)	6	9	13	16 (S:>.05)
Saline	--	20	17	20	20	20	20	20	20

* challenged ip 7 days from last injection with approximately 3.47×10^7 org./0.5 ml

\+ 2 injections 7 days apart

\# P value

TABLE 21

EFFECT OF IMMUNIZATION OF ACET-RE, RE, AND HP VACCINE PREPARATIONS IN MICE AGAINST A $10LD_{50}$ CHALLENGE OF SALMONELLA TYPHIMURIUM *

Treatment 0.5 ml, sc+	Immun. Dose	No. of Mice	Cumulative Deaths, in Days						
			1	2	3	4	5	6	7
Acet-RE	200 ug	20	1	2	7 (S:<.01)# (HP:>.05)	10	17	20	20 (S:>.05)
RE	200 ug	19	1	1	4 (S:<.01)	9	12	15	17 (S:>.05)
HP	--	20	1	1	2 (S:<.01)	5	8	13	15 (S:>.01-<.05)
Saline	--	20	16	18	20	20	20	20	20

* challenged ip 7 days from last injection with approximately 2.95×10^7 org./0.5 ml

\+ 2 injections 7 days apart

\# P value

recorded over a 3 day period in mice immunized with two 200 ug (two times the MPD) doses using the same Acet-RE vaccine preparation indicate a good protective effect when compared to the saline immunized mice following challenge. Moreover, these results seem to correlate well with the results obtained with the HP vaccine immunized mice challenged with the same $10LD_{50}$ dose. Further evidence of this correlation between the Acet-RE and HP vaccine preparations was shown by chi-square analysis. Although both of these preparations show significant protection at the one per cent level when compared to the saline control group after 3 days, the mouse protection between the Acet-RE and HP vaccine preparations was not significant (HP:$>$.05). Thus, under the conditions of these experiments, the 200 ug immunizing dose of the Acet-RE vaccine preparation, but not the 100 ug dose, yielded results not significantly different from those obtained with HP vaccine.

It is interesting to note that no significant difference in mouse protection occurs with either the RE or the Acet-RE vaccine preparations (Tables 20 and 21) throughout the observation period, whereas similarities in mouse protection with the Boivin and HP vaccine immunized mice are apparent only during the first 4 days. Moreover, the results with the saline controls suggest a reliable $10LD_{50}$ challenge dose response in both experiments during the first 24 hours. Therefore, it is apparent that all of the vaccine preparations are showing some protection when compared to the controls during the first few days of the observation period. This impression is supported by chi-square analysis of the data

at both the third and seventh day after ip challenge.

E. The immunogenic effect employing various concentrations of the Acet-RE vaccine preparation in mice against a $10LD_{50}$ challenge of S. typhimurium.

The data in previous experiments indicate that the MPD of the Acet-RE preparation is approximately 100 ug and the minimal immunogenic dose (MID) is 200 ug. The question was asked whether prolonged immunization of mice employing the MPD or lessor amounts of the same Acet-RE preparation might yield significant protection against challenge. In this experiment, seven groups of mice were immunized in the manner shown in Table 22. The effect of 3 weekly immunizations in mice employing 50 and 100 ug doses of the Acet-RE vaccine preparation followed by approximate $10LD_{50}$ challenge 7 days after the last immunizing dose is shown in Table 23. Although the single and three weekly 50 and 100 ug immunizing doses yielded little or no protective effect at the end of 72 hours, there is some protection when compared to the control mice receiving saline. However, when compared to either of the HP vaccine immunized mice, the 100 ug amount of the Acet-RE vaccine preparation is still less effective in its protective capacity in mice at the critical 3 day observation period. Treatment of these data by chi-square analysis and comparison of the various Acet-RE preparations with either the saline control mice or the HP vaccine immunized mice lend further support to these observations. That is, the level of significance of the difference in the vaccine preparations at 72 hours with three weekly sc injections of either 50 or 100 ug dose amounts of the Acet-RE preparation when

TABLE 22

MOUSE IMMUNIZATION SCHEDULE

Mouse Group	Immunizing Dose *	Weeks before challenge 3	2	1
1	50 ug			Acet-RE
2	50 ug	Acet-RE	Acet-RE	Acet-RE
3	100 ug			Acet-RE
4	100 ug	Acet-RE	Acet-RE	Acet-RE
5	9×10^8 org.			HP
6	9×10^8 org.		HP	HP
7	---			Saline

* each immunizing dose injected subcutaneously in a volume of 0.5 ml

TABLE 23

EFFECT OF IMMUNIZATION EMPLOYING VARIOUS CONCENTRATIONS OF AN ACET-RE VACCINE PREPARATION IN MICE AGAINST A $10LD_{50}$ CHALLENGE OF SALMONELLA TYPHIMURIUM *

Treatment 0.5 ml, sc	Immun. Dose	No. of Mice	Cumulative Deaths, in Days						
			1	2	3	4	5	6	7
Acet-RE[1]	50	20	16	17	20 (S:>.05)# (HP[2]:<.01)	20	20	20	20 (S:>.05)
Acet-RE[3]	50	20	9	11	12 (S: .01) (HP[2]:<.01)	14	17	20	20 (S:>.05)
Acet-RE[1]	100	18	6	11	16 (S:>.05) (HP[2]:<.01)	16	18	18	18 (S:>.05)
Acet-RE[3]	100	20	1	1	11 (S:<.01) (HP[2]:.01)	14	17	17	17 (S:>.05)
HP[1]	--	18	0	0	4 (S:<.01)	9	15	16	16 (S:>.05)
HP[2]	--	20	0	0	2 (S:<.01)	8	12	13	16 (S:>.05)
Saline[1]	--	20	14	18	20	20	20	20	20

* challenged ip 7 days from last injection with approximately 4.54×10^7 org./0.5 ml
1 one injection 7 days before challenge
2 two injections 7 and 14 days before challenge
3 three injections 7, 14 and 21 days before challenge
\# P value

compared to the saline immunized mice is at the one per cent level. In addition, these same preparations when compared to the HP vaccine are also significant at the one per cent level indicating that this latter vaccine is, under the conditions of this experiment, still more protective in mice. It is interesting to note from Table 23 that after 7 days of challenge none of the vaccine preparations have capacity to protect mice.

F. The immunogenic effect employing two 100 ug immunizing doses per week (total 400 ug) of the Acet-RE vaccine preparation in mice against a $10LD_{50}$ challenge of S. typhimurium.

In the experiment to be described, four groups of mice were immunized in the manner shown in Table 24. The 100 ug dose of the Acet-RE vaccine preparation was again arbitrarily selected for this mouse protection experiment since <200 ug of this same preparation was found previously to be nonpyrogenic in rabbits (Part I, Experimental Results). All groups of mice, including the saline treated control group, were challenged with an approximate $10LD_{50}$ one week after the last immunizing injection. By employing the DBS criteria of recording lethalities over a three day period, it can be seen in Table 25 that there is no significant difference in protection between the mice immunized with one weekly 100 ug dose and mice immunized with two per week 100 ug doses of the Acet-RE vaccine preparation. That is, there is no significant protection in mice receiving a total of 200 ug as compared to 400 ug immunizing doses over a two week period followed by the same

TABLE 24

MOUSE IMMUNIZATION SCHEDULE

Mouse Group	Immunizing Dose *	Days before challenge 18	14	11	7
1	100 ug	Acet-RE	Acet-RE	Acet-RE	Acet-RE
2	100 ug		Acet-RE		Acet-RE
3	9×10^8 org.		HP		HP
4	---				Saline

* each immunizing dose injected subcutaneously in a volume of 0.5 ml

TABLE 25

EFFECT OF IMMUNIZATION EMPLOYING TWO 100 ug IMMUNIZING DOSES PER WEEK (TOTAL 400 ug) OF AN ACET-RE VACCINE PREPARATION IN MICE AGAINST A $10LD_{50}$ CHALLENGE OF SALMONELLA TYPHIMURIUM*

Treatment 0.5 ml, sc	Immun. Dose	No. of Mice	Cumulative Deaths, in Days						
			1	2	3	4	5	6	7
Acet-RE[1]	100 ug	19	3	6	12 (S:.01)# (HP:<.01)	13	16	17	18 (S:>.05)
Acet-RE[2]	100 ug	20	5	12	14 (S:.05) (HP:<.01)	17	18	20	20 (S:>.05)
HP[2]	--	18	0	0	0 (S:<.01)	2	6	11	12 (S:>.01-<.05)
Saline[2]	--	20	15	19	20	20	20	20	20

* challenged ip 7 days from last injection with approximately 2.13×10^7 org./0.5 ml

1 two weekly injections over a two week period - 4 injection total

2 one weekly injection over a two week period - 2 injection total

\# P value

$10LD_{50}$ challenge. Although there is good evidence of mouse protection with these two Acet-RE preparations when compared with the saline control mice (S:01 and S:05, respectively), these same vaccines when compared to the HP vaccine show little protective effect (HP: $<.01$ and HP:$<.01$, respectively). Moreover, the mouse protective data seven days after challenge show that both the immunizing doses of the Acet-RE preparation (S:$>.05$) have diminished protective capacity in mice when again compared to the HP vaccine (S:$>.01 - <.05$). Thus, the cumulative deaths recorded on post-challenge days 5, 6, and 7 suggest that all of the vaccine preparations, including the HP vaccine, were less effective in mice four or more days after challenge. This latter observation has been consistently observed with all our vaccine preparations.

G. The immunogenic effect employing two weekly 500 ug immunizing doses (total 1,000 ug) of the Acet-RE vaccine preparation in mice against a $10LD_{50}$ challenge of S. typhimurium.

Since all previous results indicated that the HP vaccine is more protective in mice against a $10LD_{50}$ challenge than any of the Acet-RE immunizing doses employed (50, 100, 200 ug), the hypothesis that the Acet-RE vaccine preparation is not as immunogenic as the HP vaccine was examined. That is, rather than relying on the approximate MPD of the Acet-RE preparation as the MID to employ in mouse immunization experiments, the following considerations suggested other possibilities. Approximately 1 mg of wet bacteria contains 10^9 bacterial cells (Oginsky and Umbriet, 1959). The HP vaccine employed in our active mouse

immunization experiment contains approximately 900 x 10^6 bacteria per 0.5 ml. This value suggests that our mice are receiving anywhere from 500 to 1000 ug per immunizing dose, whereas our Acet-RE preparations have been used only in 50, 100 and 200 ug/0.5 ml immunizing doses since the MPD of this preparation is approximately 100 ug. Thus, the difference in vaccine strength between these two vaccines may be 5 to 10 times and, on a weight basis, we may be administering 5 to 10 times more of the HP vaccine than of the Acet-RE preparation. On the basis of this reasoning the following experiment was carried out. To test both the HP and Acet-RE vaccines at approximately equivalent concentrations and compare their respective immunogenic capacities under similar conditions, two weekly 500 ug mouse immunizing doses of the Acet-RE as well as the HP vaccine were given followed 7 days later by a $10LD_{50}$ challenge of S. typhimurium.

The results shown in Table 26 support the above hypothesis that there is a factor of approximately 5 to 10 in vaccine strength between the two vaccines. That is, there appears to be no significant difference in mouse mortality either at the 3 day or 7 day observation period. These data suggest that the difference between the MPD (100 ug) and the effective immunogenic dose (EID) (500 ug) is approximately 5 to 10 in rabbits and mice, respectively. In other words, it takes approximately 5 to 10 times the MPD of the Acet-RE preparation to give an immunogenic dose (EID) equal to that of the HP vaccine.

TABLE 26

EFFECT OF IMMUNIZATION EMPLOYING TWO WEEKLY 500 ug IMMUNIZING DOSES (TOTAL 1,000 ug) OF AN ACET-RE VACCINE PREPARATION IN MICE AGAINST A 10LD_{50} CHALLENGE OF SALMONELLA TYPHIMURIUM *

Treatment 0.5 ml, sc+	Immun. Dose	No. of Mice	Cumulative Deaths, in Days						
			1	2	3	4	5	6	7
Acet-RE	500 ug	20	0	0	3 (S:<.01)# (HP:>.05)	8	9	11	13 (S:.01)
HP	--	18	0	0	0 (S:<.01)	5	8	10	13 (S:>.01-<.05)
Saline	--	20	16	18	19	20	20	20	20

* challenged ip 7 days from last injection with approximately 3.23 x 10^7 org./0.5 ml

\+ 2 injections 7 days apart

\# P value

H. The immunogenic effect of potassium methylate RE, boron trifluoride RE, RE, and HP vaccine preparations in mice against a $10LD_{50}$ challenge of S. typhimurium.

