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AN INVESTIGATION OF THE PNEUMOCOCCIDAL ACTIVITY OF EXTRACTS OF TISSUES FROM RESISTANT AND SUSCEPTIBLE HOST SPECIES

by
Dora Alice Henley

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An Investigation of the Pneumococcidal Activity of Extracts of Tissues from Resistant and Susceptible Host Species

By
Dora Alice Henley

This investigation concerns the isolation, partial purification and characterization of a pneumococcidal factor from the reticulo-endothelial organs, especially the liver, of the resistant guinea pig and the susceptible rat. An attempt has been made to establish a difference in the pneumococcidal activity of the material isolated from the two species and to study the basis of this difference. It is anticipated that data obtained from this type of study may be related directly to the innate resistance of a species or of an individual of a species for a given parasite.

Using the filter pad technique similar to that employed in assaying antibiotics, antibacterial action for the Type I pneumococcus was first demonstrated with minced livers and also with crude aqueous extract of livers of the resistant guinea pig. Similar tests with livers of the susceptible rat showed the rat tissues to be markedly less active against the pneumococcus than the guinea pig tissues.

A 50 per cent ethanol soluble tissue extract was finally developed which was suitable for tube bactericidal tests. The pneumococcidal activity of this material was shown to be independent

of any bile components which might be present in liver extracts, and also independent of any blood which was present in the organs. It was further shown that the active agent was not a product derived by enzymatic activity during the processing or testing of the extract.

Extracts prepared from pooled or individual livers, and extracts prepared from spleens of the resistant guinea pig all demonstrated a marked superiority of pneumococcidal activity over similar extracts of the susceptible rat. The activity of extracts prepared from the livers of individuals in each species was found to vary within the species. Extracts of brain tissue in both species were relatively low in bactericidal activity.

The 50 per cent ethanol liver extracts were fractionated in step-wise manner with ethanol, ether and acetone. By resuspension of fractions in equivalent concentrations, it was possible to show:

(1) the active factor was soluble in ethanol, ether and acetone

(2) apparently an inhibitor or inhibitor complex was present in similar extracts of guinea pig livers. Furthermore, a persistant quantitative difference in the activity of the ethanol-ether-acetone extracted fraction and apparent differences in infrared spectrographs of this fraction in the two species points to a qualitative

difference in the active agents.

On the basis of these findings, it is suggested that this pneumococcidal agent is present and is active in vivo in the case of the guinea pig and may be one factor involved in this species' innate resistance to infection with the Type I pneumococcus. It is further suggested that in the susceptible rat, a similar factor, but not necessarily the same entity, is considerably inhibited and that this species' resistance or susceptibility to this infection may in part be a resultant of the state of the equilibrium between the active agent and the inhibitor.

Since the active factor was not isolated as a unit, chemical characterization is merely presumptive. However, on the basis of the following observations: solubility in organic solvents; resistance to temperatures of 100°C; hemolytic activity; inactivation by serum; adsorption on to protein, and the oily consistency of the acetone soluble fraction, the pneumococcidal agent appears to be most closely related to the lipids.

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INTRODUCTION

The concept of the cells of the body functioning as a resistance mechanism, particularly the cells of the reticulo-endothelial system, has been recognized and accepted ever since the early studies of Metchnikoff (1,2) on phagocytosis. Although since that time there have been a number of investigations of this and related problems, with the publishing of Ashoff's monograph in 1924 (3) there has been increasing emphasis upon the activity of the cellular elements of the body in combating invasion by a parasite.

One of the earliest investigations into the fate of injected bacteria in warm blooded animals was that of Traube and Gescheiden (4) in 1874, who noted that the blood of dogs and rabbits was sterile twenty-four to forty-eight hours after intravenous injection with putrifactive bacteria. Watson Cheyne (5) in 1879 made a similar observation that both blood and organs were free of contamination after injection of bacteria. Going a step further, he found that if the animal received a larger inoculum, the organisms were then observed in the organs. Wyssokowitsch (6), with improved techniques and a broader approach to the problem, in 1886 confirmed these earlier findings and definitely established that there is an active clearance mechanism operating in the body.

From this time until the present many investigators have demonstrated repeatedly this rapid disappearance of bacterial organisms from the blood stream and the accumulation of these organisms in organs, such as the spleen, liver, and bone marrow, which are known to be rich in fixed phagocytic cells.

Keyes (7) considered the phagocytic phenomena, occurring especially in the liver and spleen, in the case of pigeons inoculated with the Type I pneumococcus, to be one of the determining factors in this host's natural resistance to the pneumococcus. About the same time (1916) Bartlett and Osaki (8) demonstrated in dogs the clearance of Micrococcus aureus from the blood stream and its accumulation in the liver and spleen followed by rapid lysis within the fixed phagocytic cells. Gay, Clark, and Linton (9) in 1920, on the basis of their studies on streptococcus infection, concluded that the tissues most concerned in immunization, namely those of the reticulo- endothelial system, also offer greatest resistance to local lesions. Tashian (10) concurred in this view that the reticulo-endothelial system is the most important factor in local tissue resistance to infection.

According to Reiss (11), all organs rich in reticulo-endothelial tissue are not uniformly active in removing and destroying bacteria. He found the Kupffer cells of the liver to be far more active in phagocytosing bacteria than the fixed phagocytic cells of the spleen, brain, adrenals, and kidney. From this he concluded that the liver is the most important defense organ in bacterial infection. Nungester and Watrous (12) failed to note much difference between the liver and the spleen in the removal of bacteriophage injected into rats. However, Taliaferro and Cannon (13) found in monkeys that the spleen was more effective than the liver in clearing the blood of malarial parasites. Moore and Mika (14) obtained evidence that indicates there is possibly a species difference in the ability of the different reticulo-endothelial organs to clear the blood of organisms. They found the liver, in the case of the rat, and the spleen, in the case of the guinea pig,to be most efficient in clearing the blood of a virulent strain of pneumococcus.

Complete intracellular destruction of the phagocytosed parasite is a requisite if phagocytosis is to function effectively as a defense mechanism. Thus there soon arose curiosity concerning the internal metabolism of phagocytic cells, which in turn led to investigation of the bactericidal potentialities of these cells. In this connection extracts of cells and tissues were often used for the experimental approach.

In 1899 Deutsch (15) reported that of extracts prepared from various organs the spleen exhibited the most "protective" power against typhoid. He was able to show that this "protective power" of the extracts was distinct from the agglutinating power of blood serum. Two years previously Schottenfroh (16) obtained from leukocytes of rabbits and guinea pigs aqueous extracts which were strongly bactericidal for Vibrio cholerae, Staphylococcus aureus, Bacillus typhosus, and Bacillus coli.

Korschun and Morgenroth (17) in 1902 prepared extracts of organs from the mouse, guinea pig, dog, and ox which were hemolytic. He described this substance as coctostabile, alcohol soluble, and non-antigenic. (Bordet (18) considered there to be a close relationship between bacteriolytic and hemolytic materials).

Three years later Turró and Pi y Suñer (19) prepared extracts of a number of tissues from man and cold blooded animals and reported them all active in destroying organisms. They particularly cited the case of thyroid and muscle extracts destroying Bacillus anthracis in vitro.

Noguchi (20), on the basis of his experimental results, considered that the bactericidal activity of leucocytic extracts against Vibrio cholorae was due chiefly to their content of soaps of higher unsaturated acids. He extracted the blood, liver, kidney and spleen of the dog, rabbit and cow with hot alcohol. The material he obtained he described as being

active after long boiling, only weakly specific, soluble in alcohol, and soluble in cold water with turbidity which disappears on boiling and reappears on cooling. Small amounts of serum brought about inactivation of this material.

On investigating the hemolytic activity of various organ extracts in 1907, Weil (21) observed great variability and a considerable loss in hemolytic power in extracts prepared from organs "thoroughly freed from blood". Interestingly, though, he found that red cell extract, inactive per se, added to such organ extracts increased their hemolytic power.

Zinsser (22) prepared extracts of leukocytes of rabbits and guinea pigs in normal salt solution and found them to be bactericidal for the staphylococcus but not for Spirillum cholerae asiaticae or for Bacillus typhosus. Manwaring (23) in 1912 concluded that the bacteriolysin he extracted from rabbit leukocytes was not a soap or a lipid but had more of the properties of an enzyme.

Rabbit livers, poisoned with phosphorus, treated to an involved extraction and purification process using alcohol and ether, yielded a mixture of unsaturated fatty acids which Lamar (24) in 1911 found to be bacteriolytic for a virulent pneumococcus. Furthermore, he also noted a close parallel relationship between the lytic activity of these acids for red blood cells and for the virulent pneumococci.

Mackie, Van Rooyen and Finkelstein (25) suggested that there was more than one extractable bactericidal substance in leukocytes and platelets. They found that the substance effective in killing gram positive organisms was more easily extracted than that active against gram negative organisms.

A study of the effect of extracts of pancreas on four viruses, namely vaccinia, the Rous and Fujanami tumor viruses, and the "virus" of pleuropneumonia, was carried out by Pirie (26) in 1935. She worked principally with dried pig pancreas, but also with the heart and liver, and was able to isolate from each a virucidal substance having the properties of a fairly unstable fatty acid. On the other hand, Dubos and MacCleod (27) characterized a material extracted from the leukocytes, liver, pancreas, spleen, and lung of several animal species as an enzyme. However, this material did not destroy the test organism, that is the pneumococcus, but caused a destruction of the basophilic properties of the cell and an inactivation of the capsular polysaccharide antigen.

Believing that natural immunity or resistance of an organ to local infection might be related to extractable inhibitory substances within the organ, Nutini and his coworkers (28, 29, 30, 31) undertook a series of investigations in 1942 on the effect of tissue extracts on certain bacteria and certain infections. They were successful in isolating a factor, soluble in 80% alcohol, from both human and beef spleens which, in vitro, in concentrations as low as 0.1% was bactericidal for Streptococcus hemolyticus. In a similar manner they extracted from ox brain, spleen, heart and kidney tissue, a factor which was capable of altering the morphological and biochemical properties of Staphylococcus aureus and in higher concentrations was bactericidal for this organism. However, extracts of human and beef spleens, even in high concentrations, had no bactericidal effect on either Staphylococcus aureus or the Type VII pneumococcus. These same investigators found that brain extracts, 80% alcohol soluble, were effective both therapeutically and prophylactically in

Staphylococcus aureus infections induced subcutaneously, intraperitoneally, or intravenously in mice. It was noted that extracts of brain and spleen, organs rich in reticulo-endothelial tissue, were more effective than those of either the heart or kidney.

Maegraith, Martin, and Findlay (32) isolated a lytic material from blood and tissues which they considered to be enzyme-like in nature, but they failed to give definite proof that it was an enzyme.

Having evidence indicating a correlation of the resistance of an animal to tuberculosis with the ability of its tissue enzymes to split tubercle phosphatide, Gerstl, Tennant, and Pelzman (33) studied the in vitro effect of purified liver cathepsins from rabbit, guinea pigs, and mice on tuberculoprotein. With immunization of the animals they demonstrated an increase in catheptic activity (as tested on hemoglobin solutions) but noted little difference in the activity of the enzyme prepared from tissues of the three species. The liver cathepsin did not split the tuberculoprotein but these authors postulated that cathepsins provide a faster removal of the necrotic tissue resulting from antigen-antibody action in the tissues.

Various investigators have shown histones and protamines extracted from animal tissues to be bactericidal for certain organisms. Miller and associates (34) investigated the anti-bacterial properties of a histone prepared from calf thymus. They found this to be active in inhibiting oxygen uptake in the case of Escherichia coli, Group A Streptococci, Eberthella typhosa, and Type I pneumococcus but were not successful in demonstrating any bactericidal activity for these organisms. A protamine, salmine sulfate, was bactericidal for this same group of organisms.

In connection with a group of studies on infection with Bacillus anthracis, Bloom, Watson, Cromartie, and Freed (35) became interested in anthracidal extracts prepared from dog leukocytes, hog pancreas, and calf thymus. With the aid of Warburg studies, along with bacterial colony counts, these extracts were shown to be anthracidal. Furthermore, these same preparations were effective in protecting against anthrax infection. Chemical characterization of these extracts, all having similar electophoretic mobilities, indicated the active material to be a basic polypeptide with a very high lysine content. Bloom and Blake (36) prepared such a polypeptide from hog thyroid. Tested with Escherichia coli, Staphylococcus aureus, a β-hemolytic streptococcus, and Bacillus megatherium, a non-specific type of agglutination of heat killed organisms occurred. In Warburg studies this same material inhibited the oxygen uptake of this same group of organisms.

Russian workers, Kornikova, Urasova, and Osarkh (37), using a procedure similar to that used in extracting tyrothricin from soil bacteria, isolated from rabbit liver and human placenta substances which showed a bacteriostatic effect for certain gram positive organisms. They described these fractions as alcohol soluble, acetone soluble, water insoluble and coctostabile. Fractions isolated in a similar manner from bovine and rat liver, rabbit bile, blood and muscle failed to exhibit any bacteriostatic activity for the organisms tested.

Lipid fractions obtained from serum and brain tissue of several animal species, especially the horse, proved capable of inactivating certain neurotropic viruses (St. Louis, Russian Far East and Japanese encephalitis) when tested by Casals and Olitsky (38, 39). Their technique

of extraction involved the use of organic solvents, either acetone and ethyl ether used in succession, or a mixture of chloroform and methyl alcohol. They suspended their final residue in saline, and after allowing it to stand at least four weeks, showed it to be active in protecting mice against encephalitis. If this suspension was used within 10 to 12 days after preparation it was not effective in preventing infection in these animals.

More recently, Whitney, Anigstein, and Micks (40) have reported the isolation of an antibacterial agent from tryptic digests of human red blood cells which is active against many gram negative and gram positive bacteria. Tryptic digests of bovine blood completely inhibitied the growth of such organisms as Staphylococcus aureus, Sarcina lutea, Streptococcus viridans, Bacillus anthracis, and Escherichia coli, whereas similar digests of human and canine blood were slightly less active against these organisms. These authors believe the active principle in this material to be a peptide-amino acid complex.

Other hemolytic substances of tissue origin have been reported in recent years. Laser (41) identified the hemolytic substance he obtained by fractionation and purification of the serum, spleen, and liver of the horse and the brain of the human and the horse as cis 11-12 octadecenoic acid. Tyler (42, 43) has fractionated adult and fetal liver cells of the guinea pig, first by saline extraction, and then by centrifugation and found them to be hemolytic. He is of the opinion that this substance is enzymatic in nature.

