SELECTED STUDIES ON THE SOMATIC ANTIGENS OF *ESCHERICHIA COLI* O111 B4 AND O55 B5 ISOLATED AS THE IMPURE POLYSACCHARIDES

By

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A THESIS

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TABLE OF CONTENTS

INTRODUCTION

Infant gastro-enteritis has been of interest to various investigators for many years. A multiplicity of suspected viral and bacterial agents has been cited as responsible for many deaths among newborn and infants. Numerous agents have been incriminated, some of which have attracted the attention of workers on both sides of the Atlantic.

Several years ago the question arose as to whether two special serotypes of Escherichia coli could be responsible for the disease. These types were often found to be the dominating organism in sporadic and epidemic infant gastro-enteritis. The two serotypes in question have been designated as 055 and 0111 according to Kauffman (1950). Investigations in the past regarding the pathogenicity of many organisms have been questioned. Serious consideration has been given, therefore, to the toxic substances obtained from bacterial cells by chemical fractionation methods.

The polysaccharides of E. coli were obtained in 1923 as a "residue antigen" by Zinsser and Parker. Their final product gave positive carbohydrate and negative protein tests. It also precipitated homologous antisera, but was incapable of inducing protective antibodies in rabbits. Smith (1927) confirmed this information.

Boevin and Mesrobeanu (1937) prepared a glucolipid antigen from E. coli. The bacteria were extracted in the cold with trichloracetic acid and the residue removed. After dialyzation to remove the acid, the carbohydrate was precipitated with alcohol. This substance showed immunological activity in vivo and in vitro.

Wong (1938) isolated the polysaccharide of E. coli and obtained fractions, first by acid hydrolysis and then by alkaline hydrolysis. The procedure consisted of a hydrolysis with two per cent acetic acid. The final extraction was completed in alcohol. After drying the precipitate appeared as a fine white powder. A complete chemical study revealed a substance which could be hydrolyzed by mineral acids to simple hexose sugars. The two fractions differed only in minor details.

One of the early studies concerning the physiological effect of bacterial fractions upon the blood of rabbits was described by Delafield et al. (1934). A close parallel was shown to exist between fractions which were antigenic and the production of a hyperglycemic-toxic state in rabbits. All fractions were assumed to contain polysaccharides but no unaltered protein. The products were produced by tryptic digestion followed by alcohol precipitation. The greater the toxicity of the fraction, as demonstrated by injection into mice, the better immunizing agent it became when the same

animals were given subsequent injections of the fraction. When rabbits were immunized with the same antigen, further injections failed to produce the chemical changes in the blood found to occur in the non-immune animal.

A material was isolated by Dennis (1939) from NaCl infusion broth cultures of Salmonella typhosa by precipitation with alcohol. The material was not characterized chemically as a polysaccharide, though it proved to be non-protein in nature. The fraction produced leucopenia in rabbits. The effect was more pronounced in reducing the numbers of polymorphnuclear leukocytes, though it was apparent that some of them survived the effects of the toxin. The leukocytes were not markedly affected in the immune animals, while in the non-immune serious leukopenic states were observed. These reactions suggested the possibility that the leukopenia of typhoid, which is accompanied by depletion of the myelopoietic elements of bone marrow, might be due to this fraction. In addition, the lack of inflammatory cellular infiltration in the vicinity of typhoid bacilli gave further substantiation.

While working with the same organism as Dennis (op. cit. 1939) Morgan (1940) isolated a substance from S. typhosa which later (Morgan, 1941) proved to be toxic for rabbits. The in vivo destruction of leukocytes was used as a measure of toxicity. The toxic fraction was obtained by alcohol precipitation from an aqueous solution of the disintegrated

organisms and purified by dialyzation in cellophane bags. The resultant toxic material was treated by the method of Sevag (1938) to remove the protein. A light gray colloidal solution was obtained when the dried material was re-suspended in distilled water. Dosages in mice gave the following results:

Mg Dose	Number of Mice	% Fatalities
3	4	100
2	4	75
1	12	50
0.5	4	25
0.2	4	0

In rabbits, doses of 0.4 to 0.5 mg per kg injected intravenously caused prostration with dyspnea and marked diarrhea, terminating in death.

Smith (1939) discovered that, after five or six injections of a typhoid filtrate into rabbits at a time when the animal showed a very high titer of antibodies in the serum, there would follow a typical fall in the white blood cell count. Morgan (on. cit. 1940) encountered similar results when immunized animals reacted sharply to injection with a similar fraction. This change constituted a diminution in the number of neutrophiles. An interesting point

to be observed here is that animals possessing a high antibody titer to the toxic fraction were not protected from further lysis of neutrophiles upon subsequent injections of homologous antigen.

The leukopenic effect of bacterial fractions was summarized by Olitzki (1941) in four statements: (1) A general leukopenia consisting of a decrease in both neutrophiles and lymphocytes; (2) Lymphopenic-leukopenia due to a sharp decrease in the lymphocytes, associated with an increase in the neutrophiles; (3) Polynucleosis due to a sharp rise in neutrophiles, associated with a mild decrease in lymphocytes; (4) General leukocytosis due to a rise in all types. It was determined that a leukopenic resistance, which was due to the activity of neutrophiles to resist lysis by a toxic fraction, could be developed by active immunization with the fraction. This was not noted in all bacterial fractions tested.

A fraction was prepared by Favorite (1942) that, upon injection into human adult volunteers, caused chills, fever and muscular ache, followed by a leukopenia which was almost entirely due to a loss of neutrophiles. A return to normal was noted followed by a leukocytosis. Blood chemistry values showed no change in total protein, urea nitrogen, creatinine, chlorides or glucose. High titers of agglutinating and precipitating antibodies were found to be present. As was the case in the studies of Morgan (op. cit. 1941), titers of circulating antibody did not seem to be related to the level

of tolerance exhibited by the animal when injected with the toxic antigen.

The most recent work was carried out by Hays, et al. (1950). A polysaccharide substance was isolated from an untyped strain of E. coli which presumably did not contain the "B" antigen of Kauffman (1951). Reactions in rabbits to this material were investigated with interesting results. Hays (1951) demonstrated serum agglutinins for sheep erythrocytes which were previously sensitized using a polysaccharide of E. coli as the antigen.

Weter et al. (1952), although not working with a polysaccharide fraction of E. coli, showed that somatic antigens present in fluid culture media can be adsorbed on the erythrocytes of many species of animals. In a later study, Weter et al. (1952) demonstrated specific hemagglutination and hemolysis of erythrocytes previously sensitized with the somatic antigens of O55 and O111. In a subsequent investigation, Weter et al. (1952) inhibited the modification of erythrocytes by adding human or animal sera or by the addition of egg yolk or various fractions of rat liver.

The seriousness of the problem of epidemic gastroenteritis, where O55 and O111 were the suspected causative agents, led the author to consider certain fractions of these organisms which might be responsible for their toxic reaction in the host. To arrive at a clearer understanding of the

active principle involved, and to observe the effects of that principle upon various organs of the host, this study was undertaken.

REVIEW OF LITERATURE

Infant Gastro-Enteritis

In reporting his famous work in 1886, Escherich described a new bacterium which was found to be present in the intestinal tract of every mammal. He held the opinion that diarrheal disease was due to a redistribution of intestinal forms and not to a specific pathogen. Investigations since have discovered strains of E. coli known to incite disease of diarrheal nature when present in the intestinal tract of infants and experimentally in adults. (Lancet, leading article, 1952)

Since the condition of diarrhea in infants can be produced by many agents and clinical conditions, a clarification as to the various causes is necessary. Crowley et al. (1941) summed it up very well by outlining six major categories into which any case could be placed. The first includes those cases due to an outbreak of salmonellosis or dysentery. Such infections are easily identified and cause little trouble for the bacteriologist. The second group is characterized by a high mortality rate, a rapid spread among infants with loss of weight, anorexia, toxemia, diarrhea, vomiting, and dehydration. Adults are not affected.

The third is one in which one may see diarrhea but usually no vomiting. A fourth in which adults as well as children are affected seems to be common, and many times cannot be traced to any specific agent. The fifth type may be referred to as the influenzal, where mothers of infants and the hospital staff are common victims. The last group is characterized by both adults and newborn showing stomatitis in conjunction with diarrhea. This group would suggest a viral etiology (Light and Hodes, 1943).

Infantile diarrhea, more accurately termed gastro-enteritis, has been recognized for many years as one of the important causes of death in infants. Because of epidemics which have occurred in many countries of the world, particularly associated with hospital wards where crowded conditions prevailed, much interest has been aroused regarding the cause. It was not until 1945 that coliform organisms were seriously considered as causative agents. All work incriminating such agents is not yet complete. There are still many workers who take a dim view of the situation, even though the evidence is overwhelming.

The literature is abundant with references alluding to the causes of infant diarrhea. As early as 1892 Jensen et al. in Denmark reproduced a disease with diarrheal symptoms in calves. He further infected calves by feeding cultures of E. coli isolated from diseased calves. It was believed that races of E. coli existed which were pathogenic for young

calves, even though strains of such organisms were present in all normal animals.

Lovell (1937) confirmed these findings and produced a commercial polyvalent antiserum against E. coli. The same technique was applied to human infants by Hamburger (1920) who claimed success with antisera prepared against E. coli. His work was not confirmed by subsequent investigations. Shortly after Hamburger's declaration, Adam (1927) described coliform bacilli which he believed to be related to diarrheal disease in calves. Referring to these strains as "Dyspepsie-Koli", he proffered a theory of "alimentary intoxication" and described coliform organisms as related to diarrheal disease.

Following the above work, several experimenters preferentially selected the filtrate of broth cultures for examination. Filtrates from cultures isolated in connection with cases of scouring calves were studied by Smith et al. (1927) and were found to be toxic for calves one month old when given intravenously. This was not true when the same filtrates were injected intraperitoneally into guinea pigs, even when the dose, toxic for calves, was increased many times. Feeding large numbers of bacteria to guinea pigs and calves had no apparent effect.

Smith et al. (1927) further discovered that when certain strains of E. coli from the ileum of calves suffering from diarrhea or scours were grown upon agar plates, mutations occurred in which capsular substance was lost, virulence was

reduced, agglutinability was increased, as was susceptibility to phagocytosis by leukocytes. Smith (1927) also studied the interrelations between bacterial toxins and capsular substance compared with normal protective factors in the guinea pig.

Much confusion existed as to how each strain or different organism belonging to the coliform group could be specifically identified. A timely clarification appeared when Stuart et al. (1938) laid the groundwork for subsequent separations of the coliform from other groups of bacteria. Accordingly, organisms of the same genus could have varying serological reactions and at the same time be biochemically inseparable.

Still attempting to discover the causative agent of diarrheal disease in infants, Crowley et al. (1941) examined the flora of 57 normal infants. Their results revealed 80 per cent Lactobacillus bifidus, 10 per cent coliform bacteria, 4 per cent streptococci, and 4 per cent staphylococci.

In comparison, 34 infants ill with a diarrheal disease in two different outbreaks showed lactobacilli, 30 to 60 per cent, coliform, 31 to 55 per cent, with streptococci and staphylococci exhibiting little change. An E. coli etiology was not suspected in these outbreaks, but it seems significant that the coliform organisms increased at the expense of other species present in normal stools.

An increasing number of workers began to observe outbreaks of gastro-enteritis in infants, arriving at little by way of a definite cause. Lembeke et al. (1943) on the eve

of the discovery of E. coli as the agent associated with gastro-enteritis in infants, observed an epidemic in which there occurred 22 mild and 23 severe cases with three deaths. No agent was found but feces injected into baby Swiss mice proved fatal to a much greater degree than did feces from normal infants. The disease appeared selective for infants above and below normal birth weight.

In 1944, when Kauffmann began the long task of extending the work of Stuart op cit. (1938) he brought forth his publication regarding the serology of the coliform group. Here definite limitations were placed upon the various strains according to the occurrence of somatic "O", the flagellar "H" and capsular or "envelope K", antigens. This particular work made possible the present study as it proved the existence of many separate strains of E. coli. The number of strains or "O" groups of Kauffmann has been extended by Knipschildt (1945), Vahlne (1945), and Wramby (1948) to include at least 125 serologically distinct strains. Orskov (1951) examined strains of E. coli belonging to O-group, 26 isolated from cases of infant diarrhea. He correlated strains found at necropsy in newborn calves with those found in infants with gastro-enteritis, and indicated that they were probably identical. Never before had any coliform organism present in animals been suspected of being pathogenic for humans on an epidemic scale.

At the time when much of the evidence in favor of a bacterial etiology was being advanced, many investigators began to think in terms of a virus-caused diarrhea. It was only natural, in the absence of a definite agent, to explain the epidemic in terms of some virus. Lyon and Folsom (1941) correlated outbreaks of epidemic diarrhea among newborn babies with the simultaneous occurrence of virus influenzae among adults.

Light and Holes (1943) were the first to isolate a filterable agent from diarrheal stools of infants and to produce a disease in young calves by nasal administration of the virus.

Other attempts to produce diarrhea experimentally with a virus were made by Buddingh and Dodd (1944). An attempt was made to produce diarrhea in rabbits by feeding suspensions of experimentally infected cornea from other rabbits having clinical eye infections. After an incubation period of seven days the animals developed characteristic diarrheal stools. When portions of these stools were fed to normal rabbits, no symptoms were observed. The viral agent which was supposed to be the cause of the corneal infection was also credited with producing both stomatitis and diarrhea in infants. Swabs, taken from the stool of diarrheal infants, produced a disease of the cornea when rabbits were experimentally infected.

Reiman et al. (1944), while investigating an epidemic of diarrhea among students, failed to recover a filterable agent but were able to transmit the disease to adult human volunteers by having them inhale a mist of filtered naso-pharyngeal washings or filtered stools from patients showing the disease. Further, they were unable to substantiate the work of Light et al. op. cit. (1943).