In this experiment, five groups of mice were immunized with potassium methylate RE, boron trifluoride RE, saline, HP vaccine, and RE preparations, respectively. Each animal received 2 sc injections, 7 days apart, containing an arbitrary dose of 100 ug/0.5 ml. All groups of mice, including the saline control groups, were challenged ip one week after the last immunizing injection with an approximate $10LD_{50}$ dose. By employing the DBS criteria (recording deaths over a three day period), it can be seen in Table 27 that, with the exception of the HP vaccine protected mice, the potassium methylate RE immunized mice showed the most protection followed by the RE and BF_3 preparations, respectively. It is interesting to note that the 100 ug immunizing dose of the potassium methylate RE preparation gave approximately twice the protection in mice obtained with similar amounts of the BF_3, the RE and, as elaborated in the Discussion, the Acet-RE immunizing preparations. Thus, the cumulative deaths for both the potassium methylate RE and the HP vaccine preparations were very similar in their protective capacity in mice throughout the observation period, whereas the boron trifluoride RE and the RE preparations showed a definite breakdown in mouse immunity after 48 hours. The significance of these observations is seen from the respective chi-square analysis shown in Table 27 for both the third and seventh day after ip challenge. That is, there is no significant

TABLE 27

EFFECT OF IMMUNIZATION OF POTASSIUM METHYLATE RE, BORON TRIFLUORIDE RE, RE, AND HP VACCINE PREPARATIONS IN MICE AGAINST A $10LD_{50}$ CHALLENGE OF SALMONELLA TYPHIMURIUM *

Treatment 0.5 ml, sc+	Immun. Dose	No. of Mice	Cumulative Deaths, in Days						
			1	2	3	4	5	6	7
Potassium Methylate RE	100 ug	20	2	3	5 (S:<.01)# (HP:>.05)	8	11	12	13 (S:.01) (HP:>.05)
Boron Trifluoride RE	100 ug	20	7	12	16 (S:>.05) (HP:<.01)	19	20	20	20 (S:>.05)
RE	100 ug	20	2	11	11 (S:<.01)	15	17	18	18 (S:>.05)
HP	--	20	1	1	2 (S:<.01)	5	8	13	15 (S:.05)
Saline	--	20	16	18	20	20	20	20	20

* challenged ip 7 days from last injection with approximately 2.95×10^7 org./0.5 ml

\+ 2 injections 7 days apart

\# P value

difference (HP: $>.05$) in mouse protection between the potassium methylate RE and the HP vaccine preparation during the entire observation period. On the other hand, the boron trifluoride RE preparation, under similar conditions, shows very little mouse protection. Comparing the third day data between the saline control and the BF_3 treated mice respectively, there is no significant difference. However, compared to the saline control mice it is apparent that all 4 vaccine preparations are showing some protection (HP > potassium methylate > RE > BF_3) during the first 48 hours of the observation period.

I. The immunogenic effect employing various concentrations of the potassium methylate RE vaccine preparation in mice against a $10LD_{50}$ challenge of S. typhimurium.

Since it has been previously shown (Part I, Experimental Results) that the MPD of the potassium methylate RE vaccine preparation is approximately 10 ug ($>1 <10$), an attempt was made to determine whether the immunogenic effect in mice is retained by decreasing the immunizing dose of this preparation to 2 sc injections containing 10 and 50 ug/0.5 ml, respectively. Table 28 shows the results of this experiment. Compared to the saline immunized mice following challenge, the 10 ug immunizing dose of the potassium methylate RE vaccine preparation shows some protection for a 48 hour period following challenge. However, the 50 ug immunizing dose of this preparation indicates a definite protective effect in mice during the first 3 days of the observation period. In addition, these latter results seem to compare favorably

TABLE 28

EFFECT OF IMMUNIZATION EMPLOYING VARIOUS CONCENTRATIONS OF A POTASSIUM METHYLATE RE VACCINE PREPARATION IN MICE AGAINST A $10LD_{50}$ CHALLENGE OF *SALMONELLA TYPHIMURIUM* *

Treatment 0.5 ml, sc+	Immun. Dose	No. of Mice	Cumulative Deaths, in Days						
			1	2	3	4	5	6	7
Potassium Methylate RE	10 ug	20	8	11	18 (S:>.05)# (HP:<.01)	19	19	19	19 (S:>.05)
Potassium Methylate RE	50 ug	20	3	5	9 (S:<.01) (HP:.05)	14	18	19	19 (S:>.05)
HP	--	20	0	0	2 (S:<.01)	7	10	12	13 (S:.01)
Saline	--	20	16	18	19	19	19	20	20

* challenged ip 7 days from last injection with approximately 2.78×10^7 org./0.5 ml

\+ 2 injections 7 days apart

\# P value

with the results obtained with HP vaccine immunized mice challenged with the same $10LD_{50}$ dose. Chi-square analysis of these data reveal that 72 hours after challenge the difference in mouse protection between the potassium methylate RE and HP vaccine preparations is highly significant (HP: $<.01$) employing the 10 ug immunizing dose whereas the 50 ug dose is significant just at the 5% level (HP: .05). These data (Tables 27 and 28) suggest that the EID of the potassium methylate RE vaccine preparation is approximately 100 ug. However, as previously shown and elaborated in the Discussion, these results suggest a ten fold discrepancy between the MPD and the possible EID.

IV. EXPERIMENTS DESIGNED TO STUDY THE IN VITRO ANTIGENIC SPECIFICITY OF THE SALMONELLA TYPHIMURIUM ENDOTOXOID PREPARATIONS

A. The serological specificity of the various vaccine preparations as determined by the agar gel diffusion test (Ouchterlony).

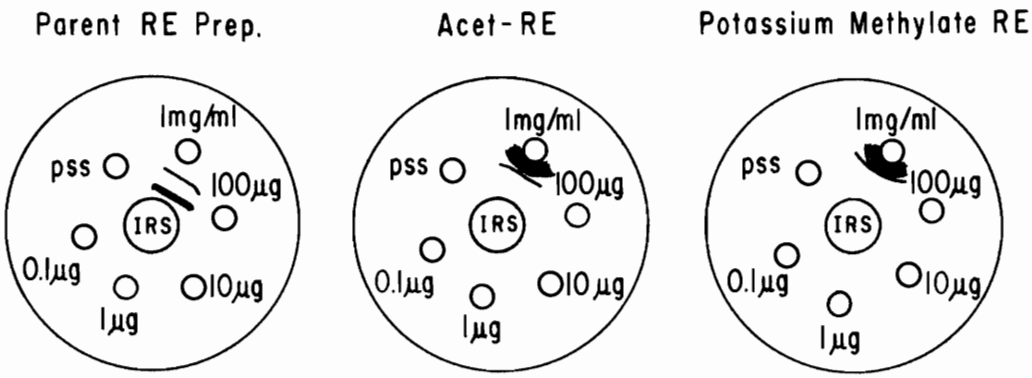
The following experiments employing the gel double diffusion test were carried out in order to determine whether an antigen-antibody reaction can occur between the altered antigens (endotoxoids) and sera obtained from rabbits which had been previously immunized with the parent RE preparation (see Part V, Materials and Methods). These studies were designed to determine whether certain antigen-antibody identities of the various endotoxoids were similar in character. Such findings would indicate that the immunogenicity of the parent RE preparation was not

altered and/or destroyed by chemical manipulation. Diagrams of the results of duplicate experiments can be seen in Figure 2 in which 10 fold dilutions of the respective endotoxoids were placed in adjacent wells around the immune rabbit antiserum (IRS). Under these experimental conditions characteristic lines of precipitation occurred with all but the boron trifluoride RE and the potassium periodate RE vaccine preparations. These data correlate well with the previous observations that both the BF_3 and the potassium periodate preparations yielded endotoxoids with poor immunogenicity in the active-immunized mouse protection tests. Duplicate plates set up with normal rabbit serum (NRS) showed no lines of precipitation with any of the endotoxoids tested further supporting the validity of these observations.

In order to characterize the antigen-antibody identities of the various endotoxoids further, immunodiffusion tests were set up as shown in Figure 3. All antigen preparations, including the Boivin antigen, were employed in concentrations of 1 mg/ml and set up in duplicate plates placed in adjacent wells around either IRS or NRS. Although the potassium periodate or the BF_3 preparations show no lines of precipitation with either the IRS or the NRS, it is obvious that the Boivin, Acet-RE, potassium methylate RE, and RE preparations show characteristic identities which appear, in some cases, to be merging with one another. Moreover, these results suggest that certain antigenic sites were completely abolished as with the boron trifluoride RE and the potassium periodate RE preparations, whereas the remaining endotoxoid

Fig. 2

SEROLOGICAL SPECIFICITY OF THE VARIOUS VACCINE PREPARATIONS AS DETERMINED BY THE AGAR GEL DIFFUSION TEST.



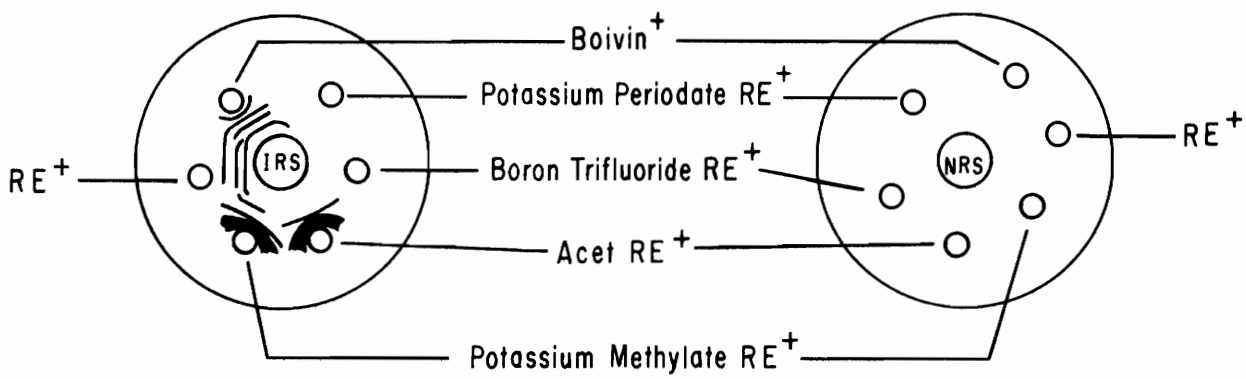
Boron Trifluoride RE
Negative at all Concentrations

Potassium Periodate RE
Negative at all Concentrations

IRS - Rabbit anti-RE Serum

Fig. 3

SEROLOGICAL SPECIFICITY OF THE VARIOUS VACCINE PREPARATIONS AS DETERMINED BY THE AGAR GEL DIFFUSION TEST.



IRS-Rabbit Anti-RE Serum

NRS-Normal Rabbit Serum

+ 1 mg/ml

preparations, with their lines of precipitation, show evidence of either alteration and/or incomplete destruction of their respective antigenic sites. These observations correlate well with those obtained with the various endotoxoids employed in mouse immunization experiments.