It would seem then that of the many antibacterial and hemolytic substances which have been derived from animal tissues, all fall into three groups; those considered enzymes or enzyme-like in nature, those character-

ized as polypeptides and those with the properties of lipids. Although these materials were obtained and studied, in some instances, with the specific idea of trying to correlate their presence with the resistance of the animal to a given infection, there seems to have been little systematic approach to this problem. Thus, although there has been collected a mass of data which might seem to lend support to the hypothesis that antibacterial tissue extractables are concerned in the resistance mechanism, under the circumstances it is difficult to evaluate and to correlate the evidence from this viewpoint

Fully realizing the complexities of the resistance process of the body and the possibility of the existence, within the tissues, of a multiplicity of mechanisms for combating different infections, it was suggested that perhaps a more valuable approach to a study of host resistance would be a comparative study of some of the defense mechanisms using a resistant host-susceptible host system.

Moore and Mika (44) found a million fold difference in the susceptibility of the white rat and the guinea pig to Type I pneumococcus. They have further shown that Type I pneumococci are not removed from the blood or organs of the susceptible rat but that these organisms do disappear rapidly from the resistant guinea pig.

With this background, a comparative study of the antibacterial activity of materials extracted from one or more of the organs of the reticulo-endothelial system of these two species seemed one logical point of attack in a study of overall resistance and one which might lead to a practical application in the development of an <u>in vitro</u> method for measuring the virulence of a parasite for man.*

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* Originally proposed by Dr. W. J. Nungester

The present study concerns the isolation, partial purification and characterization of a pneumococcidal factor from the reticulo-endothelial organs, especially the liver, of the resistant guinea pig and the susceptible rat. An attempt has been made to establish a difference in the activity of the materials isolated from the two species and to study the basis of this difference.

METHODS AND MATERIALS

Animals.

The guinea pigs used in these experiments were obtained from Parke, Davis and Company, had not been previously injected with pneumococcus antigen or antibody and may be considered as representing normal adult individuals of their species. They were maintained in an open stock pen on fortified guinea pig ration.

The white rats were adult normal individuals of the Sprague-Dawley strain. They were maintained on a complete dog ration ("Friskies").

Organism.

The test organism* was Michigan Strain 76 of Type I Diplococcus pneumoniae, which was originally isolated at the University of Chicago and designated by them as Strain A-5.

Virulence was maintained by animal passage through rats at intervals of about 3 weeks. The procedure consisted of intraperitoneal inoculation of 0.1 ml of a 6 hour culture into a rat and recovery of the organism by cardiac puncture after 12 to 24 hours. Reinoculation of a 0.1 ml portion of the blood into the next rat continued the passage. Poured plate dilutions (usually 10^{-1}, 10^{-3}, and 10^{-5}) of the blood from the last passage were used for recovery of the organism. After a 24 hour incubation period, 5 to 10 typical isolated colonies were inoculated into broth. The virulence of the organism treated in this manner was such that a 1×10^{-7}

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* Stock culture and virulence maintained by Miles G. Hench

dilution of a 6 hour culture (approximately 10-100 organisms) was lethal for rats by intraperitoneal inoculation. The LD 50 for guinea pigs by the same route of inoculation was between 1×10^7 and 1×10^8.

For routine subculture a 0.1 ml inoculum was introduced into 10 ml of Difco Brain, Heart Infusion broth (B.H.I). This was incubated for 6 hours at 37 C, and then stored at 2 to 5 C until use. A culture was never used after 24 to 48 hours storage.

Chesney (45) and Felton and Dougherty (46) showed that if subculture is made during the phase of rapid growth there will be no lag period and that such a culture possesses a greater infectivity than an older culture. For this reason the six hour culture was considered most desirable for these experiments.

Harvesting of Tissues.

Animals were sacrificed by means of ether. The tissues were removed aseptically and placed immediately in a mixture of ethanol and dry ice. The gall bladder, in the case of the guinea pig, was excised intact and discarded before freezing the tissue. Since the rat does not possess a gall bladder no such precaution was necessary for this species.

Methods for Measuring the Biological Activity of Tissue Extracts.

1. Filter Pad Technique.

In early experiments minced tissue, tissue homogenate and aqueous extracts were tested for biological activity by the filter pad technique similar to that used in assaying antibiotics. These materials could not be successfully incubated in broth suspensions because of marked tendency to coagulate.

Seeded blood plates were prepared with 10 ml of Difco B.H.I. agar, to which was added 4 per cent blood and 0.5 ml of a 10^{-4} dilution of a 6 hour culture of Type I pneumococcus. After the agar hardened, sterile 15 mm filter discs were placed on the surface and saturated with the material to be tested. These plates were then incubated for 24 hours at 37 C, after which they were examined for zones of inhibition.

2. Broth Suspensions.

A weighed amount of dried tissue extract was introduced into a tube containing 1 ml of Difco B.H.I. broth. This suspension was then inoculated with a given number of organisms and incubated at 37 C before being tested for organism survival. In some cases plate counts were made after 2 and 6 hours incubation but for the most part counts were made only at the end of the 6 hour incubation period.

In the case of highly active material either doubling dilutions or decimal dilutions of the suspended extract were made in broth. These tubes were then inoculated with a maximum inoculum of organisms (in the order of 10^7) and incubated at 37 C. When the material was of a low order of activity, as was usually the case for the rat, the concentration of the broth suspension was kept constant and the inoculum was varied from a maximum (10^7) to a minimum (10^2). The minimum concentration of extract causing complete destruction of a given inoculum in 6 hours was taken as the end-point for bactericidal activity.

Plate Counting.

After six hours' incubation of the suspended extract with the test organism, estimations of organism survival were obtained with the aid of colony counts. Decimal dilutions of the test suspensions in saline solution (0.85 per cent NaCl) were transferred in 0.1 ml quantities to sterile

petri dishes. Approximately 10 ml of 4 per cent blood-B.H.I agar was added and the samples thoroughly mixed for even distribution of colonies. After 24 hours' incubation of plates, results were recorded as number of colonies counted per ml of the test suspension.

Purification of Solvents.

For the fractionation and purification of dried tissue extracts it was essential that the solvents be as nearly anhydrous as possible as well as free of peroxides and other impurities. For this purpose the following procedures were used.

1. Absolute Ethanol.

150 gms Calcium oxide, crushed but not powdered, was added to every 300 ml of commercial ethanol. This mixture was refluxed for 1 hour and then distilled into a flask protected by a drying tube. This redistilled alcohol was then stored in a tightly stoppered bottle.

2. Ether.

Anhydrous ether, analytical reagent grade, was allowed to stand over calcium chloride (150 gms to 300 ml) overnight at 5 C. This was then distilled and stored in a tightly stoppered bottle at 5 C.

3. Acetone.

Acetone, analytical reagent grade, was treated in the same manner as the ether.

EXPERIMENTAL

I. Preparation of a Pneumococcidal Tissue Extract

Minced Liver, Liver Homogenate, Aqueous Liver Extract:

In becoming orientated in this problem of bactericidal tissue extracts, the first experimental attempts were concerned with demonstrating a pneumococcidal effect with tissues receiving a minimum of treatment and manipulation. If such an effect could be demonstrated then further steps toward the isolation and purification of this active factor would seem justified.

Fresh minced livers from four individual guinea pigs were tested for inhibitory activity by the filter pad technique, using a poured blood plate seeded with Type I pneumococcus. Figure 1a shows that the minced liver of three of the individuals produced zones of inhibition immediately surrounding the filter discs, with zones of enhanced growth beyond. The tissues of the fourth animal showed little or no such activity.

Aqueous homogenates were prepared with the livers of the same individuals used for the minced tissue test. Ten grams of liver, with 100 ml of distilled water at room temperature, were homogenized on the Waring blender for two minutes. This mixture was allowed to stand one hour at 5 C and then tested in the same manner as the minced tissues. Very weak activity for the same three individuals whose minced tissues showed activity is indicated in Figure 1b by zones of reduced growth in which there was a noticeable difference in the intensity of the greening of the colonies. Again the homogenate from the fourth animal shows little or no activity.

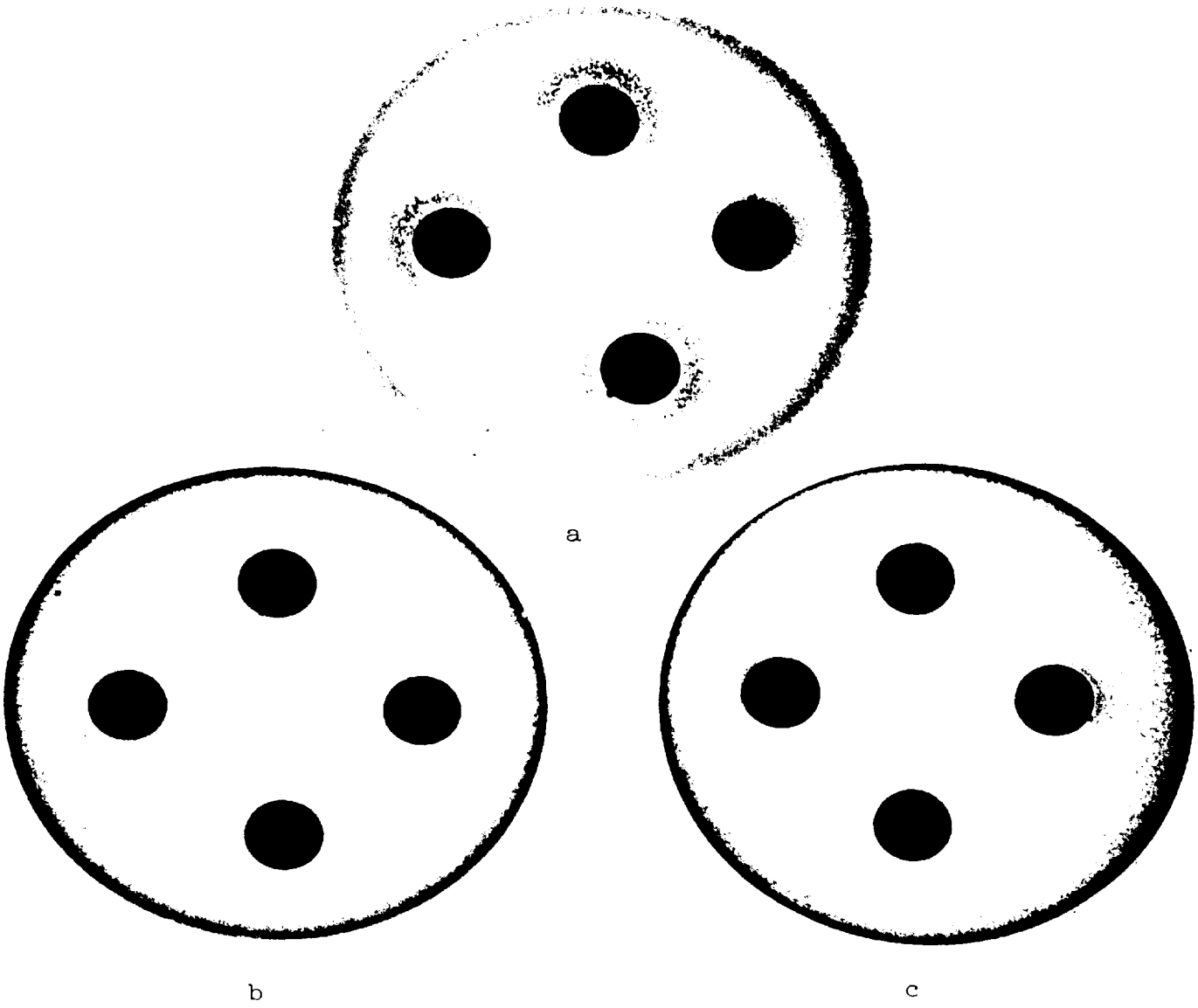


Figure 1. Zones of Inhibition on Poured Blood Plates of Type I Pneumococcus Produced by Minced Livers, Liver Homogenates, and Concentrated Aqueous Liver Extracts of Four Individual Guinea Pigs. a. Minced Livers. b. Liver Homogenates. c. Concentrated Aqueous Liver Extracts.

These same homogenates were then centrifuged at approximately 4000 x G for one hour. The supernatants were decanted, shell frozen in round bottom high vacuum flasks, and dried under vacuum. The dried residues were reconstituted to 1/20 of their original volumes with sterile distilled water. These concentrated aqueous extracts tested by the filter pad technique produced (Figure 1c) the most clear cut zones of inhibition and in two instances marked zones of hemolysis. The animal whose tissue in the other two tests had failed to exhibit much activity, on concentration, showed a definite zone of inhibition though not as large in diameter as the other three.

Pooling the livers from six or more animals, extracting with water and concentrating by freeze-drying, gave extracts which were consistent in producing the same type of inhibition as noted in the above test.

Extracts prepared in a similar manner from rat livers were variable in activity, some showing no zones of inhibition, some showing small zones of incomplete inhibition.

These results indicate that there is a biologically active material in guinea pig livers especially, which is at least weakly soluble in water. Furthermore, there appears to be a significant difference in the activity of the extracts prepared from the livers of the two species.

50 Per Cent Ethanol Soluble Extract.

It was soon realized that for comparing the activity of the extracts of the two species a more exact method of quantitation was needed. However, in order to show significant activity consistently it was necessary to concentrate the aqueous extracts and this highly concentrated material, when inoculated with the test organism and placed in the 37 C incubator,

quickly coagulated. Thus the next problem appeared to be purification and concentration of this biologically active factor.

As finally developed, the steps in preparation of the 50 Per Cent Ethanol Soluble Extract are as follows. An aqueous homogenate of guinea pig liver was prepared as before, using 10 grams of tissue to 100 ml of sterile distilled water at room temperature. This homogenate was held at 5 C for one hour. Ethanol was then added to give a final concentration of 50 per cent and this mixture was allowed to stand two hours at room temperature. A heavy precipitate formed which was filtered off and discarded. The filtrate was a clear greenish yellow which became cloudy again upon standing. The alcohol was removed by boiling under reduced pressure, (obtained with a water aspirator) in a water bath at 40 to 50 C. This latter step required from 4 to 18 hours depending upon the original volume of the material. During this step a light precipitate formed and a marked turbidity of the material developed. This necessitated centrifugation at approximately 4000 x G for one hour. At the end of this time the supernatant, which was still distinctly turbid, was shell frozen and dried under vacuum. The dried residues were stored in sterile bottles in a dessicator jar. Aseptic precautions were followed throughout the procedure. A flow diagram of this procedure is found in Figure 2.

Various modifications of this technique were tried but none yielded as satisfactory results. Increasing the ethanol to 80% concentration seemed to be of little or no value. Extraction of the tissue in undiluted ethanol failed to yield an active fraction. Extraction of the aqueous homogenate with a mixture of ethanol-ether (3:1) in place of the 50 per cent ethanol markedly decreased the biological activity of the

material. Extraction in a water-ether emulsion (2:1) failed to yield an active fraction.

