A TOXIN NEUTRALIZING SUBSTANCE FROM PENICILLIUM CYANEO FULVUM

by

Benito Baruch Diena, M.V.D.

A Thesis

Submitted to the Faculty of Graduate Studies and

Research of McGill University in Partial Fulfillment

of the Requirement for the Degree of Master of Science.

July 1954

ACKNOWLEDGMENT

The candidate wishes to express his thanks to Professor E.G.D. Murray, Chairman of the Department of Bacteriology and Immunology for the opportunity of working in the department, for his advice and interest in the investigation.

The candidate expresses his deep appreciation to Dr. J.W. Stevenson who directed the research, for his generous support and for his stimulating and critical supervision. Special thanks are expressed to Dr. Van Stratten of the Montreal General Hospital Research Institute for her assistance in the experimental work done with paper chromatography and microelectrophoresis.

The candidate wishes to express his indebtedness to Dr. F. Blank for his helpful advice and to Mr. T. Salo for his valuable suggestions throughout this investigation.

TABLE OF CONTENTS

INTRODUCTION

A mould was isolated in this laboratory by Dr. D.G. Denton in 1947 which was found to produce in culture a potent toxin neutralizing substance. The mould was isolated as a contaminant from a Lowenstein's slope which had been inoculated with sputum for the bacteriological diagnosis of tuberculosis.

It was shown that the active toxic neutralizing principle was secreted into the medium during the growth of the mould in fluid cultures and that it may be demonstrated in mycelium free culture filtrates.

Since, other than the specific antitoxins, no toxin neutralizing substances have ever been found which have proven effective in the therapy of bacterial toxaemias it was felt that an attempt should be made to isolate and characterize the active principle and to investigate its spectrum of toxin neutralizing activity.

SECTION A

TOXIN NEUTRALIZING SUBSTANCES FROM MICROORGANISMS

AN HISTORICAL REVIEW

TOXIN NEUTRALIZING SUBSTANCES DERIVED FROM MICROORGANISMS

AN HISTORICAL REVIEW

I. A GENERAL SURVEY OF DETOXIFYING AGENTS PRODUCED BY MICROORGANISMS.

In the early 1890's Metchnikoff demonstrated that Bacillus subtilis was able to produce substances which neutralized diphtheria toxin, tetanus toxin and snake venom after a period of "in vitro" combination. It was further shown that fungi like Isaries, Sporotrichous and Torula, parasites of insects and human parasites, destroyed the toxicity of such toxins.

Calmette working with a strain of Bacillus subtilis isolated by Metchnikoff, observed that it was able to diminish the toxicity of snake venom. In 1902 Emmerish, Lowe and Koeshum reported that pyocyanase, produced by Pseudomonas aeruginosa, detoxified diphtheria toxin. Nicolle, in 1907, showed that in symbiotic cultures, Bacillus subtilis detoxified the toxins produced by Clostridium tetani, Clostridium chauvei and other bacteria.

The interest in detoxifying agents was stimulated by the work of Carpenter and Barbou who in 1938 demonstrated that sulphanilamide destroyed the toxicity of gonococcal endotoxins and the toxins of Staphylococcus aureus and Clostridium welchii, so that after a combination of few hours at 37°C. they were no longer active "in vitro" or "in vivo".

With the discovery of the antibiotics many experiments have been made to find out whether they might inhibit the production of toxins from bacteria, or neutralize their toxins "in vitro" and "in vivo". Controversial results have been recorded in the medical literature and no agreement has been reached at this time in respect to their possible therapeutic use.

Ramon and Richou in 1945 caused a new wave of interest in the field of the antibiotics in general and of the detoxifying agents in particular with a series of experiments on the filtrates of Penicillium notatum, Streptomyces griseus and Bacillus subtilis. They demonstrated in the filtrates potent substances, totally different from the antibiotic which these filtrates contain, which were able to detoxify certain bacterial toxins.

The following is a more detailed account of the literature concerning the toxin neutralizing activity of fungal and bacterial derivatives. -

a) Pure and commercial penicillin

Richou (1945) showed that 200 Units of highly purified penicillin did not destroy tetanus, diphtheria and staphylococcal toxins when incubated with the toxin for 6 days at 37°C. Blair (1946) and Segalove and Hite in 1947 claimed that the haemolytic, dermonecrotic and lethal effects of staphylococcal alpha toxin were not neutralized after incubation with penicillin. Segalove in 1947, however, demonstrated inhibition of the production of staphylococcal haemolysin, lethal factor and dermonecrotoxin but not enterotoxin by growing selected naturally and artificially

resistant strains in Amigin liquid medium containing sublethal concentration of penicillin.

In regard to neutralization of the diphtheria toxin, Ercoli (1945) showed that high concentrations of penicillin such as 10,000 units/ml. after 5 hours combination with diluted diphtheria toxin corresponding to 3-4 necrotizing doses gave complete inhibition of the toxin effect as tested in the skin of the rabbit. Crystalline penicillin G had the same effect as the commercial samples. Buxbaum, Nenner and Dolgopolo (1947) reported that doses of penicillin from 5,000 to 10,000 units protected to some extent guinea pigs inoculated intradermally with the diphtheria toxin. Ehrenberg, Fisher and Lofgren (1946) showed that a commercial preparation of penicillin delayed the diphtheria toxin-antitoxin flocculation reaction, and that it was more effective in this respect if it was mixed first with the toxin rather than the antitoxin. This suggested that it combined with the toxin.

Scheneirson (1947) reported that impure penicillin incubated with the toxin of Clostridium welchii, type A, failed to neutralize its haemolytic, lecithinase, necrotizing and lethal activities, each effect being tested separately.

Dowling and Hirsh (1946) following up the work of Boor and Meads who had observed a shortening of the course of scarlet fever in patients treated with penicillin, noted that a mixture of penicillin and erythrogenic toxin when injected intracutaneously into normal subjects gave no different results than did injections of toxin with isotonic salt solutions. They observed, furthermore,

that penicillin injected into the area of rash of patients with scarlet fever did not cause blanching, and that Dick positive patients, receiving pencillin, did not become Dick negative.

In spite of this lack of effect against a number of toxins especially "in vivo", Miller, Boor, and Hawk in a series of experiments from 1945 to 1948 were able to show that penicillin in sufficient dosage could diminish the effect on mice of the endotoxins of Neisseria intracellularis, Neisseria gonorrhoeae, Salmonella paratyphi A, Salmonella paratyphi B, Salmonella typhi-murium, Salmonella enteritidis, Shigella paradysenteriae and Aerobacter aerogenes. The crude endotoxins consisted of bacterial bodies suspended in water, held at pH 8 in the refrigerator for 14 hours, then neutralized and sterilized. Such suspensions, injected intraperitoneally in appropriate doses, killed the mice regularly. By giving penicillin repeatedly the death rate could be much reduced, although it was never brought to zero. Treatment which started 1-2 hours before the injection of endotoxin and continued for 24 hours afterwards, was found to be inferior to treatment in which large repeated doses totalling from 12,000 to 20,000 units were given in the 24 hours period before the injection of endotoxin. Even if penicillin injections were stopped some hours before the endotoxin was injected so that penicillin could no longer be detected in the blood the saving of life resulted. A single injection of 30,000 units in oil and bees wax also had a considerable effect. The intraperitoneal route was more effective than the subcutaneous. The toxin neutralizing activity was found in crystalline penicillins G,X,K,

and dihydro penicillin F and was abolished by penicillinase. Impure penicillin and intermediate fractions of industrial purification, according to the above cited Miller, Boor and Hawk had an activity of from 2 to 4 times as great as that of crystalline penicillin. The enhancement was due to a heat stable factor which was apparently lost to some extent in the preparation of the crystalline product. In experiments with Salmonella typhi murium Miller, Boor and Hawk established the amount of endotoxin which gave the LD 50, and administered it to three groups of 10 mice each. The first group received three injections of crystalline penicillin 20, 18 and 2 hours before the injection of the endotoxin. The second group received impure penicillin instead of crystalline penicillin and the third group served as a control. The LD 50 of the control group was 0.05 ml, that of the group treated with crystalline penicillin 0.11 ml., and that of the group treated with impure penicillin 0.26 ml. The data show that increasing amounts of endotoxin were necessary to establish an LD 50 with increasing activity of the detoxifying agent and that impure penicillin was more effective than crystalline pencillin.

In regard to tetanus toxin Neter (1945) reported a slight reduction in the activity of tetanus toxin by contact with 1,000 units/ml. of penicillin. Manzullo (1948) found that tetanus toxin was not neutralized by penicillin.

Imbriano (1952) tested the activity of penicillin on tetanus toxin working with semi-purified precipitates of culture filtrates of Clostridium tetani. He determined the

LD 50 as the quantity of toxin which killed 50 per cent of rats of 100 gms. in 120 to 144 hours following subcutaneous injection; with 1 LD 50 of toxin the animals died in 120 to 144 hours whereas 1 LD 50 of toxin mixed with 30 mgms. of penicillin (50,000 International Units) failed to produce symptoms. 2 LD 50s of the toxin caused tetanus and death in 120 hours. Animals treated with 50,000 units of penicillin developed tetanus in 72 hours which remained localized for three days in the posterior part of the body and until the ninth day at the leg of the injected side at which time symptoms disappeared. Increasing doses of tetanus toxin required increasing doses of penicillin until with 1,000 LD 50, which constitutes the maximum amount of toxin which was administered, the controls died in 24 hours. When 1,000 LD 50's were mixed with 1,000,000 units of penicillin 60 per cent treated animals died. 1,000 LD 50's mixed with 1,250,000 units of penicillin caused at 72 hours a local tetanus at the leg which disappeared in four days. The same dose of toxin when mixed with 1,700,000 units of penicillin failed to produce tetanus.

b) Clavacin and Its Isomers

Blair and Halman (1943) found that clavacin did not affect the toxicity of staphylococcal toxins. Neter and Will (1944) and Neter (1945) found that clavacin mixed with tetanus toxin "in vitro" destroyed its toxic effect; a mixture of clavacin and lethal dose of tetanus toxin failed to kill experimental animals.

Putzer (1946) investigated the effect of clavacin, isoclavacin, dimethilisoclavacin and alpha keto beta butyrolactone on tetanus toxin. He showed that clavacin prevented tetanus and death in all the animals receiving clavacin-toxin mixtures. On the other hand neither isoclavacin nor its derivatives prevented tetanus and death, even when administered in amounts five times greater than that of clavacin.

c) Toxin Neutralizing Substances Isolated by Ramon and Richou

Ramon and Richou (1945) found that filtrates of Bacillus subtilis, Penicillium notatum and Streptomyces griseus contained a principle which was able to detoxify a number of toxins "in vitro". This active principle did not bear any relationship to the antibiotic contained in the same filtrates, i.e. subtilin, penicillin and streptomycin respectively, nor to the principles having anti-virus properties which are also contained in the filtrates.

The method used for the production of the toxin neutralizing substances was the following: the medium in which the respective mould or bacterium had been grown was filtered. The filtrate was saturated with sodium sulphate and was held for three hours in water-bath at 37°C. When a precipitate formed the material was filtered through paper, the precipitate was collected from the paper and redissolved in distilled water. A unit of the active principle was taken as the minimal quantity of filtrate which was able to neutralize 100 Burnett units of Staphylococcus haemolysin in a water bath at 37°C. in six hours.

The active principle was capable of detoxifying diphtheria toxin, the toxin of the Preisz-Nocard bacillus, staphylococcal and tetanus toxins. It had little effect on the toxin of Pasteurella pestis and Staphylococcus beta-toxin.

The principle isolated from Bacillus substilis was heat-stable, being destroyed only at a temperature of 125°C.; the principles from Penicillium notatum and Streptomyces griseus were, on the other hand, destroyed at a temperature of 75°C. The active principles, when dialysed against distilled water, were not found in the dialysate, nor within the cellophane bag but, according the authors, were rather adsorbed to the cellophane. Formalin added to a concentration of 0.05 per cent did not destroy their toxin neutralizing properties.

Cavalli (1947) following the experiments of Ramon and Richou found that different strains of Penicillium notatum produced different quantities of toxin neutralizing substance. He showed that the destruction of the toxin followed a monomolecular curve, the velocity of the reaction being proportional to the ratio of the concentration of filtrate to the concentration of toxin. Cavalli's toxin neutralizing substance was not dialysable and it was destroyed by heating at 60°C. Del Vecchio et al. (1948) grew Penicillium notatum on a Czapex-Dox medium modified by adding lactose and peptone. With one-third saturation with ammonium sulphate a precipitate was formed which conserved all of the toxin neutralizing activity of the original filtrate.

Hauduroy and Rosset (1946) using the methods of Ramon and Richou incubated for six hours at 37°C. a mixture of Penicillium notatum filtrate and concentrated tuberculin. The mixture was injected subcutaneously into guinea pigs which had been infected with tuberculosis six weeks before. They showed that the filtrate of Penicillium notatum was able to neutralize the toxicity of the tuberculin for sensitized pigs, the pigs receiving the mixture remaining alive, whereas the controls receiving only tuberculin died.

Smolens, McAleer and McLaren (1947) following the experiments of Ramon and Richou worked with a crude commercial filtrate, from which the penicillin had been extracted, and tetanus toxin containing 100,000 MLD/ml. In the "in vitro" experiments, toxin and mould filtrate were mixed at 2°C., 20°C. and 37°C., for thirteen hours. Upon adding sheep red blood cells after this time no haemolysis occurred showing that the tetanus haemolysin had been inactivated. However, in "in vitro" experiments the mould filtrates were not active against the neurotoxin. Groups of fifteen mice were injected subcutaneously with an MLD of toxin and 1 ml. of the filtrate intraabdominally. A total of 220 mgms. of detoxifying agent was given at intervals. All of the mice died within 24 hours.

Smolens et al. claimed that the detoxifying agent was dialysable and therefore could not be a protein. They further stated that the principle destroyed "in vitro" not only the toxicity but also the antigenic power of tetanus toxin. Thirty-six mice, survivors of an infection of toxin plus detoxifying

principle, when challenged with 4 MLDs of tetanus toxin three weeks later all died within 48 hours.

d) Aspergillus fumigatus

Villa Anna Maria (1950) found that the filtrates of two strains of Aspergillus fumigatus had a marked activity as a detoxifying agent on staphylococcal toxin.

Nelis (1945) supposing that the detoxifying substances secreted by some microorganisms develop better under conditions where the struggle for life is stronger, fixes three conditions for the research of active detoxifying principle.

a) isolation of microorganisms in an external medium as, for example, in his own studies river water,

b) growth in a medium closely simulating the natural habitat,

c) growth at a temperature of 22°C. to simulate natural conditions.

Nelis found three strains, that he called 027, 026, 022, which secreted substances able to detoxify stephylococcal and diphtheria toxins. The strains were not identified by the author. He found a certain correlation between the gelatinolytic and the detoxifying power of the strains. The following is the only information provided concerning the nature of the organisms.

Strain 027: Large cocci in tetreads; non motile; gram positive. On agar: round colonies, porcelain white. Sugar not fermented; no action on the milk; no production of indole. Strain 026: Small cocci in clusters; non motile; gram negative. On agar: irregular colonies with elevated centers. Sugars not

fermented; no action on milk; no indole production; no liquefaction of gelatine. Production of brown pigment which diffuses into the medium. Strain 022: Short rods; motile; gram negative. On agar: white semitransparent colonies. Gas and acid from maltose; saccharose; levulose; acid from galactose. Coagulation of milk. Strongly gelatinolytic.

II. STUDIES OF THE POSSIBLE MODE OF ACTION OF TOXIN NEUTRALIZING SUBSTANCES FROM MICROORGANISMS

a) Commercial and Purified Penicillin

Imbriano (1952) in his study of the activity of commercial and purified penicillins on tetanus toxin, suggests that the penicillin acted in an enzymatic way by blocking some important groups in the molecule of tetanus toxin.

Putzer (1946) studied the activity of clavacin and isoclavacin and showed that clavacin was able to neutralize tetanus toxin whereas isoclavacin did not. These results offered to the author the possibility of studying the correlation between the chemical structure of clavacin and its toxin neutralizing capacity. By comparing the formulae of clavacin and isoclavacin it is apparent that these compounds differ only in the position of one double bond between two atoms of carbon. However, the simple shift in the positions of the double bond produces a profound change in several of the chemical characteristics of clavacin. Thus the tetrahydro gamma pyrone ring in clavacin, becomes a dihydro gamma pyrone ring in isoclavacin, and the beta gamma unsaturated butyrolactone in clavacin changes to an alpha-beta unsaturated butyrolactone in isoclavacin. While both

compounds have a double bond in the alpha beta position to a keto group its position in relation to the rest of the molecule is different in the two compounds. Evidence that the position of the double bond markedly alters the characteristic of these compounds is indicated by the fact that the dimetyl isoclavacin, having the double bond in the same position as the isoclavacin, is more resistant to catalytic hydrogenation than clavacin. Putzer states that the toxin neutralizing activity is apparently dependent upon the position of the double bond. However, which chemical group or groups in the molecule, affected by the position of the double bond, are responsible for the detoxifying activity cannot as yet be determined.