V. EXPERIMENTS DESIGNED TO STUDY THE REPRODUCIBILITY OF THE ACETYLATION PROCEDURE IN REGARD TO PYROGENICITY, LETHALITY AND ACTIVE MOUSE IMMUNIZATION STUDIES

A. A summary of the pyrogen test responses in rabbits employing two new Acet-RE preparations.

Two new acetylated-RE preparations (Acet-RE-I and Acet-RE-II, respectively) were prepared from the original parent RE S. typhimurium preparation. The only departure from the original acetylation procedure (see Part III, Materials and Methods) was that the Acet-RE-II preparation was acetylated in a boiling water bath for 6 hours instead of the usual 4 hours and, in addition, instead of grinding the acetylated material by mortar and pestle by hand, both of the new acetylated preparations were ground to a powder (approximately 20 minutes) by employing a motor driven mortar and pestle (Fisher Mortar Grinder). This motor driven mortar grinder, however, was not employed when triturating with saline to make up the stock suspensions (50 ug/10 ml) prior to testing in animals. That is, both the Acet-RE-I and the Acet-RE-II stock suspensions were triturating in saline suspension employing a hand mortar and pestle. From an initial weight of 500 mg, the yields of both the Acet-RE-I and Acet-RE-II preparations were 421.8 mg (84.4%) and 406.6 mg (81.3%) respectively. Both final products were strongly lyophobic and were granular

in appearance. Unlike the original Acet-RE and Acet-RE-I preparations, however, the color of the final product of the Acet-RE-II preparation was rust-like instead of light tan in color. Chemical analysis of the respective acetyl contents (Australian Microanalytical Service, University of Melbourne) as $COCH_3$ (methanolic NaOH used as the saponification agent) was 11.7% for Acet-RE-I and 10.5% for Acet-RE-II.

Although the method of preparation, yields and acetyl content of these two preparations were generally the same, it was necessary to test these preparations, under similar conditions, for pyrogenicity, lethality and active mouse-protection. These experiments were designed to yield information concerning the reproducibility of the acetylation procedure by comparing these data with the data obtained from the original Acet-RE preparation. That is, does the acetylation of a crude endotoxin prepared by the RE method from a strain of S. typhimurium produce a product with consistent biological effects with relation to pyrogenicity, lethality and active mouse immunization studies? The following animal data (Tables 29, 30, and 31) indicate that the results of acetylation are reproducible.

The reduction in pyrogenicity exhibited by the three Acet-RE preparations is shown in summary form in Table 29. It is interesting to note that the two new Acet-RE preparations (Acet-RE-I and Acet-RE-II) both show MPD of 50 ug, whereas the original Acet-RE preparation shows a MPD of 100 ug. Moreover, 6 hours of acetylation (Acet-RE-II) when compared to 4 hours of acetylation (Acet-RE and Acet-RE-I) apparently

TABLE 29

A SUMMARY OF THE PYROGEN TEST RESPONSES IN RABBITS EMPLOYING TWO NEW ACET-RE PREPARATIONS

	Acetyl Content % +	Amounts Tested *	Pyrogen Test Result
Acet-RE Preparation (4 hours)	6.8	1.0	-
		10	-
		50	-
		100	-
		500	+
		1000	+
Acet-RE-I Preparation (4 hours)	11.7	1.0	-
		10	-
		50	-
		100	+
		500	+
Acet-RE-II Preparation (6 hours)	10.5	1.0	-
		10	-
		50	-
		100	+
		500	+
RE Preparation	--	1.0	+
		10	+

* ug/33 ml PFPSS

\+ calculated as $COCH_3$

TABLE 30

A SUMMARY OF MOUSE LETHALITY RESPONSES EMPLOYING

TWO NEW ACET-RE PREPARATIONS

Preparation	Dose * (ug)	No. of Mice	Cumulative Deaths, in Days 1	2	3	7
Acet-RE Preparation (4 hours)	2.5	8	0	0	0	0
	25	8	0	0	0	0
	250	8	0	0	0	0
	500	8	1	1	1	1
	2500	8	2	2	2	2
Acet-RE-I Preparation (4 hours)	2.5	8	0	0	0	0
	25	8	0	0	0	0
	250	8	0	0	0	0
	500	8	0	0	0	0
	2500	8	2	3	3	3
Acet-RE-II Preparation (6 hours)	2.5	8	0	0	0	0
	25	8	0	0	0	0
	250	8	0	0	0	0
	500	8	0	0	0	0
	2500	8	0	0	0	0
RE Preparation	2.5	8	0	0	0	0
	25	8	0	0	0	0
	250	8	0	0	0	0
	500	8	1	1	1	1
	2500	8	1	1	1	1
Saline Controls	--	8	0	0	0	0

* all preparations given in 0.5 ml saline

TABLE 31

EFFECT OF IMMUNIZATION EMPLOYING TWO NEW ACET-RE VACCINE PREPARATIONS (ACET-RE-I AND ACET-RE-II) IN MICE AGAINST A $10LD_{50}$ CHALLENGE OF SALMONELLA TYPHIMURIUM *

Treatment 0.5 ml, sc+	Immun. Dose	No. of Mice	Cumulative Deaths, in Days						
			1	2	3	4	5	6	7
Acet-RE	50 ug	20	5	12	14 (S:.05)# (HP:<.01)	17	18	19	19 (S:>.05)
Acet-RE-I	50 ug	20	6	10	16 (S:>.05) (HP:<.01)	18	19	19	20 (S:>.05)
Acet-RE-II	50 ug	20	10	13	15 (S:>.05) (HP:<.01)	19	20	20	20 (S:>.05)
RE	--	20	11	12	15 (S:>.05)	18	19	19	20 (S:>.05)
HP	--	19	0	0	1 (S:<.01)	6	8	10	12 (S:.01)
Saline	--	19	15	18	19	19	19	19	19

* challenged ip 7 days from last injection with approximately 2.96×10^7 org./0.5 ml

\+ 2 injections 7 days apart

\# P value

has little or no effect on rabbit pyrogenicity. Thus, it appears there is only a two fold decrease in rabbit pyrogenicity with both the 4 and 6 hour acetylated preparations (Acet-RE-I and Acet-RE-II, respectively) when compared to the original Acet-RE preparation.

These data suggest that the MPD of these acetylated endotoxin preparations, when compared to the MPD (1 ug) of the parent RE preparation, is >50 <100 ug as interpreted by the USP rabbit pyrogen test.

B. A summary of mouse lethality responses employing two new Acet-RE preparations.

Table 30 shows the data from experiments comparing the lethality in mice by ip injection of the various acetylated preparations with that of the parent RE endotoxin preparation. As noted earlier in Part II of the Experimental Results by comparing the parent RE preparation, which is apparently non-lethal for mice at the doses shown, with similar amounts of Acet-RE preparations suggests that acetylation may induce lethality in mice at high concentrations (>500 ug). However, the results shown for the Acet-RE-II preparation reveal no mouse lethalities even at high concentrations suggesting that 6 hours of acetylation (as compared to 4 hours) may aid in altering and/or decreasing the toxic portion induced apparently by the 4 hour acetylation procedure.

C. The immunogenic effect employing two new Acet-RE vaccine preparations (Acet-RE-I and Acet-RE-II) in mice against a 10LD_{50} challenge of S. typhimurium.

Since it had been shown previously that the MPD of the two new

Acet-RE preparations (Table 29) was approximately 50 ug, the following experiment shows data employing all of the respective Acet-RE vaccine preparations in two weekly sc 50 ug (total 100 ug) mouse immunizing doses. In addition, these data, under similar conditions, are compared with the HP vaccine as well as with 50 ug immunizing doses of the parent RE preparation. All groups of immunized mice, including the saline group, were challenged one week after the last immunizing injection with an approximate $10LD_{50}$ dose. As can be seen in Table 31 and by employing the DBS criteria of recording deaths over a three day period, there is little or no significant difference ($S:.05$ or $>.05$) in mouse protection with any of the acetylated preparations as well as with the parent RE preparation when compared to the saline control mice. Although there was very little significant difference in mouse protection with any of the Acet-RE vaccine preparations, whether at the 3 or 7 day observation period, it can be noted that the overall protection is small when compared to the HP vaccine (HP: $<.01$). That is, there is no significant difference in protection throughout the observation period with either the saline or any of the Acet-RE immunized mice.

DISCUSSION

The data reported in this dissertation show that treatment of a crude endotoxin prepared by the RE procedure from a strain of S. typhimurium with the various chemical procedures described yielded certain significant changes in biological activity. Others have reported similar data employing a more purified endotoxin as the starting material. That is, with the exception of the potassium periodate RE preparation, the results obtained with our endotoxoids compared favorably with those prepared by others with regard to yield, pyrogenicity, lethality, and mouse immunogenicity. Where differences do occur, it may be due to the various gram-negative organisms employed as well as the "purity" of the respective endotoxins used as starting materials. On the other hand, it is interesting to note that under similar conditions we have tested an RE and a Boivin preparation from an S. typhimurium strain in rabbits and that both of these preparations yielded identical lower limit fever responses of 1.0 ug per rabbit (Tables 2, 3 and 10). Since the RE method must represent primarily denaturation of bacterial protein, these results suggest that the molecular site or sites responsible for fever in rabbits are not altered to any great extent when the bacterial cell walls are extracted with TCA as is done in the Boivin procedure. However, molecular sites responsible for biological properties other than fever may be subjected to alteration upon TCA extraction. Moreover, the effects of physical

and chemical agents on the biological properties of purified entero-bacterial lipopolysaccharides indicate that the various biological activities of bacterial endotoxins are affected differentially by different physical and chemical agents (Neter et al., 1956). The latter concept is also evident in our studies. The results using the RE preparation as the parent endotoxin suggest that the molecular site or sites were not altered and/or exposed to the same extent as compared to the bacterial cell walls which were extracted with the many procedures capable of yielding a much "cleaner" preparation of endotoxin. Therefore, the differences reported that do exist between the endotoxoids studied in this thesis and those prepared by others as noted (pps. 69, 71, and 74 in Literature Review) are not of great magnitude.

As was pointed out in the Introduction, a main purpose of this study was the possible development of a less toxic enteric fever vaccine. Each endotoxoid preparation was tested for pyrogenicity in rabbits as well as mouse lethality and immunogenicity, and these data were compared with similarly obtained parent RE preparation data. With this in mind and also for convenience and clarity, each of the endotoxoid preparations studied will be discussed in the general order of rabbit pyrogenicity, followed by mouse lethality, and then mouse immunogenicity.

The pyrogen test responses of the respective Acet-RE and the potassium periodate RE preparations indicated a $>100<200$ fold reduction in pyrogenicity for both preparations when compared to similar doses of parent RE and Boivin preparations. Although these experimental data

approximate the upper and lower limits of decrease in pyrogenicity under our conditions only, it is of interest to note that the pyrogen test results with the Acet-RE preparation compare favorably with those reported by Freedman and Sultzer (1962). These investigators noted a marked reduction in pyrogenicity with their acetylated endotoxin (fraction P) of 1000 fold and 100 fold, respectively. On the other hand, the results obtained with the potassium periodate RE preparation indicated a $>10{,}000 < 50{,}000$ fold reduction in rabbit pyrogenicity when compared to the periodate-treated E. coli lipopolysaccharide of Neter and his associates (1956). These workers reported that oxidation of their E. coli lipopolysaccharide with sodium periodate, prepared by the phenol/water method, yielded a compound of low toxicity and marked pyrogenicity (MPD .0013 ug/kg). Although the possibility of using potassium periodate instead of sodium periodate as the oxidizing agent cannot be discounted entirely, the apparent wide discrepancy between the two endotoxin preparations, at least with regard to MPD, suggests that the parent RE preparation responds more readily to the periodation procedure. The difference in "purity" of endotoxin starting material in this particular case apparently determines whether the molecular site or sites responsible for fever in rabbits will be altered as a result of chemical oxidation.

Under similar conditions the potassium methylate RE preparation showed a reduced fever effect in rabbits, whereas the boron trifluoride RE preparation showed no reduction in pyrogenicity when compared to the parent RE preparation, respectively. Johnson and Nowotny (1964) noted

in their pyrogen studies that 1 ug amounts of their preparations would eliminate only the second peak in the biphasic fever responses with their boron trifluoride preparation causing the most drastic reduction in this parameter. In contrast, employing the USP rabbit pyrogen test (1961), our data indicate that the potassium methylate RE preparation reduced pyrogenicity from 1 ug (parent RE compound) to 10 ug per rabbit. However, the BF_3 preparation showed no reduction in pyrogenicity (<1 ug) as per USP interpretation. Although our results correlate well with those of Johnson and Nowotny (1964), the differences that do exist in rabbit pyrogenicity with the various endotoxoids may be due once again to the "purity" of the respective parent endotoxins. Whereas the latter authors employed a TCA extract from a strain of Serr. marcescens as their starting material, we employed an RE preparation obtained from a strain of S. typhimurium.