Sevitt (1948) doubted the virus nature of the agent studied by Light et al. op. cit. (1943) as it could not always be inactivated by boiling for five minutes.

On the other hand, Gordon et al. (1947) isolated a filterable agent from the feces and throat washings in an outbreak of gastro-enteritis among infants and achieved the transmission of the disease in adult volunteers by the oral route. However, inoculation of this filtrate into embryonated eggs by three different routes failed to show evidence of virus growth when tested by feeding these injected eggs to adult volunteers.

It cannot be denied that in many of the studies where E. coli has been the suspected agent, the presence of a viral agent has not been adequately investigated. It might even be possible that a virus is associated with a specific strain of bacteria. Regardless, many other causes of diarrhea have been sought.

For example, Keitell (1950) in the course of a study concerning an epidemic of diarrhea affecting both adults and

infants, discovered that in many cases the infants' sera gave increased titers to cold agglutinins and streptococcus M. G., both findings suggesting to him a virus infection. He did not attempt to isolate a viral agent.

The Shigella and Salmonella genera, along with many other bacteria, have been suspected as the responsible agents in infant gastro-enteritis. Among these are the paracolon group (Mushin, 1949), the genus Proteus (Mushin, 1950), the genus Klebsiella, (Mushin, 1952), Alkaligenes faecalis (ibid.), and Pseudomonas aeruginosa (ibid.).

Gram-positive bacteria have played a minor role in the causation of diarrhea in comparison with gram-negative. Such organisms that have attracted some attention have been the staphylococci (Martyn 1949, Crowley et al. 1941, and Draper et al. 1941), and the streptococci, certain strains of which were shown by Gale (1944) to produce enzymes changing tyrosine to tyramine. This substance when fed to young rats produced diarrhea. The cultures were beta hemolytic group D streptococci, and were found to exist as the predominating organisms in the stools of infants in certain outbreaks in Cambridge, England. Similar studies involving tyramine were carried out by Dack (1947) and Dack et al. (1947) who failed to obtain evidence of toxicity with tyramine fed to human volunteers per os.

Certain strains of E. coli have more recently been shown to be associated with sporadic and epidemic gastro-enteritis

in infants. Early work carried out in Great Britain and the Scandinavian countries has been voluminous. Many can be cited, such as Beeuwkes et al. (1949), Bray (1945), Bray et al. (1948), Cathie et al. (1951), Crowley et al. (1941), Christiansen et al. (1946), Giles et al. (1949), Giles et al. (1948), Holzel et al. (1949), Kauffmann (1950), Kirby et al. (1950), Magnusson et al. (1950), Payne et al. (1951), Rogers et al. (1951), Sevitt (1948), Stevenson (1952), Taylor (1951), and others.

The two predominating coliform serotypes found associated with epidemics of gastro-enteritis have been named by many investigators and present a confusing nomenclature. The first referred to as type 1 was called Bacterium coli, var. neapolitanum by Bray (1945), Bacterium coli type alpha by Giles et al. (1948), type D 433 by Taylor (1951), Bacterium coli, B. C. T. by Rogers et al. (1951), and O111 B_4, O111 B_4 H_2, and O111 B_4 H_{12} by Kauffmann (1950). The second type referred to as type 2 has been named Bacterium coli type beta by Giles et al. (1948), and O55 B_5, O55 B_5 H_6 by Kauffmann (1950).

The initial discovery of a specific serotype of E. coli (to be referred to in this thesis as O111 or O111 B_4) associated with infant gastro-enteritis was made by Bray (1945). A group of organisms was considered which was referred to as Bacterium coli var. neapolitanum. A strain, 4988, was found to be responsible for an epidemic of summer diarrhea in

infants. The strain was not found in the stools of normal infants to any significant degree, and was recovered from 42 out of 44 cases of summer diarrhea. A seminal odor was noted upon culture on artificial media, a phenomenon which has been noticed by others.

Applying the technique of slide agglutination, Bray and Beavan (1948) discovered positive cultures of the special serotype, 4933 (0111), to be present in 87.5 per cent of the cases studied. Only four per cent of the control group were found to harbor the organism. A specific antiserum prepared against the strain of Bray, 4933, was used in all tests. Biochemical separation of this strain from other coli was considered impractical, if not impossible. The agglutination studies comprised a group of 40 cases diagnosed as gastro-enteritis. The mean age of the infants was five months. Twenty-eight of the cases were severe and a definite odor was ascribed to the feces. It is interesting to note that here, as in other epidemics apparently due to the same organism, few gross lesions were noted at post mortem.

In an outbreak of infantile gastro-enteritis in Aberdeen, Scotland, Giles and Sangster (1948) recorded that the majority of the cases studied was unrelated and that no common source of infection could be definitely established. Two ward outbreaks occurred in which it was possible to trace the infection from patient to patient. Of the 159 cases, 66 were considered to be of dietetic origin, 93 appeared to belong to

the primary infective group, and the remaining 92 showed clearly to be a common strain of E. coli. The latter were serologically homogeneous.

Confirming the work of Bray op. cit. (1945), Beerukes et al. (1949) studied the role of specific strains of E. coli in epidemic infant gastro-enteritis. A year later Payne and Cook (1950) studied the incidence of a special serotype of E. coli from a group of infants over a one year period. (An investigation of gastro-enteritis in an orphans' home.) The clinical condition of the babies was under constant observation. Bacterium coli O111 B_4 was isolated from 40 of 337 rectal swabs taken. The organism was often present in pure culture on the swab plate. All the strains conformed biochemically and serologically to those of Bray op. cit. (1945). Ten out of 15 infants in one group showed the strain in their stool but exhibited no clinical symptoms.

Smith et al. (1950) associated the supposed causative agent of an epidemic with that designated O55 B_5 H_6 by Kauffmann op. cit. (1950). This type had replaced the O111 variety present in an earlier epidemic in the same geographical area. Titers of antisera against O55 appeared to be very low or negative when tested by bacterial agglutination. By the use of more recent tests these sera would, in all probability, have shown higher titers. (Weter op. cit. (1952) has shown that low titers of antibodies in the blood of human volunteers infected with O55 (experimentally) could not be

detected by the use of the bacterial agglutination test, but were sufficiently high to cause the hemagglutination of sheep erythrocytes that had been previously sensitized with the boiled culture filtrate of E. coli 055.) Cases were treated with chloromycetin with good results. The fact that treated children (those who had received antibiotic) showed weight increases over untreated is of interest in view of the present knowledge of growth stimulation due to ingested antibiotics. Sufficient numbers of infants were treated to make the observation valid.

Furthering the search for infections due to E. coli D 433 (0111), Meter et al. (1950) attempted to discover whether type D 433 is present in sporadic cases of infant diarrhea as well as in infections such as pyelitis, peritonitis, meningitis and otitis media. Cultural studies were carried out to determine whether these special serotypes resembled coliform bacteria according to their reactions on various media. The presence of D 433 in the upper respiratory tract of infants was investigated. In this regard, the organism was found in five infants with non-epidemic diarrhea. All the strains isolated were biochemically coliform and were agglutinated by specific antisera prepared against type D 433.

It should be mentioned that this serotype was not found in 33 additional infants exhibiting diarrheal symptoms. Strains of E. coli isolated from cases of peritonitis,

pyelitis, meningitis, otitis media, and septicemia were not of this special type. An infant, upon being fed a culture of D 433, experienced diarrhea and weight loss. Exposure to the patient's own strain of E. coli failed to produce symptoms.

In drawing some important conclusions, Meter et al. (1951) recorded four points: (1) Two serotypes of E. coli were found to produce sporadic cases of infant diarrhea. (2) Contact with these special types lead to symptoms of gastro-enteritis. (3) There is a carrier state in infants as the organisms had been recovered from the upper respiratory tract from which they could produce an air-borne infection. (4) These states may be closely associated with epidemic diarrheal disease of infants.

In England, Taylor (1951) made extensive investigations into outbreaks of infant gastro-enteritis. During a single study, one healthy baby excreting 055 was admitted to a residential nursery, and soon an outbreak of gastro-enteritis occurred in which the beta type organism (055) was isolated from all of five sick infants. The organism was also isolated from six of seven babies showing an increased stool frequency, and from two of five infants with abnormal stools but with no increase in frequency. Taylor concluded that this and other epidemics were due to a special type of E. coli.

In the same year Cathie et al. (1951) reported on a study of two years duration in which they recovered D 433 (0111) in 264 cases of infant diarrhea.

Very interesting and significant results were published by Rogers (1951) and Rogers et al. (1951) when they reported on the incidence of cross contamination with infant diarrhea when the patients were confined in a cubicled ward. Air was found to be contaminated with coliform organisms due to routine activities in the ward. All the articles associated with the infants were also contaminated in a like manner. The disease was seen to spread rapidly from cubicle to cubicle. The treatment of floor mops with five per cent phenol failed to prevent air contamination of clean cubicles. It was observed that a single cubicle could be completely contaminated within 18 hours after admission of an infant harboring a type strain of E. coli, and that this organism was able to remain viable in the dust of the room for 27 days. This work certainly emphasized the close association of both types of E. coli with outbreaks of infant diarrhea, especially in hospitals where intimate contact between patients is unavoidable.

After studies on the spread of such organisms within a hospital in England, Rogers et al. (ibid.) further revealed observations made on the rapidity with which these special types of coliform organisms could be traced from one hospital, known to harbor the organisms, to a second, then to a third, and still a fourth, each succeeding one previously known to be free of the strains. In an attempt to prevent further spread of the disease, the authors recommended bacterial

examination of stools from all infants entering a hospital for treatment of a diarrheal condition, vomiting, or both. In addition, the stools of each newborn infant should be examined bacteriologically.

In the United States, Ferguson and June (1952) were interested in the possibility of adult infection with O111 B_4 when the organisms were ingested. Male volunteers were selected for the study. One group was fed varying numbers of the organism; a second group served as a control. When 500,000,000 organisms or more were ingested (strain O111), symptoms similar to bacterial food poisoning were noted. Quantities less than the above number of bacteria failed to produce significant symptoms. The control organism, fed to a second group of volunteers, was a strain of E. coli isolated from the stool of a normal infant known not to harbor any special type of coliform organism. Many of the persons ingesting the strain of O111 B_4 showed agglutinins for that strain. The general conclusion was that adults are quite resistant to natural infection with O111 B_4 strain of E. coli.

Studies concerning the feeding of the 055 B_5 strain to human volunteers have also been undertaken more recently by June et al. (in press). When this work has been published, there is little doubt that findings similar to those of Ferguson et al. op. cit. (1952) will have been made.

Recent work by Modlin et al. and Stevenson (1952) has added greater impetus to the idea of an O111 and 055 etiology

of infant gastro-enteritis. Hodisa et al. (1952), over a period of seven months, observed 56 cases of diarrhea in which 80 per cent showed a specific coliform. One hundred and forty-six infants not suffering from diarrheal disease showed the organisms O111 to be present in 4.7 per cent of the cases. Ninety-three adults in the same hospital were shown not to contain the organism by bacterial cultures. The reported mortality among infants was only 10 per cent, the low figure being credited to the use of chloromycetin in treatment during the epidemic.

Pathology

Histopathologic findings in the case of infants known to be infected with O111 or O55 have been varied. The most constant finding has been a mild fatty change in the liver of infected infants.

Giles et al. (1948), in an epidemic of infant gastro-enteritis, found that among 92 cases harboring a common strain of E. coli the liver was the only organ to show consistently some evidence of damage. Changes in this organ varied from mild fatty change to a severe necrosis. The peripheral portion of the liver lobule was most affected. The spleen and kidneys were often found congested. Only a few cases appeared to have definite nephritis. Broncho-pneumonia was noted in a few cases but was considered terminal.

The presence of meningo-encephalitis in infants suffering with a similar condition was reported by Christensen et al. (1946). Giles et al. (1949) in a similar investigation found no such lesions in a large number of cases.

Sakula (1943) carried out extensive studies upon infants dead of a diarrheal disease in which O111 and O55 were the suspected agents. The pathological findings closely paralleled those found by others mentioned above. The intestines manifested little change with no ulcers of the mucosa and only slight lymphadenopathy of the mesenteric lymph glands. Only the liver consistently showed pathological changes. Most marked were fatty change, congestion, and jaundice, in that order. The fatty changes appeared peripheral in the liver lobule. There was usually evidence of early proliferation of the cells forming the bile canaliculi. The thymus gland was smaller in every case, as were the supra-renals in some cases. A spleenamegaly was observed in some. Emphysema of the lung with atelectasia was noted in four cases. These general findings only indicate a toxemia and wasting condition and are in no way specific for any given disease entity. These findings are not uncommon to many wasting diseases of children.

An examination of infants dead from a diarrheal disease was made by Kirby et al. (1950). In four of the patients no gross changes were observed in the intestines, other than a congestion of the mucosa. Mesenteric lymph nodes were normal

or only slightly enlarged. Where plasma and protein hydrolysates were given as treatment, little fatty change was noted in the liver. Changes in the brain were limited to mild congestion of the pia-arachnoid vessels and edema of the meninges. There were no lesions to compare with those found by Christensen *op. cit.* (1946).

Few other investigations have been made in the case of known epidemics of O111 or O55 infant gastro-enteritis. In an investigation in which the author took part, findings similar to those cited above were observed. Only one case out of 13 showed any appreciable pathological manifestations.

MATERIALS AND METHODS

Cultures

The strains of *E. coli* used in this study were obtained through the kindness of Dr. W. W. Ferguson, Division of Laboratories, Michigan Department of Health, Lansing, Michigan. The strains were identified as O111 B_4 and O55 B_5, numbered 5373 and 13027, respectively. Several transfers were made from the original cultures to brain heart infusion agar (Difco Laboratories) to insure the purity of the cultures. The organisms were transferred weekly and observed throughout the course of the study for roughness of colony growth. Originally, both strains were isolated from the feces of human infants ill with gastro-enteritis.