Van Heyningen (1951) claims that in the experiments of Putzer with clavacin only a few MLD's of tetanus toxin were concerned such that the molar ratio of clavacin to toxin was probably very high and doubts for this reason that one can claim any specific effect of clavacin on the toxin molecule.

In connection with the activity of clavacin in reference to the position of the double bond in the molecule, it is interesting to note that cholesterol contains certain specific groups in its molecule which are responsible for its anti-haemolytic activity. Smythe and Harris (1940) Hewitt and Todd (1939) and Cohen, Perkins and Puterman (1940) found that cholesterol was active against saponin and the oxygen-labile haemolysins, but that none of the oxygen-stable haemolysins such as Clostridium welchii alpha toxin, Staphylococcus haemolysin and Streptolysin S was inhibited by cholesterol. Berliner and

and Schonheimer (1938) studied the antihaemolytic activity of cholesterol, the stereoisomers of dihydrocholesterol and the bile acids corresponding in structure and spatial arrangement. They found that the antihaemolytic compound had a hydroxyl group at position 3 cis to the methyl group at position 10 and either a transfusion of rings A and B, or a 5-6 double bond. The bile acids whose spatial arrangement of the substituents at position 3 and 5 were opposite to those of the anti-haemolytic compounds were strongly haemolytic.

Ramon and Richou (1947) have on the basis of the thermolability of the toxin neutralizing substances found in the filtrates of Penicillium notatum, Streptomyces griseus and Bacillus subtilis suggested a possible enzymatic activity. They feel that the detoxifying activity is not a simple combination of the two substances; it is irreversible, the toxin losing its toxic and antigenic properties completely and irretrievably.

Cavalli (1947) in discussing the results of Ramon and Richou notes that the active principle from Bacillus subtilis is thermostable, being affected only by a temperature of 125°C., and suggests on this basis that enzymatic activity of this substance is postulated with difficulty. However, with respect to the active principles of Penicillium notatum and Streptomyces griseus Cavalli suggests that the curves of inactivation of the toxin, the thermolability of the substances and their non-dialyzable property point to a possible enzymatic nature, the enzyme affecting the toxic group of the toxin molecule. Cavalli states that non-

specificity of the detoxifying agent in regard to different toxic substances is highly interesting because it brings an analogy between the various toxins.

Del Vecchio (1948) in discussing the experiments of Ramon and Richou suggests that the active principle is probably a protein with high molecular weight or a prosthetic group of a protein molecule. Del Vecchio et al. (1948) attempted to see whether the activity of the detoxifying agents was to attack a specific group of the toxin, changing it to a toxoid, or if it simply destroyed the toxin by proteolysis; it is known that the filtrates contain potent proteolytic enzymes. They determined in one experiment the optimal ratio between diphtheria toxin and antitoxin by means of the flocculation test. By adding in a second experiment diphtheria antitoxin to a mixture of diphtheria toxin and detoxifying substance, in the optimal ratio already established, flocculation occurred showing that the mould filtrate had no influence on the antigenicity of the toxin. This is contrary to the findings of Ramon and Richou. Del Vecchio repeated the experiment using diphtheria toxoid instead of diphtheria toxin and showed that the mould filtrate did not destroy the antigenicity of the diphtheria toxoid measured by means of the toxoid-antitoxin reaction.

From a general point of view the problem of the mode of action of the toxin neutralizing substances is an important one in that it may throw some light on the nature and mode of action of the bacterial toxins.

The very fact that toxins of different origin and apparently different activity can be treated "in vitro" and sometimes "in vivo" with a single substance which can neutralize their toxicity suggests that the bacterial toxins may have much in common.

From the time in which Paul Ehrlich noticed the phenomenon of the spontaneous loss of toxicity by toxin, a number of substances have been shown to accellerate this process, including formaldehyde, ketene, iodine, azo compounds, ascorbic acid, carbon disulphide, pepsin, cholesterol, psychosin and others. In spite of such research, the mechanism of the mode of action of bacterial toxins and of the toxoiding process is still not understood. Follensby and Hooker (1936) suggested that the accelleration of the toxoiding process of hydroxyl ions and formaldehyde is a catalysis of a reaction that can take place spontaneously. Pillemer and others (1948) have accumulated evidence from the rate of sedimentation of crystalline tetanus toxin before and after the toxoiding process, which suggests that tetanus toxoid is a dimer of toxin molecules condensed through their toxic groups; strangely enough, this toxoiding process brought by a very low concentration of formaldehyde (0.01-0.001 per cent) is inhibited by the presence of albumin which is a inhibitor of most of the toxin neutralizing agents.

In the toxoiding process, the free amino-groups of lysine residues and the hydroxyl groups of tyrosine residues disappear, suggesting that these groups are necessary for the activity of the toxin. Cavalli (1947) discussing the experiments of Ramon and Richou, brings the hypothesis of a possible activity

of the microbial derivatives on the epsilon amino-groups of diphtheria toxin which are responsible for its toxicity according to Pappenheimer, and draws some analogies between the epsilon amino-groups of diphtheria toxin and related groups in other toxins.

A study of the mode of action of the toxin neutralizing substances from microorganisms strongly suggests they have some common factor or molecular grouping which accounts for their toxicity.

III. THERAPEUTIC IMPLICATIONS OF TOXIN NEUTRALIZING SUBSTANCES FROM MICROORGANISMS

The toxin neutralizing agents, to become useful therapeutic substances, must possess certain definite properties:

(a) The substance must have little toxicity for the intact animal body; it should produce no pathological changes when administered in quantity and frequently for prolonged periods.

(b) Even though the substance be non toxic it should act in low concentration so to render practicable its posology.

(c) It should not be antigenic because of the danger of producing allergic or anaphylactic reactions and further if antibody developed, repeated doses of the substances would become less and less effective.

(d) The substance should be active in the presence of normal and pathological body fluids. Many substances which are highly effective in ordinary media are inactivated by these

fluids. A slow rate of excretion is also necessary and the substance should not be inactivated by tissue enzymes. The latter is an important point in connection with the practical application of the detoxifying agents of microbial origin described in the literature.

Clavacin was found by Krebs (1944) to be inactivated by an enzyme present in sheep serum and by Stansfield et al (1944) to lose its activity in presence of human serum. Ramon and Richou (1947) found that "in vitro" combination for six hours at 37°C. of filtrates from Penicillium notatum, Streptomyces griseus and Bacillus subtilis with human and guinea pig serum destroyed the toxin neutralizing activity of the filtrates and neutralized at the same time certain toxic properties that they showed for the animal body.

There are certain factors involved in the inactivation of these toxin neutralizing agents in the body fluids. Eagle (1947) showed that penicillins F, G, K, X, were all inactivated by human and rabbit serum, but two qualitatively different mechanisms were involved: one was a slow inactivation of all penicillin by a relatively thermostable serum factor which was not demonstrably affected by heating for sixty minutes at 56°C. The rate of inactivation varied linearly with the concentration of the serum factor. Superimposed on this slow inactivation of the penicillin by the thermostable serum component was a much faster inactivation, observed only with penicillin K, by an highly termolabile serum factor.

Tompsett (1947) observed that the degree of inactivation was quantitatively different for the different penicillins and was caused by serum and albumin. The degree of inactivation caused by these substances was roughly in direct proportion to the degree of binding of the agents as demonstrable by dialysis. Penicillin X bound 47% and lost 47% of its "in vitro" activity in the presence of serum and albumin. Penicillin K bound 90% lost 90%. Penicillin G and dihydro F were intermediary.

Davies (1947) reported that the binding properties of the albumin depends upon the native undenaturated configuration of the albumin molecule. Serum albumin has a great capacity for tightly binding of long chain fatty acids; human serum globulin, prolamine, gelatine and crystalline ovalbumine had no effect.

The unique property of albumin in this respect is probably due to the interaction which it undergoes with many inorganic anions including sulphanilamide, anionic acids and other products. This suggests also that this property probably serves as a useful physiological function such as transport of material in the blood and protection of animal cells against toxic effect of various substances. The special capacity of the albumin implies the presence, on the surface of the molecule, of regions where the specific configuration of the amino-acid residues lead to an interaction with other substances.

Since the most distinctive feature of the serum albumin is its unusually high content of lysine and leucine, it is tentatively suggested by the author that albumin may have a number of lysine residues each of which is adjacent to several leucine

or other non-polar residues. According to Davis (1949), the chemical forces responsible for the formation of complexes between albumin or serum on one side and antibiotis and similar substances on the other, are not the covalent bonds which are represented by straight line in the symbols of organic chemistry, but includes electrostatic attraction between oppositely charged groups, dipolar interaction between undissociated groups, hydrogen bonds and Van der Waals' forces.

In the body these active principles exist in several forms as acetylated and free, and the latter may be divided into bound and unbound. The acetylated agent may or may not be permanently inactivated; the bound substance is temporarily out of combat but may constitute a reservoir of available detoxifying agent. In summary, the level of activity is proportional to the concentration of the agent, which in turn exists in equilibrium between ionized and non-ionized molecules.

At the present state of experimental evidence, insofar as therapeutical value is concerned, the situation regarding toxin neutralizing substances of microbial origin is not clear.

(1) Controversial results have been reported in the medical literature for the penicillins, crystalline and impure in combatting toxoemias.

(2) Clavacin has been proven to be not a suitable therapeutic agent on account of its toxicity (Hopkins 1943) (Brooms 1944) (Stansfield 1944).

(3) The substances isolated by Ramon, Richou and Gerbaux in the filtrates of Penicillium notatum, Streptomyces griseus and Bacillus subtilis, are now in limited use in human and veterinary medicine, but show definite disadvantages with respect to systemic therapy due to their toxity and other factors. Ramon and Richou (1947) suggested that these filtrates should be used locally on account of their inactivation by the body fluids. Richou and Gerbaux (1951) gave a review of the results of the use of these substances, which were called "Antagonistic complex". They were used as whole filtrates of cultures of Penicillium notatum, Streptomyces griseus and Bacillus subtilis because such filtrates contain the antibiotics, penicillin, streptomycin or subtilin respectively, the detoxifying agents already described and a group of enzymes which are possibly of some value. The action of the antibiotic, plus that of the detoxifying agents, plus that of the enzymes can be highly effective locally and in some cases internally, because while the antibiotic attacks the causal agent of the infection, the detoxifying agent neutralizes its toxin and the enzyme can carry on an activity on the inflammatory exudates, pus, and other substances. Gerbaux and Richou (1952) review the utilization on the antagonostic complex in human and veterinary medicine in the treatment of abscesses, wounds, skin disorders, mammary gland and gastro-intestinal infections caused by the coliforms and various other infections.

To conclude in our present state of knowledge, the field of the therapeutic detoxifying agents is rather unexplored. Some substances have been tried but with no immediate results. Other antibiotics than those referred to earlier in this review have been given very limited trial as antitoxic agents but there is little information, as yet, about them. Actinomycin A and tyrothricin have been tested by Blair and Halmann (1943) against staphylococcal toxin, chloramphenicol by Checcacci against the glucolipid-polypeptide complex of the autolysate and broth cultures of Salmonella typhi and streptomycin by Neter against tetanus toxin. All have had negative results. The reason of the failure can be ascribed partly to the inactivation by serum, partly to the fact that the detoxifying agent does not reach the toxin because of its prompt excretion from the body or because the toxin is fixed to the substrate so quickly that the detoxifying substance cannot prevent the combination and the dissociation of the toxin substrate complex, if any, takes place after irreparable damage has occurred.

SECTION B

EXPERIMENTAL PROCEDURE AND RESULTS

EXPERIMENTAL PROCEDURE AND RESULTS

1. A HISTORY AND SOME CHARACTERISTICS OF THE MOULD

The mould was isolated in this laboratory as a contaminant from a Lowenstein slope which had been inoculated with sputum for the isolation of the Mycobacterium tuberculosis. In testing the antibiotic activity of the mould it was noted that with a resistant strain of haemolytic Staphylococcus pyogenes, although there was no inhibition of bacterial growth a very marked zone of inhibition of haemolysis occurred on blood agar plates. The mould was sent to the Centraalbureau voor Schimmelcultures of Baarn (Holland) for identification and was determined to be Penicillium cyaneo-fulvum Biourge, NRRL 837.

The mould may be subcultured on Sabouraud slopes, Sabouraud dextrose slopes and on malt extract slopes and it is upon this medium that stock and seed inoculum cultures are maintained. Cultures have been maintained in the deep freeze at -21°C. for several months with no loss in essential characters or in its ability to produce a toxin neutralizing substance. The mould grows most luxuriantly at 20°C. and a maximum yield of mycelium is obtained in seven days. Fair growth may be obtained at 10°C., but at 5°C. growth is slow and sparse. Ordinary atmospheric conditions are most adequate for optimal growth. Good aeration is necessary for good growth and under anaerobic conditions no growth occurs. Stock cultures are incubated at room temperature for seven days after which they are stored in the ice-box for

periods of not longer than three weeks. During this period of time they are used as a seed culture. Some variations of the cultures in colour and other minor qualities occur after storage for one month and for this reason subcultures are made at intervals of three weeks.

Morphology and Cultural Characteristics: Colonies on malt extract grow rapidly attaining a diameter of 4.5 to 5.5 cm. in seven to eight days and a height of 1-2mm. They show a loose-textured, floccose or felt-like surface which is furrowed in radial pattern. There is a growing white margin 1-2 mm. wide which is light yellow-green in shade. Odour is not pronounced. The under surface is lightly coloured in dull orange-brown shades, becoming dark brown with age at which time pigment diffuses throughout the substrate.

The morphology is as follows: conidiophores variable in length, arising from the substrate with smooth walls. The penicilli are in general biverticillate and asymmetrical, loosely branched and irregularly shaped, bearing parallel chains of conidia. The branches are variable in length. The metulae are commonly in verticils of three to four, variable in length. Conidia globose or subglobose, of slightly varying diameter.

According to Raper and Thom (1949) Penicillium cyaneo fulvum belongs to the section of the Asymmetrica and to the subsection of Velutina in the series of Penicillium crysogenum and it is characterized by "the penicilli typically rebranched below the level of the metulae with main axes and branches terminating in verticils of metulae. Penicilli commonly long with the element loosely arranged and often divergent."

In regard to the question as to whether the described organism should be considered a distinct species, Raper and Thom (1949) state that "the validity of this species is somewhat in doubt because in studies of variations within the series, mutants of Penicillium notatum have been observed which approximate this species. However, since the general type of cultures have been obtained from widely separated natural sources and since Biourge's strains have in general retained their characters for twenty years or more in cultures, it seems best to recognize such a species as P. cyaneo-fulvum".

II. PREPARATION AND PURIFICATION OF THE TOXIN NEUTRALIZING PRINCIPLE OF P. CYANEO-FULVUM

The Culture Medium:

The toxin neutralizing factor is produced during the growth of P. cyaneo-fulvum in a fluid medium and may be demonstrated in the culture filtrate.

A number of media have been tested for optimum yield of toxin neutralizing principle: Glucose beef infusion broth, Peptone broth, Malt extract, Raulin's medium, Czapex Dox medium, Czapex Dox-Del Vecchio medium and a Casein hydrolysate medium. (The formulae of the various media are presented in the appendix). The results are presented in Tables 1 and 2, Figure 1. -

TABLE 1.