No enhancement or diminution in mouse lethality of the potassium periodate, potassium methylate, and BF_3 preparations was noted when compared to the parent RE preparation. That is, none of these preparations approached a mouse LD_{50} with the amounts tested (2,500 ug). These results show general agreement with those reported by Neter et al. (1956) and those of Johnson and Nowotny (1964) respectively. Neter and his coworkers reported that a mouse LD_{50} value for their sodium periodate preparation was approximately 30 mg/kg (or 600 ug per 20 gm mouse), whereas Johnson and Nowotny (1964) reported respective LD_{50} values for their potassium methylate and BF_3 preparations of >4,000 ug

and >2,000 ug per mouse. However, the respective parent endotoxin mouse LD_{50} values were 3.0 mg/kg, or 60 ug per 20 gm mouse (Neter et al.), and 180 ug per mouse (Johnson and Nowotny) as compared to a mouse LD_{50} of >2,500 ug for the RE preparation. The results indicated to Neter et al. (1956), as well as Johnson and Nowotny (1964), that their respective procedures were capable of reducing the mouse lethality of their parent purified endotoxins.

Of interest, however, is the finding that the mouse LD_{50} of both the Acet-RE and the RE preparations was also >2,500 ug, whereas Freedman and Sultzer (1962), by acetylating a purified endotoxin, showed data similar to those reported by the above authors. These investigators reported an LD_{50} response in mice of 250 ug with a Boivin-type endotoxin derived from S. typhosa, whereas acetylation of this same endotoxin yielded a mouse LD_{50} of approximately 2,000 ug. The mouse LD_{50} of our TCA extract of S. typhimurium was approximately 500 ug which agrees favorably with data reported by Freedman and Sultzer. These additional observations by Freedman and Sultzer lend support to the idea that the molecular site or sites of the RE preparation responsible for mouse lethality are apparently "masked". On the other hand, the purified endotoxins apparently have their site or sites exposed to such an extent that microgram amounts are capable of inducing equivalent LD_{50} results in mice. Anywhere from 10 to 50 times more of the parent RE preparation (>2,500 ug) is needed to produce the same LD_{50} response as those reported above for the respective purified endotoxins.

It is apparent that the above methods of detoxification of crude endotoxin prepared by the RE procedure from a strain of S. typhimurium yielded end products which, with the exception of the BF_3 preparation, had reduced fever effects in rabbits as well as reduced toxicity in mice. A critical evaluation of the immunogenicity of the respective endotoxoids was obtained by employing the active-immunized mouse protection test. That is, a readily reproducible method of challenge with a $10LD_{50}$ dose instead of the usual LD_{50} dose simplified the problem of evaluating immunogenicity of the respective vaccines. The reproducibility of the $10LD_{50}$ challenge dose in each of the active mouse-protection tests shown in Part III of the Experimental Results has been remarkably consistent employing these conditions of challenge. The saline control mice included in each experiment consistently showed a reproducible $10LD_{50}$ response within 72 hours after ip challenge with the test strain of S. typhimurium. Therefore, it was generally assumed that protection was dependent on the immunogen and the immunization schedule. Furthermore, this was observed in mice with all of the endotoxoid preparations when comparison was made with both the HP vaccine and the saline treated mice.

A comparison of the immunogenic effect in mice of the HP vaccine to two subcutaneous 100 ug immunizing doses of the potassium periodate (Table 19) as well as the potassium methylate, BF_3 and RE preparations respectively (Table 27) showed that the potassium methylate RE vaccine preparation gives the best protection. In contrast, both the BF_3

and the periodate preparations were the least protective. In addition, the potassium methylate preparation showed some protection in mice immunized with two weekly subcutaneous doses as low as 10 ug when compared with the saline immunized mice (Table 28). These data strongly indicate that the potassium methylate procedure when applied to an RE endotoxin preparation greatly increases its antigenicity in mice, whereas the respective BF_3 and periodate treatments of the same parent endotoxin show almost identical decreased mouse antigenicity (Tables 19 and 27). However, Johnson and Nowotny (1964) reported that, when compared to their parent endotoxin, both of their chemical methods (BF_3 and potassium methylation) resulted in decreased antigenicity in rabbits when each of these preparations was given intravenously in a single dose of 1 ug. They further reported that the BF_3 procedure appeared to be more drastic in disrupting antigenicity than the potassium methylate procedure. These results were similar to those previously published by Nowotny (1963), where it was shown that several injections of the BF_3-treated preparation into rabbits resulted in antibody levels lower than those of the potassium methylate preparation, as well as the control endotoxin. In addition, this investigator suggested that this BF_3 procedure destroyed the O antigen determinant group (carbohydrates), whereas the potassium methylate procedure appeared not to affect this component. Our data, employing these preparations as vaccines in mice, show general agreement with these observations. In contrast to Johnson and Nowotny's preparations,

our potassium methylated RE preparation showed increased antigenicity in mice when compared to the parent endotoxin (RE), whereas the BF_3 preparation indicated a decreased antigenicity. Again, these differences are probably due to the respective parent endotoxins.

The effect of decreased antigenicity of the potassium periodate RE vaccine preparation suggests that oxidation of the parent RE preparation is also destroying or, at least, modifying the specific antigenic sites involved in mouse protection. Neter et al. (1956) concluded from their in vitro studies that periodate-treated purified E. coli lipopolysaccharide had antigenic characteristics which were markedly altered. They suggested that the amount of periodate used up corresponds to 1 c-c-cleavage of every fourth sugar unit, with each cleavage resulting in 2 titerable aldehyde groups. With this type of severe oxidation, it is not surprising that our potassium periodate-treated RE preparation is poorly antigenic in mice.

The protection afforded mice by the HP vaccine as compared with two sc 100 ug immunizing doses of the Acet-RE, RE, and Boivin vaccine preparations (Table 20) suggests once again that the HP vaccine is more protective against a $10LD_{50}$ challenge of S. typhimurium. On the other hand, experiments in mice employing the Acet-RE preparation in two sc 200 ug immunizing doses (Table 21) followed one week later with the same $10LD_{50}$ challenge, strongly indicates a definite protective effect when compared to the HP vaccine. At the end of 72 hours, per DBS criteria, the HP vaccine protected 95% of the mice as compared to 75%

protection in the Acet-RE (200 ug) immunized mice. However, it should be noted that all of our endotoxoid vaccine preparations, as well as the Boivin, RE, and HP vaccines, had a diminished protective effect in mice 72-96 hours after a $10LD_{50}$ challenge dose. These observations were observed consistently and suggest that with our strain of S. typhimurium, the $10LD_{50}$ challenge dose overwhelms the mouse immunity by the fourth day since none of the vaccine preparations were protective to any great extent after 96 hours.

Of interest also is the apparent similarity between the RE and Acet-RE preparations in their protective ability in mice (see Tables 20 and 21). That is, the acetylation procedure had not destroyed the antigenic site or sites to any great extent on the original RE preparation. The exact nature of the antigenic site or sites disrupted by acetylation as well as the potassium periodate, potassium methylate, and BF_3 procedures, which leads to apparent detoxification, is as yet unknown. However, whatever the nature of the antigenic site or sites disrupted by these chemical procedures, the method of acetylation held the most promise for our purpose of trying to develop a less toxic enteric fever vaccine. Although our data indicated that the difference between the MPD (100 ug vs 10 ug) and the MID (200 ug vs 50 ug) is approximately the same for both the acetylated and the potassium methylate preparations, it was felt that the procedure of acetylation, which apparently has the greatest effect on reducing pyrogenicity, should be considered further (see below). Furthermore, the results with the

immunodiffusion tests (Figures 2 and 3) support these observations. The acetylated as well as the potassium methylate RE vaccine preparations, in contrast to the BF_3 and periodate preparations, reacted specifically with the IRS thus indicating that antigenicity was intact. Although the above considerations led us to select the Acet-RE vaccine preparation for further study in mouse protection, the observation that the potassium methylate RE vaccine preparation demonstrates twice the protective response in mice against the same $10LD_{50}$ challenge on a dose-response relationship must not be overlooked.

By comparing the protective effect in mice of the HP vaccine to one, two and even three weekly sc 100 ug immunizing doses of the Acet-RE preparation, it was apparent that the HP vaccine is more protective against a similar $10LD_{50}$ challenge of S. typhimurium (Table 23). Further evidence to support these data is seen in Table 25, which shows the effect of two per week (total 400 ug) injections as compared to one weekly injection (total 200 ug) of the Acet-RE preparation both given over a two-week period (Table 24). The apparent dose-response effect with the Acet-RE preparations as determined by comparing the respective total mouse immunizing doses of 200 ug with 400 ug suggests a twofold increase in mouse protection for the first few days of the observation period. However, as compared to one weekly 100 ug immunizing dose given over a three-week period (total 300 ug) Tables 22 and 23), the mouse protection afforded with the same dose but given in two per week immunizing injections (instead of one) showed

less overall protection when challenged with the same approximate $10LD_{50}$ dose. Moreover, the protective effect when compared to the HP is also less. On the other hand, there is a protective effect when compared to the saline control mice. These data and all previous data have indicated that the HP vaccine is more protective than any of the Acet-RE immunizing doses employed so far. However, this observation may mean that the Acet-RE preparation is weakly immunogenic at the MPD of 100 ug. By comparison, the immunizing dose of the HP vaccine employed (900×10^6 org./0.5 ml) is a much stronger dose than the previously used Acet-RE immunizing doses. An indication that this may be true was noted earlier (Table 21) when an immunizing dose of 200 ug was injected sc into mice over a two week period. At the end of the three day observation period as per DBS criteria, the HP vaccine protected approximately 90 to 95% of the mice as compared to 70 to 75% protection in the immunized mice receiving two 200 ug (total 400 ug) doses of the Acet-RE preparation. Applying this method of comparison for testing each of the vaccine preparations but at approximate equivalent concentrations and comparing their respective immunogenic capacities under similar conditions it was observed that the above assumptions (see Part G, Experimental Results) were correct. That is, on a weight basis, there appears to be a differential factor from 5 to 10 in vaccine strength between the Acet-RE preparation and the HP vaccine. That this was borne out is seen from the above experiment (Table 26) when two 500 ug immunizing doses of the Acet-RE preparation were employed. Mouse

protection results were almost identical throughout the entire observation period for both the HP vaccine and the Acet-RE preparation against the same $10LD_{50}$ challenge. Thus, these data suggest that it takes 5 to 10 times the MPD of the Acet-RE preparation to approach an approximate effective immunogenic dose (EID) equal to that of the HP vaccine. However, the difference of approximately a 50 to 100 fold reduction in rabbit pyrogenicity over that of the parent RE preparation should not be overlooked. This observation suggests a less toxic and particularly a less pyrogenic vaccine if it is to be used under the same conditions as that of the HP vaccine.