Biochemical tests were carried out in duplicate to check the cultures for typical reactions. It was found that they fermented maltose, sorbitol, dulcitol, dextrin, rhaminose, arabinose, manitol, lactose, and xylose. No fermentation of sucrose or inulin was observed. Indol was not formed, gelatin was not liquefied, and citrate was not utilized. They were Voges-Proskauer negative and methyl-red positive. After biochemical examination, the organisms were agglutinated by means of group specific antisera. All cultures were observed to be pure.

Fractionation of Cultures

The bacteria for fractionation were grown in nutrient broth at pH 7. Attempts were made to grow the organisms in a synthetic medium, but the limited growth obtained was not adequate for the purposes of the study. Many workers, undertaking similar studies, have used various types of ordinary media with success. Hays et al. (1950) utilized tryptic digest agar satisfactorily. Wong et al. (1938) used an ordinary agar medium.

To grow the cultures for fractionation two methods were used. They differed only in the amount of medium contained in a single flask and the type of shaker employed. In the first method 500 ml Erhlenmeyer flasks were used in which was placed 250 ml of nutrient broth. After seeding, the flasks were shaken continuously for 24 hours, in an incubator, at 37 C. The constant agitation was accomplished by means of a Borell shaker. Method two involved six liter flasks that contained three liters of media each. These were shaken on a platform-type machine at 37 C for 24 hours. More growth per volume of media resulted from growth in the larger flasks.

The rapid removal of the growth from the medium required the use of the Sharples centrifuge. Speeds of approximately 12,000 rpm were required to make the separation. Only a small percentage of the growth was lost in the first centrifugation and this could be reclaimed by a second centri-

fugation. The organisms were collected from the rotor with a spatula and suspended in distilled water at a ratio of about one to 100 parts water. The water suspension of bacteria was placed in a water bath at 56 C for one-half hour. After washing the cells three times by centrifugation, they were extracted three times with ethyl ether, and finally air dried. During the chemical procedures throughout the study, the bacteria were kept from as much contamination as possible by the use of clean glassware and careful covering. The dried bacteria were placed in a mortar and ground thoroughly with sand until microscopic examination failed to reveal intact cells. This period varied from batch to batch.

The disintegrated cell fragments were extracted three times with distilled water. The supernatant liquid was saved each time and the sediment discarded. It was assumed at this point that the materials sought were water soluble products. This proved to be the case. Hays et al. (1950).

The water extract containing the impure water-soluble polysaccharide fraction was shaken with a mixture of chloroform and amyl alcohol after the method of Sevag et al. (1938). The ratio of chloroform to alcohol was 0.1 part to 0.25 part, respectively. The total mixture was shaken for a period of 45 to 60 minutes, by hand, in a separatory funnel. The mixture was centrifuged in 50 ml portions at 3,000 rpm in an International Centrifuge, type PR-1, for 10 minutes. A separation of the chloroform from the water layer was noted.

At the interface a gelatinous-appearing membrane was formed. This layer contained intact protein materials and was discarded. The formation of such a layer indicating the presence of protein substances is, according to Sevag et al. (op. cit. 1938), a test for protein, sensitive to one part in 40,000. The aqueous fraction was treated in the above manner six times after which no gelatinous membrane was noted at the chloroform-water interface. The chloroform was found in the bottom of the tube. Finally, the absence of the gelatinous layer indicated that all of the protein possible had been removed from the mixture. A negative Biuret reaction (Table I, column 2), indicating the absence of the peptide linkage, substantiated the observation that little intact protein remained.

The water layer, from the above extractions with chloroform, was precipitated with two volumes of ethyl alcohol, 95 per cent, which contained a trace of sodium acetate. A fine white precipitate appeared and was recovered by centrifugation. This precipitate was dissolved in distilled water. (A ratio of about 100 parts water to one of precipitate.) Precipitation of the polysaccharide material was carried out for a total of five times. Any sediment appearing in the centrifuged water solutions was discarded each time.

The final precipitate was extracted three times with alcohol, three times with ether, and dried by evaporation.

The final material appeared as a light brown powder. The polysaccharide was weighed accurately, and portions for immediate use were dissolved in warm saline solution. In cold saline solution it formed a colloidal-like suspension which did not settle out upon standing. Other polysaccharides have been reported as being colloidal, namely those of Morgan (1940) who isolated them from a culture of S. typhosa.

Rabbits

White albino rabbits, weighing from two to three kg were used. All animals had a normal temperature prior to use in any experiment. Blood samples for total and differential leukocyte and erythrocyte counts, blood glucose and hemoglobin determinations were taken from the marginal ear vein.

Preparation of Specific Antisera

Living and heat-killed vaccines were prepared in saline solution (0.85 per cent sodium chloride, pH 7.0) from E. coli, serotypes O111 B_4 and O55 B_5. Wherever the words "saline solution" appear throughout the study, the above specifications apply unless otherwise stated. Rabbits were injected intravenously with gradually increasing doses of vaccine, on alternate days, for periods of six to twelve days. All animals were rested for at least one week prior to bleeding.

Blood was taken from the heart and the serum separated from the clot. All such sera were checked by bacterial tube agglutination for titers and were phenolized (0.1 per cent) for preservation. The various antisera were made from the blood of rabbits immunized with either boiled (100 C for one hour) or living suspensions of organisms. Some sera were prepared by injecting a mixture of boiled organisms (prepared as above) and the specific polysaccharide antigen. Sera made from rabbits receiving only boiled organisms contained only O antibodies. (Boiling of O111 or O55 strains destroys the B antigen, so that no B antibodies result from injection.) Sera made from rabbits receiving the living culture contained both O and B antibodies. (These cultures were not heated; therefore, the B antigen was not destroyed, and upon injection produced B antibodies.) Sera prepared from rabbits receiving only polysaccharide antigen contained both O and B antibodies. (The polysaccharide antigen was not made from boiled organisms.) As the cultures utilized were found to be non-motile, no consideration was made regarding H antibodies.

Alsever's Solution

All erythrocytes were collected and stored in Alsever's solution. The solution was made by dissolving 2.05 per cent dextrose, 0.8 per cent citrate, and 0.42 per cent sodium chloride in distilled water. (Alsever et al. 1941) The

erythrocytes were washed in saline solution several times by centrifugation, and resuspended in Alsever's solution. Washed cells were stored in Alsever's solution at 4 C until used. Cells showing hemolysis were discarded.

Sensitization of Erythrocytes from Various Species of Animals with Polysaccharide

Packed erythrocytes were added to a known polysaccharide-saline solution to make a five per cent suspension of cells. The tube containing the mixture of erythrocytes and polysaccharide was placed in a water bath at 37 C and incubated for one hour with frequent agitation. At the end of this period, the cells were thrown down by centrifugation at 2,500 rpm, and the supernatant fluid removed. The sensitized packed cells were then washed three times with saline solution. Finally, the sensitized washed erythrocytes were diluted to approximately one and one-half per cent with saline solution and used promptly in hemagglutination and hemolysis experiments. A final dilution of polysaccharide one to 1,000 was found optimal for sensitizing erythrocytes.

Hemagglutination Test

Erythrocytes were sensitized with the desired polysaccharide or culture filtrate. (These were both boiled and

unheated.) Serial dilutions of antisera to be tested were made in saline solution. Twenty-five hundredths ml saline-serum mixture was placed in each tube (Hahn). Control tubes included (a) no serum and (b) normal rabbit serum. Finally, each tube received 0.25 ml of sensitized erythrocytes and was shaken thoroughly. The tubes were then placed in a water bath at 37 C for two hours. The reaction was read at 30 minutes, one hour, and two hours.

After the final reading, the tubes were centrifuged at approximately 2000 rpm and again read. A positive test was indicated by a disc at the bottom of the tube, broader than the control, and having a serrated edge. The disc would not slip from position when the tube was tilted.

Hemolysis Test

Fresh guinea pig serum (pooled from the blood of several animals) was used as a source of complement. Before each test the complement was titrated with normal sheep and human erythrocytes to detect the presence of lysins for normal sheep and human blood cells. The lowest dilution of complement which showed no hemolysis was selected for use. Because the tests conducted were of a preliminary nature, it was not considered important to standardize the complement in terms of minimum hemolytic doses for normal sheep erythrocytes. Some difference in hemolytic titers was noted, due primarily

to differences in samples of complement. Serial dilutions of sera to be tested were made in the desired concentration of complement (0.25 ml was added to each tube. Kahn). The control tubes contained (1) heated serum but no guinea pig complement; (2) guinea pig complement alone. Twenty-five hundredths ml of a one per cent suspension of sensitized sheep or human erythrocytes was added to each tube. The tubes were shaken and placed in a 37 C water bath for one hour, after which a final reading was taken. The hemolytic titer was read as the highest dilution of serum in which hemolysis occurred. The hemolysis of normal sheep or human erythrocytes in the presence of specific antisera was investigated previous to the beginning of each test. No hemolysis, due to specific antisera alone, was noted in dilutions of antisera of one to one hundred or greater.

Agglutination Test

All bacterial tube agglutinations were carried out according to the technique for the Widal test for E. typhosa (Widal, 1896). Slide agglutinations were performed by placing a small amount of the culture, diluted in a saline solution, on a clean glass slide and mixing in a drop of specific antiserum. Agglutination was observed by indirect light as a clumping of the cells within a period of one to two minutes. Reactions occurring after two minutes were not considered significant.

Precipitin Test

Polysaccharides for testing were made up in saline solution to the desired concentration. (A 1-1,000 dilution gave good results.)

To perform the test, small precipitin tubes (Durham tubes) were filled to about one-third with the specific antiserum to be tested. The polysaccharide solution was layered carefully upon the serum so that no mixing of the two liquids occurred. The precipitin tubes were placed in a special rack, so constructed that it was not necessary to remove the tubes in order for readings to be made. All precipitin tubes were incubated at 37 C for 18 hours and then read. A definite cloudy layer at the antiserum-polysaccharide interface indicated a positive test. A saline-antiserum control, run at the same time as the test sample, was included.

In Vivo and In Vitro Tests of Polysaccharide

In vivo tests were made in rabbits by intravenous injection of known amounts of the specific polysaccharide suspended in saline solution. Blood samples taken for study were collected in paraffin-lined tubes containing sufficient heparin to prevent coagulation of the blood. The tubes were stoppered with paraffin-coated corks. The blood was collected from the cut marginal ear vein as it flowed freely into the

tube, and gently mixed to prevent coagulation.

In vitro tests for the lysis of polymorphnuclear leukocytes were conducted using freshly collected cord blood from human newborn infants.

Tubes for the collection of blood were prepared as above, and delivered to the obstetrical section of the hospital. The blood was collected in wax-lined, heparinized tubes from the cut umbilical cords and placed immediately in a 37 C water bath. Samples of cord blood were run in two different hospitals. Five ml of blood was taken from each of several newborn infants. Three complete cell counts were made on each sample; the total counts were averaged. Differential counts were also carried out in triplicate and averaged.

Two mg of polysaccharide in two cc of saline solution was placed in each five ml of blood sample, mixed well by gentle rotation of the tube, and then placed in a water bath at 37 C for one hour. At the completion of the incubation period, triplicate counts (as above) were made and the final average counts of all samples obtained. The results shown in Table VI are an average of the several separate blood samples investigated in the experiment.

EXPERIMENTAL RESULTS

Biochemical Tests

An adequate chemical characterization of the final polysaccharide was not carried out due to lack of the necessary equipment and the detailed chemical examinations required. Few bacterial polysaccharides with minor exceptions have been adequately studied chemically. However, basic chemical tests indicated that the material was carbohydrate in nature with many nuclei containing nitrogen in their structure. Hayworth *et al.* (1948) found that in an O antigen preparation from *S. typhosa* there was 20 per cent of a soluble nitrogenous constituent present. This type of material would not be removed by ordinary de-proteinization methods. Hays *et al.* (1950), using the same method as was used in this study, did not report the per cent nitrogen present in their polysaccharide product.

From the results of the chemical examinations shown (Table I), it can be reasonably concluded that there is a carbohydrate nucleus present. Intact protein is not present as indicated by the absence of the gelatinous layer, Sevag *et al. op. cit.* (1938), and repeated negative Biuret reactions. Nitrogen-containing compounds, probably peptide in

nature, are present to an appreciable extent. The percentage of nitrogen present in each preparation (as noted in Table I, column 3) is high when compared with polysaccharide fractions of others such as Wong et al. (1938). However, these workers did not fractionate a strain of E. coli known to possess the B antigen. It should be brought to mind that the presence of various chemical constituents in polysaccharide fractions depends greatly upon the method of isolation, and no results can be compared on an equal basis unless the same strain of organism is fractionated and the same method of extraction is employed. The chemical substances that can be isolated from a given organism, even by a single method of isolation, are numerous and should not be compared with those isolated by other methods. Therefore, the fractions discussed in this study are not compared chemically with other E. coli fractions obtained by similar or different methods.

Phenolic substances were found to be present as indicated by a positive Millon's reaction. All organic compounds which contain the hydroxy phenyl group give the Millon reaction. The test is generally considered one for the amino acid tyrosine. Desoxyribonucleic acid was found present to a significant degree (Dische test). Phosphorous and sulphur were also strongly positive. The presence of phosphorous-containing nuclei, according to Raistrick et al. (ibid.), seems to preclude a certain degree of toxicity for animals.

They stressed the importance of phosphorous and sulphur in the biologically active molecule.