YIELDS OF MOULD ALBUMEN FRACTION IN DIFFERENT MEDIA

MEDIUM	Toxin neutralizing capacity of albumen fraction equivalent of Burnett Units /ml.
Glucose beef infusion broth	3200
Malt extract medium	2000
Czapex Dox	0
Czapex Dox-Del Vecchio	0
Raulin's medium	0
Casein hydrolysate medium	320

TABLE 2

YIELDS OF MOULD NEUTRALIZING SUBSTANCE IN CRUDE FILTRATE OF DIFFERENT MEDIA

MEDIUM	Toxin neutralizing capacity of crude filtrate equivalent of Burnett Units /ml.
Glucose beef infusion broth	100 - 800
Peptone broth	100 - 400
Malt extract medium	100 - 200
Czapex-Dox	0
Raulin's medium	0
Casein hydrolysate medium	10

From the data it appears that the most suitable medium for the production of the toxin neutralizing substance is Glucose beef infusion broth, prepared according to the standard technique of this department. The method of preparation of this medium is as follows: Finely minced fresh beef heart freed of fat is mixed with distilled water in the proportion of 1 lb. of meat per litre of water. The infusion is heated at 75°C.- 80°C. for 1-1/2 hours, and filtered through paper pulp in a Buchner funnel under slight suction. Proteose peptone (Difco) is added to a concentration of 1 per cent. Salts are added as follows:-

0.25 per cent NaCl, 0.02 per cent KCl, 0.01 per cent $CaCl_2$ (kept as stock solution 25 per cent NaCl, 2 per cent KCl, 1 per cent $CaCl_2$). The pH is adjusted to 8.3 to phenol red with 10 N NaOH. The infusion is then heated at 120°C. for 20 minutes to precipitate phosphates; a visible precipitate should be produced which is removed by filtration through paper pulp. The pH is readjusted to 7.2 to phenol red with N HCl, checking the reaction of bulk after adding the calculated amount of reagent. Heat at 120°C. for 20 minutes. The material is filtered through paper pulp in a Buchner funnel with slight suction. Adjust the reaction to pH 7.2. Add 1 per cent of dry dextrose and autoclave at 120°C. for 20 minutes.

In regard to the other media, peptone broth, malt extract and casein hydrodysate media are less suitable and with Raulin's, Czapex Dox, and Czapex-Dox-Del Vecchio media no production of the detoxifying agent occurred. From the data it is indicates that glucose and peptone are essential factors for the production of the toxin neutralizing agent.

Method of Preparation of the Active Culture Filtrate:

The pure colonies of the mould are inoculated in the Glucose beef infusion broth medium. As inoculum a large standard loop of mycelium from a stock culture is used. The colonies are inoculated into 100 ml. of medium contained in a 1 liter flask to provide an adequate surface area. The flasks are allowed to stand, without shaking, at room temperature for 7 days. The

maximum yield of the antitoxic substance is produced at end of 7 days at which time the surface growth of mycelium is at a maximum. After 7 days the lysis of the mycelium progresses rapidly and there occurs at the same time a fall off in titer of toxin neutralizing substance. The pH of the medium after inoculation is 7.2. The pH falls off slowly until, in the seventh day, reaches the maximum of 5.0. After this time it rises again. (See Tables 3 and 4, Figure 2.)

The production of detoxifying agent shows a close correlation with the increase in concentration of a yellow-brown pigment which discolours the underlying fluid. The pigment has been traced (Foster 1952) to an humin like substance formed in the break down of the mycelium of penicillia composed of resistant-N. According to the same author the pigment is also found in the make-up of a respiratory enzyme of the mould which produces it.

Following the incubation period of 7 days, the medium is poured off and separated from the mycelium by filtration through Whatman No.2 paper. The antitoxic principle is strongly adsorbed by the filter paper. However, once the paper is well soaked with filtrate, no appreciable loss occurs during subsequent filtration.

Precipitation and Purification of the Detoxifying Agent.

Several methods have been used for the precipitation and purification of different substances, depending on the nature of the substances and on their properties with regard to pH, temperature, ionic concentration of the substrate and other factors. A number of different steps are ordinarly required

TABLE 3.

THE EFFECT OF AGE OF CULTURE ON THE PRODUCTION OF TOXIN NEUTRALIZING SUBSTANCE AND PH OF THE MEDIUM

DAYS	Toxin neutralizing capacity of whole culture filtrate. Burnett Units of Staphylococcal alpha toxin	pH
1	8-16	6.9
2	8-16	6.8
3	8-16	6.8
4	8-16	6.6
5	8-16	6.6
6	16-32	5.8
7	50-100	5.0
8	50-100	5.0
9	50-80	5.2
10	50-80	5.3
11	50-80	5.4
12	50-70	5.6
13	40-70	5.7

TABLE 4.

POTENCY OF CRUDE CULTURE FILTRATES OF PENICILLIUM CYANEO-FULVUM IN DIFFERENT BATCHES

Batch Number	Neutralizing capacity of Staphylococcal alpha toxin equivalent of Burnett Units /ml
1	16
2	40
3	100
4	120
5	60
6	100
7	100
8	400
9	100
10	100
11	60
12	60
13	60
14	800
15	80
16	100
17	100
18	120
19	1600
20	100

for the isolation of a pure substance as for example successive precipitations or any other manipulation to obtain the active agent free from contaminating substances of the substrate.

Many tissue substances have been extracted with water or saline, freed from the cell debris by filtration and purified with such chemical agents as acetone, ammonium sulphate, sodium sulphate and others, by fractional precipitation, fractional adsorption and more specialized procedures.

In consideration of these facts and assuming a protein-like nature of our active detoxifying principle, the method of fractional precipitation with ammonium sulphate was tested and found to yield the active toxin neutralizing principle in concentrated form on the albumen fraction. After filtration, the pH of the filtrate is adjusted to 7.0 and ammonium sulphate is added to a concentration of 48 per cent of saturation (36.9 gms. / 100 ml.) at room temperature to precipitate the globulin fraction. The globulin fraction, which is of dark grey colour, sediments during centrifugation for 20 minutes at 2000 r.p.m. and is removed. The washed globulin precipitate is devoid of toxin neutralizing properties.

The clear yellow-brown supernatant fluid, which contains all of the detoxifying agent, is carefully decanted. To the clear supernatant fluid is added ammonium sulphate (40 gms./ml. to yield full saturation at room temperature. The fluid is well shaken for 3 minutes and is left at room temperature for 15 to 30 minutes. A dark brown precipitate forms which rises to the

surface of the liquid and sticks to the walls of the glass container. An excess of ammonium salt sediments to the bottom. The liquid is then decanted with the precipitate to 250 ml. centrifuging bottles, which are centrifuged for 20 minutes at 2,000 r.p.m. to harvest the precipitate. This precipitate contains the albumen fraction. By centrifugation the albumen fraction rises at the surface of the fluid as a pellicle. The density of the material is apparently less than that of the ammonium saturated substrate. The albumen fraction may be harvested on a spatula or by membrane filtration. This fraction contains all the active toxin neutralizing principle.

The wet albumen fraction is resuspended in saline, 50 ml. for every 500 ml. of the original volume of crude filtrate, and is dialyzed against saline (0.85 per cent NaCl solution) for 48 hours at 5°C. and -6mm. of mercury.

The active principle is not dialysable, remaining entirely in the cellophane bag used for dialysis (see appendix) and it is only slightly diluted by the saline entering the bag to counteract the osmotic pressure of the ammonium sulphate-albumen fraction complex. (The increase of volume is of about 1/5 of the original volume). The final yield of "wet" albumen fraction in the dialyzing bag is 12 ml. for each 100 ml. of culture filtrate. The dry weight was determined by two methods, lyophilizing "in vacuo" in sealed ampoules 5 ml. of the mould albumen fraction and calculating the difference between the weight of the glass ampoule alone and the weight of the dry material plus the sealed glass ampoule. Dividing by five the

dry weight per ml. of active principle was obtained. Another method consisted of dispensing 5 ml. of the wet slbumen fraction in a weighing bottle of known weight and placing the bottle in an electric oven. After 2 hours at 200°C. when the material was completely sterilized and dry, the bottle was weighed again and the difference between the two data constituted the dry weight of the toxin neutralizing factor. In both calculations the dry weight was on the average of 130 ± 20 mgms. /ml. of the mould albumen fraction.

For storage the dialyzed albumen fraction is then lyophilized in vacuo in sealed glass ampoules which are ordinarily stored at 4°C. to 10°C. although there is evidence that the material is quite stable even at room temperature.

The further procedure, that is, copper sulphate precipitation of the impurities in the crude albumen fraction was not developed until late in the course of the investigation. As a result, as will be noted in sections which follow, much of the investigation of toxin neutralizing properties, properties of the active principle, its mode of action etc., were carried out with the albumen fraction.

Several attempts were made to separate the active principle from the albumen fraction by such methods as adsorption on kaolin, animal charcoal, activated charcoal (see Section IV) by chromatography and electrophoresis (see Section IV) and by precipitation with acetone and butanol. Such methods were unsuccessful.

In experiments designed to overcome the slight toxicity of the semi-purified albumen fraction, precipitation

of impurities with $CuSO_4$ was finally found to be the answer. Some antibiotic substances like kojic acid and aspergillic acid were shown by Yabuta (1912) and Tobie and Alberson (1947) to form an insoluble salt with copper sulphate. Such substances were, however, highly toxic per os and intraperitoneally for mice. On the basis of such observations and in consideration of the fact that we were dealing with a mould product an attempt was made to precipitate out from the solution of the slightly toxic albumen fraction of the mould filtrate, toxic pigments which might resemble aspergillic acid for example.
10 ml. of an acqeuos solution of albumen fraction (150 mgms./ml.) were precipitated with 1 per cent copper sulphate and gave a voluminous orange precipitate. The precipitate was redissolved and was found to possess no toxin inactivating activity with an anti-haemolytic titration (see Section 111). The clear green tinted supernatant fluid was found to possess all of the toxin inactivating activity of the albumen fraction.

The supernatant fluid was dialyzed against saline for 48 hours at 5^{o}C. at -6mm. of mercury to eliminate the copper sulphate and after dialysis it was found that the greenish colour disappeared and that the active antihaemolytic or other toxin inactivating activity was not dialysable. The final yield of the copper precipitate residue in the dialysis bag was 12 ml. showing that the copper purified material was only slightly diluted by the saline entering the bag to counteract the osmotic pressure of the material inside the bag. This fluid substance has a dry weight yield of 15.2 mgms. /ml. For

storage the dialysed copper precipitated residue is then lyophilized in vacuo in sealed glass ampoules which are ordinarily stored at 4°C. to 10°C.

The following is a summary outline of the preparation and purification of the toxin neutralizing principle. The material is presented in the form of a flow sheet in Table 5.

1. Culture: 100 ml. of McGill Glucose Beef Infusion Broth contained in a 1 liter flask are inoculated with a large loop of mycelium from a Malt Extract slope of the stock Penicillium culture. Incubated without shaking at 25°C. for 7 days.
2. Filtration through Whatman No. 2 paper to remove mycelium.
3. The filtrate is adjusted to pH 7.0 with 10 N. NaOH.
4. Ammonium sulphate is added to a concentration of 48 per cent saturation at room temperature (36.9 gms/ 100 ml.). This precipitates the globulin fraction which contains no toxin Neutralizing ability.
5. The globulin fraction is removed by centrifuging for 20 minutes at 2,000 r.p.m. The clear supernatant fluid is carefully decanted.
6. To the clear supernatant fluid is added ammonium sulphate to yield full saturation at room temperature (40 gms. /100 ml.) This precipitates the albumen fraction.
7. The albumen fraction is harvested by centrifuging for 20 minutes at 2,000 r.p.m. The supernatant fluid is decanted and discarded.
8. The wet albumen fraction is resuspended in 0.85 per cent NaCl solution (10 ml. for each 100 ml. of original culture filtrate.)

9. The saline suspension of the albumen fraction is dialysed against saline (0.85 per cent) for 48 hours at 5°C. and -6 mm. of mercury. The final yield in the dialysing bag is 12 ml. of suspension of the albumen fraction for each 100 ml. of crude culture filtrate. The dry weight of this material is 130 ± 20 mgms./ml.

10. To the dialysed saline suspension of the albumen fraction is added at room temperature copper sulphate to a concentration of 1.0 per cent. This is allowed to stand 30 minutes and centrifuged at 2800-3000 r.p.m. for 20 minutes. The clear light green supernatant fluid is carefully decanted. The precipitate which has no toxin neutralizing activity and which is highly toxic for animal is discarded.

11. The supernatant fluid is dialysed against 0.85 per cent NaCl solution for 48 hours at 5°C. and -6 mm. of mercury. The final volume of the fluid recovered from the bag is 14 ml. The dry weight of the purified fraction is 15.2 mgms. /ml. Thus the final yield of purified material is 212.8 mgms. for each 100 ml. of original crude culture filtrate.

12. The dialysed supernatant fluid of the $CuSO_4$ precipitation is lyophilized and stored "in vacuo" in sealed glass ampoules and stored at 5°C.

13. One mg. of the final product has toxin neutralizing ability equal to that of 9.8 mgms. of the crude albumen fraction, i.e., it is some 10 times more potent on a unit weight basis. (See section on "Properties of the Toxin Neutralizing Substance of P. Cyaneo-fulvum). The copper sulphate procedure removes virtually all of the inactive impurities

which are highly toxic to animals; the final product is relatively atoxic for the animal body. (See section on "Properties of the Toxin Neutralizing Substance of P. Cyaneo-fulvum).

The foregoing procedure has proven the most successful for the production and partial purification of the active principle. However, other media and methods have been tested in this respect. The method used involves one limitation, i.e., the complexity of the medium. The complexity of the medium used constitutes a serious limitation in view of its content in proteins, and the complex substances which render more difficult the purification of the active principle. However, attempts to produce the toxin neutralizing substance by growing the mould in less complex media were not fully successful (See Table 1 and Table 2). In casein hydrolysate medium, the yield of the active principle was low and in the Raulin's synthetic medium no production at all occurred of the detoxifying substance showing that the detoxifying agent cannot as such be synthesized by the mould starting from inorganic substances plus sugar and tartaric acid, even if all the required mineral ions are present in the medium, (See Table 1). These data agree with those of Del Vecchio (1948) who found best production of the toxin neutralizing substance in a Czapex Dox medium improved by the addition of sugar and peptone. As far as the method of purification is concerned,

TABLE 5

1. Culture of Penicillium McGill Strain.
   on suitable medium in thin layer at 25°C. for 7 days.

↓

2. Filtered through Whatman No.1 filter paper.
   to remove mycelium.

↓

3. Adjust filtrate to pH 7.0 with N/1 NaOH.

↓

4. Add Ammonium Sulphate to 48 per cent of Saturation.
   36.9 gms. per 100 mls. of filtrate.

↓

5. Centrifuge ——— > Discard deposit.

↓

6. Saturate supernatant with Ammonium sulphate.
   40 gms. per 100 mls. at room temperature.

↓

7. Centrifuge ——— > Discard clear supernatant.

↓

8. Suspend deposit in 0.85 per cent NaCl.
   10 mls. saline for each 100 mls. original filtrate
   under 3. in a cellophane dialysis bag.

↓

9. Dialyse against 0.85 per cent NaCl solution — > Discard dialysate

ammonium sulphate is highly effective with the glucose beef infusion broth medium: it is less effective with other media as for example, malt extract medium even though this latter medium is the best for the conversation of seed cultures and gives a good yield of detoxifying substance in the crude filtrate. When one attempts to precipitate the active principle from a culture grown in this medium a large part of the active principle (38 per cent) is lost. Concentration of the active toxin neutralizing material in the albumen fraction by fractional precipitation of a glucose beef infusion broth culture of Penicillium cyaneo-fulvum Biourge has thus become the standard procedure in this present investigation.

111. TOXIN NEUTRALIZING ACTIVITY OF CULTURE DERIVATIVES OF PENICILLIUM CYANEO-FULVUM (BIOURGE) (MC GILL STRAIN).

The whole culture filtrate, the semipurified albumen fraction and the more highly purified residue following copper sulphate precipitation have been tested for toxin neutralizing activity. The great majority of the experiments, particularly those designed to test the whole spectrum of toxin neutralizing activity with various bacterial toxins, were carried out with the albumen derivative since the more refined copper sulphate residue was not discovered until a late period in the course of the work.

1. EXPERIMENTS WITH STAPHYLOCOCCUS PYOGENES ALPHA TOXIN.

Toxin formation is a property of pathogenic strains of Staphylococcus pyogenes; considerable variation exists between different strains and if toxin is required on a large scale for experimental work or other purposes, it is important to select a strain with a high toxigenic capacity. For that reason our staphylococcal alpha toxin was prepared from the Wood 46 strain of Staphylococcus pyogenes which gives a toxin of high potency in consistent yields.