Certain steps in the time and temperatures of extractions were examined critically. An aqueous homogenate was prepared from the livers of eight guinea pigs. This homogenate was divided into three equal portions, each subsequently being treated differently. To the first sufficient ethanol to give a final concentration of 50 per cent was added immediately and allowed to stand three hours at room temperature before firltering. The second sample of the homogenate was held at 5 C for one hour and then ethanol was added to a final concentration of 50 per cent. This was allowed to stand at room temperature for two hours before filtering. The ethanol from these two filtrates was then removed by boiling under reduced pressure. The third sample of homogenate was held at 5 C for one hour, precipitated with ethanol as already described, but stood overnight at room temperature before filtering. Removal of ethanol, centrifugation and drying was as has been described and was the same for all three samples. The results, as presented in Table 1, indicate that for obtaining this extract at maximum pneumococcidal potency the time and temperature of extraction as noted in the flow diagram (Figure 2) are the most satisfactory.

Blood Control.

Because the liver is such a vascular organ, and because blood is known to possess antibacterial properties, a blood control was definitely indicated. Two methods were used for establishing such a control.

Perfusion: The animals were placed under deep ether anesthesia, the thorax opened, and 10 ml of 1 per cent citrate in saline was injected

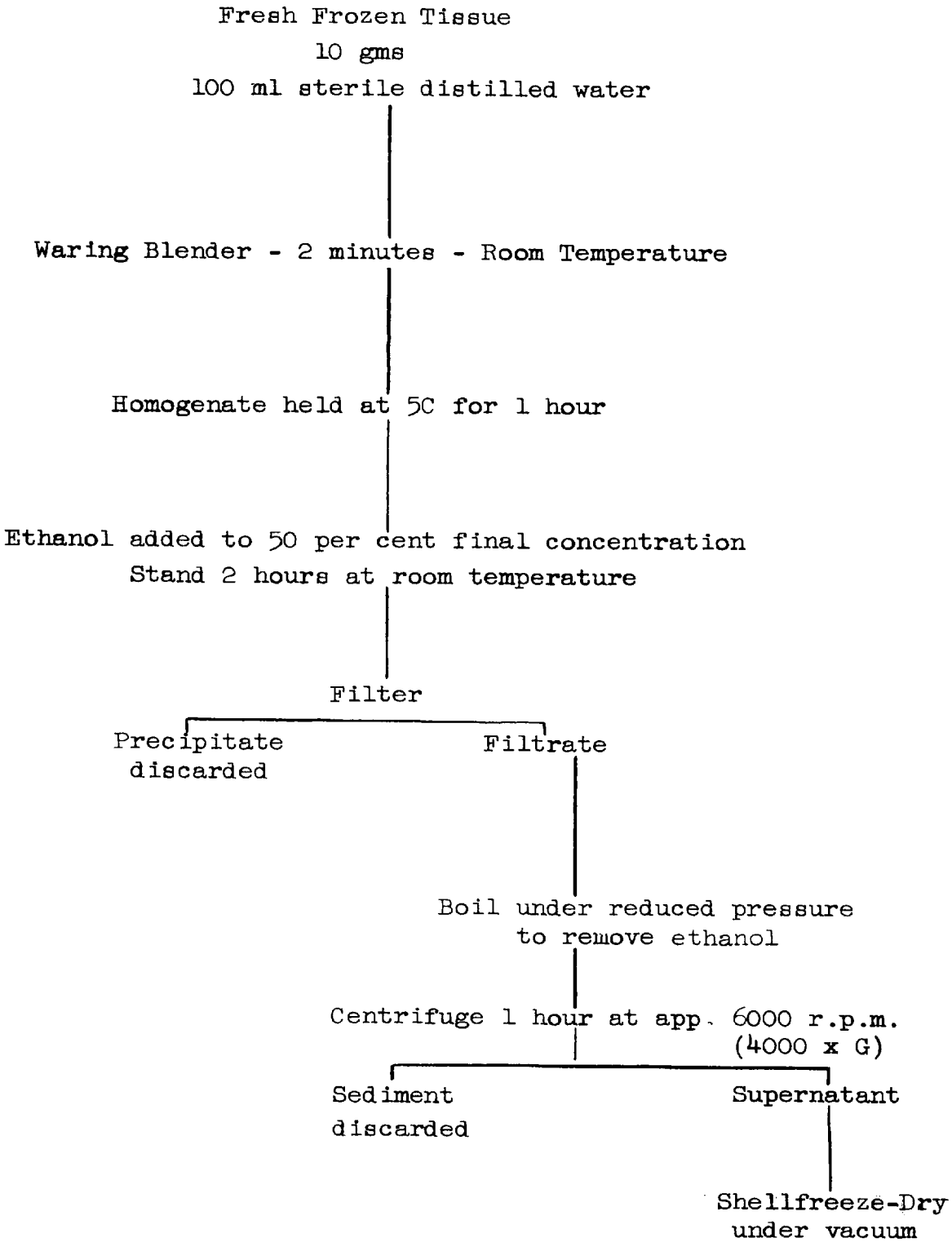


Figure 2. Flow Diagram for the Preparation of 50 Per Cent Ethanol Soluble Tissue Extracts.

TABLE 1

THE EFFECT OF VARYING CONDITIONS OF EXTRACTION UPON THE PNEUMOCOCCIDAL ACTIVITY OF A 50 PER CENT ETHANOL SOLUBLE EXTRACT OF GUINEA PIG LIVER

Extract Concentration, mg per ml	Initial Inoculum, Colony Count per 0.1 ml	Conditions of Extraction		
		Ethanol Direct	Ethanol after 1 hour at 5 C	Ethanol with Overnight Standing
10	1×10^8	0*	0	0
5	1×10^8	0	0	0
2.5	1×10^8	0	0	0
1.2	1×10^8	3×10^3	0	0
0.6	1×10^8	4×10^5	0	0
0.3	1×10^8	3×10^6	0	1×10^1
0.15	1×10^8	1×10^7	0	2×10^4
0.01	1×10^8	---	0	---
0.001	1×10^8	---	4×10^6	---

* These figures represent colonies per ml after 6 hours incubation of the extract with the inoculum at 37 C.

into the left ventricle of the heart. At the same time the ascending vena cava was cut. About 200 ml of saline solution was introduced into the left ventricle until marked blanching of the liver occurred. The tissue was then removed and a 50 per cent ethanol extract prepared as usual. Extracts prepared from livers treated in this manner still exhibited a high degree of pneumococcidal activity.

Whole Blood Extracts: Perfusion is a time consuming operation, and therefore not considered practical for routine use. It was felt that an extract of whole blood prepared from the same animal, at the same time and in the same manner as an extract of liver and then both tested simultaneously for pneumococcidal activity would be an adequate control on the blood in the liver. Therefore, blood and liver 50 per cent ethanol soluble extracts were prepared from the tissues of four individual guinea pigs. Results are presented in Table 2.

Liver extracts from each of the four animals showed a high degree of pneumococcidal activity. On the other hand, not one of the blood extracts was pneumococcidal. Two of the blood extracts could be considered inhibitory but as compared to the activity of the liver extracts this was negligible.

Extracts prepared from samples of blood pooled from 14 guinea pigs on three different occasions failed to show any pneumococcidal activity. Therefore, it would seem that any pneumococcidal activity due to blood in the liver may be disregarded.

Bile Control.

The Type I pneumococcus is a bile soluble organism and the liver is a biliary organ. Any extract prepared from the liver, therefore, might

TABLE 2

A COMPARISON OF THE PNEUMOCOCCIDAL ACTIVITY OF 50 PER CENT ETHANOL SOLUBLE EXTRACTS OF THE BLOOD AND OF THE LIVER FROM FOUR INDIVIDUAL GUINEA PIGS

Tissues	Extract Concentration, mg per ml	Initial Inoculum, colony count per 0.1 ml	Animals			
			1	2	3	4
	10	1.7×10^8	0*	0	0	0
	5	1.7×10^8	0	0	0	0
Liver	2.5	1.7×10^8	2×10^1	0	0	0
	1.25	1.7×10^8	1.5×10^3	3×10^2	0	0
	0.6	1.7×10^8	1×10^5	2×10^4	4×10^2	0
	10	6.8×10^7	TNC	6×10^7	TNC	6×10^7
Blood	10	6.8×10^4	9×10^6	1×10^5	1×10^7	8×10^5
	10	6.8×10^2	2×10^3	1×10^2	1×10^4	4×10^2

* These figures represent colonies per ml after 6 hours incubation (at 37 C) of extract with the inoculum.

possibly contain certain bile salts which could be responsible for the pneumococcidal activity of the extract. The following experiments were carried out in an attempt to show the presence of a pneumococcidal factor in liver extract which was not dependent on bile for its activity.

No direct qualitative tests for the presence of bile in the liver extract were found suitable. The Pettenkofers' test for bile acids was unsatisfactory due to the presence of other substances in the liver extract which gave a color reaction with sulfuric acid. The pigment in guinea pig bile is biliverdin, therefore, the common tests for bilirubin were not applicable. An indirect approach, therefore, seemed to offer the most easily available information.

A comparison of the pneumococcidal strength of guinea pig bile and of guinea pig liver extract was first undertaken. The bile and livers were removed from 20 guinea pigs. The pooled bile was shell frozen immediately and dried under vacuum. An extract (50 per cent ethanol soluble) of the pooled livers was prepared as usual. Dried bile and dried liver extract were suspended in B.H.I. broth in the same concentration by weight. Decimal dilutions in B.H.I. broth were inoculated with a maximum inoculum and the test carried out as usual. Results are recorded in Table 3.

The 10 mg of dried bile in 1 ml of B.H.I broth represented about 1/3 of the concentration of undiluted gall bladder bile. Gall bladder bile is generally considered about 10 times more concentrated than hepatic bile. 10 mg of dried extract in 1 ml represent 1/10 of the original concentration of the liver in the homogenate. 1 mg of dried extract was capable of destroying completely 5×10^7 pneumococci whereas 1 mg of dried bile failed to destroy completely this same inoculum. Considering the probability that any biliary constituents present in the 1 mg of extract

TABLE 3

A COMPARISON OF THE PNEUMOCOCCIDAL ACTIVITY OF GUINEA PIG BILE AND OF GUINEA PIG 50 PER CENT ETHANOL SOLUBLE LIVER EXTRACT

Concentration, mg per ml	Initial Inoculum, Colony Count per 0.1 ml	Bile	Extract
10	5×10^7	0*	0
1	5×10^7	3×10^4	0
0.1	5×10^7	TNC	TNC
0.01	5×10^7	TNC	TNC

* These figures represent colonies per ml after 6 hours incubation (at 37 C) of extract with the inoculum.

are far less concentrated than they are in the 10 mg of dried bile, these results suggest that the pneumococcidal factor in the extract is something other than bile or bile salts.

It has been observed on blood plates that both bile and liver extract are hemolytic. Based on the same principle of concentrations as for the pneumococcidal test, it was felt that a hemolytic titration of the two might provide further evidence that the liver extract and bile are not related. Suspensions of extract and of bile were prepared and diluted as for the bactericidal test except that dilutions were made in physiological saline (0.85 per cent NaCl) in 1 ml final volume. One drop of fresh blood (human) was added to each tube directly from the syringe. The tubes were mixed and allowed to stand at room temperature. Readings were made at 1 hour and at 24 hours. In the case of both extract and bile, hemolysis was well under way in the first tube (10 mg per ml) in one hour but not complete until 24 hours. In the second tube (1 mg per ml) bile showed some hemolysis after 24 hours but most of the cells were still intact. In the second tube of extract, after 24 hours there was a very faint trace of hemolysis but not quite as evident as in the case of the bile. The hemolytic titres do not appear to parallel the bactericidal titres of these two materials, though the difference may be explained by the larger volume of blood cells present as compared to the pneumoccal cells. In general, the hemolytic property of the bile and of the extract appears to offer little information concerning the relation of these two materials.

Experiments using the filter pad technique and the original concentrated aqueous extract of liver had indicated that boiling (100 C) considerably diminished its pneumococcidal activity. If it could be shown that bile

reacted differently to boiling, this would be further evidence that bile was not responsible for the pneumococcidal activity of the liver extract.

Since the original experiments were carried out with very concentrated suspension of extract, it was decided to prepare suspensions of varying concentrations of 50 per cent ethanol soluble extract, boil each for 30 minutes and then titrate as usual, along with an untreated sample of extract, to determine the effect of boiling on the pneumococcidal activity. Undiluted fresh guinea pig bile was boiled at the same time and then titrated with an untreated sample. Results are found in Tables 4 and 5.

Boiling appears to have no effect on the pneumococcidal strength of guinea pig bile. When guinea pig extract, in concentrated suspension (100 mg per ml), is boiled for thirty minutes its pneumococcidal activity is markedly diminshed. However, when the concentration is reduced to 1 mg per ml, boiling appears to have no effect on this biological activity of the material.

It was noticed that in the tubes containing the more concentrated suspensions of extract a heavy coagulum formed on boiling. Possibly the loss of activity might be accounted for by the adsorption of the active material onto the coagulum. This was found to be the case when the coagulated material was separated by centrifugation, suspended in B.H.I. broth in such a manner as to break up the coagulum, and tested for pneumococcidal activity. These results are found in Table 6. Most of the activity is concentrated in the sediment, leaving little suspended in the supernatant. The reduced surface area of the coagulum probably accounts for the fact that this material is not as active as the untreated extract.

The livers were removed from 10 guinea pigs, pooled and treated as usual until after the final centrifugation step. At this point, one

TABLE 4

THE EFFECT OF BOILING (100 C) ON THE PNEUMOCOCCIDAL ACTIVITY OF VARYING CONCENTRATIONS OF A 50 PER CENT ETHANOL SOLUBLE EXTRACT OF GUINEA PIG LIVER

Extract Concentration mg per ml	Initial Inoculum, Colony Count per 0.1 ml	Treatment of Varying Concentrations			
		100 mg in 1 ml Room Temp.	100 mg in 1 ml 100 C	10 mg in 1 ml 100 C	1 mg in 1 ml 100 C
100	8.5×10^7	0*	0	---	---
10	8.5×10^7	0	1×10^2	0	---
1	8.5×10^7	0	2×10^4	1×10^3	0
0.1	8.5×10^7	1×10^8	---	3×10^8	3×10^8

* These figures represent colonies per ml after 6 hours incubation (at 37 C) of extract with the inoculum.

TABLE 5

THE EFFECT OF BOILING (100 C) ON THE PNEUMOCOCCIDAL ACTIVITY OF GUINEA PIG BILE

Bile Dilutions	Initial Inoculum, Colony Count per 0.1 ml	Treatment of Bile	
		Room Temperature	100 C
0	8.5×10^7	0*	0
1:2	8.5×10^7	0	0
1:4	8.5×10^7	1×10^2	1×10^2
1:8	8.5×10^7	2×10^2	2×10^2
1:16	8.5×10^7	5×10^2	2×10^2

* These figures represent colonies per ml after 6 hours incubation (at 37 C) of extract with the inoculum.