Reference to Table I, column 7, will show that the substances were hydrolized with sulfuric acid and, upon neutralization, yielded reducing sugars. These sugars were not characterized as to their specific nature. Further chemical analysis of such materials should be undertaken to give more exact information regarding their structure. Until such is the case, one can only characterize these materials as impure polysaccharides.

Until more workers undertake the isolation of polysaccharide fractions from the E. coli known to possess the B antigens, it is the opinion of the author that the presence of these antigens, which render the bacterial cells O inagglutinable, is of tremendous importance both from the chemical and immunological point of view. As no one to date has isolated the B antigens, it might be well to consider them as a chemical entity, which, with careful chemical extraction methods, could be isolated. There is no indication at this point to consider them as being entirely of a polysaccharide nature.

In Vivo Studies

Effect of Polysaccharide on Rabbits

Intravenous injection of 0.3 mg per kg body weight of the polysaccharide in saline solution proved lethal in each of five rabbits. Table III contains a record of the leukocyte count, erythrocyte count, blood glucose, and temperature after injection, of two rabbits. Rabbit number 2 is included in the above five. It can be seen that a dose of 0.3 mg per kg of polysaccharide proved lethal to rabbit number 2; while rabbit number 3 survived a dose of 0.4 mg per kg. In rabbit number 2 a marked reaction developed within two to three hours and was characterized by prostration and diarrhea. The peripheral circulation was depressed as evidenced by a paleness and blueing of the ears. Death occurred 22 hours after the onset of the symptoms. The heart blood was subsequently collected from four other rabbits that had been injected with a lethal dose of the polysaccharide and examined. There existed a marked leukopenia and an elevated blood glucose. (The normal blood glucose for rabbits is from 90 to 115 mg per 100 ml of blood. (Mays et al. 1930)) The temperature rose only slightly in each rabbit. Tissues were taken of various organs after death and fixed in 10 per cent formalin.

As much as 15.0 mg of the polysaccharide administered per os to normal rabbits failed to elicit any noticeable response. Similarly, baby mice fed in the same manner showed no obvious reactions.

Injections into non-immune rabbits of 0.4 mg of polysaccharide per kg body weight did not prove lethal to any rabbits injected.

Changes in the Cells and Glucose Level of Venous Blood of Normal and Immune Rabbits Following Intravenous Injection of Polysaccharide

When 0.4 mg of polysaccharide per kg body weight was injected intravenously into normal rabbits, marked symptoms occurred. After a two-hour period, samples of blood showed a leukopenia accompanied by an increased blood glucose. The leukopenia was characterized by a disappearance of polymorphnuclear cells from the blood. About 24 hours after injection the rabbits developed a leukocytosis, characterized by an increase of the polymorphnuclear cells. In 72 hours the blood picture was essentially normal. (Rabbits 1 and 3 found in Tables II and III.) (See figures 1 and 3).

Two hours after injection of polysaccharide the blood glucose increased in proportion to the size of the injected dose of toxic material (see figure 2).

Tests were made to determine whether a rabbit having antibodies against the cell vaccine or the polysaccharide

would react in a manner similar to the non-injected animal. Rabbit number 4 (Table IV) was injected intravenously with one ml doses containing two mg each of the polysaccharide from O111 on every other day for a total of nine times. These injections were given in preparation for the test, results of which are given in Table IV. A check on the blood two hours after each injection showed the usual leukopenia and increased glucose. After the above series of injections with polysaccharide from O111, the animal was rested for a period of three weeks. Antibodies against the polysaccharide injected were tested for and demonstrated by bacterial agglutination (Table XIV). It is shown that the polysaccharide antiserum contained O and B antibodies, as living, intact bacterial cells were agglutinated.

A second series of injections of O111 polysaccharide was then given to rabbit number 4, the results of which are summarized in Table IV (see figure 4). On the first day, two hours after injection, a characteristic leukopenia was found. Approximately 35 per cent of the leukocytes had disappeared, 42 per cent of which were neutrophiles. A leukocytosis was noted on the day after the initial injection. Two hours after injection on the second day an even more marked leukopenia was produced. An 85 per cent reduction occurred in the total number of leukocytes, associated with a 92 per cent reduction in neutrophiles. The results on the third day were similar to those above. No injection was

given on the fourth day; however, a mild leukocytosis was found. On the fifth day an increase in the total leukocyte count was observed two hours after injection. This rise was due to an increase in the lymphocyte level, as an actual decrease in the numbers of neutrophiles occurred. The rabbit, (number 4) on the sixth day, once again presented a high leukocyte count but was not injected. A tolerance to the injections began to be shown by the seventh day, as the polysaccharide produced only slight changes in the total leukocyte count, represented by an eight per cent reduction. The loss, though small, was made up entirely by neutrophiles. Examination on the eighth day revealed a normal blood picture. A blood glucose determination on the seventh day, and two hours after injection, however, showed the usual increase. No apparent tolerance had been established for this mechanism.

The same animal as above was rested for a period of eight weeks and given a 2.0 mg dose intravenously of the same polysaccharide. Within two hours an examination of the blood revealed a leukopenia and an increased blood glucose. Whatever tolerance the animal had previously was no longer in evidence.

A rabbit (number 5, Table V) which had been previously given a cell vaccine prepared from E. coli O111, was injected intravenously with O111 polysaccharide (0.4 mg per kg body weight), three and one-half weeks after the last intravenous injection of cell vaccine. The blood serum from this animal

at that time possessed an agglutination titer of one to 640. The response of the rabbit to the injection is tabulated in Table V. Two and one-half hours after the injection a leukopenia was established, characterized by the disappearance of polymorphnuclear cells. In addition, the lymphocytes were markedly reduced. The total leukocytes were reduced about 70 per cent; the lymphocytes, 50 per cent; the polymorphnuclear cells, 86 per cent. The blood glucose increased sharply. After a period of 11 hours, a mild leukocytosis existed with a continued increased blood glucose. Within a 24-hour period a definite leukocytosis occurred, accompanied by a nearly normal blood glucose. A complete return to normal was achieved in 72 hours after the injection of the polysaccharide. Monocyte estimations resulted in no definite conclusions. Hemoglobin values were not significantly altered.

In Vitro Studies

Lysis of Leukocytes *In Vitro* by O111 Polysaccharide

The technique employed by Dennis *et al.* (1939) and Hays *et al.* (1950) was used to determine the ability of the polysaccharide from O111 to lyse leukocytes *in vitro*. Freshly collected cord blood of five human infants at birth was mixed

with polysaccharide in saline solution. After a two-hour period of incubation at 37 C, the results found in Table VI were obtained.

A 42 per cent reduction in the total numbers of leukocytes occurred compared to the number present in the control sample. The neutrophiles were reduced 83 per cent, while the lymphocytes showed a 35 per cent apparent increase.

The polymorphnuclear cells observed in blood films made at the termination of the incubation period were interesting. These cells were found in various stages of dissolution. Some appeared greatly ballooned and others appeared broken, with a pouring out of the granular material. Other cells possessed only a nucleus; the cytoplasm had passed out through the fractured cell wall. The copious amount of debris found was accounted for by the fracture of the leukocytes, in this case neutrophiles. Lymphocytes appeared essentially normal; only a few showed any alteration in morphology. The erythrocytes were intact in all samples observed.

Agglutination and Precipitation Tests

Following the preparation of antisera, made by injecting a rabbit with the polysaccharide from O111 or O55, it was necessary to determine if the antisera contained both the somatic O and the "envelope" B antibodies. As a pure B antiserum has never been prepared, it was thought that possibly

one made by injections of polysaccharide would be free of O antibodies. To investigate this possibility an experiment was made in which an antiserum was prepared by injecting a mixture of O111 polysaccharide and organisms, that had been previously boiled (O111 organisms), into a rabbit, while a second rabbit received only the polysaccharide. If the polysaccharide were pure B antigen, only B antibodies would be produced. By the same rule, if the polysaccharide were pure B antigen, mixing it with a boiled culture would supply an antigen complete with both O and B antigens. Such an antigen when injected into a rabbit would result in an antiserum having both O and B antibodies. The results of such an experiment can be seen in Table VII.

As is evident, the boiled organisms were agglutinated in a 1 to 2560 dilution by the polysaccharide-cell vaccine antiserum, the unheated to 1 to 320 dilution. Antisera prepared against the polysaccharide alone agglutinated the boiled organisms in a 1 to 640 dilution. Had the polysaccharide antiserum contained only B agglutinins, it would not have agglutinated the boiled organisms, which possessed only O antigen. The B antigen on the bacteria had been destroyed by boiling for one hour. (Kauffman, 1950) It was, therefore, concluded that the polysaccharide was made up of both O and B antigens.

Having established that the polysaccharide was made up of both O and B antigens, which upon injection would produce

O and B agglutinins, a precipitation reaction was investigated to determine if the OB antigen of the polysaccharide would precipitate cell vaccine antisera possessing OB precipitins, as well as those having only O precipitins. The assumption was made that the injection of boiled organisms into rabbits formed precipitins specific for the O antigen. A precipitin reaction took place between the cell vaccine OB antiserum and the polysaccharide OB antigen (Table XII). However, unexpected results were obtained when the polysaccharide OB antigen failed to precipitate the cell vaccine O antiserum. The possibility was therefore presented that injection of boiled organisms into a rabbit does not produce O precipitins. It might also be concluded that only the B antigen present in the polysaccharide is able to elicit the precipitation reaction. Since only one animal was used to produce this antiserum, it might be possible that the animal used was not capable of producing O precipitins.

Hemagglutination and Hemolysis Experiments

Many workers in the past have shown that erythrocytes are capable of adsorbing various antigens which render them agglutinable by specific serum antibody. Reactions of this type have been reported by Keogh et al. (1947), Kravchenko et al. (1947), Middlebrook et al. (1948), Hays et al. (1950), Fisher (1950), Fisher et al. (1951), and Meter et al. (1952).

Polysaccharides from various species of bacteria have been adsorbed by the red blood cells of many species of animals. The polysaccharide antigens of an untyped strain of E. coli have been adsorbed by the erythrocytes of animals as recently as 1951 by Mays et al.. Kravchenko op. cit. (1947) adsorbed the polysaccharides of various bacteria onto human type O erythrocytes.

To determine whether the polysaccharides from E. coli O111 and O55 could be adsorbed onto the red cells of various species of animals, the following experiment was undertaken. The erythrocytes of a cow, rabbit, dog, man, sheep, and chicken were washed and sensitized with the polysaccharide from O111. The results are presented in Table VIII which gives the reactions for the antigens of E. coli O111; however, the test was repeated for the serotype O55 with similar results. A like reaction was carried out by Weter et al. (1952) using the antigens present in culture filtrates from both O111 and O55 that had been boiled for one hour. It can be observed that the various blood cells adsorbed the polysaccharide antigen and were agglutinated by homologous antisera to about the same titer. Antisera containing O and OB antibodies were equally effective in producing hemagglutination.

Adsorbtion of Two Antigens Simultaneously

In order to determine whether red blood cells are capable of adsorbing the antigens of polysaccharides O111 and O55 simultaneously, the following experiment was carried out. Sheep red cells were modified by (a) incubation with the polysaccharide from O111, (b) incubation with the polysaccharide from O55, and (c) incubation with the polysaccharides from O111 and O55 simultaneously. The results of the experiment are presented in Table IX.

Red blood cells treated as in a and b above were agglutinated by their homologous antisera. Cells that had adsorbed both antigens were agglutinated by either antiserum, indicating that each cell had adsorbed both antigens. Repeated experiments gave similar results. Moreover, when cells prepared as in a and b were mixed together, heagglutination with a single antiserum (one for O111 or O55) caused only partial agglutination of the blood cells. Agglutination of cells sensitized as in c produced a complete reaction using either antiserum. Further evidence of the simultaneous adsorbtion of antigens was gained when sensitized cells, as in a or b, were mixed with untreated cells and agglutinated with the homologous antiserum. Only partial agglutination was observed, indicating that only the sensitized cells were involved.

The Subsequent Treatment of Sheep Red Blood Cells with Polysaccharide

If the polysaccharide antigens could be adsorbed onto red cells independently and simultaneously, the question arose as to whether the presence of one polysaccharide on the red cell would exclude the adsorbtion of another. A blocking effect might be present to prevent subsequent adsorbtion of the polysaccharide antigens. To investigate this question, repeated experiments were undertaken in which washed sheep rod cells were first treated with the polysaccharide from O111 followed by the polysaccharide from O55 and vice versa. The reactions are shown in Table X. Almost identical titers were produced by the various reactions. No blocking of hemagglutination was noted in any reaction. Blood cells treated with one antigen did not hemagglutinate with the heterologous antiserum. Titers were similar to those obtained when sheep red blood cells were treated simultaneously.

Hamagglutination and Hemolysis by Addition of Complement

Fisher and Keogh in 1950 reported that erythrocytes sensitized by bacterial antigens would undergo lysis in the presence of complement. To determine whether this same phenomenon could be demonstrated using the antigens of E. coli (polysaccharides) O111 and O55, the following experiment was undertaken.

Sheep and human erythrocytes were sensitized by the polysaccharide antigens from O111 and O55. To carry out a test, sensitized erythrocytes (having adsorbed specific polysaccharide) were added to tubes containing two-fold serial dilutions of the homologous antiserum. Complement made from pooled sera of several guinea pigs was added in a final dilution of 1 to 40 to each tube containing sensitized erythrocytes and specific antiserum. The tubes were gently shaken to insure mixing and then were incubated in a water bath at 37 C for one hour. Lysis occurred in tubes containing sheep erythrocytes but failed to take place in tubes containing human red cells. Lysis of sheep or human erythrocytes did not occur in the absence of complement. When complement was mixed with sensitized erythrocytes of man or sheep in the absence of antiserum, complement dilutions of 1 to 30 and greater did not cause lysis of the erythrocytes. A final dilution of 1 to 40 of complement was selected, therefore, as optimum for hemolysis tests. Antisera employed in the hemolysis tests were heated at 56 C for 30 minutes in order to destroy complement normally present in serum.