The reproducibility of the acetylation procedure determined by comparing the original Acet-RE preparation data (Tables 29, 30 and 31) with the data obtained with the two new Acet-RE preparations (Acet-RE-I and Acet-RE-II) has indicated the feasibility of using this method for detoxifying the parent RE endotoxin. That is, with regard to rabbit pyrogenicity, mouse lethality, and active mouse immunization, the acetylation of a crude endotoxin prepared by the RE method from a strain of S. typhimurium produced, in general, consistant biological results. However, some discrepancies with regard to both the rabbit pyrogen and mouse lethality data are worthy of note. The rabbit pyrogen data (Table 29) suggested a MPD of 50 ug for both the Acet-RE-I and Acet-RE-II preparations respectively, whereas the original Acet-RE preparation, under similar conditions, yielded a MPD of 100 ug. These results are of interest when one considers the acetyl contents

of the respective acetylated preparations. The acetyl content of the two new Acet-RE preparations was approximately double that (I = 11.7% and II = 10.5%) of the original Acet-RE preparation (6.8%); thus suggesting a decrease in pyrogenicity as the acetyl content is increased in the parent RE preparation. As pointed out earlier within Part V, Experimental Results section, the changes incorporated with the two new Acet-RE preparations were the use of a power-driven mortar and pestle for grinding both preparations to a fine powder. In addition, the Acet-RE-II preparation was acetylated in a boiling water bath for 6 instead of 4 hours. It is obvious this latter change in experimental procedure has had some effect on the acetyl content between the Acet-RE-I and Acet-RE-II preparations respectively but not enough to affect the MPD (50 ug) in rabbits. Thus, the apparent two fold difference in MPD between the original and the two new Acet-RE preparations is thought to be due to the use of the power driven mortar and pestle. Although Freedman and Sultzer (1962) reported a reduction in pyrogenicity with one of their acetylated Boivin endotoxin preparations as 1000 fold, it is interesting to note that a second identical preparation of theirs showed a reduction in rabbit pyrogenicity of only 100 fold. These authors indicated that their results represented the upper and lower limits of decrease in pyrogenicity for all of their preparations. On the other hand, our results with acetylated crude RE endotoxin indicate (Table 29) a reduction in pyrogenicity of $>50<100$ ug.

Although the effect of 6 hours acetylation (Acet-RE-II), when

compared to Acet-RE-I, appears to have little or no effect on either the acetyl content or on the MPD in rabbits, it does apparently influence mouse lethality. As noted in the above experiments on mouse lethality (Table 30), the effect of 4 hours of acetylation, when compared to the parent RE preparation, may induce an increase in mouse toxicity. This observation was particularly true with the Acet-RE-I preparation which revealed that an ip injection of 2.5 mg per mouse tended to approximate an LD_{50} dose, whereas the same injection of the parent RE preparation resulted in only one death under similar conditions. This latter observation with the parent RE preparation has been consistently observed in several mouse lethality experiments. On the other hand, the effect of 6 hours acetylation (Acet-RE-II) apparently reduced lethality of the material for the mouse. That is, no deaths were observed with any of the amounts injected throughout the entire observation period. Although these differences between the two new Acet-RE preparations appear to be real, it remains to be determined whether the effect of 6 hours acetylation may aid in altering and/or decreasing the toxicity observed for the 4 hours acetylation procedure since the parent RE preparation is apparently non-toxic also.

The immunogenicity in mice afforded by the two new Acet-RE preparations (Acet-RE-I and Acet-RE-II, respectively) against a $10LD_{50}$ challenge of S. typhimurium indicated no significant protection with either of these preparations when the MPD (50 ug) is employed as the MID. Moreover, the mouse protective effects of all the acetylated as well as

the parent RE preparations (Table 31) were almost identical. It should be noted that this latter observation gives evidence that the above alterations in the two methods of acetylation do not appear to alter the antigenicity of the parent RE preparation. On the other hand, the mouse protection afforded by employing two 50 ug MPD of these acetylated preparations as immunizing doses is low when compared to HP vaccine. However, as earlier data showed (Table 26) when compared with the HP vaccine, the EID with the original Acet-RE preparation is approximately 5 to 10 times greater than the MPD. Since all of these acetylated preparations appear to be identical in their mouse immunogenicity, at least at the 50 ug level, it seems not unreasonable at this point to assume a similar relation between the HP vaccine and the two new Acet-RE preparations. That is, a current working assumption is that the ratio between the EID (500 ug) and the MPD (50 ug) when compared to the HP vaccine, is approximately 10 for the Acet-RE-I and Acet-RE-II preparations, respectively.

SUMMARY

Chemical modification of a crude endotoxin prepared by the RE procedure from a strain of S. typhimurium yielded certain significant changes in biological activity. The chemical procedures employed were oxidation by boron trifluoride and potassium periodate, acetylation, and methylation. The results obtained with these endotoxoids, with the exception of the potassium periodate RE preparation, compared favorably with those reported by other investigators with regard to yield, pyrogenicity, lethality, and mouse immunogenicity. Where differences occurred these may have been due to the different strains of gram-negative organisms employed as well as the "purity" of the respective endotoxins used as starting materials.

A main purpose for the use of chemically altered crude endotoxins was the possible development of a less toxic enteric fever vaccine. Each endotoxoid preparation was tested for pyrogenicity in rabbits as well as mouse lethality and immunogenicity, and these data were compared with similarly obtained parent RE preparation data as well as standard vaccine.

The pyrogen test results with Acet-RE and the potassium periodate RE preparations indicated that these, on a weight basis, were approximately 100 times less pyrogenic than the preparations of Boivin and parent RE. Under similar conditions the potassium methylate RE preparation showed an approximate 10 fold reduction in fever effects

in rabbits, and the boron trifluoride RE preparation showed no reduction in pyrogenicity.

No enhancement or diminution in mouse lethality of the potassium periodate, potassium methylate, Acet-RE, and boron trifluoride preparations was noted when compared to comparable doses of the parent RE preparation. That is, none of these preparations approached a mouse LD_{50} with the amounts tested (2,500 ug).

Studies were also undertaken to determine immunogenicity in mice by comparing Boivin, RE, and the previously mentioned endotoxoid preparations with a heat-killed, phenol-preserved (HP) vaccine prepared from the same strain of S. typhimurium. With the exception of using $10LD_{50}$ instead of an LD_{50} challenge, the immunogenicity of the respective vaccines was determined for the most part by active-immunized mouse protection tests as outlined by the Division of Biological Standards, Public Health Service. Although two 100 ug immunizing doses of the Boivin, RE, and the respective endotoxoid preparations varied in mouse protection (potassium methylate RE > Boivin > RE > Acet-RE > boron trifluoride RE > potassium periodate RE), it was obvious, with the exception of the potassium methylate preparation, that the HP vaccine yielded greatest protection against the $10LD_{50}$ challenge with S. typhimurium. Further mouse protection studies indicated the minimal immunogenic dose (MID) of the potassium methylate RE vaccine preparation to be approximately 50 ug. These data suggested an approximate five fold difference between the minimal pyrogenic dose (MPD) of 10 ug and

the MID (50 ug).

The procedure of acetylation, which apparently has the greatest effect on reducing rabbit pyrogenicity, received major experimental attention in the effort to develop a less toxic enteric fever vaccine. Active-immunized mouse protection experiments in mice employing the Acet-RE vaccine preparation in two 200 ug and 500 ug immunizing (pyrogenic) doses indicated a definite protective effect against a $10LD_{50}$ challenge when compared to the HP vaccine. Furthermore, the apparent similarity between the parent RE and Acet-RE vaccine preparations in their protective ability in mice suggested that acetylation had not altered immunogenically the antigenic sites to any great extent on the original RE preparation. These results indicated at most a five fold difference between the MPD in the rabbit and the approximate effective immunogenic dose (EID) in the mouse. Results with both the Acet-RE and potassium methylate RE vaccine preparations should be considered further in the search for a less toxic enteric fever vaccine.

BIBLIOGRAPHY

1. Allan, S. M. 1943. An outbreak of typhoid fever. Lancet 1:708-710.

2. Anacker, R. L., R. A. Finkelstein, W. T. Haskins, M. Landy, K. C. Milner, E. Ribi and P. W. Stashak. 1964. Origin and properties of naturally occurring hapten from Escherichia coli. J. Bacteriol. 88:1705-1720.

3. Ashcroft, M. T. 1963. Immunization against typhoid fever. West Ind. Med. J. 12:82-89.

4. Ashcroft, M. T. 1964. Immunization against typhoid and paratyphoid fevers. Clin. Pediat. 3:385-393.

5. Ashcroft, M. T., J. M. Ritchie and C. C. Nicholson. 1964. Controlled field trial in British Guiana school children of heat-killed—phenolized and acetone-killed lyophilized typhoid vaccines. Am. J. Hyg. 79:196-206.

6. Atkins, E. 1960. Pathogenesis of fever. Physiol. Rev. 40:580-646.

7. Barnes, F. W., M. Dewey, S. S. Henry and H. Lupfer. 1947. Chemical detoxification of flexner dysentery antigen. I. Survey of results. J. Immunol. 56:255-261.

8. Batson, H. C. 1948. The relative efficiency of varied immunizing and challenge doses in measuring the mouse-protective potency of typhoid vaccine. Proc. Soc. Am. Bacteriol. 1:55.

9. Batson, H. C. 1949. Typhoid fever prophylaxis by active immunization. Pub. Health Rep., Supplement 212, 1-34.

10. Beeson, P. B. 1947a. Tolerance to bacterial pyrogens. I. Factors influencing its development. J. Exper. Med. 86:29-38.

11. Benenson, A. S. 1964. Serological responses of man to typhoid vaccines. Bull. Wld. Hlth. Org. 30:653-662.

12. Bensted, H. J. 1940. Bacterium typhosum. The development of Vi-antigen and Vi-antibody. J. Roy. Army Med. Corps 74:19-35 (Original not seen; cited from Batson, 1949).

13. Bensted, H. J. 1941. Immunization against bacterial infections. J. Roy. Inst. Pub. Health and Hyg. 4:9-14 (Original not seen; cited from Batson, 1949).

14. Bhatnagar, S. S. 1935. Phagocytosis of B. typhosus in relation to its antigenic structure and to the antibody components of the sensitizing serum. Brit. J. Exptl. Pathol. 16:375-383.

15. Boivin, A., L. Mesrobeanu and J. Mesrobeanu. 1933. Technique pour la preparation des polysaccharides microbiens specifiques. Compt. rend. Soc. Biol. 113:490-492.

16. Boivin, A. and L. Mesrobeanu. 1937. Recherches sur les toxines des bacilles dysenteriques. Sur les principes toxiques du bacille de flexner. Compt. rend. Soc. Biol. 124:1078-1081.

17. Boor, A. K. and C. P. Miller. 1944. A carbohydrate-lipid fraction of gonococcus and meningococcus. J. Infect. Diseases 75:47-57.

18. Bowdoin, C. D. and L. M. Petrie. 1940. The efficacy of immunization in an acute water-borne outbreak of typhoid fever. Med. Times 68:53-56.

19. Boyd, J. S. K. 1943. Enteric group fevers in prisoners of war from the western desert. Brit. Med. J. 1:719-721.

20. Brown, M. H. 1936. Criteria for the selection of suitable strains of B. typhosus for use in the preparation of typhoid vaccine. Canad. J. Pub. Health 27:171-175 (Original not seen; cited from Batson, 1949).

21. Burrage, T. J. 1932. Typhoid fever in the vaccinated. Mil. Surgeon 70:231-237.

22. Castellani, A. 1937. Medical aspects of the Italo-Ethiopian War. Mil. Surgeon 81:1-16.

23. Climie, H. 1942. Immunization against typhoid and paratyphoid with alcohol-killed, alcohol-preserved and heat-killed, phenol-preserved vaccine. J. Hyg. 42:411-415.

24. Cluver, E. 1929. Oral immunization against typhoid in South Africa. Lancet 1:1302-1304.

25. Corwin, L. M. and W. E. Farrar, Jr. 1964. Nature of the endotoxin-inactivating principle in guinea-pig liver. J. Bacteriol. 87: 832-837.

26. Creech, H. J., E. R. Breuninger and G. A. Adams. 1964. Polysaccharide-peptide complexes from Serratia marcescens cells. Canad. J. Biochem. 42:593-603.

27. Cruickshank, J. C., B. C. Hobbs, A. M. McFarlin and I. Maier. 1942. The disinfectant action of phenol and of alcohol in T.A.B.C. vaccine. Brit. Med. J. 2:182-183.

28. Cvjetanović, B. B. 1957. Field trial of typhoid vaccines. Am. J. Pub. Health 47:578-581.

29. Davison, W. C. 1918. The superiority of inoculations with mixed triple vaccine (B. typhosus, B. paratyphosus A, and B. paratyphosus B) Arch. Int. Med. 21:437-509.

30. Destouches, L. F. 1934. Oral immunization against typhoid. Lancet 1:1088.

31. Downs, C. M. and G. C. Bond. 1937. Occurrence of O and H agglutinins following subcutaneous and oral administration of typhoid vaccine. Am. J. Pub. Health 27:889-892.