TABLE 6

A COMPARISON OF THE PNEUMOCOCCIDAL ACTIVITY OF FRACTIONS OBTAINED AFTER BOILING A 50 PER CENT ETHANOL SOLUBLE EXTRACT OF GUINEA PIG LIVER

Extract Concentration, mg per ml	Initial Inoculum, Colony Count per 0.1 ml	Untreated Extract	Sediment after Boiling (100 C)	Supernatant after Boiling (100 C)
100	4×10^7	0*	0	0
10	4×10^7	0	0	3×10^5
1	4×10^7	0	0	TNC
0.1	4×10^7	0	2×10^5	TNC
0.01	4×10^7	0	TNC	TNC
0.001	4×10^7	TNC	TNC	TNC

* These figures represent colonies per ml after 6 hours incubation (at 37 C) of extract with the inoculum.

aliquot was passed through a Seitz filter before drying, the other was treated as usual. These two extracts were titrated for pneumococcidal activity. Results are recorded in Table 7.

Seitz filtration of guinea pig liver extract (50 per cent ethanol soluble) appears to remove completely the pneumococcidal factor. Guinea pig bile when tested for pneumococcidal activity before and after Seitz filtration remains unchanged. These results indicate a difference in the physical nature of the pneumococcidal factor in bile and of that in the guinea pig liver extract.

It has been reported that bile acids, desoxycholic, cholic, dihydrodesoxycholic, and dehydrocholic, in decreasing order are bacteriolytic for Staphylococcus aureus (47). Filter pad tests with guinea pig liver extract had indicated that this material was not biologically active against the Staphylococcus aureus. To confirm this quantitatively, two different lots of guinea pig liver extract (50 per cent ethanol soluble) of known pneumococcidal strength, were tested simultaneously with the Type I pneumococcus and Staphylococcus aureus (a virulent strain, designated P-209). These results are recorded in Table 8.

These two liver extracts fail to inhibit the growth of Staphylococcus aureus whereas they are strongly bactericidal for the Type I pneumococcus. It appears then that either the liver extract does not contain the bile salts under consideration or that they are present in such low concentrations as to be of no consequence.

Though evidence has been presented which supports the belief that the pneumococcidal factor present in liver extract is not of biliary origin, it was felt that this could be more conclusively confirmed if such a factor could be demonstrated in extracts prepared from non-biliary

TABLE 7

THE EFFECT OF SEITZ FILTRATION ON THE PNEUMOCOCCIDAL ACTIVITY OF A 50 PER CENT ETHANOL SOLUBLE EXTRACT OF GUINEA PIG LIVER

Extract Concentration, mg per ml	Initial Inoculum, Colony Count per 0.1 ml	Untreated Extract	Seitz Filtered Extract
10	1×10^7	0*	6×10^8
1	1×10^7	0	TNC
0.1	1×10^7	0	TNC
0.01	1×10^7	2×10^5	TNC
0.001	1×10^7	TNC	TNC

* These figures represent colonies per ml after 6 hours incubation (at 37 C) of extract with the inoculum.

TABLE 8

A COMPARISON OF THE BACTERICIDAL ACTIVITY OF TWO GUINEA PIG 50 PER CENT ETHANOL SOLUBLE LIVER EXTRACTS FOR THE TYPE I PNEUMOCOCCUS AND FOR STAPHYLOCOCCUS AUREUS

Organism	Extract Concentration, mg per ml	Initial Inoculum, Colony Count per 0.1 ml	Extract Sample	
			1	2
Type I Pneumococcus	10	1×10^8	0*	0
	1	1×10^8	0	2×10^2
	0.1	1×10^8	TNC	7×10^7
	0.01	1×10^8	TNC	TNC
Staphylococcus Aureus	10	2×10^7	1×10^8	1×10^9
	10	2×10^4	4×10^8	1×10^9
	10	2×10^2	1×10^8	1×10^9

* These figures represent colonies per ml after 6 hours incubation (at 37 C) of extract with the inoculum.

tissues. Such an experiment is described in Section II and the data recorded in Tables 21, 22, and 23. Thus, significant pneumococcidal activity has been demonstrated in extracts prepared from two non-biliary organs, the brain and the spleen, though the activity of the brain extract is considerably less than that of the liver or spleen.

Enzyme Control.

In the consideration of the pneumococcidal property of tissue extracts, a third control seemed essential, namely, a control of the activity of the enzyme systems of the material. The activity which had been observed possibly could be due to a product derived by action of tissue enzymes during processing or by bacterial enzymes during incubation of the extract with the inoculum. Such a derived product, then, would not necessarily exist as such in the tissue, and therefore, the *in vitro* activity observed might have little relation to an *in vivo* process.

If the activity of the extract were due to enzymatic break down of some material which might otherwise be inactive, incubation of the aqueous homogenate at 37 C for one hour before the addition of ethanol should yield an extract more active than one held at 5 C for one hour before the addition of ethanol.

The livers from 20 guinea pigs were pooled and an aqueous homogenate prepared as usual. This homogenate was divided into four aliquots. Two aliquots were incubated at 37 C, one for 1 hour, the other overnight. The other two aliquots were incubated at 5 C, one for 1 hour, the other overnight. The remainder of the processing was as usual and the same for all four samples. The dried extracts were then titrated for pneumococcidal activity. The results are recorded in Table 9.

TABLE 9

THE EFFECT OF VARYING TIME AND TEMPERATURES OF INCUBATION OF THE AQUEOUS HOMOGENATE ON THE PNEUMOCOCCICIDAL ACTIVITY OF THE FINAL DRIED 90 PER CENT ETHANOL SOLUBLE EXTRACT OF GUINEA PIG LIVER

Extract Concentration, mg. per ml	Initial Inoculum, Colony Count per 0.1 ml	Treatment of Aqueous Homogenate			
		1 hour 37 C	Overnight 37 C	1 hour 5 C	Overnight 5 C
10	1×10^8	0*	0	0	0
1	1×10^8	1×10^5	4×10^5	0	TNC
0.1	1×10^8	TNC	TNC	1×10^5	TNC
0.01	1×10^8	TNC	TNC	TNC	TNC

* These figures represent colonies per ml after 6 hours incubation (at 37 C) of extract with the inoculum.

Incubation of the aqueous homogenate at 37 C for 1 hour or overnight appears to diminish rather than to enhance the pneumococcidal activity of the extract. Marked enhancement would be expected if the active factor were an enzymatically derived product. Furthermore, holding the homogenate at 37 C or at 5 C overnight also diminishes the activity of the final extract.

It was still possible that certain enzymes were not completely inactivated by the 50 per cent ethanol and enzymatic breakdown could be taking place while the ethanol was being removed by boiling under reduced pressure in a 40 C water bath.

An ethanol extract was prepared from the pooled livers of two guinea pigs. One half of this extract was freed of ethanol by boiling under reduced pressure and then dried as usual. The other half was placed in a boiling water bath for 30 minutes. A slight coagulum formed and then toward the end of the boiling period (after most of the ethanol had boiled off) a marked clouding occurred which was similar to that which forms when ethanol is removed by the water aspirator method. The dried extract was prepared from this material in the usual manner.

As observed in Table 10 there was no difference in the pneumococcidal titres of the two extracts receiving different treatment. However, if the active factor were being released by enzymatic action during the interval of ethanol removal, the ethanol extract which in processing had been treated in the boiling water bath for 30 minutes should be significantly less active than the one which was treated in the usual manner. Since this is not the case, it seems that, at least at this stage, enzymes are not liberating the pneumococcidal factor.

TABLE 10

THE EFFECT OF THE TREATMENT OF THE ETHANOL FILTRATE IN A BOILING WATER BATH ON THE PNEUMOCOCCIDAL ACTIVITY OF THE FINAL DRIED 50 PER CENT ETHANOL SOLUBLE EXTRACT OF GUINEA PIG LIVER

Extract Concentration, mg per ml	Initial Inoculum, Colony Count per 0.1 ml	Treatment of Ethanol Filtrate	
		Boiled under Reduced Pressure	Boiled 100 C
10	8×10^7	0*	0
1	8×10^7	0	0
0.1	8×10^7	4×10^3	3×10^4
0.01	8×10^7	TNC	TNC

* These figures represent colonies per ml after 6 hours (at 37 C) incubation of extract with inoculum.

During the incubation of the extract suspension with the test organisms, presumably enzymes produced by the bacterial cells could cause a breakdown and a release of a substance in the extract which might only then become toxic. Such a process would require time, thus a study of the time and mode of action of the pneumococcidal extract seemed indicated.

Suspensions were prepared using a 50 per cent ethanol soluble extract of known pneumococcidal strength (0.01 mg was capable of destroying completely 10^8 organisms in 6 hours). These tubes were inoculated with a maximum inoculum (4×10^7) and colony counts and gram stains were made after 5 minutes, 2 hours and 6 hours incubation at 37 C. Table 11 is representative of the results obtained in several such tests.

These results indicate that the pneumococci are killed almost immediately, especially in the suspensions of higher concentration of extract. Lysis of the cells was not conspicuous until after the 5 minute incubation period which suggests that the cells are killed (as evidenced by the reduced count at 5 minutes) and then lysed.

The immediate killing action of the extract which was demonstrated in this experiment is not compatible with the hypothesis that a toxic factor is released from the extract through enzymatic action of bacterial enzymes.

Effect of Serum on Pneumococcidal Activity.

Because of reports that serum inhibited the antibacterial properties of some tissue extracts, especially those of lipid nature, (20,24) it was felt that such an experiment using the 50 per cent ethanol soluble extract might help in characterizing the active factor present in this extract.

TABLE 11

THE TIME AND MODE OF ACTION OF A 50 PER CENT ETHANOL SOLUBLE EXTRACT OF GUINEA PIG LIVER ON THE TYPE I PNEUMOCOCCUS

Extract Concentration, mg per ml	Initial Inoculum, Colony Count per 0.1 ml	Incubation Time					
		5 minutes		2 hours		6 hours	
		Colony Count per ml	Gram Stain	Colony Count per ml	Gram Stain	Colony Count per ml	Gram Stains
10	4×10^7	1×10^1	Many Intact Gram positive diplococci	0	No Intact Cells	0	No Intact cells
1	4×10^7	2×10^5	Many Intact Gram positive diplococci	0	No Intact Cells	0	No Intact Cells

Thus, a 50 per cent ethanol soluble extract of guinea pig liver of known pneumococcidal activity was suspended in weighed amounts in 1 ml volumes of pooled serum from four guinea pigs. These tubes, with serum controls (no extract added) and B.H.I. broth controls (extract suspended in B.H.I. broth) were inoculated with pneumococci and tested in the usual manner. These data are presented in Table 12.

Under these experimental conditions, homologous serum appeared to reduce the activity of the liver extract 1000 fold. Serum alone, by this method of testing, had no antibacterial effect. The extract suspended in serum in high concentration (100 mg per ml) was capable of destroying a maximum inoculum (10^7) but in lower concentrations it was completely ineffective.

Hemolytic Artifact.

During the course of these experiments, from time to time, certain extract preparations seemed to be causing some change in the colony growth of the pneumococcus. In such cases, instead of complete inhibition of the growth of the colonies, tiny pinpoint β-hemolytic in place of α-hemolytic colonies seemed to persist. However, with gram stain no intact organisms could be demonstrated nor would subculture of the excised colonies produce growth. Subsequently it was shown (Figure 3) that this phenomenon was an artifact since the same tiny hemolytic, colony-like forms appeared on poured plates of such extract suspensions which had not been inoculated with the test organism. Apparently these suspensions, for some reason, contained tiny clumps of protein onto which the active principle (shown to be both bacteriolytic and hemolytic) had been adsorbed and these small clumps then in blood plates produced forms and hemolytic areas which were indistinguishable from pinpoint colonial growths.

TABLE 12

THE EFFECT OF SERUM ON THE PNEUMOCOCCIDAL ACTIVITY OF A 50 PER CENT ETHANOL SOLUBLE EXTRACT OF GUINEA PIG LIVER

Samples	Extract Concentration, mg per ml	Initial Inoculum, Colony Count per 0.1 ml	Colonies per ml after 6 hours incubation at 37 C
	0	9×10^7	TNC
Serum	0	9×10^4	2×10^6
	0	9×10^2	1×10^4
	100	9×10^7	0
Extract	10	9×10^7	TNC
in	1	9×10^7	TNC
Serum	0.1	9×10^7	TNC
	0.01	9×10^7	TNC
	100	9×10^7	0
Extract	10	9×10^7	0
in	1	9×10^7	0
B.H.I.	0.1	9×10^7	0
	0.01	9×10^7	4×10^5

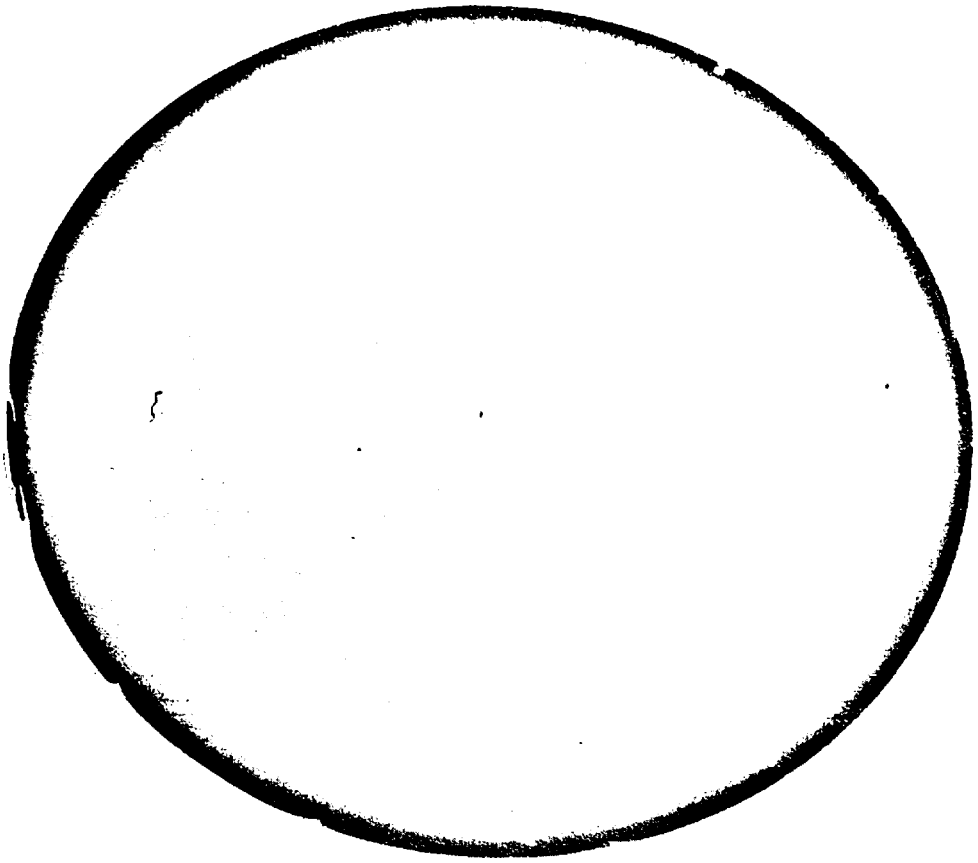


Figure 3. Hemolytic Colony-like Forms on a Poured Blood Plate of a 50 Percent Ethanol Soluble Extract of Guinea Pig Liver.