Table XI indicates that hemolytic titers were higher than corresponding hemagglutination titers. Antisera used in these experiments were produced from cell vaccines used as antigens. Hemolysis experiments were also conducted using antisera prepared against the specific polysaccharide antigens. Hemolysis titers in these cases were consistently lower than

when cell vaccine antisera were employed. The hemolysis test appeared to be more sensitive than the hemagglutination reaction. It is interesting to point out that no lysis of human blood cells took place. During the test human cells were washed in the same manner as sheep cells, but apparently small amounts of contaminating serum remained to block the reaction. Neter et al. (1952) have shown that small amounts of serum present with human red cells in the sensitizing medium can prevent the adsorbtion of antigens onto the erythrocytes. Hemagglutination reactions, on the other hand, were not affected. Repeated tests yielded the same results. It was also noted that treatment with more than one polysaccharide antigen did not prevent the lysis of sheep red cells in the presence of homologous antiserum and complement.

The Effect of Heat Upon Polysaccharides

Neter et al. op. cit. (1952) showed that in order to made an antigen from O111 or O55 active (so that it would sensitize erythrocytes for hemagglutination), boiling for one hour was necessary (Table XV). It has been shown in this thesis that boiling of polysaccharide-saline solutions was not necessary in order to render them active for red cell sensitization. An experiment was undertaken to determine whether boiling of polysaccharide antigens would prevent them from being adsorbed by erythrocytes and whether they would be

able to act specifically in hemagglutination reactions if adsorbed. Table XIII shows the effect of heat upon the adsorbtion of the polysaccharide from 055. Hemagglutination titers of erythrocytes sensitized with the boiled polysaccharide antigens from 055 were nearly equal to those of the unboiled. However, it can also be observed that when a culture filtrate was used for erythrocyte sensitization, it was necessary to boil it in order for the antigens to be adsorbed by the erythrocytes. A test using unheated filtrate showed a titer of 1 to 100 dilution. It can be observed from Table XIII that the polysaccharide antigens produced better sensitization of erythrocytes than the boiled filtrate.

Pathology

Five rabbits, all of which were normal half-grown animals, were given doses of polysaccharide in excess of the amount known to be tolerated. Eight-tenths mg per kg body weight proved fatal to each rabbit injected. The animals were prostrate within a few hours following the injection. The animals were necropsied soon after death and sections of the various organs taken for histological examination. These were fixed in 10 per cent formalin.

Gross Examination

The lesions found upon gross examination of each animal were those suggestive of a rather severe toxemia. The animals were bleeding from the nostrils. Green watery feces covered the hind parts of three of the rabbits.

The lymph nodes, particularly those of the bronchial area, showed a marked degree of hyperemia. The spleen was also very hyperemic but otherwise generally normal in appearance. Petechial hemorrhages were seen in the thymus gland of three animals.

A mild hyperemia was noted in the intestines upon being opened which suggested the presence of catarrhal enteritis.

The heart in all rabbits was flabby and lighter than normal in color.

The condition of the lungs varied from nearly normal to some in which areas of emphysema could be detected. The condition was more pronounced in the basal lobes.

Examination of several livers revealed small focal areas of necrosis well distributed over the organ. The organ appeared generally lighter in color than the normal.

The kidneys, with the exception of a slightly adherent capsule, were grossly normal.

Microscopic Examination

Lymph nodes. Many of these were hyperemic and some showed minute hemorrhages.

Spleen. The spleen was congested but no other changes were observed.

Intestines. Sections of intestine showed a hypersecretory epithelium with an increase in the number of goblet cells. Masses of mucin were present covering the columnar epithelial cells. A definite increase in the size of the intestinal capillaries was seen.

Liver. Microscopic examination showed the result of a toxic reaction. Areas of necrosis were scattered throughout the section, and were associated with the inner third of the lobules, chiefly in the region of the central veins. There was coagulation, caseation and some liquefaction necrosis present in the liver cord cells. The portal canals were congested but the areas around them were free from necrosis. The sinusoids, when not disrupted by necrosis, were filled with erythrocytes. Fatty changes were present in the hepatic epithelium of lobules where little necrosis had taken place. Thrombi were found in many of the central veins. Other veins showed normal erythrocytes in the lumen with an occasional one containing fibrin.

Kidneys. Coagulation necrosis was visible in the proximal and distal convoluted tubules. Some glomeruli were hyalinized. Nuclei, where recognizable in the tubules, displayed pyenosis, karyolysis and karyorrhexis. Many convoluted tubules contained debris and occasionally albuminous casts.

Lungs. The lungs in three cases showed emphysema. This condition could have been terminal. Hemorrhage was present in areas surrounding many of the bronchioles, which contained numerous erythrocytes. The capillaries of the lung contained pink staining material having the appearance of hyalin. There was no inflammatory cellular infiltration of the alveoli.

Heart. Vacuolated areas among the bundles of fibers indicated fatty change. These were not extensive. Limited necrosis was seen in small focal areas and where present the bundle fibers showed loss of striation. Occasional hemorrhage was present to a mild degree in the bundles of fibers. In such areas the fibers appeared to have undergone myolysis. Again no inflammatory cellular infiltration was seen.

DISCUSSION OF RESULTS

Isolation of Polysaccharides

It was recognized at the beginning of the study that a method of isolation must be utilized that would produce as little degradation of the fractions desired as possible. Many methods used in the past have involved strong acids or alkalis or extreme temperatures. The method chosen for this study was mild and the polysaccharides obtained were not subjected to acids, alkalis or high temperatures. For the disruption of the bacteria, the grinding method of Langner and Forrester (1939) was used. Good results were obtained, judged on the basis of microscopic examination of the fractured cells.

Inasmuch as the polysaccharides from O111 and O55 reacted chemically and biologically so nearly alike, discussion of the polysaccharides will apply to both, unless otherwise stated.

Purity of the Polysaccharide Fractions

Various agents have been used to extract the O antigens of the *E. coli*. Among these are, to mention a few, trichlor-

acetic acid, trypsin, glycine, diethylene glycol, phenol, and guanidien, followed in most cases by alcohol precipitation. These preparations have not been demonstrated to be pure polysaccharides, but mixtures of many chemical groups such as D-glucosamine, phospholipins, glucuronic acids, polypeptides, phosphatides and some sulphur-containing nuclei. All of them, however, have been positive to the Molisch reaction, although this would not necessarily prove that they were carbohydrate in the strict sense of the word but merely would indicate the presence of the carbohydrate radical. In the Molisch reaction, the carbohydrate radical remains intact as an aldehyde group present in a new grouping known as a furfural. Raistrick et al. (1934) stated that there is no justification to assume that bacterial fractions obtained by present methods are pure polysaccharides. In addition to what has already been said in discussing the biochemical aspects of the fractions, a few points are to be made regarding their purity. Few polysaccharides have been isolated that can be termed pure carbohydrate. Tal et al. (1943) stated in describing antigens isolated from Shigella dysenteriae that the leukopenia present, in tests conducted, was produced by a conjugated protein and an undegraded polysaccharide fraction, both substances having a phosphorous group attached. Removal of this group rendered the fractions inactive. The fractions of O111 and O55 have been shown to possess this group.

Raistrick et al. (1934) produced a fraction from Bacterium aertrycke which contained the specific polysaccharide in an "antigenically" pure form, though many split or degraded protein products were present. It was emphasized that the fractions were certainly not pure substances chemically, but were considered to be phosphatide as well as polysaccharide. Their exact structure is still a matter of conjecture.

Delafield et al. (1934) claimed a fraction that was a polysaccharide, containing no unaltered protein. However, altered protein products were in evidence.

Hays et al. (1950) found two fractions from E. coli to be of a polysaccharide nature but possessing many other chemical nuclei. The fractions from O111 and O55 compare chemically with those found by other investigators in most respects in that (1) they contain reducing sugars in easily detectable amounts, (2) they do not contain undegraded protein, (3) they possess phenolic substances, and (4) many nuclei containing sulphur and phosphorous with (5) moderate amounts of nitrogen in various non-protein arrangements. Haworth and Stacey (1949) stated that an antigen of S. typhosa contained 50 to 60 per cent polysaccharide, 15 per cent insoluble polypeptide, 10 to 20 per cent of a soluble nitrogenous constituent, and 3 to 4 per cent of a soluble nitrogenous constituent, and 3 to 4 per cent of a lipoid component. D-glucosamine was contained as a unit.

A preparation by Morgan et al. (1941) proved to be a polysaccharide-protein complex which was both toxic for rabbits and also antigenic.

Injection of Polysaccharide Fractions

Reference to Tables II and III indicates that the most marked reaction of rabbits to intravenous injection of polysaccharide O111 or O55 was a rapid reduction of the leukocytes of the venous blood. The granulocytes are affected to the greatest extent. A quick rise in the blood glucose level was also marked.

Leukopenic effects of various bacterial fractions, some of which have been polysaccharide in nature, were first reported by Kolmer (1891), Kanthack (1892), Loewit (1892), and Golscheider and Jacob (1894). The leukopenia in most cases was followed by a rise in blood glucose levels. More recent work (referred to elsewhere) has offered similar findings, the present study not excepted.

Not all fractions have been alike in their selective action upon certain cells of the blood. Those reported in this study were more pronounced in reducing the numbers of polymorphnuclear leukocytes. Such results can probably be attributed to the phagocytic action of the neutrophiles, as cells generally considered to be non-phagocytes are not reduced in appreciable numbers.

The fact that large doses of polysaccharide when fed to rabbits and mice did not produce symptoms seems to indicate that the material was either (1) not absorbed from the digestive tract, or (2) inactivated by digestive enzymes present there, or (3) not absorbed rapidly enough to reach a toxic level in the blood. The possibility of the detoxification of certain amounts by the liver should not be overlooked. Studies upon the blood of animals that ingested the fractions were not indicated.

The selective destruction of the granulocytes of the blood has been reported by Dennis op. cit. (1939), Morgan op. cit. (1940), Favorite op. cit. (1942), and Hays et al. op. cit. (1950). The various mechanisms responsible for such a reaction have each been questioned. The possibility of the response being due to an adrenalin increase was considered. In two papers by Frey et al. (1913) and Frey (1914) the results of injecting rabbits and humans with adrenalin did not parallel the findings of the present study. In both investigations an immediate relative and absolute rise in all leukocytes occurred upon injection of 1-1000 dilution of adrenalin. No leukopenia was observed in any case and the increase in leukocytes was immediate.

Other considerations as to the cause of the cellular response must be mentioned. Leukopenias produced by the injection of nucleic acid derivatives have been reported by Doan et al. (1928). These studies indicate a highly specific

stimulatory effect, limited to the granulocytes of the blood. Injections produced marked increases in the total circulating neutrophiles. No leukopenia or reduction in any other cellular element was noted. In another study Doan et al. (1936) failed to produce reactions in rabbits by the intravenous injection of 200,000,000 living organisms. Only mild transient constitutional and hematologic reactions were observed.

Many workers have attempted to produce leukopenias similar to those produced by the polysaccharides of O111 and O55 by the injection of various protein substances into rabbits. Notable among these was Wiseman (1931) who administered such proteins as egg albumin. Foreign protein from a variety of sources, when given intravenously in rabbits, has exhibited the common property of stimulating lymphopoiesis in as specific a manner as the nucleic acids (Doan et al. op. cit. 1928) appeared to increase the myeloid elements.

The response of rabbits injected with the polysaccharide from O111 or O55 (Tables II-V) does not simulate results obtained by workers with protein substances, while injection of various polysaccharide fractions by a considerable number of persons has revealed similar reactions. Therefore, it does not appear that protein substances produce results comparable to those found upon injection of polysaccharide fractions intravenously into rabbits. Moreover, the hyperglycemic state reported here has not been observed in protein-injected animals. However, the hyperglycemia reported could

be due to an adrenalin response, though this is not compatible with the leukopenia observed.

Historically, the first case of a bacterial agranulocytosis in this country was reported by Lovett (1924), in which Bacillus pyocyaneous was the infecting agent. Later, Meyer et al. (1934) failed to produce an agranulocytosis by injecting bacterial toxins into rabbits. A toxin from B. pyocyaneous was the only one found to be active in this manner. No doubt, investigations would show that the ineffectiveness of these fractions would be due to the method of fractionation.

Alterations in the leukopenic response have been studied extensively. Tables IV and V show the results of injecting polysaccharide into cell vaccine and polysaccharide immune rabbits. Immunity to the leukopenia or hyperglycemia was not observed. The response of the vaccine immune rabbit (Table V) indicated some attenuation of the effect of the polysaccharide. Studies in which no immunity could be demonstrated to a leukopenic response are those of Favorite et al. op. cit. (1942), Hays et al. op. cit. (1950), and Morgan et al. op. cit. (1941), though Morgan gave no indication of the time between the last immunizing dose and the challenging dose. Workers claiming to have produced an immunity to cellular and chemical changes of the blood are Dennis et al. op. cit. (1939), Delafield et al. op. cit. (1934), and Olitski et al. op. cit. (1941). Any consideration, however,

of the toxic or immune response to injection of bacterial fractions must be tempered by the realization that the toxic and immunizing substances present in such fractions cannot be assumed to represent the total toxic and immunizing potentialities of the intact bacterial cells. However, injection of whole organisms has been shown to produce little change in the blood of rabbits (Doan et al. op. cit. 1936).