Various methods are used in the production of this toxin. In a fluid medium it developes rather slowly, as tested by Nesser and Wechsberg (1901). Burnet's technique (1930) of growing the organisms on 0.8 per cent nutrient agar for 24 hours in air containing 10-20 per cent CO_2 and extracting the toxin from the agar with saline was widely followed either in

its original or in a slight modified form. (Parish and Clark 1932 Dolman 1932). The method followed in this work to produce the toxin is that of Roy (1937). One heaping small loop of culture from an agar slope of Staphylococcus phogenes (strain Wood 46) is placed into 10 ml. of glucose beef infusion broth contained in a one hundred and twenty five ml. flask. The flask is well shaken and incubated for 7-8 hours at 37°C. At the same time plates of semi-solid agar (the medium is described in appendix) are prepared by placing about 60 ml. of semi-solid agar into each of four large Petri dishes. The plates are chilled thoroughly in the cold for four or five hours, and inoculated with three ml. of the above culture for each of the large petri dishes. The plates are placed in a McIntosh anaerobic jar with 45 per cent carbon dioxide and 55 per cent oxygen. The jar is incubated for three days at 37°C. After this time it is necessary to separate the toxin from the growing organisms. Although the toxin can traverse a Seitz filter, some of its activity is lost in the passage, and it is therefore advisable to separate the organisms from the toxin by centrifugation. In our procedure the toxin was centrifuged at 2000 rpm. for 25 minutes at 5°C. and then filtered through an E.K. Seitz filter at room temperature, placed in sterile vials and stored at 4°C. to 6°C. The titer of the toxin was measured according to the method of Burnet (1929). Halving dilutions of the toxin, in broth saline (1 part of peptone broth and 9 parts of saline) were placed in waterbath at 37°C. with rabbit red blood cells.

(1 ml. of toxin and 1 ml. of 2 per cent suspension of rabbit cells, such that the final concentration of rabbit red blood cells was of 1 per cent).

Following incubation for 1 hour at 37°C. the tubes were read for 50 per cent haemolysis, after centrifugation, by comparing them with a control tube prepared to show 50 per cent of haemolysis. This gave the rough titer of the toxin which was followed by the fine test in which the toxin was placed in 10 tubes in harmonic dilutions containing respectively 1 ml. of toxin, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2 and 0.1 ml., starting from a dilution close to the titer previously established by the rough test. Rabbit red blood cells were added to a concentration of 1 per cent and the haemolysis was read with the method already described.

It was found that the batches of toxin used in our experimental work varied in potency from 1600 to 6000 Burnett units per ml., taking as a Unit the minimal amount of toxin which gave 50 per cent haemolysis of a 2 per cent suspension of rabbit red blood cells in a total volume of 2 ml. after incubation for 1 hour at 37°C. It was shown that the titer of the toxin remained for several weeks at a high level, provided that sterile precautions were taken when using the toxin.

a) Antihaemolytic Effects of the Albumen Fraction

Some 20 experiments have been performed to determine the toxin neutralizing activity of different batches of the semi-purified mould albumen concentrate on staphylococcal alpha-haemolysin . The results are presented in Table 6.

TABLE 6

CAPACITY OF DIFFERENT BATCHES OF ALBUMEN FRACTION TO NEUTRALIZE STAPHYLOCOCCAL ALPHA TOXIN IN RELATIONSHIP TO PROVISIONAL INTERNATIONAL UNITS OF STAPHYLOCOCCAL ANTITOXIN.

Batches No.	Neutralizing capacity expressed as equivalent of Burnett units / 150 mgms. of mould derivative.	Potency expressed as equivalent to I.U. of antitoxin per 150 mgms of mould derivative.
1	3200	Not done
2	1600	"
3	1600	"
4	12.800	"
5	1600	"
6	1600	"
7	1600	"
8	1600	"
9	3200	17
10	3200	13
11	3200	18
12	3200	13
13	12800	52
14	3200	16
15	3200	13
16	1600	9
17	1600	9
18	3200	16
19	3200	13
20	3200	16

For all such tests the lyophilized mould is dissolved in 0.85 per cent NaCl to yield a concentration of 150 mgms.of the dry powder per ml. of solution.

Antihaemolysin titres were performed according to the technique of Hartley and Smith (1935), by mixing 0.25, 0.5, and 1 ml.of the mould albumen solution (as above) with 1 ml. aliquots of whole undiluted toxin and 1 ml. aliquots of the mould solution with serially diluted samples of toxin in halving dilutions from 1/2 to 1/256. The mixtures were incubated in water-bath at 37°C. for 1 hour. After this time 0.5 ml.of a 5 per cent suspension of rabbit red blood cells was added to each tube. The tubes were incubated in water-bath at 37°C. for another hour and shaken at the end of the first 30 minutes. After the incubation period the tubes were centrifuged for 20 minutes at 2000 r.p.m. and 50 per cent haemolysis was read by comparing the haemolysis within the tubes with that of a known standard 50 haemolysis. A typical experiment is shown in Table 7.

In our experiment it was shown that 1 ml. of albumen fraction containing 150 mgms. is capable of neutralizing 3200 $\pm$ 1600 Burnett Units of staphylococcal alpha haemolysin.

A direct comparison was established between the semi-purified albumen fraction and staphylococcal alpha antitoxin, in regard to their antihaemolytic activity. The following method was employed. For a staphylococcal alpha-toxin of known potency which was used for the antihaemolytic test with the mould albumen fraction, the LH dose was calculated according to the method of Hartley and Smith (1935) as follows: Harmonic

TABLE 7

ALPHA HAEMOLYSIN INACTIVATION TITRE OF SEMI-PURIFIED MOULD DERIVATIVE.

TOXIN ACTIVITY CONTROL.

*Dilution of Staph. Alpha Haemolysin in 1 ml.	1	1	1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
	1 ml. of 2 per cent Rabbit RBC's to each tube and incubated 1 hr. at 37°C.										
Haemolysis	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++

TOXIN INACTIVATION TEST.

Staph. Alpha Haemolysin ml.	1	1	1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
Dilution of Mould Derivative (150 mgm. in 1 ml.)	1/4	1/2	1	1	1	1	1	1	1	1	1
	Incubated 1/2 hr. at 37°C. 1 ml. 2 per cent Rabbit RBC's to each tube and incubated 1 hr. at 37°C.										
Haemolysis	++	-	-	-	-	-	-	-	-	-	-

* The titre of the toxin was 1600 Burnett Units/ml. Therefore 1 ml. of the mould derivative neutralizes 3200 Burnett units of staphylococcal alpha toxin or 150 mgm. of the lyophilized mould derivative neutralizes 3200 Burnett Units.

dilutions of the toxin containing in a total of 1 ml., 1 ml., 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2 and 0.1 ml. of toxin were set up and each tube received 1 ml. of antitoxin containing 1 Provisional International Unit. The range for the dilutions was calculated from the haemolytic titer of the toxin, by assuming that 200 Burnett units are neutralized by 1 Unit of International Standard Antitoxin. The mixture of toxin and antitoxin was kept in water-bath at 37° C. for 1 hour and after this time 0.5 ml. of a 5 per cent suspension of rabbit red blood cells was added to each tube. After shaking the tubes were incubated for one hour at 37° C. and placed in the ice box overnight before reading. The end point was considered to be the mean value between the last tube showing haemolysis and the first tube free from haemolysis.

By this experiment was determined the LH dose of the staphylococcal toxin as the minimal amount of toxin which was neutralized by 1 Provisional International Unit of Antitoxin. By direct comparison between the antihaemolytic effect of the mould albumen fraction and that of standard antitoxin, it was shown that 1 ml. of mould concentrate (150 mgms. of dry material) is equivalent on the average in neutralizing capacity to 13 Provisional Units of Antitoxin. Thus 11 mgms. of dry material is the equivalent of 1 Provisional International Unit of Antitoxin.

In the various experiments with different batches of mould albumen fraction (Table 6) it has been shown that 11 mgms. of dry albumen fraction have a neutralizing capacity equivalent to from 0.5 to 4 Provisional International Units of Antitoxin,

depending on the batch. The reason for the rather wide variation in the potency of different batches has not been determined as yet, but several factors have been shown to influence the optimum of production, like choice of a properly stabilized stock culture for the inoculation of the liquid medium, avoidance of denaturation of the detoxifying factor during the manipulation in the preparation of the purified product and care that it is not lost by adsorption to filter paper.

b) Neutralization of the Dermonecrotic Effect of Staphylococcal Alpha Toxin.

After it had been shown that the mould albumen fraction neutralized the haemolytic activity of staphylococcal alpha toxin, it was tested to determine its ability to neutralize the other major properties of staphylococcal alpha toxin, i.e., the dermonecrotic and the lethal effects. There has been, and still is, much contention as to whether the alpha toxin as a single molecule has haemolytic, dermonecrotizing and lethal effect or whether the alpha toxin is a complex substance each of these effects being due to a different toxic molecule. If the mould derivatives were able to neutralize all three toxic effects simultaneously, it would lend support to the more popular theory that the exotoxin is simply one substance having different effects on different tissues.

To test for antidermonecrotic effect, a rabbit was shaven over the right side and the space was divided in two rows. Each row was divided into six portions so that a total of twelve portions were obtained. The first row received intradermal injections of serial dilutions of staphylococcal toxin in 0.5 ml.

volumes. (800 Burnett Units - 400 B.U. - 200 B.U. - 100 B.U. - 50 B.U.) each dilution having been incubated for 1 hour at 37°C. with 1 Provisional International Unit of antitoxin contained in 0.5 ml. of serum. One portion was allowed to be the control of the toxin alone (50 B.U.). The second row received serial dilutions of mould albumen fraction in a volume of 0.5 ml. (75 mgms., 37.5 mgms., 18.75 mgms., 9.37 mgms. and 4.68 mgms.) incubated for 1 hour at 37°C. with 0.5 ml. of toxin containing 800 Burnett Units. One portion was allowed to be the control for the mould derivative (75 mgms.).

The results (Table 8) show that 800 Burnett Units constitute the minimal dose of staphylococcal alpha toxin which produced a dermonecrotic effect in an area of 2 cm. of diameter when mixed with 1 Provisional International Unit of antitoxin, while the same amount of toxin is able to produce the same effect only when mixed with 1/32 of ml. of albumen fraction or 4.68 mgms. Thus the semi-purified albumen fraction had about the same potency as 16 P.I.U. of antitoxin; this result correlates well with the inactivationof haemolytic activity of the toxin.

It was attempted to determine whether the neutralization of the dermonecrotizing effect of staphylococcal toxin occurred also when the mould albumen fraction was given after a lapse of time following injection of toxin rather than being mixed with the toxin "in vitro", that is, if it exerted an "in vivo" neutralization.

A rabbit was shaven over the right side and the space divided into six squares. In each square 0.25 ml. of toxin was injected intradermally (0.25 ml. containing 200 Burnett Units). One

TABLE 8

NEUTRALIZATION OF THE DERMONECROTIC EFFECT OF STAPHYLOCOCCAL ALPHA-TOXIN BY THE MOULD ALBUMEN FRACTION.

SQUARES	1	2	3	4	5	6
Staph. toxin in Burnett Units	800	400	200	100	50	50
Staph. Antitoxin in P.I. Units	1	1	1	1	1	-
Saline solution in ml.	-	-	-	-	-	0.5
Reaction*	+	-	-	-	-	-

SQUARES	1	2	3	4	5	6
Staph. toxin in Burnett Units	800	400	200	100	50	-
Mould albumen fraction in mgms.	75.0	37.5	18.75	9.37	4.68	75
Saline solution in ml.	-	-	-	-	-	0.5
Reaction*	-	-	-	-	+	-

* + = Dermonecrosis
\- = No dermonecrosis

TABLE 9

DERMONECROTIC EFFECT OF STAPHYLOCOCCAL ALPHA TOXIN WITH DELAYED INJECTION OF THE ALBUMEN FRACTION

SQUARES	1	2	3	4	5	6
Staph. toxin in Burnett units	200	200	200	200	200	200
Saline solution in ml.	-	-	-	-	-	0.5
Albumen fraction in mgms.	75	75	75	75	75	-
Time between injection of toxin and mould (in hours)	2	5	24	24	24	-
Reaction *	tr.	+	+	+	+	+

* + = Dermonecrosis
tr = Traces of dermonecrosis

square was left as a control with only the toxin. The other five squares received 75 mgms. of mould albumen fraction, in a volume of 0.5 ml., with a capacity to neutralize 750 B.U. of staphylococcal toxin. The first square received the detoxifying substance after 2 hours. The second square after 5 hours and the other squares after 24 hours.

Dermonecrosis occurred in all the squares except in the first where only a slight reaction occurred. (Table 9).

It is obvious from the foregoing experiments that although the dermonecrotizing factor is efficiently neutralized "in vitro" once the toxin is fixed to the tissues the albumen fraction is not too effective in accomplishing its toxin neutralizing activity. Presumably by the time that the mould product had been injected the damage was done. It is to be noted that in that square where the toxin neutralizing substance was injected very shortly after the injection of toxin, the toxin effect was diminished.

c) Neutralization of the Lethal Effect of Staphylococcal Alpha Toxin.

It was attempted to determine if together with the neutralization of the haemolytic and dermonecrotizing effect, the mould albumen fraction was able to neutralize also the lethal effect of staphylococcal toxin "in vitro" and "in vivo".

Animal Experiments.

One experiment has been performed with one rabbit and two experiments with mice. In the one experiment a mixture of 1 ml. of staphylococcal toxin containing 3200 Burnett Units and of 150 mgms. of mould albumen derivative containing the equivalent to

TABLE 10

"IN VIVO" EXPERIMENTS WITH STAPHYLOCOCCAL ALPHA TOXIN AND THE SEMI-PURIFIED ALBUMEN FRACTION FROM PENICILLIUM CYANEO FULVUM

Experiments	Control*	Death time	Test*	Death time	Treated animals remaining alive
1 (Rabbits)	3200 B.U. to 1 rabbit	1 hour	3200 B.U.and 150 mgms. AF to 1 rabbit	-	1
2 (mice)	400 B.U. to 2 mice	1 hour	400 B.U. and 37.5 mgms.AF to 2 mice	-	2
3 (mice)	400 B.U. to 2 mice	1 hour	400 B.U. and 37.5 mgms.AF to 2 mice	-	2
4 (mice)	79.00 B.U. to 5 mice	24 hours	79. B.U. and 15 mgms. AF to 5 mice	24 hours	none
5 (mice)	79 B.U. to 2 mice	24 hours	79 B.U. and 6 mgms. AF to 5 mice	6 hours	none
6 (mice)	37.5 mgms. AF to 2 mice	1 hour	37.5 mgms.AF and 79 B.U. and 0.5 ml. of neoantergan to 4 mice	24 hours	2
7 (rabbits)	3500 B.U. to 1 rabbit	1 hour	3500 B.U. and 225 mgms. of AF to 3 rabbits	1 hour	none
8 (rabbits)	1800 B.U.to 1 rabbit	1 hour	1800 B.U. and 225 mgms.AF to 1 rabbit. 1800 B.U. and 300 mgms.AF to 1 rabbit. 1800 B.U. and 375 mgms.AF to 1 rabbit	1 hour 1 hour 1 hour	none
9 (rabbits)	900 B.U. to 1 rabbit	24 hours	900 B.U. and 750 mgms.AF to 1 rabbit 900 B.U. and 1125 mgms.AF to 1 rabbit	24 hours 24 hours	none

* AF - Albumen fraction from Penicillium cyaneo fulvum.

B.U. - Burnett Units of staphylococcal alpha toxin

neutralize the 3200 Burnett Units was incubated at 37°C. for 1 hour. After incubation the mixture was injected intravenously into a rabbit weighing 4 Kgs., while another rabbit was the control receiving only the same quantity of toxin.

The toxin control died within 1 hour since for a 4 kg. rabbit the minimal lethal dose is calculated in 1400 ± 200 Burnett Units (i.e., 350 ± 50 B.U. per kilogram). The treated rabbit remained alive.

In two experiments with mice a mixture was prepared with 0.25 ml. of toxin containing 400 Burnett Units and 37.5 mgm. of mould albumen derivative in a volume of 0.5 ml., an amount sufficient to completely neutralize the staphylococcal toxin. The mixture was left in water-bath at 37°C. for 1 hour and then injected to each of two mice. Two controls received only the same dose of toxin. As in the rabbit experiment the toxin controls died within 1 hour while the treated animals remained alive and healthy for the period of observation. (7 days). (Table 10)

From the foregoing experiments it is seen that the mould albumen derivative is able to neutralize "in vitro" the haemolytic, dermonecrotic and lethal effect of the staphylococcal alpha-toxin and that the relationship between the toxin and the detoxifying agent is the same in the neutralization of the three different activities of the toxin, the inactivation of one effect correlating very well with the inactivation of the others.