II. Comparison of the Pneumococcidal Activity of Tissue Extracts of the Rat and the Guinea Pig

Extracts of Pooled Tissues.

The filter pad tests with concentrated aqueous liver extract had indicated a marked superiority of guinea pig liver extracts over rat liver extracts in inhibiting the growth of Type I pneumococci. Because of the tendency of the aqueous extract to coagulate on incubation, further investigation and more exact quantitation of this observed difference in the two species was practical only after the development of the 50 per cent ethanol soluble extract. This extract is easily suspended in a liquid medium and shows no tendency to coagulate even in high concentrations.

To supply a nutrient base which was not in itself toxic, B.H.I broth was selected for suspending and testing the biological activity of the extracts. Growth curve experiments on the 1 ml medium--0.1 ml inoculum system indicated that six hour exposure of organisms to extract was the most suitable. Whereas most active extracts destroyed the maximum inoculum completely in two hours, weakly active extracts (as those of the rat) did not show maximum activity until six hours. At the end of 24 hours marked autolysis had occurred in the control broth tube of 1 ml B.H.I. broth inoculated with 0.1 ml of pneumococci, whereas maximum growth in this tube was noted in six hours.

50 per cent ethanol soluble extracts of pooled liver of the guinea pig and of the rat were prepared and tested for pneumococcidal activity. The number of animals represented in the pools varied and the extracts were not all prepared or tested at the same time. However, treatment of the animals (18-24 fasting) and method of processing the material was the same in all cases.

An examination of Table 13, which summarizes these data reveals both a variation of activity within the species and a marked difference in activity between the species. Whereas the extracts of guinea pig liver exhibited a consistently high degree of pneumococcidal activity, comparably the activity of the rat extracts was of a very low order.

Extracts of Individual Tissues.

The observation that there was variation within the species in the activity of the extracts prepared from pooled tissues, prompted an investigation of the pneumococcidal effect of extracts prepared from a number of individuals of the two species.

A total of ten 50 per cent ethanol soluble extracts were prepared from the livers of ten guinea pigs. The livers of ten rats were treated in the same manner. Generally, as many as six extracts, representing three guinea pigs and three rats, were prepared and tested at the same time under identical conditions. These data are presented in Tables 14, 15, 16, and 17.

Table 18 summarizes the results of these individual animal tests. Extracts prepared from six out of ten guinea pigs, in concentrations as low as 6.25 mg of extract per ml, were capable of destroying completely an inoculum of 10^7 or more pneumococci, whereas none of the rat extracts could kill this number of organisms even at higher concentrations. 25 mg of one guinea pig extract was required to kill a 10^7 inoculum of pneumococci; no rat extract at the same concentration was capable of destroying a similar inoculum. However, 25 mg of one rat extract completely destroyed 10^6 pneumococci. One guinea pig extract was effective in killing 10^6 organisms only in a concentration of 50 mg per ml, whereas extracts of two

TABLE 13

A COMPARISON OF THE PNEUMOCOCCIDAL ACTIVITY OF 50 PER CENT ETHANOL SOLUBLE EXTRACTS PREPARED FROM POOLED SAMPLES OF GUINEA PIG LIVER AND FROM POOLED SAMPLES OF RAT LIVER

Number of Animals Represented		Minimum Concentration Destroying completely, mg per ml		Maximum Inoculum Completely Destroyed in 6 hours, Colony Count per 0.1 ml	
Guinea Pig	Rat	Guinea Pig	Rat	Guinea Pig	Rat
10	8	1.75	50	10^7	10^3
14	8	0.1	50	10^7	10^3
14	14	1.0	10	10^7	10^4
14	14	0.1	50	10^7	10^2
14	14	0.3	50	10^7	10^2
14	14	0.3	10	10^7	10^2
6	14	7.0	10	10^6	10^2
8	6	0.01	10	10^8	10^2
16	12	3.0	100	10^7	10^7
10	14	0.1	10	10^7	10^2
20	32	1.0	10	10^7	10^2
2	8	1.0	50	10^7	10^3

TABLE 14

A COMPARISON OF THE PNEUMOCOCCIDAL ACTIVITY OF 50 PER CENT ETHANOL SOLUBLE EXTRACTS OF THREE INDIVIDUAL GUINEA PIGS AND OF THREE INDIVIDUAL RATS

Species	Extract Concentration, mg per ml	Initial Inoculum, Colony Count per 0.1 ml	Animal Number		
			1	2	3
Guinea Pig	50	4×10^7	0*	0	0
	25	4×10^7	0	0	0
	12.5	4×10^7	0	0	0
	6.25	4×10^7	0	0	0
Rat	50	4×10^7	2×10^5	2×10^6	6×10^4
	50	4×10^4	1×10^3	1×10^4	4×10^2
	50	4×10^3	1×10^2	4×10^2	4×10^1
	50	4×10^2	0	0	0

* These figures represent colonies per ml after 6 hours incubation (at 37 C) of extract with the inoculum.

TABLE 15

A COMPARISON OF THE PNEUMOCOCCIDAL ACTIVITY OF 50 PER CENT ETHANOL SOLUBLE LIVER EXTRACTS OF THREE INDIVIDUAL GUINEA PIGS AND OF THREE INDIVIDUAL RATS

Species	Extract Concentration, mg per ml	Initial Inoculum, Colony Count per 0.1 ml	Animal Number		
			4	5	6
Guinea Pig	50	5×10^7	0*	0	0
	25	5×10^7	0	0	0
	12.5	5×10^7	1×10^2	0	0
	6.25	5×10^7	1.3×10^2	0	0
Rat	50	5×10^7	1×10^6	1×10^7	4×10^6
	50	5×10^4	2×10^4	1×10^4	2×10^5
	50	5×10^3	1×10^3	1×10^3	2×10^4
	50	5×10^2	1×10^2	1×10^2	2×10^2

* These figures represent colonies per ml after 6 hours incubation (at 37 C) of extract with the inoculum.

TABLE 16

A COMPARISON OF THE PNEUMOCOCCIDAL ACTIVITY OF 50 PER CENT ETHANOL SOLUBLE EXTRACTS OF THREE INDIVIDUAL GUINEA PIGS AND OF THREE INDIVIDUAL RATS

Species	Extract Concentration, mg per ml	Initial Inoculum, Colony Count per 0.1 ml	Animal Number		
			7	8	9
Guinea Pig	50	6×10^6	1×10^4*	4×10^3	0
	50	6×10^3	0	0	0
	25	6×10^6	3×10^4	1×10^4	1×10^3
	12.5	6×10^6	2×10^4	1×10^4	1×10^4
	6.25	6×10^6	1×10^4	3×10^4	3×10^4
Rat	50	6×10^6	0	1×10^3	3×10^4
	50	6×10^3	0	0	3×10^1
	50	6×10^2	0	0	0
	25	6×10^6	0	---	---
	12.5	6×10^6	3×10^1	---	---
	6.25	6×10^6	1×10^2	---	---

* These figures represent colonies per ml after incubation of extract with the inoculum for 6 hours at 37 C.

TABLE 17

A COMPARISON OF THE PNEUMOCOCCIDAL ACTIVITY OF 50 PER CENT ETHANOL SOLUBLE EXTRACTS OF ONE GUINEA PIG AND OF ONE RAT

Species	Extract Concentration, mg per ml	Initial Inoculum, Colony Count per 0.1 ml	Animal Number
			10
Guinea Pig	50	1×10^8	0*
	25	1×10^8	0
	12.5	1×10^8	0
	6.25	1×10^8	0
Rat	50	1×10^8	4×10^8
	50	1×10^7	1×10^7
	50	1×10^4	5×10^4

* These figures represent colonies per ml after incubation of extract with the inoculum for 6 hours at 37 C.

TABLE 18

A SUMMARY OF THE PNEUMOCOCCIDAL ACTIVITY OF 50 PER CENT ETHANOL SOLUBLE LIVER EXTRACTS PREPARED FROM TEN INDIVIDUAL RATS AND FROM TEN INDIVIDUAL GUINEA PIGS

Minimum Concentration Completely Destroying, mg per ml	Maximum Inoculum Completely Destroyed Colony Count per 0.1 ml	Number of Individuals Having This Effect	
		Guinea Pig	Rat
6.25	10^7	6	0
25.0	10^7	1	0
25.0	10^6	0	1
50.0	10^6	1	0
50.0	10^3	2	1
50.0	10^2	0	4
50.0	0	0	4

guinea pigs, in the same concentration, destroyed completely only 10^3 pneumococci. 50 mg of one rat extract destroyed completely 10^3 organisms. 50 mg of four rat extracts destroyed completely only 10^2 organisms whereas extracts of four rats at a maximum concentration (50 mg per ml) were not effective in killing even a minimum inoculum (10^2).

It is apparent from these data that there is, within the species, a variation of the pneumococcidal activity of these liver extracts. Furthermore, it is again observed that the extracts of guinea pig livers are significantly superior to those of rat livers in pneumococcidal effect. A record was kept of individual weights of the livers used in these experiments but no correlation could be drawn between the weight of the tissue and its pneumococcidal activity.

Extracts of Liver, Brain and Spleen.

Liver extracts of the rat and of the guinea pig have shown a marked difference in pneumococcidal activity but it has been reported that there might be a species difference in the efficiency with which the reticulo-endothelial organs function in the clearance mechanism (14). Possibly then the difference noted in the biological activity of the liver extracts of the two species might not hold true for other organs or the difference might be in inverse relation.

Three tissues, the liver, brain, and spleen, were removed from 14 guinea pigs, and after the pooling of each, 50 per cent ethanol soluble extracts were prepared in the usual manner. Similar extracts were prepared from the tissues of 14 rats. The extracts of both species were tested simultaneously. This experiment was repeated three times; thus three different lots of pooled extracts, representing a total of 42 animals in each

species, were prepared and in the case of each lot, extracts from both species were tested simultaneously for pneumococcidal effect.

The data from these experiments (Tables 19, 20, and 21) reveal in each species a close parallelism in pneumococcidal effect of extracts prepared from the liver and the spleen, but the extracts of both of these tissues in the case of the guinea pig are superior in activity to those of the rat. The brain extract in both species is of a comparatively low order of activity.

III. Fractionation of Dried 50 Per Cent Ethanol Soluble Extract.

The 50 per cent ethanol soluble extract is, admittedly, a very gross fraction. To gain information concerning the nature of the active principle and to study the basis of the difference in the extracts of the two species it was necessary to attempt to further purify this gross extract.

Certain properties of the 50 per cent ethanol soluble extract, such as the fact that it is coctostabile, only weakly soluble but easily suspendable in an aqueous medium, soluble in 50 per cent ethanol and non-Seitz filterable, suggested that the chemical nature of the active principle might be that of a lipid.

Orientation fractionation experiments with 50 per cent ethanol soluble extracts of guinea pig liver and non-purified solvents revealed that the active factor was both ethanol soluble and ether soluble. When the ether soluble fraction was precipitated with acetone the active factor was found in the acetone soluble fraction. The mass of the material was considerably reduced during this fractionation since a large proportion of the original mass was insoluble in absolute ethanol; 1000 mg of

TABLE 19

A COMPARISON OF THE PNEUMOCOCCIDAL ACTIVITY OF 50 PER CENT ETHANOL SOLUBLE EXTRACTS OF THREE ORGANS OF THE RAT AND OF THE GUINEA PIG

Species	Extract Concentration, mg per ml	Initial Inoculum, Colony Count per 0.1 ml	* Liver	Spleen	Brain
Guinea Pig	100	7×10^7	0**	0	4×10^5
	100	7×10^4	0	0	0
	100	7×10^2	0	0	0
	10	7×10^7	0	---	---
	1	7×10^7	0	---	---
	0.1	7×10^7	0	---	---
	0.01	7×10^7	1×10^4	---	---
Rat	100	7×10^7	6.9×10^2	---	2×10^1
	100	7×10^4	1×10^1	0	0
	100	7×10^2	0	0	0

* Pooled tissues of 14 animals

** These figures represent colonies per ml after incubation of extract with the inoculum for 6 hours at 37 C.

TABLE 20

A COMPARISON OF THE PNEUMOCOCCIDAL ACTIVITY OF 50 PER CENT ETHANOL SOLUBLE EXTRACTS OF THREE ORGANS OF THE RAT AND OF THE GUINEA PIG

Species	Extract Concentration, mg per ml	Initial Inoculum Colony Count per 0.1 ml	* Liver	Spleen	Brain
Guinea Pig	50	3×10^7	0**	0	1×10^5
	50	3×10^4	0	0	5×10^1
	50	3×10^2	0	0	0
	10	3×10^7	0	0	---
	1	3×10^7	0	0	---
	0.1	3×10^7	0	0	---
	0.01	3×10^7	6×10^3	TNC	---
Rat	50	3×10^7	5×10^3	1×10^6	8×10^2
	50	3×10^4	2×10^1	1×10^2	0
	50	3×10^2	0	0	0

* Pooled tissues of 14 animals.

** These figures represent colonies per ml after incubation of extract with the inoculum for 6 hours at 37 C.

TABLE 21

A COMPARISON OF THE PNEUMOCOCCIDAL ACTIVITY OF 50 PER CENT ETHANOL SOLUBLE EXTRACTS OF THREE ORGANS OF THE RAT AND OF THE GUINEA PIG

Species	Extract Concentration, mg per ml	Initial Inoculum, Colony Count per 0.1 ml	* Liver	Spleen	Brain
	10	5×10^7	0**	0	1×10^6
	10	5×10^4	0	0	1×10^3
Guinea	10	5×10^2	0	0	0
Pig	1	5×10^7	0	0	---
	0.1	5×10^7	3×10^3	7×10^1	---
	0.01	5×10^7	2×10^5	3×10^3	---
	10	5×10^7	5×10^7	7×10^6	5×10^4
Rat	10	5×10^4	1×10^5	5×10^4	1×10^2
	10	5×10^2	1×10^2	0	0

* Pooled tissues of 14 animals.

** These figures represent colonies per ml after incubation of extract with the inoculum for 6 hours at 37 C.

dried 50 per cent ethanol soluble extract yielded 50 mg or less of the final fraction (ethanol-ether-acetone extracted). The insoluble fractions of this stepwise fractionation of 50 per cent ethanol soluble extract were not found to be bactericidal.

Fractionation Procedure.