The reduction in the numbers of leukocytes in vitro has been reported by Dennis op. cit. (1939) and Hays op. cit. (1950). The destruction of leukocytes in each case took place at the expense of the granulocytes. A reduction in granulocytes has been shown to take place in vitro in the cord blood of infants (Table VI). The effect was marked and the cellular destruction was complete in the cells affected. An interesting question arises as to whether infants suffering from a gastro-enteritis due to O111 or O55 show a leukopenia at any time during the course of the disease. Though the author has no information in this regard, it is very likely that the absorption of the bacterial endo-toxin is relatively slow so that a leukocytosis develops without a leukopenic state. Were a human infant injected with a large dose of the polysaccharide fraction from either O111 or O55, it is reasonable to assume that leukopenia, followed by a leukocytosis, would result.

Serological Reactions

Voluminous material is found in the literature dealing with hemagglutination reactions. Usually reference is made to direct bacterial or viral hemagglutination. However, it has been shown that the antigens of many microorganisms are capable of being adsorbed by red cells, thus rendering them agglutinable by specific antisera. This type of reaction has been called indirect bacterial hemagglutination. Keogh et al. (1947), Middlebrook et al. (1948), Hays et al. (1950), Fisher (1950), Hays (1951), Fisher et al. (1951), Meter et al. (1952), and others, have demonstrated the phenomenon.

Meter (1952) and Meter et al. (1952) reported a hemagglutination reaction, produced by adsorbing the soluble antigens present in the filtrate from O111 or O55 cultures, onto erythrocytes. These antigens were not described chemically. Meter found that adequate sensitization of erythrocytes for the indirect hemagglutination reaction occurred only if the culture filtrate was heated at 100 C for one hour (Table XV). The author stated that the mechanism of rendering the O antigen capable of adsorption by red cells might be due to the inactivation of the B antigen by boiling. Two conclusions in this regard can be drawn from the results of the present study; (1) B antigen did not appear to be inactivated by boiling as boiled polysaccharide suspensions seemed to produce reactions equal to the unboiled (Table

XIII); (2) it might be assumed that heating of the culture or culture filtrate to "inactivate" B antigen brings about a modified fractionation of the protein from the polysaccharide, allowing the polysaccharide fractions to be adsorbed onto erythrocytes. It might be of interest to test boiled culture filtrates of the two serogroups for the presence of polysaccharides.

The O inagglutinability of E. coli strains that possess the B antigen (Kauffmann, 1950) has been recognized for some time (Table XV). This reaction, thought to be due to the presence of a B antigen on the surface of the organism, results in interference with O agglutination and erythrocyte sensitization. Kauffmann proved the absence of a capsule, since no swelling occurred when the organism was treated with a specific antiserum. Antigens present in the six sero-groups of E. coli containing the B antigen were referred to as "envelope" antigens. The sero-groups O111 and O55 possess the B_4 and the B_5 antigens, respectively. The sero-groups mentioned can also be found to possess an H antigen, in which case they are motile.

Various serological reactions which have been reported by Meter op. cit. (1952) have been possible due to the adsorption of the O antigen from boiled filtrates on the surface of the erythrocytes. However, in the present experiments there was good indication that similar reactions were due to the B antigen as well as the O antigen. Meter also

pointed out that the adsorption of the O antigens from culture filtrates by erythrocytes was blocked by the presence of soluble B antigen in the filtrate. The polysaccharide antigens did not exhibit this failure to be adsorbed by erythrocytes, even though they have been shown (Table VII) to possess the B antigen in serologically active form.

Erythrocytes sensitized with the polysaccharide antigens showed consistently higher homagglutinating titers than red cells treated in the same manner with heated cultures or heated culture filtrates. It should be emphasized that at no time during the fractionation process, in the preparation of the polysaccharide, did the temperature rise above 56 C. Culture filtrates, on the other hand, required boiling for one hour in order to destroy the blocking effect (mentioned above) of the B antigen.

The presence of the B antigen in the polysaccharide fraction was demonstrated when, mixed with boiled organisms and injected into a rabbit, there was produced an OB antiserum. The B antibodies could come only from B antigen present in the polysaccharide, inasmuch as B antigen was destroyed by boiling in the bacterial cells injected. The above antiserum agglutinated living organisms (Table VII).

As pure B antisera have not been produced, the exact identity of the B antigen is unknown. Whether it exists as a coating on the surface of the living bacterial cell or is distributed throughout are matters for further investigation.

It appears that a general distribution of the antigen throughout the bacterial cell would explain most satisfactorily its presence in the polysaccharide fractions, and also in culture filtrates.

In all hemagglutination reactions an optimum serum dilution was observed for agglutination. Complete inhibition of hemagglutination was not observed to be due to a low concentration of serum. For any given antiserum, hemagglutination tests using sera produced against the vaccine, showed consistently higher titers than sera produced against the polysaccharide.

The length of time the erythrocytes were exposed during sensitization and the concentration of the polysaccharide were important. The sensitization times reported by Hays *op. cit.* (1950) appeared optimum for use in this study. Noter *op. cit.* (1950) found the same intervals for sensitizing erythrocytes to be satisfactory, using boiled culture filtrates. Concentrations of polysaccharide used to sensitize erythrocytes were higher than levels found to be optimum by Hays *op. cit.* (1951). Longer periods for sensitization than one hour did not appear to increase the hemagglutination or hemolysis titers, employing the same antisera.

From observations made during this study, it can be postulated that only a small portion of the total area of a given erythrocyte is covered at any one time by a polysaccharide adsorbed to it. Indications that this might be the

case are presented in Tables IX and X, where it was demonstrated that more than one polysaccharide could be adsorbed, either simultaneously or subsequently, by erythrocytes. The facts presented seem to indicate an adherence to certain colloidal principles. Whether the polysaccharides actually existed as colloids cannot be stated. Though the total number of polysaccharides that can be adsorbed by a single erythrocyte has not been determined, Hays op. cit. (1951) succeeded in adsorbing four at one time and in demonstrating their presence as specific antigens in hemagglutination reactions.

Kravchenko (1947), who reported the reaction of polysaccharides with erythrocytes, stated that human erythrocytes apparently combine with specific polysaccharide by adsorption. In explaining the phenomenon, the formula of Freundlich was given, in which $X = Kc^n$, where X is the amount adsorbed per gram of adsorbing substance, c is the concentration in solution, and K and n are constants. He postulated that if this rule held, erythrocytes should be able to adsorb polysaccharide and transfer it to other erythrocytes or to a suspending medium. A future test of the principle would be of interest.

Several problems arise as a result of the investigations into polysaccharide adsorption; namely, (1) what is the minimum amount of adsorbed polysaccharide necessary for hemagglutination to occur in a high titer antiserum, (2) what is the

optimum amount of polysaccharide that must be adsorbed on the erythrocyte surface to bring about the highest titer possible, (3) how much of the erythrocyte surface is occupied by the presence of one polysaccharide, (4) is the polysaccharide bound strongly to the erythrocyte surface, or could it be eluted so homagglutination with specific antiserum would not be possible?

It would appear that the answer to many of these problems lies in the fields of colloid chemistry and immunology.

SUMMARY AND CONCLUSIONS

The isolation and properties of the somatic antigens of Escherichia coli, sero-groups O111 B_4 and O55 B_5, are described. These antigens are shown to possess specific polysaccharide substances, as well as other chemical groupings containing sulphur, phosphorus, and nitrogen. Phenolic groups are present.

Rabbits injected intravenously show a marked reaction, which is characterized by leukopenia and hyperglycemia. The leukopenia is due essentially to the loss of granulocytes from the blood.

Rabbits immunized by injections of the specific polysaccharide antigen develop a tolerance to repeated injections, but when given a period of rest, and again injected, develop a leukopenia and hyperglycemia equal to that of a non-immune animal. Prostration and diarrhea are also noted. Rabbits immunized with the cell vaccine of O111 B_4, when rested, and then injected with a non-lethal dose of the specific polysaccharide, show the characteristic leukopenia and hyperglycemia.

The leukocytes present in the cord blood of newborn infants are lysed *in vitro* by the polysaccharide of O111 B_4. The loss of leukocytes is principally one of granulocytes.

Red blood cells of man, pig, chicken, dog, cow, and sheep are shown to become sensitized by the polysaccharide antigens and agglutinated in the presence of homologous antiserum. Preparations containing the B antigen are effective in rendering erythrocytes agglutinable by their specific antisera. A polysaccharide lacking the B antigen was not prepared.

Erythrocytes of sheep are shown to adsorb more than one antigen either simultaneously or in succession. No blocking of reactions, due to the presence of more than one of the polysaccharides on the erythrocyte, is noted.

Polysaccharide antigens adsorbed on sheep erythrocytes are shown to combine with specific antibody, and, in the presence of complement, produce hemolysis. Titers of hemolysis reactions are generally higher than are those of hemagglutination reactions.

Cell vaccine antisera are more effective in producing higher titers in the hemagglutination reaction than polysaccharide antisera. Boiled polysaccharide preparations appear to have as good sensitizing ability for erythrocytes as unheated preparations.

The intravenous injection of polysaccharide antigen from O111 B_4 into rabbits in lethal doses caused changes to occur in various organs. Among these, the principal effects in the liver are: (1) fatty changes, (2) coagulation and caseation, and liquefaction necrosis, and (3) thrombi in many of the central veins. In the kidney necrosis of the proximal and

distal convoluted tubules occurred. Petechial hemorrhages are found in the thymus gland. A catarrhal enteritis is produced in the intestine. Atelectasis and emphysema are shown to occur in the lung.

It appears that fractions such as are described would be useful in the diagnosis of gastro-enteritis in infants, particularly in hospitals where bacteriological procedures are not feasible. It is probable that antibodies could be demonstrated to be present in the blood of infants suffering from gastro-enteritis of coliform origin, by utilization of the hemagglutination or hemolysis reactions, in which a polysaccharide preparation might act as the sensitizing antigen.

APPENDIX

Tables

Graphs

TABLE I

BIOCHEMICAL DATA ON THE
POLYSACCHARIDES OF 0111 AND 055

Molisch		Biuret		Nitrogen[1]		Phosphorus[2]		Sulphur[3]		Dische[4]		Reducing Sugars[5]	
0111	055	0111	055	0111	055	0111	055	0111	055	0111	055	0111	055
+++	++	-	-	28	24.5	++++	++++	+++	+++	++++	++++	++++	++++

[1]Kjeldahl nitrogen based on weight of suspended solid in sample and expressed as per cent.

[2]Phosphorus by formation of ammonium phosphomolybdate.

[3]Sulphur by precipitation with barium chloride.

[4]Dische test for desoxyribonucleic acid.

[5]Reducing sugars by Benedict's qualitative test.

TABLE II

RESPONSE OF A NORMAL RABBIT (RABBIT NUMBER ONE)
TO INTRAVENOUS INJECTION WITH
E. COLI O111 POLYSACCHARIDE

Rabbit	Time of Injection	Total[1] Leukocytes	Lymphocytes: Per Cent	Lymphocytes: Absolute	Neutrophiles: Per Cent	Neutrophiles: Absolute
Rabbit #1 0.4 mg/kg body wt	before inj	7,000	66	4,620	33	2,310
	3 hours	2,300	82	1,886	14	322
	6 hours	4,200	49	2,058	51	2,142
	24 hours	20,000	12	2,400	88	17,600
	48 hours	9,800	36	3,528	62	6,276
	69 hours	7,500	40	3,000	53	3,975

[1]Leukocytes to the nearest 100

[2]Erythrocytes to the nearest 100,000 per cmm blood

[3]Hemoglobin in grams per cent

[4]Glucose in grams per cent (blood)

TABLE II (continued)

Monocytes		Erythrocytes[2]	Hb.[3]	Blood Glucose[4]	Temperature (Rectal)
Per Cent	Absolute				
1	70	5.8×10^6	14.1	102	102.0
4	92	5.5×10^6	14.0	210.0	103.4
0	0	4.8×10^6	13.6	142	104.3
0	0	4.7×10^6	13.2	134	104.7
2	196	3.4×10^6	12.2	105	102.2
7	525	3.1×10^6	10.1	97	100.1

TABLE III

RESPONSE OF NORMAL RABBITS (NUMBERS TWO AND THREE) TO INTRAVENOUS INJECTION OF E. COLI 055 POLYSACCHARIDE

Rabbit	Time of Injection	Total Leukocytes[1]	Lymphocytes: Per Cent	Lymphocytes: Absolute	Neutrophiles: Per Cent	Neutrophiles: Absolute
Rabbit #2 0.8 mg/kg body wt	before inj.	8,800	54	5,010	40	3,520
	2½ hours	2,100	66	1,400	33	700
	6 hours	7,700	41	3,200	59	4,500
	24 hours dead	—	—	—	—	—
Rabbit #3 0.4 mg/kg body wt	before inj.	5,200	70	3,640	28	1,456
	2½ hours	1,300	82	1,066	14	182
	6 hours	4,400	24	1,050	76	3,344
	25 hours	15,200	14	2,128	86	13,072
	48 hours	10,100	42	4,242	50	5,050
	72 hours	6,500	68	4,420	30	1,950

[1]Leukocytes to the nearest 100

[2]Erythrocytes to the nearest 100,000

[3]Hemoglobin in grams per cent (blood)

[4]Glucose in grams per cent (blood)

TABLE III (continued)

Monocytes		Erythrocytes[2]	Hb.[3]	Blood Glucose[4]	Temperature
Per Cent	Absolute				(Rectal)
6	528	5.3×10^6	14.0	104	103.0
0	0	5.1×10^6	14 gm	223.0	105.0
3	231	4.5×10^6	13.5	184.0	103.0
-	-	--	--	--	--
2	104	6.5×10^6	14.5	115	102.5
8	104	5.6×10^6	13.9	139	103.0
0	0	5.4×10^6	13.5	104	103.1
0	0	5.2×10^6	13.0	90	102.0
8	808	4.4×10^6	11.9	87	99.0
2	130	4.5×10^6	12.1	86	98.5