The results have been shown to be reproducible and the inactivation of staphylococcal toxin by the mould derivative is a definite conclusion which arises from all these experiments, provided that the two substances are mixed in a test tube and

incubated for a period of time, usually from half an hour to one hour, although on several occasions it was found that even after immediate combination the toxin was effectively neutralized by the albumen fraction. Quite in contrast, however, neutralization of the toxin is not accomplished in a very efficient manner if the toxin and mould product are injected into the animal body without prior "in vitro" admixture. In the following animal experiments staphylococcal toxin and the mould detoxifying agent were inoculated at different times and sometimes by different routes to test for "in vivo" neutralization.

Experiment 1.

It has been shown that the greatest accuracy, with most economy in the use of animals, is obtained if we try to find for a toxin not the dose which will kill the animals, but the dose which will kill 50 per cent of them (Gaddum J.H. 1933). This is called the LD 50. There are a number of ways to estimate the LD 50. In our experiment, we followed the method suggested by Reed and Munch (1938). When the number of animals in each group is small, as in this example, there is a number of accidental departures from a regular progression of mortalities.

To compensate for this, in so far as possible, Reed and Muench proposed cumulative mortalities for all the groups in succession. Since it may be assumed that a mouse surviving a given amount of toxin could have survived any smaller amount, the survivors are successively added, beginning at the bottom of the table, for each different dose (Column (d), next page). Deaths are similarly added column (e) and the percentage mortality computed at each step (Column (f)).

In our experiment 5 groups of 3 mice of 20 gms. were inoculated with different doses of Staphylococcal alpha toxin of a potency of 1800 Burnett Units, or 18 LH / ml.

Group 1, received 28.125 Burnett Units, in 0.5 ml., intraperitoneally.

Group 2, received 56.25 Burnett Units, in 0.5 ml., intraperitoneally.

Group 3, received 112.5 Burnett units, in 0.5 ml., intraperitoneally.

Group 4, received 225.0 Burnett units, in 0.5 ml., intraperitoneally.

Group 5, received 450.0 Burnett units, in 0.5 ml., intraperitoneally.

According to our results, the point of 50 per cent mortality lies between 56.25 and 112.5 Burnett Units.

The exact point can be calculated from the formula:

$$\frac{50 - (\text{per cent mortality at amount next below})}{(\text{per cent mortality next above}) - (\text{per cent mortality next below})} = \text{proportional distance.}$$

In the present case this gives

$$\frac{50 - 0}{100 - 0} = \frac{1}{2} = 0.5$$

FIFTY PER CENT END POINT

(a) Dose in Burnett Units	(b) Lived	(c) Died	(d) Lived	(e) Died	(f) Per Cent Mortality
28.125	3	0	6	0	0
56.250	3	0	3	0	0
112.5	0	3	0	3	100
225.0	0	3	0	6	100
450.0	0	3	0	9	100

Column d is formed by adding column b from the bottom, and entering each sub-total.

Column e is formed similarly by adding column c from the top.

The percentage mortality is calculated from columns d and e.

Since the amounts increase on a logarithmic scale, it is necessary to carry out the final calculation as follows :

log. 56 (lower amount)	$\bar{1}.7482$
+ 0.5 (propor. dist.) x log. 2 (dil.factor)	0.1505
= sum (log of end point)	$\bar{1}.8987$

which gives for the end point an amount of approximately 79.20 Burnett Units. The LD 50 as determined by this procedure was used as a statistical end-point in most of the experiments where mice were used in any appreciable numbers.

Experiment 2

After the estimation of the LD 50 as the basis to work in the animal experiments, a number of experiments have been made to see whether the albumen fraction was able to neutralize the staphylococcal toxin in vivo.

Method and results. Two groups of 5 mice each were inoculated with the LD 50 of staphylococcal toxin, intraperitoneally. Group a) was the control. Group b) received, intraperitoneally 15 mgms. of albumen fraction 1 hour before the injection of staphylococcal toxin. All of the mice died within 24 hours.

In another experiment, two groups of mice were inoculated with the LD 50 of staphylococcal toxin, intraperitoneally. Group a) (2 mice) acted as a control receiving only the toxin. Group b) (5 mice) received intraperitoneally 6 mgms. of albumen fraction, 2 hours before the LD 50 of staphylococcal toxin. Another group (2 mice) received intraperitoneally only 6 mgms. of albumen fraction, and acted as a control for detoxifying substance. After 6 hours, the 5 mice of group b) were dead, and after 24 hours the

2 mice of group a) were also dead.

Only the group which acted as a control for the albumen fraction remained alive, showing that the albumen fraction "per se" was not toxic in the amount administered.

Since in these experiments it appeared that the albumen fraction did not offer any protection "in vivo" and indeed seem to add some toxicity to that of staphylococcal toxin, a third experiment was devised to see if it was possible to protect the animals from the toxic effect of the albumen fraction where each mouse was given a dose of the albumen fraction large enough to neutralize in vivo several LD 50s of staphylococcal toxin along with the anti-histaminic "Neoantergan" as first proposed by Richou and Gerbaux (1952).

Two groups of mice were injected with 37 mgms. of albumen fraction in 0.25 ml. of volume for each mouse, intraperitoneally (neutralizing in vitro 800 Burnett Units of staphylococcal alpha toxin). Group A (2 mice) served as controls. Group B (4 mice) received 0.5 ml. of the synthetic anti-anaphylactic "neonantergan", in a dose 1/3700 that used in human medicine (calculated on a basis weight of 75 kgs. against 20 gms. for the mice), 30 minutes before the injection of the albumen fraction.

The controls died within 1 hour, while the "neoantergan" treated animals were protected from the effect of the albumen fraction and remained well during the period of observation of 7 days. Group B was then challenged after 3½ hours with the LD 50 of staphylococcal toxin. Of the 4 mice, 2 died in 24 hours while 2 mice remained alive and well during the period of observation of 7 days.

Experiment 3

Four rabbits were inoculated with 1 ml. of staphylococcal toxin containing 3500 Burnett Units, or 2.5 lethal doses, intravenously. One rabbit was the toxin control. The other three rabbits were inoculated 1 hour prior to the toxin injection with 1.5 ml. (225 mgms. of dry material) of the albumen fraction, an amount sufficient to neutralize 4800 Burnett Units. All four rabbits died within 1 hour.

In a similar experiment four rabbits were inoculated intravenously with 1800 Burnett Units, each (1 lethal dose for 5 kg. rabbit). One rabbit was the toxin control. The other rabbits received intravenously 1 hour previously, increasing amounts of the albumen fraction as follows:

No.1. 1.5 ml. (225 mgms. of dry material, neutralizing 4800 Burnett Units).

No.2. 2.0 ml. (300 mgms., neutralizing 6400 Burnett Units).

No.3. 2.5 ml. (375 mgms., neutralizing 8000 Burnett Units).

All four rabbits died within 1 hour.

In another such experiment three rabbits were challenged intravenously with 0.5 ml. of staphylococcal toxin containing 900 Burnett Units ($\frac{1}{2}$ lethal dose for 5 kgs. rabbits). One rabbit was the toxin control. The other two rabbits received increasing doses of the albumen fraction, 3 hours before the injection of the toxin, as follows:

No.1. 5 ml. of albumen fraction (750 mgms. neutralizing 8.8 lethal doses).

No.2. 7.5 ml. of albumen fraction (1125 mgms., neutralizing 13.3 lethal doses).

All three rabbits died within 24 hours.

Protection of the animals with the Residue of Copper Sulphate Precipitation.

Experiment 4

Two groups of 10 mice each were injected with 1 LD 50 of staphylococcal toxin (90 Burnett Units in 0.5 ml.), intraperitoneally. Group A served as controls with the 1 LD 50 of staphylococcal alpha toxin alone. Group B was inoculated 2 hours before the toxin injection with 7.6 mgms. of the copper sulphate precipitated residue for each mouse. 7.6 mgms. contained sufficient amount of the detoxifying agent to neutralize 1600 Burnett Units.
All the 20 mice died.

The lack of protection from the detoxifying substance was attributed to the very rapid activity of the staphylococcal alpha toxin, which, once fixed to its substrate, could not be removed or affected by the toxin neutralizing substance.

Experiment 5

Because of the rapid death following inoculation with staphylococcal alpha toxin, experiments were undertaken with a culture of C. diphtheriae to produce a local infection with a gradual release of toxin. For the virulence test of C.diphtheriae the inoculation of the growth of a pure culture of C. diphtheriae on Loeffler slope, suspended in broth or saline, was recommended by Andrews (1924), Parish et al. (1932) and others.

For our experiments, a pure culture of C. diphtheriae 1388 was received from Dr. G. Kalz of this Department. The culture was inoculated on Loeffler slopes and after 24 hours of incubation

at 37°C., the growth was washed off with 4 ml. of saline. Guinea pigs injected subcutaneously with 0.5 ml. of this suspension died regularly in 36-48 hours. At autopsy intense edema of the subcutaneous tissues was noted at the site of the injection, often haemorragic, together with a marked congestion of the adrenal cortices accompanied by haemorrage.

Two experiments were undertaken to test for neutralization of diphtheria toxin by the mould albumen derivative. In the first experiment three guinea pigs were injected with 0.5 ml. of the suspension of C. diphtheriae, subcutaneously. One guinea pig was the control for the C. diphtheriae. The other two were treated with 150 mgms. of dry albumen fraction in a volume of 1 ml., each, intraperitoneally at the same time as the culture injection. All the three guinea pigs died at the same time.

In the second experiment seven guinea pigs were injected subcutaneously with 0.5 ml.of the suspension of C. diphtheriae. Two were the controls for C. diphtheriae. Five guinea pigs were injected 4 hours previously with 150 mgms. of dry albumen fraction in a volume of 1 ml. subcutaneously. The control died, while two treated animals remained alive and well for the time of observation (10 days).

Experiment 6

Four guinea pigs were injected with 0.5 ml. of the suspensions of C. diphtheriae. One guinea pig was the control with C. diphtheriae alone.

The other three guinea pigs were treated as follows:

No.1) Two hours before the injection of C. diphtheriae with 15.2 mgms. each of the copper precipitated residue, intra-

peritoneally.

No. 2) At the same time of the injection of C. diphtheriae, with 15.2 mgms. each of the detoxifying agent, subcutaneously.

No. 3) 18 hours later, with the same amount of detoxifying substance, subcutaneously.

This copper precipitated residue had the potency in 15.2 mgms. to neutralize 1000 B.U. of staphylococcal alpha toxin.

Two guinea pigs of the treated group remained alive and well whereas the controls died within 36 hours.

Experiment 7

Eleven guinea pigs were injected with the same quantity of C. diphtheriae. Two guinea pigs were the controls with toxin alone. Four guinea pigs were treated 2 hours before the injection of C. diphtheriae with 15.2 mgms. of copper precipitated residue intraperitoneally and with the same amount of substance subcutaneously at the time of injection of C. diphtheriae.

Five guinea pigs were treated with 150 mgms. albumen fraction each intraperitoneally 2 hours before the injection of C.diphtheriae and with 150 mgms. subcutaneously at the time of injection of C. diphtheriae.

The potency of the copper precipitated residue was the same as in Experiment 6 and the albumen fraction had the same overall potency as the copper precipitated residue in view of the larger amount used.

Experimental data show that 2 out of 5 animals treated with the semi-purified albumen fraction and 3 out of 4 animals treated with the copper precipitated residue remained alive and well during the period of observation (7 days).

In summary of the animal experiments, the fact appears that the mould albumen fraction, although possessing neutralizing activity "in vitro", is not too effective "in vivo". In the experiments done with the detoxifying factor and staphylococcal toxin in mice, rabbits and guinea pigs, the animals were not protected by the mould albumen fraction. With diphtheria toxin only partial protection was obtained. It is possible that staphylococcal alpha toxin, having a great affinity for its substrate in the living body is immediately bound to tissue where it does its damage and does not offer any opportunity for the detoxifying agent to act upon it. It has been further demonstrated in a preceding section that such body fluids as serum, particularly the albumen fraction of normal serum destroys "in vitro" the toxin neutralizing principle. This may also help to explain the failure of "in vivo" efficacy of the toxin neutralizing principle.

In regard to the experiments done with <u>C. diptheriae</u>, it may be said that due to the gradual release of the toxin from the bacteria, the detoxifying agent may become free from the complex formed with the body fluid or with the tissues and partially act on the toxin while it is released by the bacterial cell and before it attacks the substrate. Moreover, in the experiments done with <u>C. diphtheriae</u> the protection given by the mould albumen fraction could also be explained as an antibiotic effect against the bacterial cell, although this does seem most unlikely in that no antibacterial effect of the toxin neutralizing principle has been demonstrated "in vitro".

2. EXPERIMENTS WITH TOXINS OTHER THAN STAPHYLOCOCCAL ALPHA TOXIN

The toxin neutralizing capacity of the albumen derivative of Penicillium cyaneo-fulvum has been tested on a variety of toxins other than staphylococcal alpha toxin as :

a. Staphylococcal Beta Toxin

b. Staphylocoagulase

c. Streptolysin O

d. Streptolysin S

e. Clostridium Welchii alpha toxin

f. Clostridium septicum haemolysin

g. Clostridium histolyticum haemolysin

h. Clostridium tetani haemolysin.

An introductory remark should be made here; in dealing with bacterial haemolysins other than staphylococcal alpha toxin for which a well defined unit of haemolytic activity exists, a standard haemolytic unit had to be adopted. There is at present no uniform standard unit in this respect. For this reason the principle of the Burnett unit has been used for all haemolysins and is referred to simply as the haemolytic unit; one unit is that amount of toxin which causes 50 per cent haemolysis of 1 ml. of a 2 per cent suspension of red blood cells in a total volume of 2 ml. in 1 hour at 37°C.

a) STAPHYLOCOCCAL BETA HAEMOLYSIN

The occurrence of a beta lysin of Staphylococcus pyogenes was first noted by Glenny and Stevens (1935) who demonstrated

that it acts on sheep, ox and human red cells but not on rabbit red blood cells and that it causes lysis only after the tubes have stood at room temperature or in the ice-chest overnight - the so-called "hot-cold lysis". It is more resistant to formalin and heat than the alpha haemolysin; it is antigenically distinct from the alpha lysin and it is specifically neutralized by the antiserum. It does not produce a dermonecrotic reaction, but only an erithematous flush. (Smith and Price 1938). Strains producing beta-lysin are predominantly of bovine origin (Minett 1936).

Method and results: Staphylococcal beta toxin was kindly provided by Dr. J. Rubles. By titration with the usual method it was shown to contain 256 haemolytic units / ml.

Neutralization by the albumen fraction: Four experiments were carried out to test for neutralization of the staphylococcal beta toxin by the albumen fraction using the antihaemolytic method previously described. The data are presented in Table 11.

TABLE 11

NEUTRALIZATION OF STAPHYLOCOCCAL BETA TOXIN BY THE SEMI-PURIFIED ALBUMEN FRACTION FROM PENICILLIUM CYANEO-FULVUM

Experiments	Number of Haemolytic Units neutralized by 150 mgms. of mould albumen fraction
1	128
2	128
3	128
4	128

It was found that 128 haemolytic units were neutralized by 150 mgms. of albumen fraction, i.e., 1 haemolytic unit is neutralized by 1.1 mgm. of dry material. (Table 12).

b) STAPHYLOCOAGULASE

The ability of certain staphylococci to coagulate citrated or oxalated plasma was first noted and described by Loeb (1903) and Much (1908).

Coagulase is formed almost exclusively by strains of Staphylococcus pyogenes aureus var. albus variants of aureus strains. Cruickshank (1937) and subsequent workers maintain that coagulase production constitutes the most convenient and reliable single test for estimating the pathogenicity of a given strain.

The method of coagulase action is not yet clearly understood. According to Smith and Hale (1944) coagulase is a thermostable substance, filtrable through a gradocol membrane having an ADP of 0.31mm . It appears to be the precursor of a thermolabile thrombin-like substance, the production of which depends on the participation of an activator present in the plasma of some animals. The staphylocoagulase reaction resembles normal thrombin formation from prothrombin under the influence of thrombokinase, except that calcium is not required.

Method and result: For the demonstration of coagulase activity 0.1 ml. of an overnight broth culture of Staphylococcus aureus is mixed with 1 ml. of a freshly prepared 1/10 dilution of rabbit plasma, in saline. The mixture is incubated at 37°C. for 4 hours.

TABLE 12

TOXIN INACTIVATION OF MOULD DERIVATIVE AGAINST VARIOUS BACTERIAL HAEMOLYSINS.