32. Drysdale, A. 1947. A comparative study of phenolized and alcoholized T.A.B. vaccines. J. Hyg. 45:46-49.

33. Duncan, T. G., J. A. Doull, E. R. Miller and H. Bancroft. 1946. Outbreak of typhoid fever with orange juice as the vehicle, illustrating the value of immunization. Amer. J. Publ. Health 36:34-36.

34. Edsall, G., S. Gaines, M. Landy and W. D. Tigertt. 1954. Typhoid fever in chimpanzees infected orally with Salmonella typhosa. Fed. Proc. 13:490.

35. Edsall, G., M. C. Carlson, S. B. Formal and A. S. Benenson. 1959. Laboratory tests of typhoid vaccines used in a controlled field study. Bull. Wld. Hlth. Org. 20:1017-1032.

36. Edsall, G., S. Gaines, M. Landy, W. D. Tigertt, H. Sprinz, R. J. Trapani, A. D. Mandel and A. S. Benenson. 1960. Studies on infection and immunity in experimental typhoid fever. I. Typhoid fever in chimpanzees orally infected with Salmonella typhosa. J. Exptl. Med. 112:143-166.

37. Edwards, P. R. 1951. Preparation of antisera for detection of the somatic antigens of Salmonella cultures. Publ. Health Rept. 66:837-839.

38. Edwards, P. R. and W. H. Ewing. 1962. Second Edition. Identification of Enterobacteriaceae. Burgess Publishing Co., Minneapolis, Minn. p. 110-111.

39. Elledge, L. C., M. A. Kennedy and K. MacDonald Cumming. 1943. Comparative study of immunity produced in typhoid and associated diseases by subcutaneous and oral vaccinations. M. Bull. Vet. Adm. 20:56-57.

40. Feemster, R. F. 1932. The effect of typhoid fever and of previous typhoid vaccination on the antibody response to inoculation with typhoid vaccine. J. Inf. Diseases 50:121-133.

41. Felix, A. 1941. A new type of typhoid and paratyphoid vaccine. Brit. Med. J. 1:391-395.

42. Felix, A. 1951b. The preparation, testing and standardization of typhoid vaccine. J. Hyg. 49:268-287.

43. Felix, A. and E. S. Anderson. 1951c. The immunizing potency of alcohol-killed and alcohol-preserved typhoid vaccine after storage for ten years. J. Hyg. 49:288-298.

44. Felix, A. and S. S. Bhatnagar. 1935. Further observations on the properties of the Vi antigen of B. typhosus and its corresponding antibody. Brit. J. Exptl. Pathol. 16:422-434.

45. Felix, A. and G. F. Petrie. 1938. The preparation of anti-typhoid serum in the horse for therapeutic use in man. J. Hyg. 38:673-682.

46. Felix, A. and R. M. Pitt. 1934. A new antigen of B. typhosus. Its relation to virulence and to active and passive immunization. Lancet 2:186-191.

47. Felix, A. and R. M. Pitt. 1935. Virulence and immunogenic activities of B. typhosus in relation to its antigenic constituents. J. Hyg. 35:428-436.

48. Felix, A. and R. M. Pitt. 1936. The Vi antigens of various salmonella types. Brit. J. Exptl. Pathol. 17:81-86.

49. Felix, A. and R. M. Pitt. 1951a. The pathogenic and immunogenic activities of Salmonella typhi in relation to its antigenic constituents. J. Hyg. 49:92-110.

50. Felix, A., S. G. Rainsford and E. J. Stokes. 1941. Antibody response and systemic reactions after inoculation of a new type of T.A.B.C. vaccine. Brit. Med. J. 1:435-440.

51. Freeman, G. G. 1942. The preparation and properties of a specific polysaccharide from Bact. typhosum T_{y2}. Biochem. J. 36:340-355.

52. Freeman, G. G. 1943. The components of the antigenic complex of Salmonella typhimurium. Biochem. J. 37:601-612.

53. Freedman, H. H., B. M. Sultzer and W. Kleinberg. 1961. Detoxification of bacterial endotoxin with retention of ability to stimulate non-specific resistance to infection. Proc. Soc. Exper. Biol. Med. 107:819-821.

54. Freedman, H. H. and B. M. Sultzer. 1962. Dissociation of the biological properties of bacterial endotoxin by chemical modification of the molecule. J. Exper. Med. 116:929-942.

55. Fukushi, K., R. L. Anacker, W. T. Haskins, M. Landy, K. C. Milner and E. Ribi. 1964a. Reaction of Escherichia coli endotoxin with lithium aluminum hydride. Proc. Soc. Exper. Biol. Med. 115:133-139.

56. Fukushi, K., R. L. Anacker, W. T. Haskins, M. Landy, K. C. Milner and E. Ribi. 1964b. Extraction and purification of endotoxin from Enterobacteriaceae: a comparison of selected methods and sources. J. Bacteriol. 87:391-400.

57. Fuller, A. T. 1938. The formamide method for the extraction of polysaccharides from hemolytic streptococci. Brit. J. Exper. Path. 19:130-139.

58. Gaines, S., M. Landy, G. Edsall and R. Trapani. 1956. Experimental typhoid fever in chimpanzees. II. Prophylactic immunization. Fed. Proc. 15:588.

59. Gaines, S., M. Landy, G. Edsall, A. D. Mandel, R. J. Trapani and A. S. Benenson. 1961. Studies on infection and immunity in experimental typhoid fever. III. Effect of prophylactic immunization. J. Exptl. Med. 114:327-342.

60. Gay, F. P. 1918. Typhoid fever. Macmillan Co., New York, p. 147, (Original not seen, cited from Marmion et al., 1953).

61. Gladstone, G. P. 1937. The antigenic composition and virulence of Bact. typhosum grown on a chemically defined medium. Brit. J. Exptl. Pathol. 18:67-82.

62. Goebel, W. F. and G. T. Barry. 1958. Colicine K. II. The preparation and properties of a substance having colicine K activity. J. Exper. Med. 107:185-209.

63. Goebel, W. F., F. Binkley and E. Perlman. 1945. Studies on the flexner group of dysentery bacilli. I. The specific antigens of Shigella paradysenteriae (flexnor). J. Exper. Med. 81:315-330.

64. Goldsmith, A. W., A Miller and G. Buchanan. 1938. Endotoxoid inoculation against typhoid. Abst. in Bull. Hyg. 13:186.

65. Grasset, E. 1939. Typhoid endotoxoid vaccine. A review of the results of preventive typhoid inoculation in an inoculated population of 400,000. Brit. Med. J. 2:58-61.

66. Grasset, E. and W. Lewin. 1936. The production and persistence of H and O agglutinins and complement-fixing bodies in persons inoculated with typhoid endotoxoid vaccine. Brit. J. Exptl. Pathol. 17:179-186.

67. Grasset, E., W. Lewin and T. VanDerMerwe. 1937. Immunization against typhoid fever by means of a single injection of typhoid endo-toxoid vaccine. South African Med. J. 11:660-662 (Original not seen; cited from Batson, 1949).

68. Greenwood, M. and G. U. Yule. 1915. The statistics of anti-typhoid and anti-cholera inoculations, and the interpretation of such statistics in general. Proc. R. Soc. Med. 8:113-194 (Original not seen; cited from Wilson and Miles, 1955).

69. Griffiths, B. W., B. B. Diena and L. Greenberg. 1963. The separation and purification of Vi and O antigens of S. typhi using a column precipitation technique. Canad. J. Biochem. Physiol. 41:249-259.

70. Griffitts, J. J. 1944. Laboratory method of determining the potency of typhoid vaccine. Pub. Health Rep. 59:1515-1528.

71. Grinnell, F. B. 1930. A study of the comparative value of rough and smooth strains of B. typhosus in the preparation of typhoid vaccines. J. Immunol. 19:457-464.

72. Grinnell, F. B. 1931. Studies on the relationship of certain variants of B. typhosus. I. Agglutination and agglutinin absorption. J. Exptl. Med. 54:577-584.

73. Grinnell, F. B. 1932. A study of the dissociation of the Rawlings strain of Bacterium typhosum with special reference to its use in the production of antityphoid vaccine. J. Exptl. Med. 56:907-918.

74. Guerner, N. 1927. A comparison of the subcutaneous and oral methods of antityphoid vaccination. Abst. in Bull. Hyg. 2:818.

75. Halliday, C. H. and M. D. Beck. 1928. Typhoid fever epidemic, Santa Ana, California. J. Prev. Med. 2:49-67.

76. Haskins, W. T., M. Landy, K. C. Milner and E. Ribi. 1961. Biological properties of parent endotoxins and lipoid fractions with a kinetic study of acid-hydrolyzed endotoxin. J. Exper. Med. 114:665-684.

77. Haskins, W. T., E. Ribi, M. Landy, R. L. Anacker and K. C. Milner. 1963. Preparation and properties of an aluminum citrate-endotoxin complex from Salmonella enteritidis. Proc. Soc. Exper. Biol. Med. 112:113-119.

78. Hawn, C. B., J. D. Hopkins and F. M. Meader. 1919. Outbreak of typhoid fever among American troops in England. J.A.M.A. 72:402-406.

79. Henderson, D. W. 1939a. The protective value of the Vi and the O antibody in relation to the virulence of strains of Bact. typhosum. Brit. J. Exptl. Pathol. 20:1-10.

80. Henderson, D. W. 1939b. Experiments with the Vi antigen of Bact. typhosum and with two new antigenic substances extracted from strains of this organism. Brit. J. Exptl. Pathol. 20:11-24.

81. Hitch, F. G., L. S. Ashcroft and G. A. Green. 1943. Antigenicity of T.A.B.C. vaccine after admixture with tetanus toxoid for various periods. J. Hyg. 43:207-213.

82. Hodgson, C. H. 1945. Typhoid and paratyphoid fever; experience with 84 cases. Proc. Staff Meetings, Mayo Clinic 20:257-262.

83. Hoffstadt, R. E., C. L. Martin and R. L. Thompson. 1929. Immunological studies of typhoid vaccination by mouth. III. Agglutinins and complement fixation reactions formed in persons treated with typhoid capsules. Am. J. Hyg. 9:37-46 (Original not seen; cited from Batson, 1949).

84. Hoffstadt, R. E. and R. L. Thompson. 1929a. Immunological studies of typhoid vaccination by mouth. I. Agglutinins formed in persons treated orally with triple typhoid bacterin. Am. J. Hyg. 9:1-20 (Original not seen; cited from Batson, 1949).

85. Hoffstadt, R. E. and R. L. Thompson. 1929b. Immunological studies of typhoid vaccination by mouth. II. Complement fixations and precipitin reactions in persons treated orally with bile and triple typhoid bacterin. Am. J. Hyg. 9:21-36 (Original not seen; cited from Batson, 1949).

86. Hogg, C. A. and O. Latham. 1926. The control of enteric fever in mental hospitals. Lancet 2:377-378.

87. Holt, R. L. 1948. Current status of immunization procedures: Typhoid fever. Am. J. Pub. Health 38:481-484.

88. Hook, E. W. and R. R. Wagner. 1959. The resistance-promoting activity of endotoxins and other microbial products. I. Their effect on Salmonella typhosa infections of mice. J. Immunol. 83:302-309.

89. Jennings, A. F. and Mathieson, D. R. 1940. Typhoid fever. Tice's Practice of Medicine, W. F. Prior Co., Inc., Hagerstown, Md., p. 451-608 (Original not seen; cited from Batson, 1949).

90. Johnson, A. G. and A. Nowotny. 1964. Relationship of structure to function in bacterial O antigens. J. Bacteriol. 87:809-814.

91. Jordan, J. and H. E. Jones. 1945. Typhoid fever in immunized personnel. Lancet 2:333-335.

92. Jude, L. V. R. 1934. Enteric fever in the French Army prior and subsequent to preventive inoculation. Abstr. in Bull. Hyg. 9:808-809.

93. Keene, W. R. 1962. Detoxification of bacterial endotoxin by soluble tissue extracts. J. Lab. and Clin. Med. 60:433-438.