TABLE IV

RABBIT[1] NUMBER FOUR IMMUNIZED WITH C111 POLYSACCHARIDE - RESPONSE TO FURTHER INJECTIONS OF POLYSACCHARIDE

Dose	Day	Time of Count	Total Leukocytes[2]	Lymphocytes	
				P.C.	Absolute
2 mg	1st	before injection	6,800	40	2,720
		2 hours	2,400	82	1,968
2 mg	2nd	before injection	13,500	6	1,110
		2 hours	2,700	51	1,377
2 mg	3rd	before injection	9,700	34	3,298
		2 hours	1,200	82	904
--	4th	--	12,700	65	8,255
2 mg	5th	before injection	8,400	59	4,956
		2 hours	10,700	70	7,490
--	6th	--	15,100	38	5,738
2 mg	7th	before injection	9,500	42	3,990
		2 hours	8,700	46	4,002
--	8th	--	7,100	42	2,982

[1]Titer of rabbit number 4 = 1/640

[2]Leukocytes per cmm of blood

TABLE IV (continued)

Polymorphonuclear Leukocytes		Monocytes	
P.C.	Absolute	P.C.	Absolute
56	3,808	4	272
14	336	4	96
91	16,835	3	545
43	1,296	1	270
64	6,208	2	194
10	120	8	960
34	4,318	1	127
40	3,360	1	84
28	2,996	1	107
60	9,060	2	302
58	5,510	0	—
54	4,698	0	—
56	4,218	0	—

TABLE V

RABBIT NUMBER FIVE INJECTED WITH E. COLI O111 VACCINE - RESPONSE TO INTRAVENOUS INJECTION OF E. COLI O111 POLYSACCHARIDE[1]

Time of Count	Total Leukocytes[2]	Lymphocytes P.C.	Lymphocytes Absolute	Polymorphnuclear Leukocytes P.C.	Polymorphnuclear Leukocytes Absolute
Before injection	6,800	54	3,672	46	3,128
2½ hours	2,000	76	1,520	22	440
11 hours	8,500	15	1,275	85	7,225
24 hours	12,500	32	4,000	68	8,500
48 hours	9,000	51	4,590	47	4,230
72 hours	7,200	58	4,176	42	3,024

[1] 2 mg polysaccharide intravenously

[2] Leukocytes to the nearest 100

[3] Erythrocytes to the nearest 100,000

[4] Hb em as gram per cent (blood)

[5] Blood glucose in gms per 100 ml of blood

TABLE V (continued)

Monocytes P.C.	Monocytes Absolute	R.B.C.[3]	Hb.[4]	Blood Glucose[5]	Rectal Temperature
0	0	4.3×10^6	12.5	152	101.1
2	40	4.3×10^6	12.5	232	101.0
0	0	4.1×10^6	12.2	185	102.1
0	0	4.1×10^6	12.4	158	102.2
2	180	4.2×10^6	12.3	160	102.1
0	0	4.2×10^6	12.5	165	102.0

TABLE VI

IN VITRO LYSIS OF LEUKOCYTES OF HUMAN CORD BLOOD[1] BY THE POLYSACCHARIDE ANTIGENS FROM E. COLI O111

	Saline Control			1-1000[3]				
					Lymphocytes		Neutrophiles	
Time (Hours)	Total Leukocytes[2]	Lympho-cytes	Neutro-philes	Total Leukocytes	Per Cent	Absolute	Per Cent	Absolute
0	14,700	29	71	14,700	29	4,263	71	10,437
2	14,500	27	73	8,500	78	6,630	22	1,784

[1]Results are the average of five separate tests; blood collected from the cord of infants at birth.

[2]Total leukocytes to the nearest 100; lymphocytes and neutrophiles expressed in per cent.

[3]Dilution of polysaccharide 1-1000.

TABLE VII

AGGLUTINATION OF BOILED AND UNHEATED E. COLI O111 BY TWO DIFFERENT ANTISERA

Antiserum - Boiled Organisms Plus Polysaccharide	Boiled O111 Organisms	Unheated O111 Organisms
1/20	≠	≠
1/40	≠	≠
1/80	≠	≠
1/160	≠	≠
1/320	≠	≠
1/640	≠	-
1/1280	≠	-
1/2560	≠	-
1/5120	-	-

[1]Serum prepared by injecting polysaccharide of O111 into rabbits.

TABLE VII (continued)

Antiserum from Polysaccharide Only[1]	O111 Boiled Organisms	Unheated O111 Organisms
1/20	≠	≠
1/40	≠	≠
1/80	≠	≠
1/160	≠	≠
1/320	≠	-
1/640	≠	-
1/1280	-	-
1/2560	-	-
1/5120	-	-

TABLE VIII

HEMAGGLUTINATION OF RED CELLS FROM DIFFERENT ANIMAL SPECIES SENSITIZED WITH E. COLI O111 POLYSACCHARIDE ANTIGEN

	Cow	Rabbit	Dog	Man	Sheep	Chicken
O111 Antisera						
1/100	≠	≠	≠	≠	≠	≠
1/200	≠	≠	≠	≠	≠	≠
1/400	≠	≠	≠	≠	≠	≠
1/800	≠	≠	≠	≠	≠	≠
1/1600	≠	≠	≠	≠	≠	≠
1/3200	≠	≠	≠	≠	≠	≠
1/6400	≠	≠	≠	-	≠	≠
1/12800	-	-	-	-	-	-
1/25600	-	-	-	-	-	-
Serum Control	-	-	-	-	-	-
Saline Solution Control	-	-	-	-	-	-
055 (Antiserum)	-	-	-	-	-	-

TABLE IX

SIMULTANEOUS ADSORPTION OF THE POLYSACCHARIDE ANTIGENS OF E. COLI O111 AND O55 BY SHEEP RED BLOOD CELLS

E. Coli Antisera	Agglutination by E. Coli Antisera of Red Blood Cells Treated with Polysaccharide Antigens		
	O111 Polysaccharide	O55 Polysaccharide	O111 and O55 Polysaccharide
O111			
1/100	≠	-	≠
1/200	≠	-	≠
1/400	≠	-	≠
1/800	≠	-	≠
1/1600	≠	-	≠
1/3200	≠	-	≠
1/6400	≠	-	≠
1/12800	-	-	-
O55			
1/100	-	≠	≠
1/200	-	≠	≠
1/400	-	≠	≠
1/800	-	≠	≠
1/1600	-	≠	≠
1/3200	-	≠	≠
1/6400	-	≠	-
1/12800	-	-	-

TABLE X

THE EFFECT OF SUBSEQUENT TREATMENT OF SHEEP RED BLOOD CELLS WITH THE POLYSACCHARIDE ANTIGENS FROM E. COLI 0111 AND 055 ON THE ADSORPTION OF THESE FRACTIONS

E. Coli Antisera	Agglutination of Sheep Red Blood Cells by E. Coli Antisera after Treatment with E. Coli Polysaccharides			
	0111	055	055 followed by 0111	0111 followed by 055
0111				
1/100	+	-	+	+
1/200	+	-	+	+
1/400	+	-	+	+
1/800	+	-	+	+
1/1600	+	-	+	+
1/3200	+	-	+	+
1/6400	-	-	-	-
1/12800	-	-	-	-
055				
1/100	-	+	+	+
1/200	-	+	+	+
1/400	-	+	+	+
1/800	-	+	+	+
1/1600	-	+	+	+
1/3200	-	-	-	+
1/6400	-	-	-	-

TABLE XI

HEMAGGLUTINATION AND HEMOLYSIS OF ERYTHROCYTES SENSITIZED WITH THE POLYSACCHARIDE ANTIGENS FROM E. COLI 0111 AND 055

Antisera Specific for Antigen	Hemagglutination of 0111 Polysaccharide Sensitized Erythrocytes		Hemagglutination of 055 Polysaccharide Sensitized Erythrocytes	
	Human	Sheep	Human	Sheep
0111	1,600	3,200	-	-
OB111	6,400	12,800	-	-
055	-	-	1,600	3,200
OB55	-	-	1,600	1,600
Controls				
Complement*	-	-	-	-
Saline only	-	-	-	-

*1-40

TABLE XI (continued)

Hemolysis of O111 Polysaccharide Sensitized Erythrocytes		Hemolysis of O55 Polysaccharide Sensitized Erythrocytes	
Human	Sheep	Human	Sheep
-	12,800	-	-
-	25,600	-	-
-	-	-	51,200
-	-	-	51,200
-	-	-	-
-	-	-	-

TABLE XII

PRECIPITIN REACTION OF ANTISERA, PREPARED FROM BOILED AND UNHEATED O111 AND O55 E. COLI ORGANISMS, WITH THE POLYSACCHARIDE ANTIGENS FROM O111

Sera having Precipitins	Polysaccharide Antigen O111
O111 OB[1]	+
O[2]	-
O55 OB[1]	-
O[2]	-

[1]Antiserum made from living culture

[2]Antiserum made from boiled culture

TABLE XIII

SENSITIZATION OF HUMAN RED BLOOD CELLS[1] WITH THE BOILED AND UNHEATED ANTIGENS OF E. COLI CULTURES AND POLYSACCHARIDES

Vaccine[3] Antisera of:	Hemagglutination of human type O red blood cells sensitized with the antigens:			
	055 Polysaccharide Heated	055 Polysaccharide Unheated	055 Filtrate[2] Heated	055 Filtrate Unheated
055 1/100	≠	≠	≠	≠
1/200	≠	≠	≠	-
1/400	≠	≠	≠	-
1/800	≠	≠	≠	-
1/1600	≠	≠	-	-
1/3200	≠	≠	-	-
1/6400	-	-	-	-
0111 1/100	-	-	-	-

[1]Human type O blood cells

[2]Filgrate from broth cultures of organisms

[3]Sera containing OB agglutinins

TABLE XIV

AGGLUTINATION[1] OF UNHEATED O111 AND O55 E. COLI ORGANISMS BY SERA CONTAINING POLYSACCHARIDE AGGLUTININS OF E. COLI O111 AND O55

Serum having Agglutinins	Agglutination of Unheated O111 Cells	Agglutination of Unheated O55 Cells
O111 OB	+	-
O55 OB	-	+

[1]Agglutination by the slide technique

TABLE XV

AGGLUTINATION OF BOILED AND UNHEATED E. COLI O111 ORGANISMS WITH ANTISERA CONTAINING BACTERIAL O AND OB AGGLUTININS

Serum with Agglutinins	Agglutination of Boiled Organisms[1]	Agglutination of Unheated Organisms
OB	+	+
O	+	-

[1]Culture boiled for one hour at 100 C

Figure 1. A graph to show the leukocyte and glucose response of a rabbit to the intravenous injection of 0.4 mg of the polysaccharide from O111. Rabbit number 1, Table II.

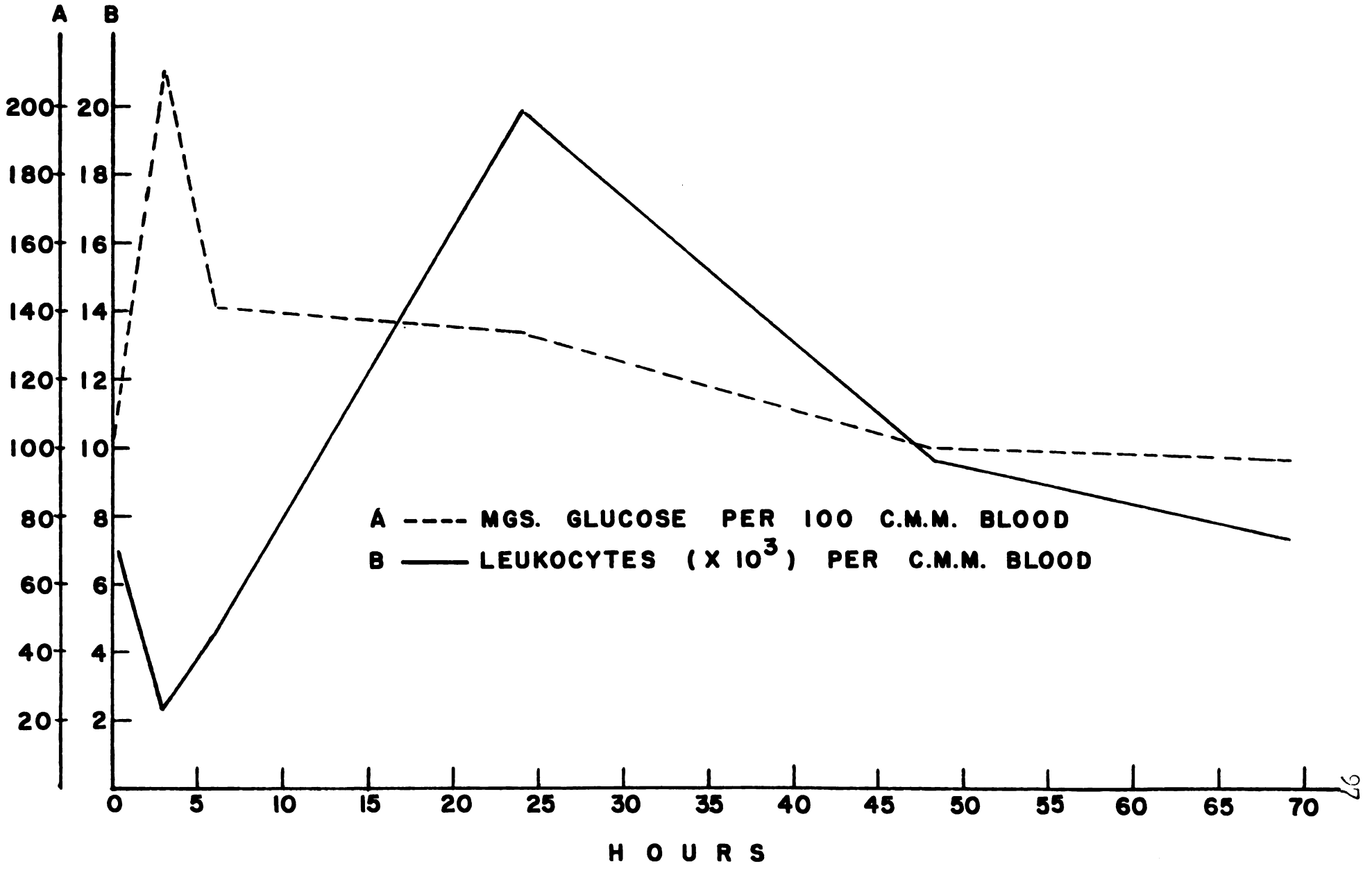

A
B
200
180
160
140
120
100
80
60
40
20
20
18
16
14
12
10
8
6
4
2
A ---- MGS. GLUCOSE PER 100 C.M.M. BLOOD
B —— LEUKOCYTES (X 10^3) PER C.M.M. BLOOD
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
HOURS

Figure 2. A graph showing the leukocyte response to injections of the polysaccharides from O111 and O55 in two rabbits, numbers 1 and 3, Tables II and III.