Haemolysins	Titer of Toxin Haemolytic Units per ml.	Titer of Toxin after Incubation with 150 mgms. of Albumen Fraction	Number of Units of Haemolysin Neutralized by 150 mgms. of Albumen Fraction	Mgms. of Albumen Fraction Neutraling 1 Haemolytic Unit of Toxin.
Staph. Alpha Haemolysin	3200	0	3200	0.046
Staph. Beta Haemolysin	256	128	128	1.1
Streptolysin O	16	8	8	18.75
Streptolysin S	1024	512	512	0.29
Cl. welchii Alpha toxin	160	0	160	0.93
Cl. septicum Haemolysin	20	0	20	7.5
Cl. histolyticum Haemolysin	64	0	64	2.34
Tetanolysin	80	0	80	1.87

If no clot has appeared by this time it should be left overnight at room temperature and again examined. A control was set up, containing plasma alone diluted with saline.

Neutralization by the Albumen Fraction: In the test 75 mgms. of the albumen fraction in a volume of 0.5 ml. were mixed to coagulase and the plasma in the described amounts. One control was prepared with coagulase and plasma, and another control with plasma alone. Experimental evidence shows that albumen fraction had no effect on staphylococcal coagulase activity, because the tube with plasma, coagulase and albumen fraction and the tube with plasma and coagulase (control) did clot in 6 hours.

c) STREPTOLYSIN O

Todd (1934, 1938, 1939) demonstrated the production of Streptolysin O from Streptococcus.

The O lysin is oxygen labile at ordinary temperature but can be reactivated by reduction with 0.1 per cent sodium hydrosulphyte; provided that it is protected from the air, it remains stable in the ice-chest for years. It is inactivated by incubation at 37°C. for 2 - 4 hours. The O lysin is formed by strains of Streptococcus belonging to Group A, C (human) and G.

Method and results: 100 ml. of Todd's broth (see appendix) were inoculated with Streptococcus pyogenes (Todd WPRL strain) and incubated for 16 hours at 37°C. After 16 hours the medium was centrifuged and the supernatant tested for Streptolysin O.

Titration: Serial dilutions of the fluid were made in saline in 1 ml. volume. To each tube was added 1 ml. of a 2 per cent rabbit red blood cells suspension. The tubes were shaken and incubated at 37°C. for 1 hour, following which the tubes were

centrifuged down and the supernatant read for 50 per cent haemolysis.

With this method Streptolysin O was obtained with a titer of 16 Haemolytic Units / ml.

Neutralization by the mould albumen fraction: 1 test was carried out for the neutralization of the Streptolysin O by the albumen fraction, using the antihaemolytic test already described in a previous section. The test shows that 8 Haemolytic Units are neutralized by 150 mgms. of albumen fraction, i.e., 1 Haemolytic Unit was neutralized by 18.75 mgms. of dry material. (See Table 12).

d) STREPTOLYSIN S

Todd (1934, 1938, 1939) demonstrated the production of the S lysin from Streptococcus by shaking the streptococci with serum and found that it is inactivated by incubation at 37°C. for 2 - 4 hours and that it cannot be reactivated by reduction. It is very sensitive to both heat and acid and can be preserved only by storage at very low temperature (-37°C.) The S lysin appears to be formed by strains of all the groups of streptococci, but the type of lysin produced is group specific, an antiserum to the S lysin of group A strains failing to neutralize S lysin formed by other groups. The streptolysin S has been purified and found to be a lipoprotein haptene incapable of stimulating the formation of antibodies.

Bernheimer (1948, 1949) was unable to find streptolysin S in any sonically disintegrated streptococci and suggested that lysin S is not produced until streptococci, either growing or resting, are brought in contact with certain essential factors.

Method and results: 100 ml. of Todd's broth (see appendix) were inoculated with Streptococcus pyogenes (Todd WPRL strain) and incubated for 16 hours at 37°C. After 16 hours the medium was centrifuged and the cells harvested. The cells were washed twice with saline to wash out all the Streptolysin O and 10 ml. of sterile horse serum were added to the cells. The suspension was placed in a shaking machine apparatus and shaken for 2 hours at room temperature. Following centrifugation for 20 minutes the clear supernatant fluid was tested for Streptolysin S.

Titration: Serial dilutions of the supernatant were made in saline in 1 ml. volume. To each tube was added 1 ml. of a 2 per cent rabbit red blood cells suspension. The tubes were shaken and incubated at 37°C. for 1 hour, following which the tubes were centrifuged down and the supernatant read for 50 per cent haemolysis.

With this method Streptolysin S was obtained with a titer of 1024 haemolytic units / ml.

Neutralization by the mould albumen fraction: Three tests were carried out for the neutralization of the Streptolysin S by the albumen fraction, using the antihaemolytic test already described in a previous section. Experimental evidence shows that 512 haemolytic units were neutralized by 150 mgms. of the albumen fraction, i.e., 1 haemolytic unit was neutralized by 0.29 mgms. of dry material. (Table 12 and Table 13).

TABLE 13

NEUTRALIZATION OF STREPTOLYSIN S BY THE SEMI-PURIFIED ALBUMEN FRACTION FROM PENICILLIUM CYANEO FULVUM

Experiments	Number of Haemolytic Units neutralized by 150 mgms. of mould albumen fraction.
1	512
2	512
3	512

EXPERIMENTS DONE WITH TOXINS FROM THE CLOSTRIDIA

e) CLOSTRIDIUM WELCHII ALPHA TOXIN

The alpha toxin of Cl. Welchii is a thermostable substance lethal for mice, guinea pigs, rabbits, pigeons and sheep, and when given intradermally produces a necrotic lesion. It produces haemolysis of the red blood cells of most laboratory animals and it is a powerful lecithinase. McFarlane and Knight (1941, 1942) demonstrated the identity of alpha toxin and of lecithinase showing a quantitative splitting of lecithine by alpha toxin into phosphocholine and agliceride and the necessity for Ca or Mg ions in the reaction. They suggested the alpha toxin to be lecithinase C and its nature to be that of a phosphatase.

Method and results: A semi-purified alpha-toxin of Clostridium welchii was supplied by Dr. J.W. Stevenson. It contained 400 MLD /ml. for 20 gms. mice by intraperitoneal injection. By haemolytic titration it was shown to contain 160 haemolytic units / ml.

Neutralization by albumen fraction: Five tests were performed

with the anti-haemolytic method already described to see whether the alpha toxin from the Cl.welchii was neutralized by the albumen fraction (See Table 14). It is seen that an average of 160 haemolytic units were neutralized by 150 mgms. of albumen fraction i.e. 1 haemolytic unit of alpha toxin is neutralized from 0.93 mgms. of dry material of albumen fraction (Table 12). Since it is known that the lethal activity of Cl. welchii alpha toxin resides in the same molecule as haemolytic activity, i.e., lecithinase C. it can be assumed that lethal activity is neutralized by the mould product.

TABLE 14

NEUTRALIZATION OF CLOSTRIDIUM WELCHII ALPHA TOXIN BY THE SEMI-PURIFIED ALBUMEN FRACTION OF PENICILLIUM CYANEO FULVUM

Experiments	Number of Haemolytic Units neutralized by 150 mgms. of mould albumen fraction.
1	160
2	160
3	32
4	160
5	160

f) CLOSTRIDIUM SEPTICUM HAEMOLYSIN

Filtrate of Cl. septicum have been shown to have in addition to a lethal effect, haemolytic activity. (Menk 1932, Beruheimer 1944). Beruheimer (1944) showed that lethal activity of the cultures of Cl. septicum was directly proportional to their

haemolytic activity and that the two effects could not be separated by fractionation with ammonium sulphate. Furthermore, he showed that the antihaemolytic titres of a number of horse sera were proportional to their antilethal titres.

In regard to the mode of action of the lysin, Bernheimer (1944, 1947) suggested that the action is enzymatic; the maximum rate of haemolysis is directly proportional to the concentration of toxin and the critical thermal increment is 12700 calories per mole. The addition of haemolysin to the red cells does not result in immediate lysis, but there is always an induction period during which the reaction takes place between the toxin and the red cells. Once the reaction has taken place lysis proceeds spontaneously without further participation of the toxin.

Method and results: Cl. septicum lysin was prepared with the medium used for the preparation of the other toxins from Clostridia. (See appendix). Using the conventional titration method it was found that the lysin contained from 2-20 haemolytic units/ ml. depending on the batches.

Neutralization by albumen fraction: Using the standard anti-haemolytic titration method it was shown in five tests that 20 haemolytic units are neutralized from 150 mgms. of albumen fraction, i.e., 1 haemolytic unit from 7.5 mgms. of dry material. (Table 12 and Table 15).

TABLE 15

NEUTRALIZATION OF CLOSTRIDIUM SEPTICUM HAEMOLYSIN

BY THE SEMI-PURIFIED ALBUMEN FRACTION

FROM PENICILLIUM CYANEO FULVUM

Experiments	Number of Haemolytic Units neutralized by 150 mgms. of mould albumen fraction.
1	20
2	64
3	20
4	20
5	20

g) CLOSTRIDIUM HISTOLYTICUM HAEMOLYSIN

Besides being a potent producer of a proteolytic enzyme, Cl. histolyticum produces a lethal and dermonecrotic toxin and an haemolysin (Stewart 1936, Waldbum and Reyman 1938). The toxin is moderately thermolabile being destroyed by heating at 70°C. for 30 - 60 minutes.

Method and results: Cl. histolyticum haemolysin was prepared with the medium used for the preparation of the other toxins from the Clostridia. (See appendix).

Using the haemolytic titration method already described it was found that the haemolysin contain 2-32 haemolytic units / ml. Neutralization by albumen fraction: with the anti-haemolytic method three tests were made; it was shown that 32 haemolytic units are neutralized by 75 mgms. of albumen fraction, i.e., 1 haemolytic

unit was neutralized by 2.34 mgms. (Tables 12 and 16).

TABLE 16

NEUTRALIZATION OF CLOSTRIDIUM HISTOLYTICUM HAEMOLYSIN BY THE SEMI-PURIFIED ALBUMEN FRACTION FROM PENICILLIUM CYANEO FULVUM

Experiments	Number of Haemolytic Units neutralized by 150 mgms. of mould albumen fraction.
1	64
2	64
3	64

h) TETANOLYSIN

Whole cultures filtrates of Cl. tetani contain, besides tetanospasmin, another substance discovered by Ehrlich and named by him "tetanolysin" (Kerrin 1930).

Tetanolysin has the power of causing haemolysis of the red blood cells of various animals and it is entirely distinct from the neurotoxin. It may be separated from the neurotoxin by treating the toxic broth with red blood cells to adsorb the haemolysin and it is more thermolabile than is tetanospasmin. It is antigenic and can be inactivated by oxygen or mild oxidizing agents (Neill 1926). This reaction is reversible with reduction. It is inhibited by cholesterol and by the specific antitoxin to any one of the other oxygen labile haemolysin, i.e., theta toxin of Cl. welchii, pneumolysin and streptolysin O (Van Heyngen 1950).

Method and results: Tetanolysin was prepared with the medium used for the preparation of the other toxins from the Clostridia. (See Appendix). Using the haemolytic method it was found the lysin contained from 2 to 64 haemolytic units/ ml. depending on the batches.

Neutralization with the albumen fraction: Five tests were performed to determine the anti-haemolytic titer of the albumen fraction for tetanolysin. It was shown that an average of 40 haemolytic units were neutralized by 75 mgms. of albumen fraction, i.e., 1 haemolytic unit was neutralized by 1.87 mgms. (See Table 12, Table17 and Figure 3).

TABLE 17

NEUTRALIZATION OF TETANOLYSIN
BY THE SEMI-PURIFIED ALBUMEN FRACTION
FROM PENICILLIUM CYANEO FULVUM

Experiments	Number of Haemolytic Units neutralized by 150 mgms. of mould albumen fraction.
1	64
2	80
3	80
4	80
5	80

IV. PROPERTIES OF THE TOXIN NEUTRALIZING SUBSTANCE OF PENICILLIUM CYANEO-FULVUM

1. Antibiotic versus Detoxifying Activity.

It had been shown previously that a whole culture filtrate of the mould had an antibiotic spectrum similar to that of penicillin (D.G. Denton, 1947) but that the semi-purified detoxifying agent does not have antibiotic activity. Thus it would appear that this species of Penicillium (i.e., Penicillium cyaneo-fulvum) produces penicillin or a penicillin-like substance and a toxin neutralizing substance which is quite distinct from the antibiotic.

To examine this fact "clarase" was tested against our active principle. "Clarase" a penicillinase preparation, was found by Lawrence (1943, 1944, 1945) a suitable agent to bring about the inactivation of penicillin in clinical diagnostic work. Because of its antipenicillin activity clarase was tested against the toxin neutralizing substance in an attempt to confirm the fact that the antibiotic and detoxifying agent were distinct and separate substances.

0.5 ml. of 0.4 per cent clarase in peptone broth was mixed with 0.5 ml. of the semi-purified albumen fraction and incubated for 1 hour at 37°C. After 1 hour the mixture was tested with an antihaemolytic titration against staphylococcal alpha toxin according the technique described in section lll and was proven to be fully effective in its detoxifying activity. Clarase in the concentration employed inactivates the antibiotic penicillin whereas it is seen that it has no effect on the

toxin neutralizing principle.

2. Adsorption of the Toxin Neutralizing Principle on Various Agents.

One of the main problems in dealings with bacteria and moulds is the problem of contamination and spreading of the spores in the laboratory particularly in dried products. In our experiments it was not possible to sterilize the filtrate or its active derivative with heat, since the active principle is destroyed at sterilization temperature (Table 18 and Figure 4). Seitz filtration could not be used because the Seitz filter adsorbed the detoxifying agent removing it completely from the solution. By using the standard antihaemolytic titration, 2 tests were performed which showed that 150 mgms. of dry albumen fraction, with a potency to neutralize completely 3200 Burnett Units of staphylococcal alpha-toxin, lost after Seitz filtration 100 per cent of the toxin neutralizing ability. This suggested the possibility that the active principle might be removed from the filtrate by adsorption on some agent as a step in concentration. Tests were performed in an attempt to purify the detoxifying substance by means of successive adsorption on several substances as kaolin, animal charcoal and activated charcoal. Method and results: In the experiment with activated charcoal, in each of 2 tubes containing 2 ml. of the mould albumen fraction (dry weight 300 mgms.) was placed 0.1 gm. of activated charcoal. The albumen fraction was able to neutralize 1600 Burnett units per ml. as shown in a control. One tube was placed in a shaking machine for 1 hour to get a better mixture and the other was held

at the same time under normal conditions. The two tubes were kept overnight in the ice-box at 5°C. The following morning the tubes were centrifuged and the supernatant fluid of each was tested by a conventional antihaemolytic titration (See section 111) for the content of detoxifying substance against staphylococcal alpha toxin (1600 Burnett units /ml.).

The sedimented parts were resuspended in buffer solution of pH 7.0, shaken for 1 hour, in the shaking machine apparatus and were placed in the ice-box for 4 hours. At the end of this second period both tubes were centrifuged and the supernatant fluids were tested for detoxifying activity by the conventional antihaemolytic titration against staphylococcal toxin (1600 Burnett units /ml.). It was shown that approximately 80 per cent of the detoxifying activity remained in the supernatant fluid and only 20 per cent was adsorbed to the charcoal.

Experiments with kaolin and animal charcoal gave the same negative results, with regard to the possibility of purifying the active material by means of adsorption.

3. <u>The Effect of Temperature on the Toxin Neutralizing Principle.</u>

A study was undertaken on the effect of temperature on the toxin neutralizing principle.

Method and results: Several tubes containing 1 ml. of a saline solution (150 mgm. dry weight) of albumen fraction of a known potency (150 mgms. neutralizing 1600 Burnett units of staphylococcal alpha toxin) were placed in waterbath at the following temperatures: 50°C., 55°C., 60°C., 65°C., 70°C., 75°C., 80°C., 85°C., 90°C. The time of exposure was in each instance

30 minutes. After 30 minutes of exposure the tubes were tested for content of detoxifying active principle by means of an antihaemolytic titration against staphylococcal alpha toxin. The results (Table 18) show that the toxin neutralizing activity decreases in 30 minutes starting from an exposure at 60°C. and completely disappears after exposure to 75°C. for 30 minutes. Thus the thermolability of the product is approximately of the same degree as that of many proteins. (See Figure 4).