94. Kim, Y. B. and D. Watson. 1964. Inactivation of gram-negative endotoxins by papain. Proc. Soc. Exptl. Biol. Med. 115:140-142.

95. Kolmer, J. A. and F. Boerner. 1945. Fourth Edition. Approved Laboratory Technic. D. Appleton-Century Co., New York, N. Y., p. 493-495.

96. Kolmer, J. A., A. Bondi, Jr. and A. M. Rule. 1942. Persistence of antibodies one year after active immunization of human beings with a mixed heat-killed vaccine of B. typhosus, Br. abortus and Br. melintensis. J. Inf. Diseases 70:54-57.

97. Kunin, C. M. 1963. Separation, characterization, and biological significance of a common antigen in Enterobacteriaceae. J. Exptl. Med. 118:565-586.

98. Landy, M. 1952. Studies on Vi antigen. III. Immunological properties of purified Vi antigen derived from Escherichia coli 5396/38. J. Immunol. 69:143-154.

99. Landy, M. 1953. Enhancement of the immunogenicity of typhoid vaccine by retention of the Vi antigen. Am. J. Hyg. 58:148-164.

100. Landy, M. 1954. Studies on Vi antigen. VI. Immunization of human beings with purified Vi antigen. Am. J. Hyg. 60:52-62.

101. Landy, M., S. Gaines, J. R. Seal and J. E. Whiteside. 1954. Antibody responses of man to three types of antityphoid immunizing agents: heat-phenol fluid vaccine, acetone-dehydrated vaccine, and isolated Vi and O antigens. Am. J. Publ. Health 44:1572-1579.

102. Landy, M. and A. G. Johnson. 1955. Studies on the O antigen of Salmonella typhosa. IV. Endotoxic properties of the purified antigen. Proc. Soc. Exper. Biol. Med. 90:57-62.

103. Landy, M., A. G. Johnson, M. E. Webster and J. F. Sagin. 1955. Studies on the O antigen of Salmonella typhosa. II. Immunological properties of the purified antigen. J. Immunol. 74:466-478.

104. Leishman, W. B. 1910. Anti-typhoid inoculation. J. Roy. Inst. Pub. Health and Hyg. 18:385-401 (Original not seen; cited from Batson, 1949).

105. Long, A. P. 1955. Typhoid-paratyphoid vaccination. Preventive Medicine in World War II. Office of the Surgeon General, Department of the Army, Washington, D. C., p. 272-280.

106. Longfellow, D. and G. F. Luippold. 1940. Typhoid vaccine studies. Revaccination and duration of immunity. Am. J. Pub. Health 30:1311-1317.

107. Longfellow, D. and G. F. Luippold. 1943. Typhoid vaccine studies. VII. Typhoid-paratyphoid vaccine. Am. J. Pub. Health 33:561-568.

108. Luippold, G. F. 1944. Typhoid vaccine studies. IX. Intracutaneous versus subcutaneous vaccination for initial immunization. Am. J. Pub. Health 34:1151-1162.

109. Luippold, G. F. 1946. Typhoid vaccine studies. X. The suitability of Vi antigen as an auxiliary immunogen for typhoid vaccine. Am. J. Pub. Health 36:15-25.

110. Luippold, G. F., D. Longfellow and M. Toporek. 1947. Typhoid vaccine studies. XI. The effect and effectiveness of three 0.5-ml doses of TAB vaccine. Am. J. Hyg. 45:355-362.

111. Lull, G. F. 1934. Fevers of the typhoid group in members of the Civilian Conservation Corps during 1933. Amer. J. Pub. Health 24:631-632.

112. MacLean, I. H. and L. B. Holt. 1940. Combined immunization with tetanus toxoid and TAB. Lancet 2:581-583.

113. Mainland, D., L. Herrera and M. I. Sutcliffe. 1956. Statistical tables for use with binomial samples - contingency tests, confidence limits, and sample size estimates. New York University, New York, N. Y., p. VI.

114. Malbin, B. 1940. Typhoid fever occurring in immunized persons. J.A.M.A. 115:33-36.

115. Marmion, D. E., G. R. E. Naylor and I. O Stewart. 1953. Second attacks of typhoid fever. J. Hyg. 51:260-267.

116. Martin, W. J. and S. Marcus. 1964a. Relation of pyrogenic and emetic properties of enterobacteriaceal endotoxin and of staphylococcal enterotoxin. J. Bacteriol. 87:1019-1026.

117. Martin, W. J. and S. Marcus. 1964b. Studies on bacterial pyrogenicity. III. Specificity of the United States Pharmacopeia rabbit pyrogen test. Appl. Microbiol. 12:483-486.

118. Mergenhagen, S. E. and G. R. Martin. 1964. Properties of a lysozyme-dissociated endotoxic fraction from Escherichia coli. J. Bacteriol. 88:1169-1174.

119. Mergenhagen, S. E., G. R. Martin and E. Schiffmann. 1963a. Studies on an endotoxin of a group C Neisseria meningitidis. J. Immunol. 90:312-317.

120. Mergenhagen, S. E., E. Schiffmann, G. R. Martin and S. B. Jensen. 1963b. Stimulation of resistance to infection by lipoid derived from Neisseria meningitidis endotoxin. J. Inf. Diseases 113:52-58.

121. Miles, A. A. and N. W. Pirie. 1939. The properties of antigenic preparations from Brucella melitensis. I. Chemical and physical properties of bacterial fractions. Brit. J. Exper. Path. 20:83-98.

122. Miller, L. C. and M. L. Tainter. 1944. Estimation of the ED_{50} and its error by means of logarithemic-probit graph paper. Proc. Soc. Exptl. Biol. Med. 57:261-264.

123. Miller, A., J. H. G. VanBlommestein and L. S. Williams. 1936. Symposium on typhoid. Abst. in Bull. Hyg. 11:898.

124. Milner, K. C., R. L. Anacker, K. Fukushi, W. T. Haskins, M. Landy, B. Malmgren and E. Ribi. 1963. Symposium on relationship of structure of microorganisms to their immunological properties. III. Structure and biological properties of surface antigens from gram-negative bacteria. Bacteriol. Rev. 27:352-368.

125. Morgan, H. R. 1941. Immunologic properties of an antigenic material isolated from Eberthella typhosa. J. Immunol. 41:161-180.

126. Morgan, H. R. 1945. Active immunization with purified somatic antigens of Eberthella typhosa, Salmonella paratyphi, and Salmonella schottmuelleri. Am. J. Pub. Health 35:614-620.

127. Morgan, W. T. J. 1936. Studies on immunochemistry. I. The preparation and properties of a specific polysaccharide from B. dysenteriae (shiga). Biochem. J. 30:909-925.

128. Morgan, W. T. J. 1937. Studies in immunochemistry. II. The isolation and properties of a specific antigenic substance from Bact. dysenteriae (shiga). Biochem. J. 31:2002-2021.

129. Morgan, W. T. J. and S. M. Partridge. 1940. Studies in immunochemistry. 4. The fraction and nature of antigenic material isolated from Bact. dysenteriae (shiga). Biochem. J. 34:169-191.

130. Morgan, W. T. J. and S. M. Partridge. 1941. Studies in immunochemistry. VI. The use of phenol and of alkali in the degradation of antigenic material isolated from Bact. dysenteriae (shiga). Biochem. J. 35:1140-1163.

131. National Institutes of Health. 1953. Minimum requirements for typhoid vaccine. Second Revision. Bethesda, Maryland.

132. Naumer, H. A. and L. Nerb. 1943. Reactions and agglutinin responses to various methods of typhoid immunization. Arch. Pediat. 60:63-68.

133. Neter, E., O Westphal, O. Lüderitz, E. A. Gorzynski and E. Eichenberger. 1956. Studies of enterobacterial lipopolysaccharides. Effects of heat and chemicals on erythrocyte-modifying, antigenic, toxic and pyrogenic properties. J. Immunol. 76:377-385.

134. New York City Department of Health. Annual Report. 1916. Typhoid fever in New York City during 1916. (Original not seen; cited from Batson, 1949).

135. Nichols, H. J. 1916. Possible reasons for lack of protection after antityphoid vaccination. Mil Surgeon 38:263-268 (Original not seen; cited from Batson, 1949).

136. Noll, H. and A. I. Braude. 1961. Preparation and biological properties of a chemically modified Escherichia coli endotoxin of high immunogenic potency and low toxicity. J. Clin. Invest. 40:1935-1951.

137. Nowotny, A. 1963. Endotoxoid preparations. Nature 197:721-722.

138. Nowotny, A. 1963b. Relation of structure to function in bacterial O antigens. II. Fractionation of lipids present in Boivin-type endotoxin of Serratia marcescens. J. Bact. 85:427-435.

139. Nowotny, A. 1964. Chemical detoxification of bacterial endotoxins, p. 29-37. In W. Braun and M. Landy (ed.), Symposium on bacterial endotoxins. Rutgers University Press, New Brunswick, N. J.

140. Nowotny, A., J. Wein, T. Shikata and R. Kinosita. 1962. Detoxified endotoxins. VIIIth International Congress for Microbiology, Abstracts, p. 104.

141. Nowotny, A. M., S. Thomas, O. S. Duron and A. Nowotny. 1963a. Relation of structure to function in bacterial O antigens. I. Isolation methods. J. Bact. 85:418-426.

142. Oginsky, E. L. and W. W. Umbriet. 1959. An introduction to bacterial physiology. W. H. Freeman and Co., San Francisco, p. 16.

143. Orskov, I., F. Orskov, B. Jann and K. Jann. 1963. Acidic polysaccharide antigens of a new type from E. coli capsules. Nature 200:144-146.

144. Osborn, M. J., S. M. Rosen, L. Rothfield, L. D. Zeleznick and B. L. Horecker. 1964. Lipopolysaccharide of the gram-negative cell wall. Science 145:783-789.

145. Ouchterlony, O. 1948. In vitro method for testing the toxin-producing capacity of diptheria bactiera. Acta Pathol. Microbiol. Scand. 25:186-191.

146. Palmer, J. W. and T. D. Gerlough. 1940. A simple method for preparing antigenic substances from the typhoid bacillus. Science 92:155-156.

147. Perry, H. M., H. T. Findlay and H.J. Bensted. 1933. Antityphoid inoculation. The role of Bacterium typhosum, strain Rawlings. J. Roy. Army M. Corps 60:241-254 (Original not seen; cited from Batson, 1949).

148. Perry, H. M., H. T. Findlay and H. F. Bensted. 1934. Antityphoid inoculation. Observations relative to the immunological value of different cultures of "Bacterium typhosum". J. Roy. Army M. Corps 63:1-12 (Original not seen; cited from Batson, 1949).

149. Perry, R. M. 1937. Comparison of typhoid "O" and "H" agglutinin responses following intracutaneous and subcutaneous inoculation of typhoid, paratyphoid A and B vaccine. Am. J. Hyg. 26:388-393.

150. Pharmacopeia of the United States. 1960. Sixteenth Revision, Mack Printing Co., Easton, Pa., p. 887.

151. Pryor, J. C. 1928. Antityphoid vaccination in the United States Navy; Experience of 14 years. U. S. Naval Med. Bull. 26:57-63.

152. Rainsford, S. G. 1939. Further studies on the sterilization of T.A.B.C. vaccine. J. Hyg. 39:131-142.

153. Rainsford, S. G. 1942. The preservation of Vi antigen in T.A.B.C. vaccine - With a note on combined active immunization with T.A.B.C. vaccine in tetanus formal-toxoid. J. Hyg. 42:297-322.

154. Raistrick, H. and W. W. C. Topley. 1934. Immunizing fractions isolated from Bact. aertrycke. Brit. J. Exper. Path. 15:113-130.

155. Rauss, K. 1942. Comparative researches on the immunizing value of precipitated typhoid antigens and typhoid vaccines. Abst. Bull. Hyg. 17:883-884.

156. Reed, L. J. and H. Muench. 1938. A single method of estimating fifty per cent endpoints. Am. J. Hyg. 27:493-497.

157. Ribi, E., R. L. Anacker, K. Fukushi, W. T. Haskins, M. Landy and K. C. Milner. 1964a. Relationship of chemical composition to biological activity, p. 16-28. In W. Braun and M. Landy (ed.), Symposium on bacterial endotoxins. Rutgers University Press, New Brunswick, N. J.