The fractionation technique finally developed is diagramed in Figure 4. A weighed amount of dried 50 per cent ethanol soluble extract was further extracted with purified absolute ethanol by grinding on the ball mill for 18 to 24 hours. The insoluble material was removed by centrifugation at approximately 4000 x G for one hour. An aliquot of this ethanol extracted fraction was held for drying and testing. The remainder was dried by boiling under reduced pressure in a 40-50 C water bath. To this was added a measured amount of purified ethyl ether. The ether extraction was carried out by allowing the mixture to stand at 5 C for 18 to 24 hours. The ether insoluble fraction was removed by centrifugation. An aliquot of the ethanol-ether-extracted fraction was held for drying and testing. The remainder of this fraction was concentrated by boiling under reduced pressure. This concentrated ethanol-ether extracted fraction was then added to an excess of cold purified acetone. The precipitate which formed was removed by centrifugation. The clear supernatant was dried by boiling under reduced pressure.

Pneumococcidal Activity of Fractions.

For testing the pneumococcidal activity of the three fractions, it was necessary to suspend the materials by homogenizing in B.H.I. broth by means of Potter-Elvejhem type glass homogenizers. In order to

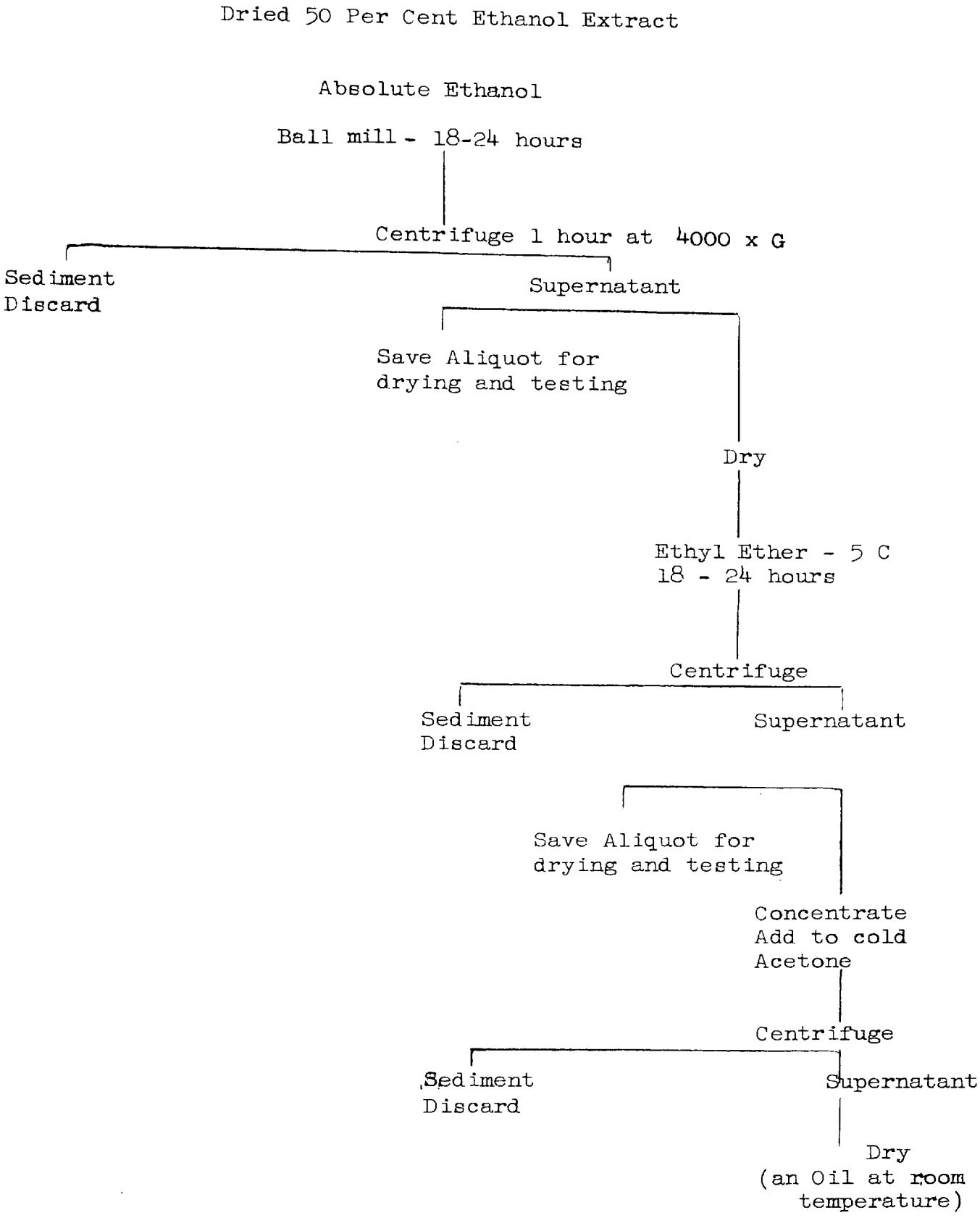


Figure 4. Flow Diagram of the Fractionation of 50 Per Cent Ethanol Soluble Extract with Organic Solvents.

have a basis for comparison of the pneumococcidal activity of each fraction with that of the dried 50 per cent ethanol extract, the final concentration of each suspension was made equivalent to that of the original dried extract. Thus, if 1 ml of broth contained 10 mg of dried extract, 1 ml of the ethanol, ether, or acetone soluble suspensions contained the equivalent of 10 mg of dried extract.

Such fractions were prepared from dried guinea pig liver extracts and dried rat liver extracts. These fractions, with the original 50 per cent ethanol soluble extract, were tested simultaneously for pneumococcidal activity. Three experiments such as this were carried out for each species on three different lots of extract. Representative data are recorded in Table 22.

This type of experiment reveals an interesting difference in the guinea pig fractions and the rat fractions. Whereas the same degree of activity is evident in all the guinea pig fractions, purification of the 50 per cent ethanol soluble extract of rat liver markedly enhances its pneumococcidal effect. There had been some indication of this in the earlier orientation experiments and it was again shown consistently in the three experiments of this type which have just been described. It should be pointed out, however, that in all three experiments there was still a quantitative difference between the pneumococcidal activity of the final fraction (ethanol→ ether→ acetone extracted) of the guinea pig and that of the rat. In all three instances there was a tenfold difference in favor of the guinea pig fraction.

Infrared Analysis.

In order to have some control, other than a biological one, upon the reproducibility of organic solvent fractions obtained in the break-

TABLE 22

A COMPARISON OF THE PNEUMOCOCCIDAL ACTIVITY OF ORGANIC SOLVENT FRACTIONS OF THE 50 PER CENT ETHANOL SOLUBLE EXTRACTS OF GUINEA PIG LIVER AND OF RAT LIVER

Species	Equivalent Concentration, mg per ml	Initial Inoculum Colony Count per 0.1 ml	*50 Per Cent Ethanol Soluble Extract	Ethanol Extracted Fraction	Ethanol, Ether Extracted Fraction	Ethanol Ether, Acetone Extracted Fraction
Guinea Pig	10	3×10^7	0**	0	0	0
	1	3×10^7	0	0	0	0
	0.1	3×10^7	1×10^1	9×10^3	1×10^4	5×10^2
	0.01	3×10^7	2×10^5	6×10^5	1×10^6	5×10^5
	10	3×10^7	1×10^5	5×10^3	0	0
	10	3×10^4	6×10^4	0	0	0
Rat	10	3×10^2	0	0	0	0
	1	3×10^7	---	2×10^5	2×10^5	1×10^5
	0.1	3×10^7	---	1×10^6	1×10^9	3×10^5

* The pH of all fractions in suspension had a range of 7.1 - 7.3.

** These figures represent colonies per ml after incubation of fractions with the inoculum for 6 hours at 37 C

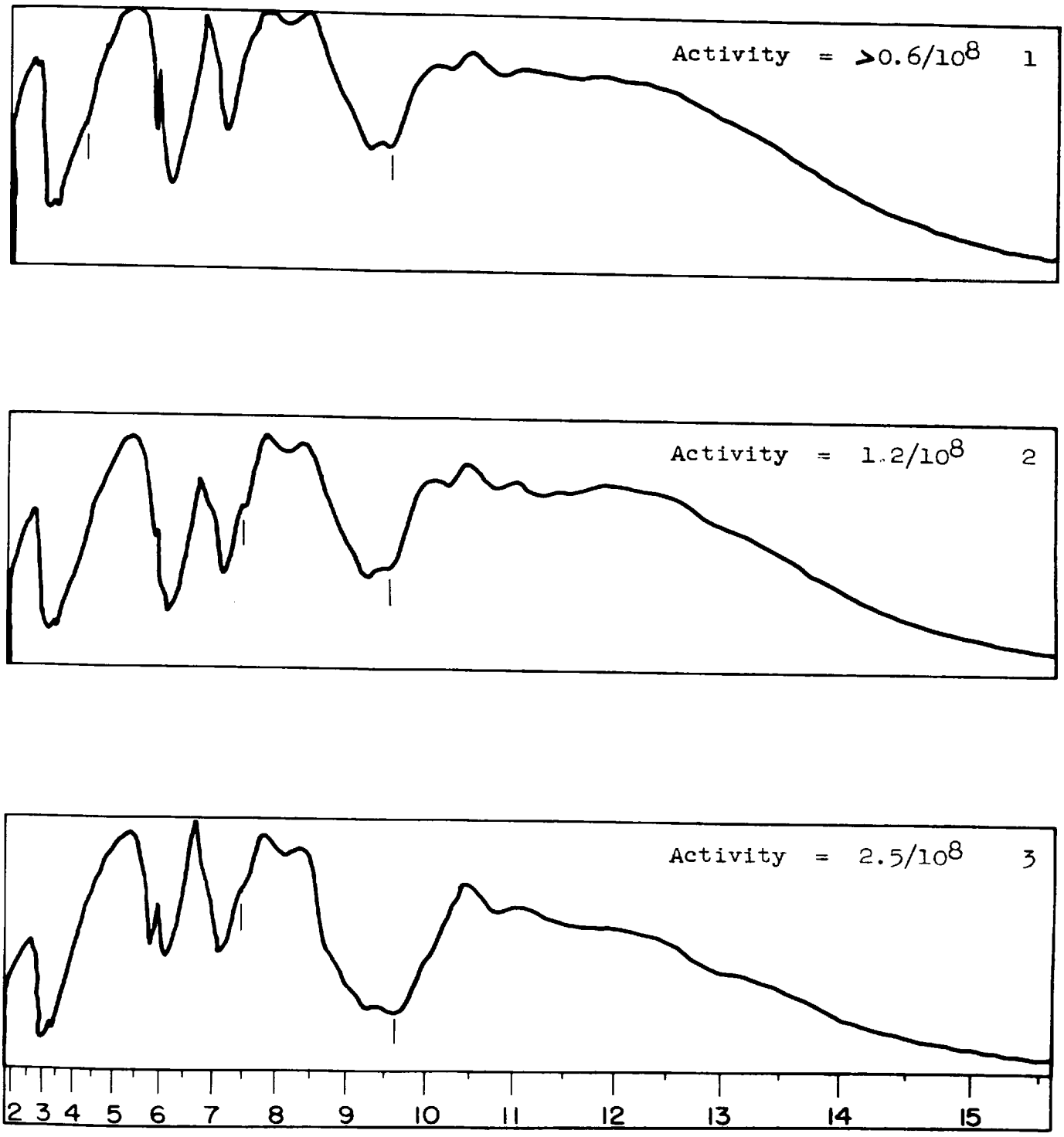
down of dried 50 per cent ethanol soluble extracts, infrared spectrographs were made on three fractions obtained from two different lots of guinea pig liver extracts and of rat liver extracts of known pneumococcidal strength. The material was prepared for analysis according to the method of Randall, et al (48)*

Earlier infrared spectrographs of the 50 per cent ethanol soluble extract (three different lots) indicated some degree of reproducibility even though this admittedly is an extremely gross fraction. These three spectrographs are shown in Figure 5. In record 1 there is an absorption at 4.23μ which is not present in the other and in record 2 absorption at 7.48μ is stronger than in the other two. Beyond 9.6μ there appears to be no similarity in the three graphs. Records 1 and 2, which, in general, are the most similar, are from lots of extracts having very nearly the same degree of biological activity, while record 3, which is most different, is from the lot of extract the least active biologically.

In Figure 6, records 1 and 2 were obtained from the ethanol extracted fraction of two different lots of dried 50 per cent ethanol soluble extracts of guinea pig liver. It is apparent that they are essentially the same. Records 3 and 4 represent the ethanol-ether extracted fraction obtained from the same two lots of liver extract. Here again the similarity of the two graphs indicates a high degree of reproducibility. Records 5 and 6 represent the ethanol-ether-acetone extracted fraction obtained from the original two lots of extract. Any differences between these two are very minor.

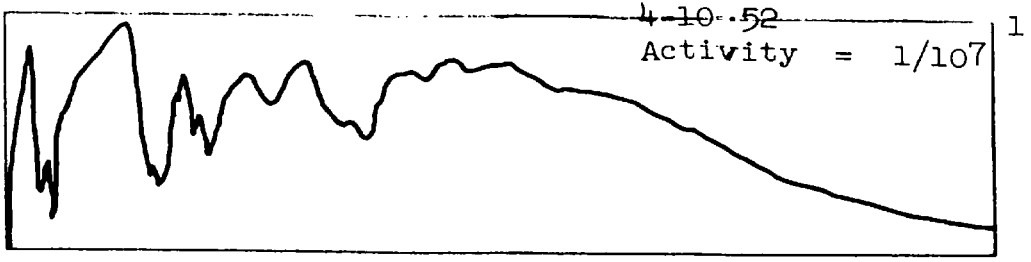
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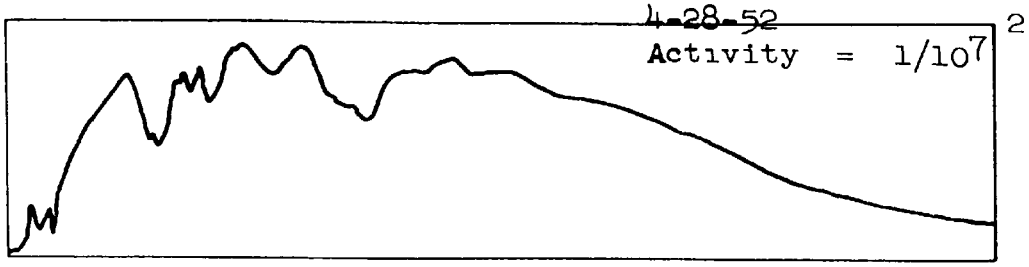
* Infrared spectrographs and interpretations were made in the laboratory of Professor H. M. Randall. His cooperation and that of his staff is greatly appreciated.