TABLE 18

EFFECT OF TEMPERATURE ON A SALINE SOLUTION OF LYOPHILIZED MOULD DERIVATIVE, USING 150 MGMS. /ML. AGAINST 3200 BURNETT UNITS OF STAPHYLOCOCCAL ALPHA TOXIN AFTER HEATING AT THE DESIGNATED TEMPERATURE FOR 30 MINUTES

Temperature (in C°.)	50	55	60	65	70	75	80	85	90
Per cent of inactivation.	0	0	65	80	90	100	100	100	100

4. The Effect of Normal Serum on the Toxin Neutralizing Principle.

One of the features of antibiotic and detoxifying substances is that unfortunately they are often neutralized totally or partially by body fluids, particularly blood serum. This fact, very important in regard to therapeutic possibilities, has been discussed in an earlier section of the thesis - the "Historical Review" (Section A).

Method and Results: In a series of ten experiments the albumen fraction of the mould filtrate was tested against guinea pig, horse and sheep serum. Tests were carried out with normal serum, serum filtered through a Seitz filter, and serum heated at 60°C. for 30 minutes.

In each experiment 0.5 ml. of a saline solution of the albumen fraction containing 75 mgms. on a dry weight basis, an amount sufficient to neutralize 800 Burnett units of staphylococcal alpha haemolysin, was mixed in a Wasserman tube with 0.5 ml. of the serum sample (respectively normal, filtered and heated serum of each of the animal species). The mixtures were incubated in a waterbath at 37°C. for 1 hour, with 0.5 ml. of staphylococcal alpha haemolysin containing 800 Burnett units. Following incubation 0.5 ml. of a 5 per cent suspension of rabbit red blood cells were added to each tube, together with 0.5 ml. of saline to make up the total volume to 2.5 ml. The mixtures were read for 50 per cent haemolysis after incubation at 37°C. for 1 hour. From the experimental data it was shown that the guinea pig and horse serum whether normal, filtered or heated, destroyed 100 per cent of the toxin neutralizing activity. The same was true of normal and heated rabbit serum, but filtered serum gave variable results which cannot be explained at the present time. The data are presented in table 19A. In a second group of experiments done with the same materials when the serum was placed 15 minutes after the combination of staphylococcal toxin and detoxifying agent no inhibition occurred of the toxin neutralizing principle by the serum, suggesting that the serum plays a competitive role to the albumen

TABLE 19

(A) EFFECT OF DIFFERENT SERA ON THE SEMI-PURIFIED ALBUMEN FRACTION FROM PENICILLIUM CYANEO FULVUM

Type of Serum	Neutralizing capacity of albumen fraction before treatment. Burnett Units/150 mgms. equivalent of staphylococcal alpha toxin.	Neutralizing capacity of albumen fraction after treatment. Burnett Units /150 mgms. equivalent of staphylococcal alpha toxin.	Per ce of in- activat
fresh guinea pig	1600	0	100
heated guinea pig	1600	0	100
filtered guinea pig	1600	0	100
filtered horse	1600	0	100
heated horse	1600	0	100
fresh sheep	1600	0	100
heated sheep	1600	0	100
filtered sheep	1600	0	100
fresh rabbit	1600	16	99
heated rabbit	1600	0	100
filtered rabbit	1600	0-1600*	0-100

* Filtered rabbit serum did not give uniform results.

(B) EFFECT OF NORMAL SERUM ON THE COPPER PRECIPITATED RESIDUE FROM PENICILLIUM CYANEO FULVUM

Type of Serum	Neutralizing Capacity of albumen fraction before treatment. Burnett Units /150 mgms. equivalent of staphylococcal alpha toxin.	Neutralizing capacity of albumen fraction after treatment. Burnett units /150 mgms. equivalent of staphylococcal alpha toxin.	Per ce of in- activati
Fresh Rabbit Serum.	1600	16	99

fraction in regard with its combination with the toxin.

A third experiment was devised to study what constituent of the serum was responsible for its activity. Using the method described above, purified albumen* and purified alpha, beta and gamm globulins* were tested against the albumen fraction. In the experiment 0.5 ml. of each of these substances (in a dilution of 2 per cent in saline) was mixed with 0.5 ml. of a saline solution of the albumen fraction containing 75 mgms. on a dry weight basis, an amount sufficient to neutralize 800 Burnett Units of staphylococcal alpha toxin. The mixtures were incubated in a waterbath for 1 hour with 0.5 ml. of staphylococcal alpha haemolysin containing 800 Burnett units. Following incubation 0.5 ml. of a 5 per cent suspension of rabbit red blood cells was added to each tube together with 0.5 ml. of saline to make up the total volume to 2.5 ml.

The results show that only the albumen part of the serum possessed the property to neutralize the mould antitoxic principle. Alpha, beta and gamma globulins were completely negative in this respect. The data are presented in Table 20.

The copper precipitated residue is inactivated by fresh normal serum. In this respect it does not differ from the crude semi-purified albumen fraction as it is shown from Table 19B.

* These substances were obtained from Dr. Van Straten of the Montreal General Hospital Research Institute.

TABLE 20

EFFECT OF CRYSTALLINE ALBUMIN AND ALPHA, BETA AND GAMMA GLOBULINS ON THE SEMI-PURIFIED ALBUMEN FRACTION FROM PENICILLIUM CYANEO FULVUM

Serum constituent	Neutralizing capacity of albumen fraction before treatment. Burnett Units /150 mgms. equivalent of staphylococcal alpha toxin.	Neutralizing capacity of albumen fraction after treatment. Burnett Units per 150 mgms. equivalent of staphylococcal alpha toxin.	Per cent of inactivation
albumin	1600	0	100
alpha globulin	1600	1600	0
beta globulin	1600	1600	0
gamma globulin	1600	1600	0

5. Toxicity of the Toxin Neutralizing Factor for Experimental Animals:

Rabbit and guinea pigs are insusceptible to relatively large doses of the mould derivative in the form of the concentrated albumen fraction. Guinea pigs of 400 gms. withstand, with no effect, a single intraperitoneally dose of 900 mgms. of the dry concentrate. However, the next higher dose tested, i.e., 1200 mgms. was lethal for guinea pigs.

The rabbit tolerates a single intravenous dose of 1500 mgms. and 2400 mgms. administered intravenously in 300 mgm. doses over a period of 3 weeks produces no symptoms. The minimal lethal dose for the rabbit was not established because of the lack of sufficient purified material.

Mice, in contrast with guinea pigs and rabbits, are relatively

susceptible to the mould albumen fraction. 6 mgms. administered intraperitoneally is not toxic for 20 gm. mice. 15 mgms. results in symptoms of shock with death in approximately 1 hour. A dose of 37 mgms. administered intraperitoneally kills mice within 1 minute with symptoms of acute shock. Administered subcutaneously the same dose results in death in about 1 hour. The tissues at the site of infection show marked oedema and congestion.

The antihistaminic Neo Antergan (Poulenc) protects mice against this effect. Further data in this respect are presented on page 56 (Section 111).

The copper precipitated residue is toxic for mice in amount of 30 mgms. whereas it is quite free of toxicity in amount of 15 mgms.

The effectiveness of the copper sulphate precipitation may be shown in that 15 mgms. of albumen fraction, which is toxic for mice, only inactivates in vitro 320 Burnett units of staphylococcal toxin. On the other hand, 15.2 mgms. of copper precipitated substance, a dose non-toxic for mice, inactivates in vitro 3,200 Burnett units of staphylococcal toxin. Thus for purpose of therapeutic administration the purified material is more than ten times less toxic than the crude albumen fraction.

6. <u>Antigenicity of the Mould Albumen Fraction.</u>

It is of considerable importance that the detoxifying principle should not be antigenic if it is to be used for therapeutic purposes, because of the danger of anaphylactic reaction and because it would become less and less effective

with repeated injections since the body would form antibodies to it which would neutralize its activity. For these reasons the albumen fraction was tested for possible antigenicity.

Method and results: A rabbit weighing 5 kg. was injected intravenously every three days with 300 mgms. of the albumen fraction until a total of 1200 mgms. had been given. One week after the last injection a reinforcement dose of 300 mgms. was given as a secondary stimulus. After two weeks the rabbit was bled from the marginal ear vein, the blood was left at room temperature for 24 hours for separation of the serum which was then obtained by centrifugation. The serum was tested for antibody content in two ways:

a) The precipitation reaction: the ring test was performed by preparing halving dilutions in 10 tubes starting with a 1:10 dilution of the albumen fraction in a volume of 0.5 ml. Serial dilutions were set up from 1/10 to 1/5120 in 0.5 ml. volumes. On a dry weight basis the first tube contained 7.5 mgms. of the albumen fraction, the last tube 0.014 mgms. In each tube 0.5 ml. of the antiserum was carefully layered over the albumen fraction. The tubes were held at room temperature for a period of three hours and overnight observing for the formation of a precipitate at the interface of the fluids. No precipitate occurred at any time during this period. (Table 21)

b) An antihaemolytic titration was set up in a rack of 10 tubes with halving dilution of staphylococcal alpha toxin of a potency of 1600 Burnett units / ml. in 0.5 ml. volumes, to which 75 mgms. of albumen fraction (0.5 ml. of volume) were added in each tube

TABLE 21

PRECIPITATION REACTION BETWEEN MOULD ALBUMEN FRACTION AND THE SERUM OF RABBITS IMMUNIZED WITH THE ALBUMEN FRACTION

Tubes	1	2	3	4	5	6	7	8	9	10	CM*	CA*
Dilution of albumen fraction in 0.5 ml. (75 mgms.)	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10	-
Antiserum in ml.	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	-	0.5
Saline	-	-	-	-	-	-	-	-	-	-	0.5	0.5
Flocculation	-	-	-	-	-	-	-	-	-	-	-	-

* CM - Control Albumen Fraction

CA - Control Antiserum

and 0.5 ml. of rabbit antiserum. After combination for 1 hour at 37°C. rabbit blood cells were added (0.5 ml. of 5 per cent suspension), and 0.5 ml.of saline to make up the total volume to 2.5 ml. in each tube. A control tube without rabbit antiserum was also prepared. The tubes were read for 50 per cent haemolysis after 1 hour combination at 37°C.

No haemolysis occurred in any tube indicating that the mould was permitted to act on the toxin and detoxifying it, without being neutralized by antibody present in the rabbit serum.

This titration was repeated three times because of a doubtful result which appeared in one titration where haemolysis occurred in all tubes but not in the control without antiserum. This was interpreted as being the result of the mould albumen fraction being neutralized not by the antiserum, but by the fresh serum, a property demonstrated in part 4 of this section. The other two titrations confirmed the results that no haemolysis occurred. Thus it would appear that antibody had not been formed which might counteract the activity of the toxin neutralizing principle.

7. A preliminary Study of the Chemical Nature of the Detoxifying Agent.

An attempt was made to define some of the chemical properties of this toxin neutralizing factor. This part of our problem is still in an early stage of development due to the complexity of the semi-purified material, which will require further purification before the chemical nature of the active principle can be studied adequately. At the present time only a preliminary study of the reacting groups through biochemical tests is possible, together

with other techniques as paper chromatography and micro-electrophoresis which can also aid in the purification and identification of the substance. This preliminary study was carried on on the semi-purified albumen fraction since the more highly refined residue of the copper sulphate precipitation procedure was not available until very late in the course of the investigation.

(i) The xantoproteic test was positive denoting the presence of phenyl, or tyrosine or tryptophane groups.

(ii) The sulphur tests for the presence of cysteine were negative.

(iii) The ninhydrin reaction was positive showing the presence of free carboxyl and alpha amino groups.

(iv) A micro-kieldhal analysis for nitrogen determination showed that there were 22.16 mgms. of nitrogen per 150 mgms. of the albumen fraction.

(v) A test was performed to detect the presence of gelatinase, as a representative of proteolytic enzymes in the albumen fraction, since Ramon and Richou (1947) and Richou and Gerbaux (1952) showed that semi-purified filtrates of Penicillium notatum contained powerful proteolytic enzymes. The following procedure was employed. 1 ml. of a sterile saline solution containing 150 mgms. of albumen fraction on a dry weight basis in halving dilutions (1/1 -1/512) was added aseptically to 10 tubes containing 5 ml. of a 1 per cent solution of gelatin. The tubes were incubated for 24 hours at 37°C. and then

placed in the ice-box for 1 hour before reading to allow any non-hydrolyzed gelatin to solidify. It was shown that the albumen fraction in 150 mgms. contained enough gelatinase to bring about proteolysis of the gelatin (First tube). The control tube, to which saline had been added, was not liquefied.

(vi) Fehling's reaction for presence of reducing sugars was negative.

(vii) 300 mgms. of albumen fraction in a volume of 2 ml. of saline were treated with trypsin added to a concentration of 0.3 per cent and left for three hours at 37°C. A control not treated with trypsin was run at the same time. An anti-haemolytic titration was set up with 0.5 ml. of the trypsin treated mould product in a Wasserman tube to which 0.5 ml. of staphylococcal alpha haemolysin (1600 Burnett units per ml.) was added. A control tube was set up with the non-treated mould in the same experimental conditions. After 1 hour incubation in water-bath at 37°C., 0.5 ml. of a 3 per cent suspension of rabbit red blood cells was added in each tube, and 50 per cent haemolysis was read after 1 hour at 37°C. Result shows that no haemolysis occurred suggesting that the trypsin did not inactivate the mould neutralizing activity. In a second experiment a group of mice was inoculated with the treated and the untreated albumen fraction, with 0.5 ml. of material for each mouse. All

the mice died showing that the trypsin did not inactivate the toxicity of the albumen fraction; however, it should be noted that the mice may have died also from residual toxin neutralizing substance not inactivated by the trypsin.

(viii) A precipitation test was set up to establish whether the albumen fraction in combining with the staphylococcal toxin might form a precipitate as part of the mechanism of the reaction. Serial dilutions of the albumen fraction were mixed together with constant amounts of toxin in one experiment, while in a second test serial dilutions of the toxin were mixed with constant amounts of the detoxifying principle (Table 22). There was no evidence that a precipitate was formed in the union of the two substances.

(ix) Paper Chromatography and Microelectrophoresis. Two techniques are available whereby proteins may be caused to move on filter paper: chromatography and microelectrophoresis. The former method involves the movement of proteins by capillary ascent. A mixture of proteins diffuses from a spot into a streak by the action of an acqueous developing solvent. The latter technique, based on the Tiselius method, makes use of the different mobility of the various proteins in an electrical field.

Several tests have been made to examine the possibility of separating the mould albumen fraction into different components in an attempt to isolate and study the active principle.

Paper chromatography and microelectrophoresis have been performed according to the methods of Kalz, Telner, Quastel and Van

TABLE 22

PRECIPITATION REACTION BETWEEN MOULD ALBUMEN FRACTION AND STAPHYLOCOCCAL ALPHA TOXIN

Tubes	1	2	3	4	5	6	7	8	9	10	CM*	CT*
Dilutions of albumen fraction (150 mgms./ml.) in 1 ml.	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10	-
Dilution of toxin 1600 B.U. /ml. in 1 ml.	1/10	1/10	1/10	1/10	1/10	1/10	1/10	1/10	1/10	1/10	-	1/10
Saline in ml.	-	-	-	-	-	-	-	-	-	-	1.0	1.0
Flocculation	-	-	-	-	-	-	-	-	-	-	-	-

Tubes	1	2	3	4	5	6	7	8	9	10	CM	CT
Dilutions of toxin 1600 B.U. /ml. in 1 ml.	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	-	1/10
Dilutions of albumen fraction (150 mgms. /ml.) in 1 ml.	1/10	1/10	1/10	1/10	1/10	1/10	1/10	1/10	1/10	1/10	1/10	-
Saline in ml.	-	-	-	-	-	-	-	-	-	-	1.0	1.0
Flocculation	-	-	-	-	-	-	-	-	-	-	-	-

* CM - control albumen fraction

CT - control toxin

Straten (1953) and Franklin and Quastel (1949). *

Method and results: The electrophoresis apparatus consists of a pair of plastic troughs (Perspex) 17" x 4" x 3-1/4" each divided longitudinally into two compartments. Electrical contact between the buffer solution in the outer and inner compartment is made through small wicks protruding through holes near the upper edge of the dividing wall. Carbon electrodes are placed in the outer compartments and connected through an ammeter and voltmeter to a source of DC current from a selenium rectifier with a variable AC input. The filter paper strips are supported on a plastic frame fitted onto the troughs so that the ends of the papers dip into the buffer solution in the two inner compartments of the troughs. The whole set up is enclosed in a glass tank with a heavy glass lid resting on a rubber rim. Veronal buffer pH 8.6 consisting of 0.05 M sodium diethyl barbiturate and 0.01 M diethyl barbituric acid is used as the electrophoresis buffer solution. A potential of 130 volts is applied across the papers for about 18 hours, after which the papers are dried. The results will be treated together with those of chromatography.