158. Ribi, E., R. L. Anacker, W. T. Haskins, M. Landy, K. C. Milner and R. Smith. 1964b. Physico-chemical properties of haptenes and cell wall extracts from Escherichia coli. Bacteriol. Proc., p. 20.

159. Ribi, E., W. T. Haskins, M. Landy and K. C. Milner. 1961. Preparation and host-reactive properties of endotoxin with low content of nitrogen and lipid. J. Exper. Med. 114:647-663.

160. Ribi, E., W. T. Haskins, K. C. Milner, R. L. Anacker, D. B. Ritter, G. Goode, R-J. and M. Landy. 1962. Physiochemical changes in endotoxin associated with loss of biological potency. J. Bact. 84:803-814.

161. Roberts, R. S. 1949. The endotoxin of Bact. coli. J. Comp. Path. Therap. 59:284-304.

162. Rudbach, J. A. and A. G. Johnson. 1962. Changes in serologic reactivity of endotoxin by fraction IV$_1$ (Cohn) of normal human serum. Proc. Soc. Exptl. Biol. Med. 111:651-655.

163. Russell, F. F. 1910. Anti-typhoid vaccination. The immediate results of the administration of 3600 doses. Bull. Johns Hopkins Hosp. 21:51.

164. Russell, F. F. 1912. Some results and fields of usefulness of antityphoid vaccination. J.A.M.A. 59:1362-1364.

165. Russell, F. F. 1913. Antityphoid vaccination. Am. J. Med. Sc. 146:803-833.

166. Seckinger, D. L. 1933. Epidemiological studies upon typhoid fever in Georgia: problems associated with its control. Southern Med. J. 26:933-941.

167. Sen, N. K. 1947. Typhoid-paratyphoid (Vi) vaccine. Lancet 1:425.

168. Shear, M. J. and F. D. Turner. 1943. Hemorrhage-producing bacterial filtrate fraction. J. Natl. Cancer Inst. 4:81-95.

169. Siler, J. F. 1936. Typhoid vaccine studies. Investigation of virulence and antigenic properties of selected strains of the typhoid organism. Am. J. Pub. Health 26:219-228.

170. Siler, J. F. and G. C. Dunham. 1939. Duration of immunity conferred by typhoid vaccine. Results of re-vaccination by intracutaneous injection of typhoid vaccine. Am. J. Pub. Health 29:95-103.

171. Siler, J. F., G. C. Dunham, A. P. Hitchens, M. R. Livesay, F. H. K. Reynolds, W. S. Stone, F. E. Council, D. Longfellow, C. P. Canby, J. R. Wood and G. F. Luippold. 1937. Protective antibodies in the blood serum of individuals after immunization with typhoid vaccine. Mil. Surgeon 80:91-104.

172. Siler, et al. 1941. Immunization to typhoid fever. The Johns Hopkins Press, Baltimore, Md.

173. Smith, E. E., S. H. Rutenburg, A. M. Rutenburg and J. Fine. 1963. Detoxification of endotoxin by splenic extracts. Proc. Soc. Exper. Biol. Med. 113:781-784.

174. Smolens, J., S. P. Halbert, S. Mudd, B. W. Doak and L. M. Gonzalaz. 1946. Studies with the somatic antigen of Shigella paradysenteriae (flexnor). J. Immunol. 52:41-58.

175. Sprinz, H., M. Landy, S. Gaines and G. Edsall. 1956. Experimental typhoid fever in chimpanzees. III. Pathogenesis. Fed. Proc. 15:614-615.

176. Stebbins, E. L. and E. Reed. 1937. Carrier-borne typhoid fever in New York State. Amer. J. Pub. Health 27:233-240.

177. Stroebe, F. 1929. Typhoid fever following inoculation with remarks on typhoid inoculation. Abst. in Bull. Hyg. 4:58-59.

178. Sultzer, B. M. and H. H. Freedman. 1962. The stimulation of non-specific host resistance to infection by chemically modified endotoxin. J. Exper. Med. 116:943-956.

179. Syverton, J. T., R. E. Ching, F. S. Cheever and A. B. Smith. 1946. Typhoid and paratyphoid A in immunized military personnel. J. Amer. Med. Assn. 131:507-514.

180. Tauber, H., H. Russell and W. J. Guest. 1961. Nature of polysaccharides obtained from endotoxins by hydroxylaminolysis. Proc. Soc. Exper. Biol. Med. 107:964-965.

181. Taylor, R. 1937. Typhoid fever in the Basque refugee camp. Brit. Med. J. 2:760-761.

182. Tennent, D. M. and W. H. Ott. 1953. Tolerance to bacterial pyrogens in the rabbit. J. Am. Pharm. Assoc. (Scient. Ed.) 42:614-618.

183. Thone. 1938. New practical suggestions on the subject of anti-typhoid vaccination in the Belgian Army. Abstr. in Bull. Hyg. 13:622-623.

184. Topley, W. W. C., H. Raistrick, J. Wilson, M. Stacey, S. W. Challinor and R. O. J. Clark. 1937. The immunizing potency of antigenic components isolated from different strains of Bact. typhosum. Lancet 2:252-256.

185. Trapani, F-J., V. S. Waravdekar, M. Landy and M. J. Shear. 1962. In vitro inactivation of endotoxin by an intracellular agent from rabbit liver. J. Infect. Diseases 110:135-142.

186. Treffers, H. P. 1946. The detoxification by acetylation of soluble antigens from Shigella dysenteriae (shiga) and E. typhosa. Science 103:387-389.

187. Treffers, H. P., B. A. Rubin and C. A. Bell. 1946a. The detoxification by acetylation of soluble antigens from Shigella dysenteriae and E. typhosa. Feder. Proc. 5:255.

188. Tribby, W. W., A. H. Stock and F. B. Warner. 1948. Typhoid fever in inoculated U. S. soldiers. Mil. Surgeon 103:210-216.

189. Tuft, L. 1931. Active immunization against typhoid fever, with particular reference to an intradermal method. J. Lab. and Clin. Med. 16:552-556.

190. Tuft, L., E. M. Yagle and S. Rogers. 1932. Comparative study of the antibody response after various methods of administration of mixed typhoid vaccine. J. Inf. Diseases 50:98-110.

191. Tully, J. G. and S. Gaines. 1961. H antigen of Salmonella typhosa. J. Bacteriol. 81:924-932.

192. Tully, J. G., S. Gaines and W. D. Tigertt. 1963. Studies on infection and immunity in experiment typhoid fever. IV. Role of H antigen in protection. J. Inf. Diseases 112:118-124.

193. Valentine, E., W. H. Park, K. G. Falk and G. McGuire. 1935. A study of agglutinin response to typhoid vaccine. Am. J. Hyg. 22:44-64.

194. Van Heyningen, W. E. 1950. The toxins of gram-negative bacteria. Bacterial Toxins. Blackwell Scientific Publications, Oxford, England, p. 89-93.

195. Walker, J. 1940. A method for the isolation of toxic and immunizing fractions from bacteria of the Salmonella group. Biochem. J. 34:325-329.

196. Webster, M. E., M. Landy and M. E. Freeman. 1952. Studies on Vi antigen. II. Purification of Vi antigen from Escherichia coli 5396/38. J. Immunol. 69:135-142.

197. Webster, M. E., J. F. Sagin, M. Landy and A. G. Johnson. 1955. Studies on the O antigen of Salmonella typhosa. I. Purification of the antigen. J. Immunol. 74:455-465.

198. Westphal, O. 1957. Pyrogens, in Polysaccharides in Biology. Trans. 2nd Macy Conference, (George F. Springer, editor). Madison Printing Co., Madison, New Jersey, p. 115.

199. Westphal, O., I. Beckmann, U. Hämmerling, B. Jann, K. Jann and O. Lüderitz. 1964. Recent investigations on the polysaccharide component of enterobacterial endotoxins (S and R forms), p. 1-15. In W. Braun and M. Landy (ed.), Symposium on bacterial endotoxins, Rutgers University Press, New Brunswick, N. J.

200. Westphal, O. and O. Lüderitz. 1954. Chemische erforschung von lipopolysacchariden gramnegativer bakterien. Angew. Chem. 66: 407-417 (Original not seen; cited from Ribi et al., 1961).

201. Williams, W. C. and E. L. Biship. 1936. The typhoid control program and results of 13 years' work in Williamson County, Tennessee, 1922-35, Pub. Health Rpts. 51:1-15.

202. Wilson, G. S. and A. A. Miles. 1964. Fifth Edition, Topley and Wilson, Principles of Bacteriology and Immunity. The Williams and Wilkins Co., Baltimore, Md., p. 1833-1875.

203. Wright, A. E. 1896. On the association of serous hemorrhages with conditions of defective blood coagulability. Lancet 2:807-809.

204. Wright, A. E. 1896. On the blood changes in 17 persons following typhoid inoculation. Brit. Med. J. 1:16 (Original not seen; cited from Russell, 1913).

205. Yoshioka, M. and A. G. Johnson. 1962. Characteristics of endotoxin altering fractions derived from normal human serum. J. Immunol. 89:326-335.

206. Yugoslav Typhoid Commission. 1962. A controlled field trial of the effectiveness of phenol and alcohol typhoid vaccines. Bull. Wld. Hlth. Org. 26:357-369.

207. Yugoslav Typhoid Commission. 1964. A controlled field trial of the effectiveness of acetone-dried and inactivated and heat-phenol-inactivated typhoid vaccines in Yugoslavia. Bull. Wld. Hlth. Org. 30:623-630.

RESEARCH PROPOSALS

submitted

by

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RESEARCH PROPOSALS

1. It has been reported by investigators that endotoxins can increase non-specific resistance in the host animal against challenge to several types of pathogenic microorganisms. Moreover, the acetylation of a Boivin preparation has been reported to have retained the property of stimulating non-specific resistance in mice. The use of the endotoxoids described should be studied for their effect in animals on induction on non-specific resistance.

2. Hemorrhagic necrosis of tumors in mice may be brought about by injection of endotoxins in the animal. The effect of the parent RE and the respective endotoxoids should be investigated also for mouse tumor necrosis.

3. Dermal hypersensitivity to endotoxins in both rabbits and guinea pigs has been reported by many workers. By employing this biological parameter, it would be of interest to determine whether an RE preparation as well as endotoxoid RE preparations can induce dermal hypersensitivity in the host animal.

4. Procedures such as double diffusion analysis, immunoelectrophoresis, and chromatography should be employed to analyze the sugar and protein contents of both the parent RE and the endotoxoid preparations by comparing these results with known standards.

5. The chick embryo bioassay procedure has been recently reported as an extremely sensitive method for the detection of lethality against

endotoxin. The lethal effect, if any, of the RE preparation as well as the detoxified RE preparations should be investigated employing this bioassay procedure.

6. Comparative determinations of the antibody response in mice to immunization by RE preparations and the associated endotoxoids should be carried out. Antibody should be determined by a standard procedure as well as methods which will detect nonagglutinating components.

7. The stability or keeping qualities of any vaccine is significant particularly if large volumes are shipped and stored under field conditions. All vaccine preparations mentioned in this thesis should be subjected to identical environmental situations for varying periods of time and then retested for pyrogenicity, lethality, and immunogenicity.

8. At present Landy's acetone-inactivated vaccine has shown superiority over all other vaccines in protection against typhoid fever as reported by the World Health Organization. Since the parent RE preparation (alcohol-acetone inactivated) suggests similarity to the acetone-inactivated vaccine, the possibility of detoxifying the latter vaccine should be investigated.

9. The preparation and immunological properties of detoxified bacterial endotoxins prepared by the RE method from a strain of Salmonella typhosa should be carried out.

10. The final test of a vaccine is its ability to protect man against typhoid fever with a minimum of systemic reactions. Carefully controlled studies employing human volunteers should be attempted

in order to compare the immunogenicity as well as the side reactions with the RE and the most promising of the endotoxoid preparations.