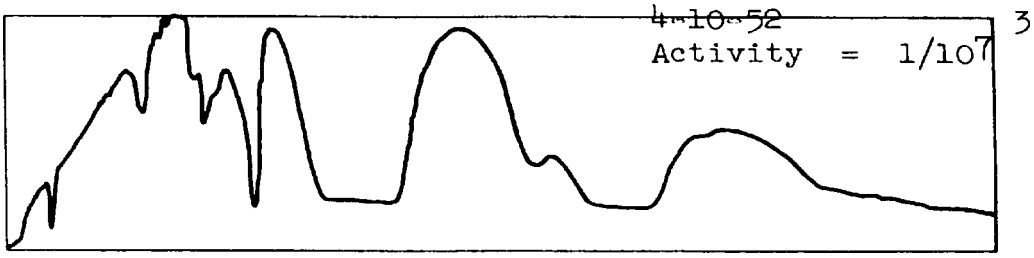
Activity = mgs/maximum inoculum destroyed

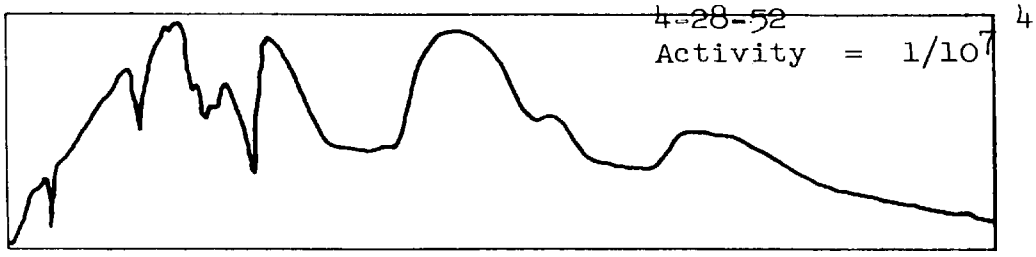
Figure 5. Infrared Spectrographs of 50 Percent Ethanol Soluble Extracts of Guinea Pig Liver.



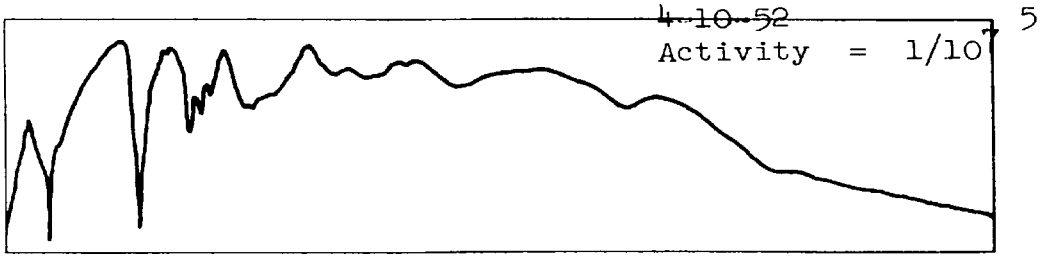


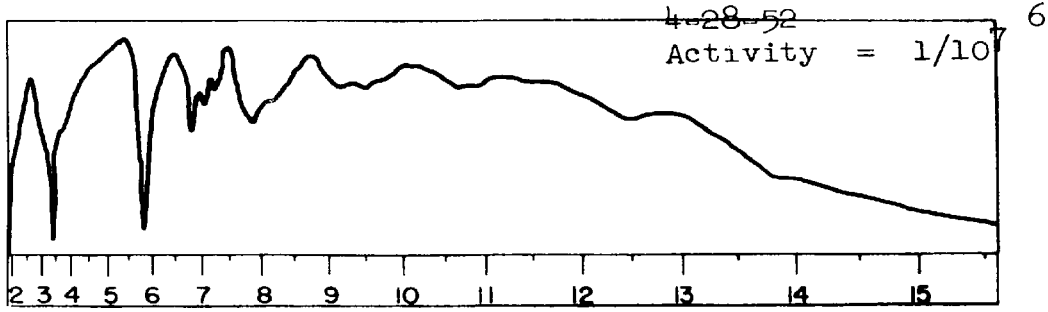
Ethanol Extracted Fraction





Ethanol-Ether Extracted Fraction





Ethanol-Ether-Acetone Extracted Fraction

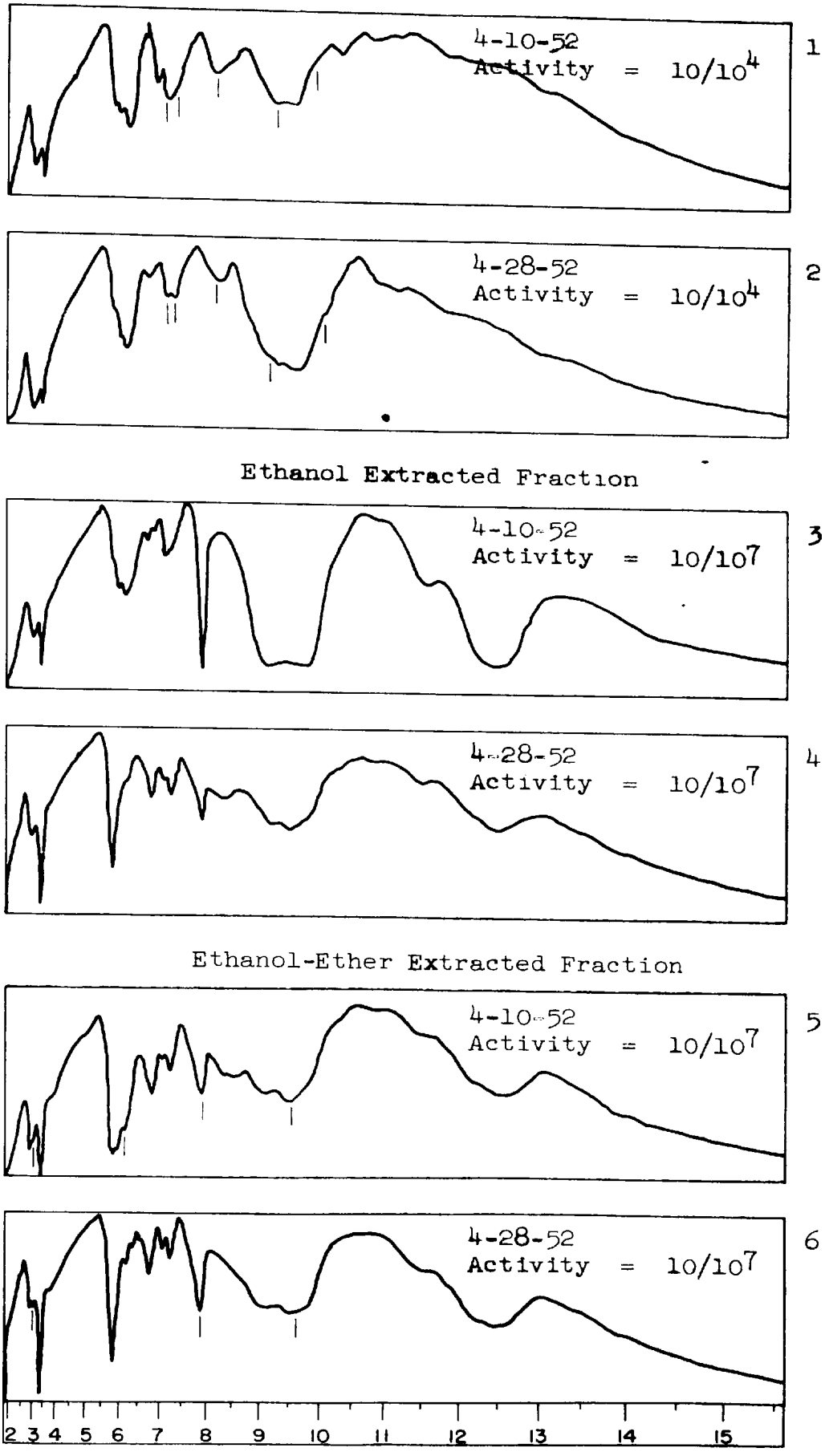
Activity = Equilivant mgs/maximum inoculum destroyed

Figure 6. Infrared Spectrographs of Organic Solvent Fractions of Two 50 Percent Ethanol Soluble Extracts of Guinea Pig Liver.

The rat liver fractions present a different picture. Each set of fractions was prepared simultaneously with and under identical conditions as for the corresponding set of guinea pig fractions. And, as for the guinea pig, these fractions are derived from the 50 per cent ethanol soluble extract of rat liver.

In Figure 7 records 1 and 2 are made from the ethanol extracted fractions. There is some question of the reproducibility of two bands, 7.12μ and 7.30μ and some question of the intensity of these two bands. There are differences between 8.13μ and 9.24μ and a number of differences from 10μ on. Thus the overall reproducibility of this fraction is poor Records 3 and 4, representing the ethanol-ether extracted fraction prepared as described, show obvious differences which indicate extremely poor reproducibility. In records 5 and 6, made from ethanol-ether-acetone extracted fractions of the same two lots of extract, absorption at 2.95μ is stronger in record 6 than in record 5. However, this apparent difference at 2.95μ and another at 6.15μ may be due to incomplete removal of water from the samples during preparation of the material for the spectrographic analysis. With the exception of minor differences in intensities between 7.92μ and 9.77μ the remainder of these two graphs are quite similar.

As has been shown with biological methods, the 50 per cent ethanol soluble liver extracts obtained from the guinea pig and from the rat are quite different quantitatively. The stepwise fractionation of this rat extract has yielded an ethanol soluble-ether soluble-acetone soluble fraction which more nearly approaches the biological activity of the corresponding fraction of the guinea pig extract. Perhaps, then, a comparison of the infrared spectrographs of the three fractions obtained from the tissue extracts of the two different species might prove valuable in



Ethanol-Ether-Acetone Extracted Fraction

Activity = Equilivant mgs/maximum inoculum destroyed

Figure 7. Infrared Spectrograph of Organic Solvent Fractions of Two 50 Percent Ethanol Soluble Extracts of Rat Liver.

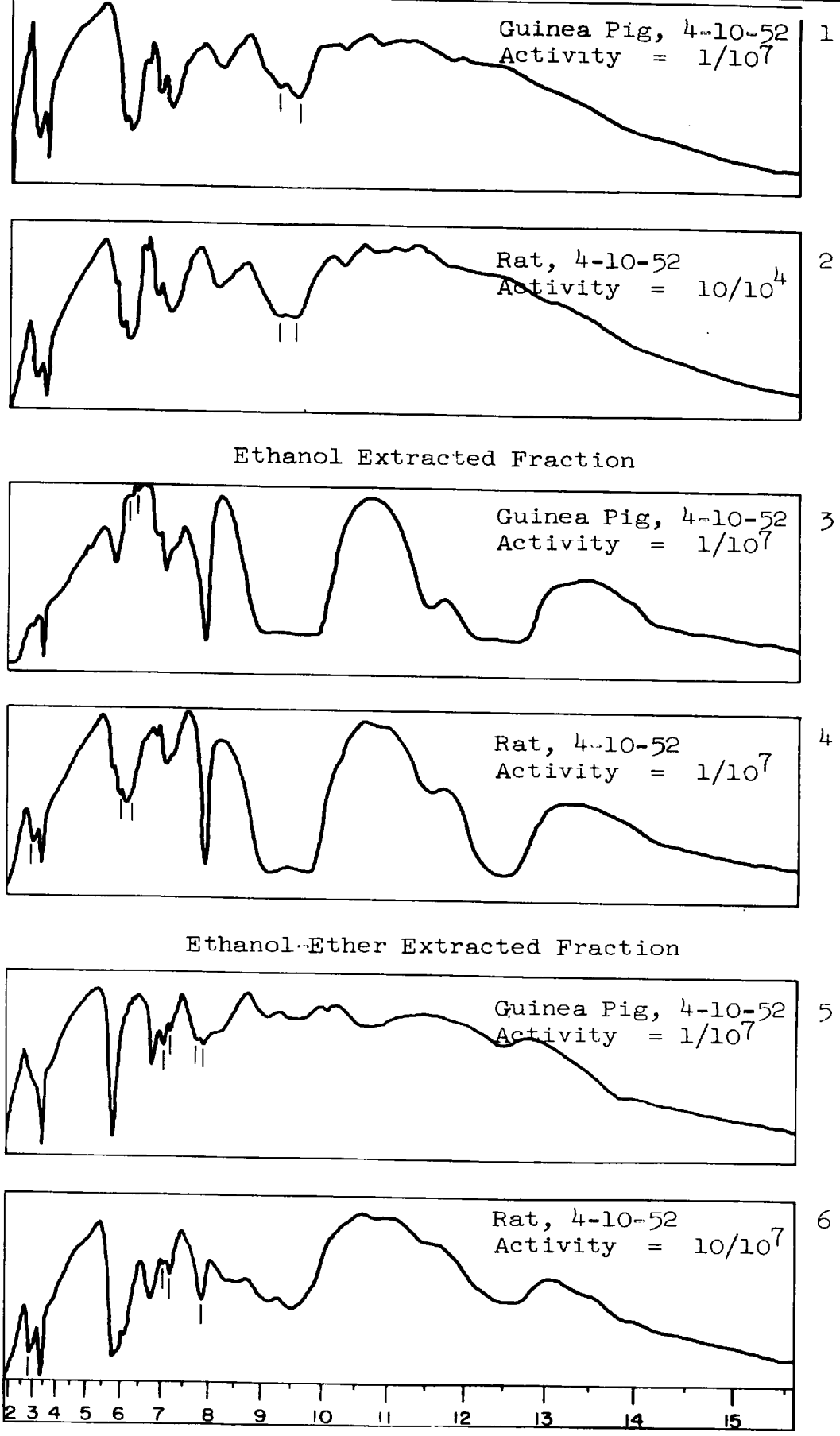
detecting any qualitative similarities or differences.

Thus, in Figure 8, a comparison of the graphs of the ethanol extracted fractions of the guinea pig extract and of the rat extract, shows, in the case of the rat, a pair of bands at 9.24μ and 9.52μ more nearly equal in intensity than in the case of the guinea pig. In the guinea pig graph the 9.52μ band is uniformly the most intense. However, grossly the chemistry of the two fractions appears to be very similar.

The ethanol-ether extracted fractions show a high degree of similarity between the rat and the guinea pig provided record 4 in Figure 7 is disregarded (this preparation, judging from the spectrographs of both the ethanol extracted fraction and the fraction just mentioned, is the one most different). In spite of this similarity there is an absorption at 3.01μ in the rat fraction which is not present in the guinea pig fractions. Furthermore, still disregarding record 4 in Figure 7, the intensity of bands from 5.97μ to 6.27μ is greater in the case of the rat than in the case of the guinea pig.

Comparing the graphs of the ethanol-ether-acetone extracted fraction, there is a more intense absorption at 2.96μ for the rat than for the guinea pig. For the guinea pig there is a more intense absorption at 7.05μ than at 7.23μ, while for the rat the reverse is true. For the rat there is a strong single absorption at 7.88μ. For the guinea pig this absorption is weaker and is accompanied by a still weaker band at 7.90μ. Beyond 7.92μ there are obvious differences in contour indicating these two substances are not entirely similar chemically.