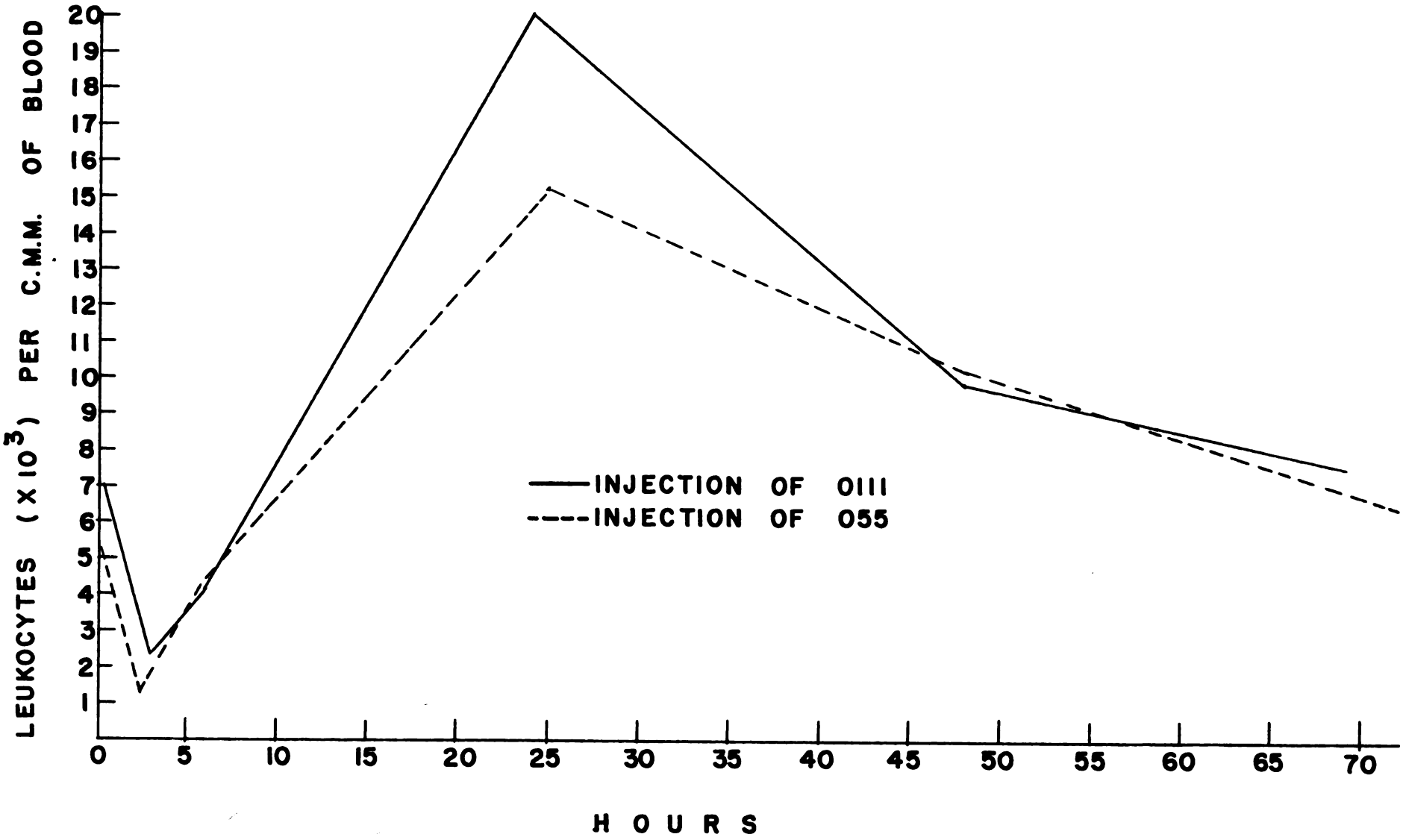

LEUKOCYTES (X 10^3) PER C.M.M. OF BLOOD
20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
HOURS
INJECTION OF 0111
INJECTION OF 055

Figure 3. A graph showing the lymphocyte response to injection of 0.4 mg of polysaccharide from O111 compared to the polymorphnuclear leukocyte response to the same injection. Rabbit number 1, Table II.

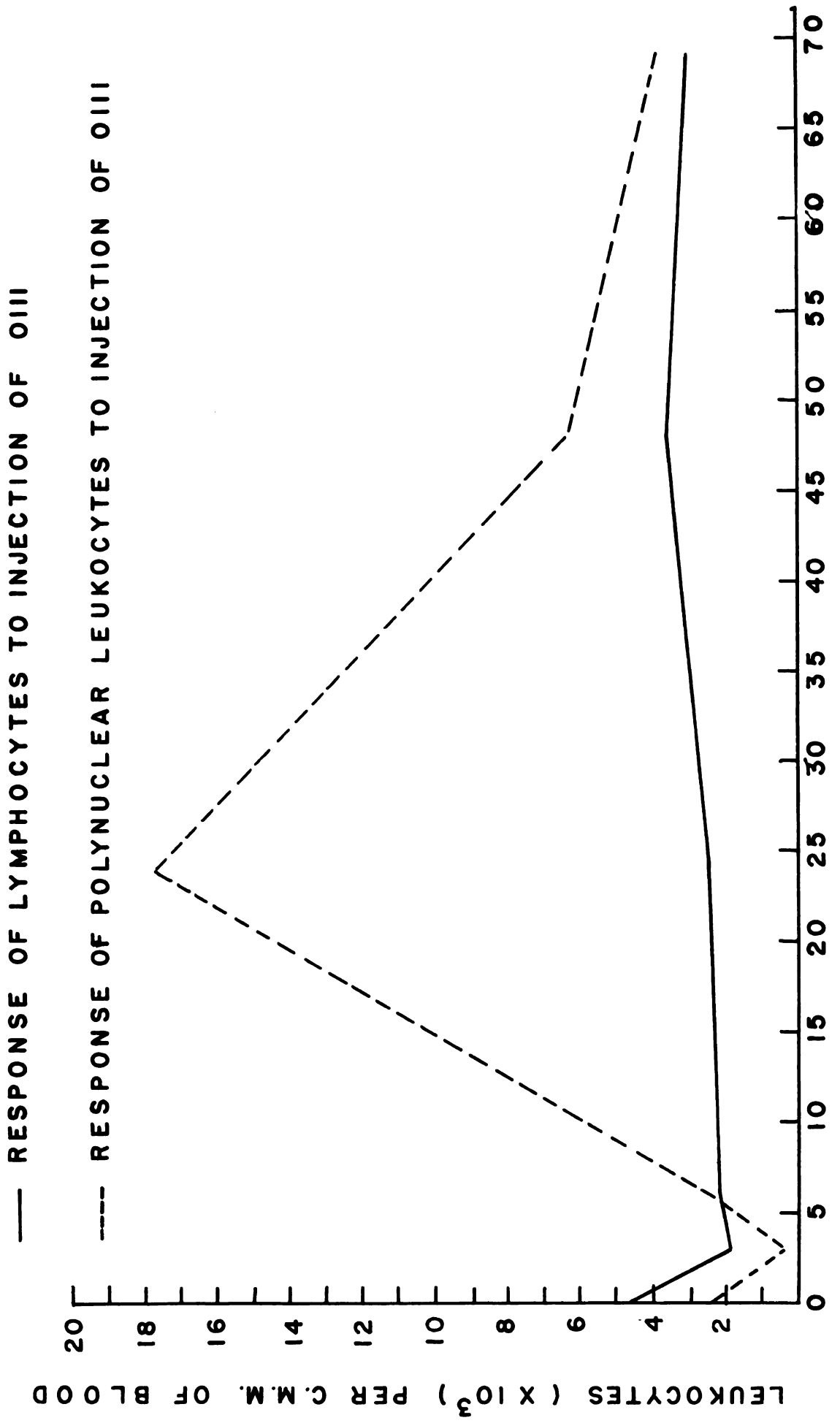
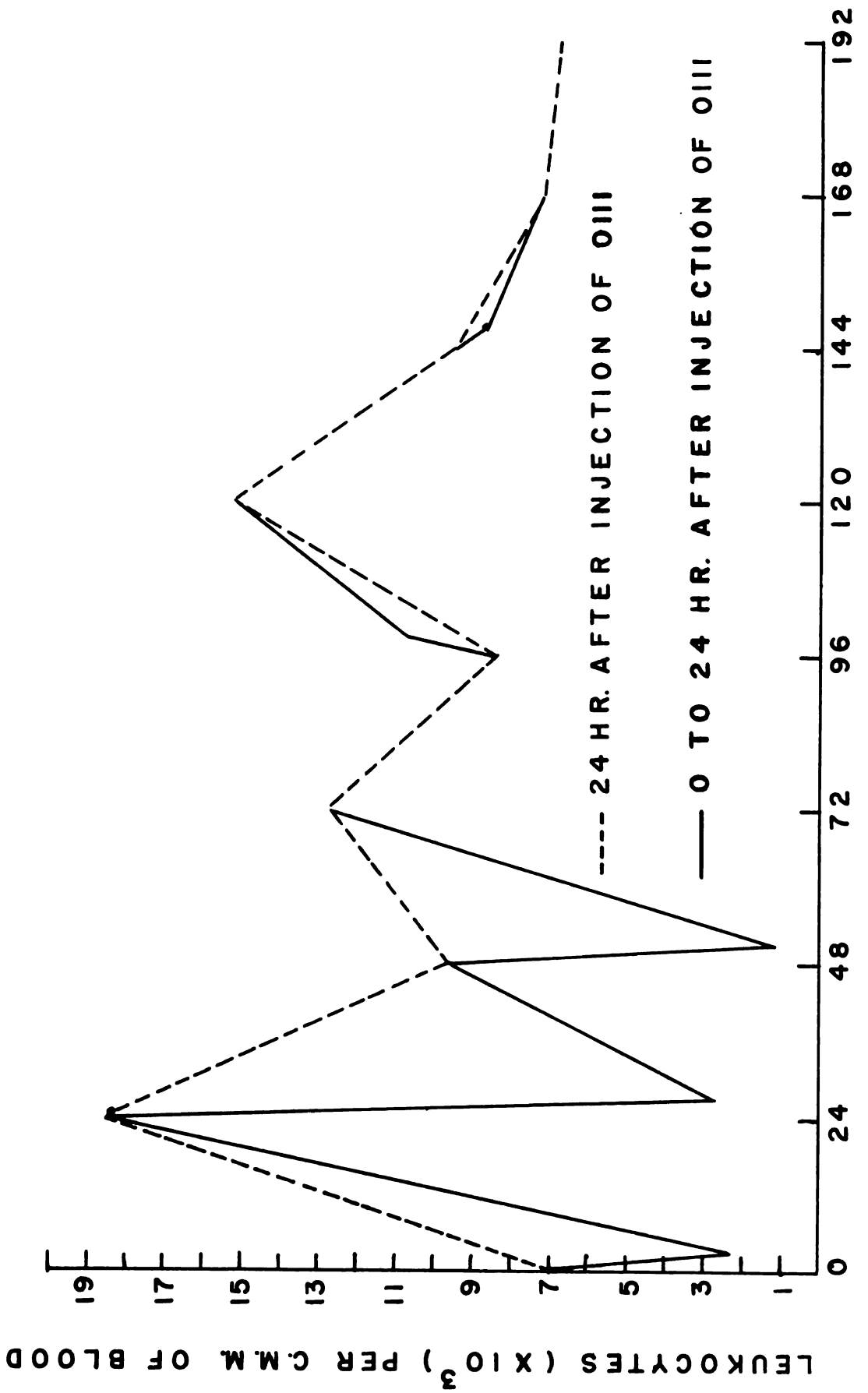

RESPONSE OF LYMPHOCYTES TO INJECTION OF OIII
RESPONSE OF POLYNUCLEAR LEUKOCYTES TO INJECTION OF OIII
LEUKOCYTES (X 10³) PER C.M.M. OF BLOOD
20
18
16
14
12
10
8
6
4
2
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
HOURS

Figure 4. A graph illustrating tolerance to injections of the polysaccharide from O111. The solid line represents the total leukocyte level each day before injection and two hours after. The broken line represents the total leukocytes each day preceding each injection. A final approximation of the two lines indicates a developed tolerance to the injections. (see Table IV)


LEUKOCYTES (X 10³) PER C.M.M. OF BLOOD
1
3
5
7
9
11
13
15
17
19
0
24
48
72
96
120
144
168
192
HOURS
---- 24 HR. AFTER INJECTION OF OIII
—— 0 TO 24 HR. AFTER INJECTION OF OIII

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