In summary these infrared spectrographs indicate that the fractionation technique is sufficiently refined to yield spectrographically reproducible fractions in the case of the guinea pig. In the rat, even



Activity = Equilivant mgs/maximum inoculum destroyed

Figure 8. A Comparison of Infrared Spectrographs of Organic Solvent Fractions of a 50 Percent Ethanol Soluble Extract of Quinea Pig Liver and of Rat Liver.

though from the standpoint of biological activity the fractions are reproducible, according to the infrared spectrographs there are some differences.

A comparison of infrared spectrographs of the fractions from the two different species seems to point to a qualitative as well as a quantitative difference between the ethanol-ether-acetone extracted fractions, this being the most purified of all the fractions. However, it should be pointed out that this fraction is still not a single component and that with the infrared spectrographic method of analysis, the impurities present which are not concerned in the biological activity of the material may tend to obscure the true picture.

DISCUSSION

The fundamental basis for the natural immunity or innate resistance of a species or of an individual of a species for a given parasite is an involved and complex problem which thus far has defied full clarification. It was with the hope of possibly contributing data directly bearing on this question and in so doing opening a way for the development of an indirect means for measuring man's resistance to a given infective agent that this present study was undertaken.

The very nature of the pathogenesis of infection with the pneumococcus, the fact that it is primarily an invasive organism, propagating within the tissues and causing damage there through its endotoxicity rather than its exotoxicity, makes it a particularly suitable organism for studies of tissue resistance.

For a comparative study of resistance to this type of infection, two species having a marked disparity in susceptibility to pneumococcal invasion were desired, and the white rat, as the susceptible host and the guinea pig, as the resistant host, were chosen as appropriate for these experimental purposes.

The tissues rich in fixed phagocytic cells have also been shown to be the tissues most resistant to local infection (9), and it has many times been shown that such tissues play an important role in the removal of injected bacteria from the blood stream and in the ultimate destruction of these organisms (10, 11, 12, 13). Consequently, the reticulo-endothelial tissues were the ones chosen for study.

Adami (49) concluded that phagocytosis was an important mechanism in the destruction of Bacillus anthracis but postulated also an extracellular destruction as a result of disintegration of phagocytes with the liberation of bactericidal substances. The inference here is that there is a chemical killing agent which functions extracellularly as well as intracellularly.

The primary goal of the present investigation was the isolation of a bactericidal agent from the tissues of a resistant host which exhibited a significantly higher degree of activity for the Type I pneumococcus than a material obtained in the same manner from the tissues of a susceptible host. If it could be postulated then, that this bactericidal factor existed as such in the tissue under consideration and that it could function in vivo as it did in vitro, being active in the resistant animal and relatively inactive in the susceptible animal, it might then be considered as one of the mechanisms involved in the innate resistance of the species, or of an individual, to infection with the Type I pneumococcus.

The data which have been presented in this study definitely point to the presence of a pneumococcidal factor in certain tissues of the resistant species. In the susceptible species the situation appears to be quite different. Either the factor is present in this species in low concentration, is present under certain circumstances as to be relatively inactive or is a different entity of inferior pneumococcidal power. As to which of these situations is the most likely one will be discussed later.

All of the evidence, to date, has been obtained from in vitro studies and thus far there has been no direct evidence which would permit

a correlation of the *in vitro* activity of tissue extracts with a possible *in vivo* function of this material. However, even though at present such direct evidence is not available, certain findings seem to lend support to the concept of this pneumococcidal factor existing in a functional capacity within the tissues and presumably within the cells of these tissues.

The evidence that this active agent can be extracted with water or with 50 per cent ethanol without previous incubation of the tissues and that it is not dependent on blood or bile for its activity is strongly suggestive that this material exists and is active *in vivo*. Ponder (50) is of the opinion that certain lytic substances, which have been extracted from blood and tissues not previously incubated, are present and function as such in normal blood and plasma and "by inference in normal tissue". And with this same line of reasoning and with the strong suggestive evidence, it is easy to visualize in the tissue juice and within the phagocytic cells of the liver of the guinea pig this agent which acts to destroy the invading pneumococci which have escaped the other defenses of the body. Thus, this pneumococcidal agent could play an important role in this species' overall resistance to infection with the Type I pneumococcus. Whereas in the tissue juice and cells of the rat this agent is either present in very low concentration, is present but together with an inhibitor complex, or is a different material of relatively low pneumococcidal power. If any one or more of these conditions should exist in the rat liver then that species would be less able to dispose of pneumococci which had evaded the other defenses and thus would be susceptible to infection.

With the evidence obtained from the studies of this particular resistant host-susceptible host system such a concept as the one just discussed would seem tenable and certainly a basis for further investigation using the same organism but different resistant and susceptible hosts. Or, if this pneumococcidal factor is found to be effective in killing other organisms, then the problem could be investigated using one of these organisms and a corresponding resistant host-susceptible host system. With such a broad approach to the problem, data might then be accumlated on bactericidal tissue extracts which could be correlated with the resistance of a given species for a given infection.

There was considerable speculation as to the basis for the difference in the pneumococcidal activity of the extracts prepared from the tissues of the two species. A comparison of the pneumococcidal strength of the fractions obtained by the stepwise fractionation of the 50 per cent ethanol soluble extract seems to give some insight into this question. It was found that such a fractionation of rat liver extracts significantly enhanced the pneumococcidal effect of this material. Thus the ethanol-ether-acetone extracted fraction obtained from the rat liver extract, in equivalent concentration, was far more active than the original dried extract. This was not found to be true in the case of the guinea pig as all fractions, in equivalent concentration, were equally as active as the original extract. Apparently then, in the 50 per cent ethanol soluble extract (and by inference in the water soluble extract and in the liver itself) of the rat liver there exists an inhibitor or an inhibitor complex which interferes with the pneumococcidal activity of the active agent. The overall activity of the extract, which has been shown to

vary to some extent among individuals of the species would then depend upon the ratio of the active agent to the inhibitor or inhibitor complex.

An analogy for such a hypothesis may be found in the work of Tyler (42, 43, 51) and Ponder (50, 53). In connection with hemolytic systems they both postulate the in vivo existence of an easily dissociable lysin-inhibitor complex and further that the hemolytic activity is dependent on the concentration of the free lysin together with the "buffering" effect of the inhibitor.

Although thus far there is no evidence that such an inhibitory mechanism operates in the guinea pig liver, fractionation of guinea pig extracts of relatively low pneumococcidal strength might reveal that such a situation may exist in individuals of this species. On the other hand, in the guinea pig, the variation within the species of the pneumococcidal strength of liver extracts may be due solely to a quantitative difference in the active agent.

It is further suggested, and to some extent supported by the infrared studies as well as by the biological tests, that other than the presence of an inhibitor complex in the rat extract there also may be a qualitative difference between the active agents in the extracts obtained from the two species. In the three fractionation experiments reported, though there was a definite enhancement in the pneumococcidal activity of the final fraction in the case of the rat, this fraction was yet only 1/10 as active as the corresponding fraction in the case of the guinea pig. Furthermore, on examination of the infrared spectrographs of these two fractions it is seen that there is enough difference between the two to suggest a chemical difference in the active agents. Possibly then, the active agent in the

rat extracts is of the same chemical nature as that in the guinea pig but is enough different, molecularly or configuratively, to be inferior in its ability to destroy the pneumococcus. However, as pointed out in the section on infrared studies, this ethanol-ether-acetone extracted fraction is not a single unit and under these circumstances the infrared spectrographs may reflect impurities which are not concerned in the biological activity of the material, and thus the differences noted in the spectrographs of this fraction from the two species may be more apparent than real. If the active agent could be isolated as a single component, then infrared spectroscopy should be most helpful in more conclusively answering this question.

On further reviewing the infrared spectrographs of fractions of the 50 per cent ethanol soluble extracts, the most surprising observation is the very poor reproducibility of the rat fractions as compared to the guinea pig fractions. This becomes increasingly difficult to explain when it is recalled that each set of fractions, that is, the three fractions each for the two species, were prepared simultaneously, with the same solvents and under identical conditions. Furthermore, the biological activity of these fractions is reproducible. It would appear then, that even though the duplicate fractions of the rat extract are different spectrographically, these differences are not reflected in the biological activity of the material. Moreover, since the guinea pig fractions are so closely reproducible according to the infrared spectrographs and the rat fractions obtained under identical conditions are not, this again points to a fundamental difference in the chemistry of the extracts prepared from the two species. Perhaps in the rat 50 per cent ethanol soluble

extract there are components, different from those in the corresponding guinea pig extract and not concerned in the biological activity of the material, which are labile to slight changes in the conditions of fractionation and this lability is then reflected in the spectrographs but not in the biological tests. The components of the guinea pig fraction, by the same reasoning, are not so labile and therefore spectrographic reproducibility of these fractions is more easily obtained.

While the observations seem to support this explanation for the apparent difficulty in spectrographically reproducing the fractions of rat liver extract, it should be pointed out that thus far the use of infrared spectroscopy as a control measure in these fractionations can only be considered as an orientation study. Analyses of many more samples would have to be made before any definite conclusions could be drawn concerning the reproducibility of fractions. From the standpoint of good chemistry, of course, it is highly desirable that the fractions be reproducible in spite of the fact that the spectrographic reproducibility or lack of reproducibility does not seem to be reflected in the biological activity of the material. Any correlation between the infrared spectrographs and the chemistry or the biological activity of the pneumococcidal agent must, of course, await further purification of the active agent.

As has been emphasized throughout this discussion the pneumococcidal agent has not been sufficiently purified to permit an exact chemical characterization. However, on the basis of solubilities, in 50 per cent ethanol and in purified organic solvents, and also on the basis of coctostability, and certain apparent physical characteristics, such as inactivation by serum, adsorption onto protein, and the oily consistency of the

acetone soluble fraction, the pneumococcidal agent appears to be most closely related to the lipids and within that group, to the fatty acids or fatty acid esters. Although fatty acids are generally considered highly insoluble in water, and even in 50 per cent ethanol, exceptions to this may be found especially in the hydroxy acids and certain esters of the fatty acids.

There have been a number of reports concerning the bactericidal nature of lipids, particularly the fatty acids and soaps. Noguchi (20) considered the bactericidal agent he obtained from tissues to consist of soaps of the higher unsaturated fatty acids. Lamar (24) studied the action of potassium soaps of oleic, erucic, linoleic, and linolenic acid on the pneumococcus. He found potassium linoleate and potassium linolenate to be particularly active in lysing this organism. Avery (54) incorporated sodium oleate into media and found it to inhibit the growth of certain gram positive organisms, particularly the pneumococci and streptococci. Walker (55) found Staphylococcus aureus resistant to all soaps, whereas sodium laurate in 0.002 per cent concentration killed the pneumococcus in 15 minutes.

Of a number of fatty acids and fatty acid esters tested by Dubos (56), oleic, ricinoleic, linoleic, linolenic and arachidonic proved to have the most pronounced bacteriostatic and bactericidal effect, particularly on the human tubercle bacillus. Of the esters, he found water soluble esters of lauric acid and palmitic acid both bacteriostatic and bactericidal in concentrations of 0.01 to 0.001 per cent whereas the esters of stearic and oleic became inhibitory only in higher concentration. Kodicek and Worden (57, 58) found linolenic, linoleic, and oleic, in that

order, to inhibit the growth of Lactobacillus helveticus and other gram positive bacteria.

Lipids and fatty acids have also been reported to be virucidal. Casals and Olitsky (38, 39) showed that lipids from brain and serum were capable of inactivating certain neurotropic viruses. Pirie (26) checked certain commercial fatty acids and found that of the higher fat acids only the unsaturated ones were virucidal. She obtained a mixture of fatty acids from dried pancreas which inactivated certain viruses. Oleic, linoleic, and linolenic acids were found by Stock and Francis (59) to be effective in inactivating the virus of epidemic influenza.

There have also been some attempts to relate the bactericidal activity of soaps or fatty acids to an in vivo defense mechanism. Klotz (60), using histological techniques, demonstrated the accumulation of soaps and fat acids in inflammatory foci. Rothman and his coworkers (61) attributed the immunity of the adult scalp to tinea capitis to the fungistatic activity of certain fatty acids isolated from adult hair fat. Kligman and Ginsberg (62), although failing to agree in entirety with the Rothman group, did find an increase in hair fat after puberty and did concede that the fatty acids may play some role in the adult resistance to ringworm infection.

According to most of these reports the bactericidal power of the fatty acids or their esters appears to be directly related to their degree of unsaturation. Bloor (63) states that the fatty acids of the liver are more unsaturated than those of the fat stores. Possibly then, the pneumococcidal property of the 50 per cent ethanol soluble liver extracts is due to the presence of unsaturated fatty acids or fatty acid

esters released from tissue juice and cells of the liver during the process of homogenization and extraction.

It is recalled that with organic solvents the active factor appears in the acetone soluble fraction. In a lipid fractionation, neutral fat, cholesterol, free fatty acids or their esters could be found in the acetone soluble fraction. However, by first extracting the liver with 50 per cent ethanol much of the neutral fat and cholesterol have been left behind. Therefore, in the acetone soluble fraction derived from the 50 per cent ethanol soluble extract there should be a minimum of neutral fat and cholesterol, leaving a mixture composed principally of free fatty acids and fatty acid esters. And it is somewhere within this mixture that the pneumococcidal agent should be found.

SUMMARY

A tissue extract, bactericidal for the Type I pneumococcus, was prepared from certain tissues of the guinea pig and rat.

The pneumococcidal agent was shown to be weakly soluble in water, considerably more soluble in 50 per cent ethanol, resistant to boiling, non-Seitz filterable, and inactivated by serum.

The activity of this material was found to be independent of any pneumococcidal activity of the blood or bile. Also it was shown that the active agent is not a product derived from the tissues by enzymatic action.

50 per cent ethanol soluble extracts prepared from the pooled livers of guinea pigs were found to be significantly more pneumococcidal than such extracts prepared from pooled rat livers.

Extracts prepared from the tissues of individuals within each species were variable in pneumococcidal activity. Again, however, a higher degree of activity was noted in guinea pig extracts.

Spleenic extracts tend to parallel liver extracts in pneumococcidal activity, but extracts of brain were found to be of a low order of activity.

The 50 per cent ethanol soluble liver extract was partially purified by stepwise fractionation with organic solvents. The active agent was found in the ethanol-ether-acetone extracted fraction.

Evidence was presented for the existence of an inhibitor complex in the 50 per cent ethanol soluble extract of rat liver. No such inhibitory mechanism was demonstrated in such extracts of guinea pig liver.

Orientation studies on the use of infrared spectroscopy for controlling the reproducibility of fractions were presented. The use of infrared spectroscopy in detecting a chemical difference in the active agents isolated from the two species was discussed.

The possible chemical nature of the pneumococcidal agent was suggested.

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