Chromatography: 0.2 ml. of a saline solution of mould albumen fraction containing 30 mgms. on a dry weight basis, are placed at the lower left-hand corner of filter paper squares. (Eight inch sheets of Whatman No.1 filter paper are used). The papers are mounted on suitable rack or stapled into cylinders, after the

* (Paper chromatography and microelectrophoresis have been performed with the help of Dr. Van Straten of the Montreal General Hospital Research Institute).

spots are dried at room temperature, and then placed in a shallow dish containing 0.1 M sucrose solution - the developing agent used in the first dimension. After the papers are thoroughly wetted by capillary action (about 1-1/2 hours), they are dried at room temperature, turned at right angles, and placed into the second dimensional developing solvent (0.1 M sodium potassium tartrate solution) until the liquid has travelled approximately 2/3 up the paper.

In the chromatographic analysis prepared in our department 1-dimensional chromatography was prepared with filter paper strips, 1 inch large, supported by a metal frame was employed as an alternate method.

After the filter papers were dry, different techniques were used to detect any substance present following both the chromatographic and electrophoretic procedures :-

a) ultraviolet examinations

b) staining reactions

c) antihaemolytic titrations.

a) Examination under ultraviolet light revealed some bands in the electrophoresis developed filter paper at about 1 inch from the origin, while in the chromatogramms the bands were at 2/3 of the total distance from the origin, i.e., about 6 inches.

b) The filter papers after electrophoresis and chromatography were stained :-

1) with a hot acqueous solution of Solvay purple (0.05 per cent), containing 0.5 per cent sulphuric acid. After about 5 minutes the papers were washed with warm water to

remove excess dye, and dried. 2) with bromophenol blue. 3) with freshly prepared benzidine reagent, (equal volumes of 3 per cent hydrogen peroxide, and a saturated alcoholic solution of benzidine acidified with glacial acetic acid) with a fresh solution of ninhydrin, prepared according to the technique of McFarren (1952). The colour is developed by placing the chromatogram in an oven at 60°C. for exactly 15 minutes. All these staining reactions in (b) gave negative results, and no spots or bands appeared in the filter paper; only in two experiments with ninhydrin was it possible to see a spot at about 1/3 of the total distance, in the chromatogram, from the origin.

c) Antihaemolytic titration: Dry filter paper from electrophoresis and chromatograms were cut into pieces of 1 square inch each (about 10 pieces for each filter paper), placed in 1.5 ml. of saline, macerated and left for 1 hour at room temperature. After 1 hour 1 ml. of staphylococcal alpha haemolysin containing 160 Burnett units was placed in each tube; the mixture was left for 1 hour in a water-bath at 37°C. and then 0.5 ml. of 5 per cent suspension of red blood cells was added. The tubes were read for 50 per cent haemolysis after 1 hour incubation at 37°C.

In all the experiments done, but once with a staphylococcal toxin of 10 Burnett units /ml., no toxin neutralizing substance was found, since haemolysis occurred in every tube. Thus little success has been achieved at this time in adapting paper chromatography and microelectrophoresis to the study of the

albumen fraction or in resolving its components. Several explanations are possible in this respect: The detoxifying substance may be totally adsorbed to the filter paper because of the small amount placed at the origin (0.2 ml., containing 30 mgms. of dry albumen fraction). The detoxifying substance may be only partially adsorbed, but the active portion which remains may not be sufficient to be detected with staining reactions, nor to neutralize even the small amount of toxin in the antihaemolytic titrations. It is possible that the mould albumen fraction does not move appreciably from the origin because of a very high molecular weight. It is also possible that in the travelling from the origin, those groups of the molecule responsible for the staining reactions and for the detoxifying activity might be covered by other groups present in the semi-purified substance.

V. MODE OF ACTION OF THE TOXIN'S NEUTRALIZING SUBSTANCE

As has been reported in the "Historical Review" of this paper, Ramon and Richou (1945-47) suggest that the detoxifying factors are active in an enzymatic way; Cavalli (1948) held the same hypothesis identifying the activity of the toxin neutralizing factor from the Penicillium notatum and Streptomyces griseus as being enzymatic in nature and acting on the toxic group of the toxin molecule. Del Vecchio (1947-1948) working with the same substances states that in inmunological reactions there is no loss in combining power of the toxin when the toxin is mixed with that neutralizing agent. There is some confusion in the litherature in this respect, others having reported a loss in combining power. The albumen fraction of P. cyaneo-fulvum tested in our experiments acts as an efficient toxoiding agent as is seen in the following experiments.

1. The effect of the toxin neutralizing principle on the antigenicity of Staphylococcal Alpha Haemolysin.

Method and results: Staphylococcal alpha toxin with a titre of 1600 Burnett Units per ml. was completely inactivated with 75 mgms. of dry albumen fraction per ml. of toxin, by combination of the two components in water-bath at 37°C. for 1 hour. Very careful tests were performed to ensure that no active haemolysin remained. A rabbit weighing 4 kgs. was injected with a mixture of neutralized staphylococcal alpha toxin and albumen fraction with increasing doses according to the immunization regime pro-

posed by Dolman (1932, 1935) for the usual formolized staphylococcal toxoid. The doses were of 0.25, 0.30, 0.35 and 0.50 ml. of the mixture with a four day interval between each injection. Ten days after the last injection a reinforcement dose of 0.5 ml. was given as a secondary stimulus.

Before the booster dose, the rabbit was bled and the serum analysed with an anti-haemolytic titration and with a flocculation test for content of antitoxin with the methods already described. It was shown that the serum contained the equivalent of 1 Provisional International Unit of antitoxin per ml. Two weeks after the booster dose the rabbit was challenged with 1 ml. of staphylococcal alpha toxin containing 1800 Burnett Units (1.2 lethal dose) intravenously. Three control rabbits received only the staphylococcal toxin in the same amount and by the same route. Result: The rabbit immunized with the mixture of staphylococcal toxin and albumen fraction remained alive and well over the observation period of ten days while the controls died within 1 hour. This result was interpreted as showing that the albumen fractionof P. cyaneo-fulvum acted as an efficient toxoiding agent. But the hypothesis arose that the protection from alpha staphylococcal toxin could arise from free toxin present in non-measurable amounts, so that another experiment was devised to overcome this difficulty.

The experiment was designed to test the actual antigenicity of a staphylococcal alpha-toxin of a known potency before admixture with the mould albumen fraction and after, to determine to what extent the albumen fraction might act in destroying the

antigenicity of the toxin. The method of Dolmann (1935) was followed: 15 test tubes received 0.4 ml. each of staphylococcal antitoxin such that the amount of antitoxin in each tube was 7 Provisional International Units of antitoxin. To the tubes in the series staphylococcal toxin was added in the following amounts: 0.05, 0.07, 0.09, 0.11, 0.14, 0.17, 0.20, 0.24, 0.28, 0.32, 0.38, 0.46, 0.56, 0.66, 0.90 ml. respectively. The mixtures were incubated at 45°C. and flocculation was observed after 15, 30, 60, 120, 180 minutes and overnight (19 hours). The first tube showing flocculation was recorded. With the same method another group of test tubes were prepared containing the same amounts of antitoxin but instead of intact staphylococcal alpha toxin the same dilutions of a staphylococcal alpha toxin completely detoxified by contact for 1 hour with the albumen fraction were added. (150 mgms. of albumen fraction in 1 ml. volume to detoxify 1 ml. of staphylococcal alpha toxin containing 1600 Burnett units). Incubation and observation were carried out as in the control test.

Results showed that with fresh toxin flocculation appeared at 30' incubation, whereas the tubes with antitoxin-detoxified toxin mixture, flocculation occurred after 120 minutes. In one experiment the tubes first showing flocculation was the same and the amount of Lf units /ml. for the toxin was 41 for the fresh toxin and 41 for the detoxified toxin. (Table 23). In a second experiment the fresh toxin showed to contain 50 Lf /ml., while the detoxified toxin contained 20 Lf /ml. (Table 24). The same tubes showing first flocculation also showed the greatest amount

TABLE 23

COMPARATIVE FLOCCULATIONS OF INTACT STAPHYLOCOCCAL ALPHA TOXIN AND OF STAPHYLOCOCCAL ALPHA TOXIN DETOXIFIED BY THE SEMI-PURIFIED ALBUMEN FRACTION FROM PENICILLIUM CYANEO FULVUM.

Tubes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	CT*	CA*
Intact Staph. Alpha Toxin (in ml.)	0.05	0.07	0.09	0.11	0.14	0.17	0.20	0.24	0.28	0.39	0.38	0.46	0.56	0.66	0.90	0.90	-
P.I.U. of Staph. Alpha Antitoxin (in 0.4 ml.)	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	-	7
Flocculation	-	+	++	+++	+++	+++ **	+++	+++	++	+	-	-	-	-	-	-	-

Tubes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	CT	CA
Detoxified Staph.Alpha Toxin (in ml.)	0.05	0.07	0.09	0.11	0.14	0.17	0.20	0.24	0.28	0.32	0.38	0.46	0.56	0.66	0.90	0.90	-
P.I.U. of Staph. Alpha Antitoxin (in 0.4 ml.)	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	-	7
Flocculation	-	+	++	+++	+++	+++ **	+++	+++	+++	+	-	-	-	-	-	-	-

* CT = Control Toxin
CA = Control Antitoxin

** First tube showing flocculation

TABLE 24

COMPARATIVE FLOCCULATIONS OF INTACT STAPHYLOCOCCAL ALPHA TOXIN AND OF STAPHYLOCOCCAL ALPHA TOXIN DETOXIFIED BY THE SEMI-PURIFIED ALBUMEN FRACTION FROM PENICILLIUM CYANEO FULVUM

Tubes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	CT*	CA*
Intact Staph. Alpha Toxin (in ml.)	0.05	0.07	0.09	0.11	0.14	0.17	0.20	0.24	0.28	0.32	0.38	0.46	0.56	0.66	0.90	0.90	-
P.I.U. of Staph. Alpha Antitoxin (in 0.4 ml.)	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	-	7
Flocculation	-	++	++	+++	+++**	+++	++	+	-	-	-	-	-	-	-	-	-

Tubes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	CT	CA
Detoxified Staph. Alpha Toxin (in ml.)	0.05	0.07	0.09	0.11	0.14	0.17	0.20	0.24	0.28	0.32	0.38	0.46	0.56	0.66	0.90	0.90	-
P.I.U. of Staph. Alpha Antitoxin (in 0.4 ml.)	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	-	7
Flocculation	-	-	+	++	+++	+++	+++	+++**	++	++	+	-	-	-	-	-	-

* CT = Control Toxin
CA = Control Antitoxin

**First tube showing flocculation

of precipitation overnight. Only one zone of flocculation was found. As the overall result, it was shown that the flocculating power of the staphylococcal alpha toxin was the same as the flocculating power of the detoxified staphylococcal toxin; thus the albumen fraction of P. cyaneo-fulvum while destroying the toxicity did not destroy the antigenicity of the staphylococcal toxin. Another test was performed using Salmonella typhosa H antigen (containing 500 x 10^6 cells /ml.) to see whether the mould albumen fraction might destroy the antigenicity of the antigen after 1 hour incubation at 37°C. The test showed that the mould albumen fraction did not interfere with the antigenicity of Salmonella typhosa H antigen as measured by means of agglutination with the homologous antiserum. (Table 25).

An attempt was made to determine with what part of the haemolytic system the mould reacts, in view of its ability to detoxify without bringing any damage to the antigenicity of the toxin. The mould product might act directly on the toxin molecule, presumably on the toxin group since antigenicity is retained or it might, of course, act on the substrate in such a way as to block contact of the toxin with substrate.

Experiment 1.

75 mgms. of the mould albumen fraction in 1 ml. of volume were mixed with 0.5 ml. of a 5 per cent rabbit red blood cells suspension. The mixture was left at 37°C. for 1 hour and centrifuged. The clear supernatant fluid was discarded and the sedimented red blood cells were washed twice with saline. To the red blood cells was then added 1 ml. of staphylococcal alpha toxin containing 1600 Burnett Units, an amount which is completely

TABLE 25

COMPARATIVE AGGLUTINATIONS OF INTACT SALMONELLA TYPHOSA H ANTIGEN BY HOMOLOGOUS ANTISERUM AND OF SALMONELLA TYPHOSA H ANTIGEN TREATED WITH THE SEMI-PURIFIED ALBUMEN FRACTION FROM PENICILLIUM CYANEO FULVUM

Tubes	1	2	3	4	5	6	7	8	9	10	CAG*	CAS*
Dilution of Antiserum (in 1 ml.)	1/60	1/120	1/240	1/480	1/960	1/1920	1/3840	1/7680	1/15360	1/30720	1/60	-
Intact Antigen (500x 10^6 cells/ml.) (in ml.)	1	1	1	1	1	1	1	1	1	1	-	1
Agglutination	++++	++++	++++	++++	++++	++++	+++	+	0	0	0	0

Tubes	1	2	3	4	5	6	7	8	9	10	CAG	CAS
Dilution of Antiserum (in 1 ml.)	1/60	1/120	1/240	1/480	1/960	1/1920	1/3840	1/7680	1/15360	1/30720	1/60	-
Treated Antigen (500 x 10^6 cells / ml) (in ml.)	1	1	1	1	1	1	1	1	1	1	-	1
Agglutination	++++	++++	++++	++++	++++	++++	+++	+	0	0	0	0

* CAG = Control Antigen
CAS = Control Antiserum

neutralized by 75 mgms. of albumen fraction. A control was seen in this respect. The mixture was incubated at 37°C. for 1 hour and then centrifuged. As a result haemolysis occurred in the two tubes showing that the albumen fraction is not combining with the red blood cells.

Experiment 2.

From the foregoing experiment it was suggested that the albumen fraction acted on the toxin combining with the toxic group, or simply that it acted competitively toward the substrate in a freely reversible reaction with the substrate. The test was designed to ascertain whether the mould albumen fraction could exert its activity even after the toxin had combined with the substrate but before visible lysis occurred. In this experiment staphylococcal beta haemolysin was used because it requires a longer period of time for its "hot-cold" type of haemolysis; it is possible to have this toxin fixed to the substrate red cells for as long as 1 hour in water-bath at 37°C. without the appearance of haemolysis.

1 ml. of staphylococcal beta toxin of a potency of 800 haemolytic units was placed in halving dilutions (1/2 - 1/1024) in each of 10 tubes; 0.5 ml. of a 5 per cent suspension of red blood cells was added to each tube. The mixture was kept at 37°C. for 1 hour. After 1 hour 150 mgms. of the mould albumen fraction in 1 ml. volume were added to each tube and the tubes were incubated at 37°C. for another hour. The entire rack was placed overnight in the ice-box and centrifuged. Haemolysis occurred according to the titer of haemolysin (800 units), as shown in a parallel

untreated control series. Thus, once the toxin is fixed to its substrate the toxin neutralizing principle is not able to displace it, nor to prevent the haemolysis of the sheep red blood cells. It may be, of course, that once the toxin contacts the red blood cells substrate the damage which eventually leads to lysis is done.

Experiment 3.

A further type of experiment was devised to study the mode of action of the toxin neutralizing agent.

It has been shown by Neter et al (1952 a & b, 1953) that the somatic antigens of Escherichia coli serogroups 111, 55 and 26 are adsorbed by the red blood cells of various species of animals and render such modified erytrocytes agglutinable by homologous bacterial antibodies. In the presence of complement modified sheep red blood cells are lysed by the respective bacterial antisera in high dilution. Neter et al have outlined the conditions that must be met if lysis of red blood cells by anti-bacterial antisera is to take place: (1) A suitable bacterial antigen must be present in appropriate state and quantity and in a favourable environment to be adsorbed by red blood cells. (2) The red blood cells must possess reactive groupings (receptors) on the surface to be capable of adsorbing the bacterial antigen and (3) The adsorbed antigen on the red blood cells must be able to react with the homologous bacterial antibodies.

It was further shown that certain materials as lecithin and cholesterol inhibited the haemagglutination and the haemolysis. The authors suggested that the inhibitory effect of such agents

is largely due to the action of the agents on the bacterial antigen in such a way as to interfere with the adsorption of the antigen by the erytrocytes.

In our experiment the somatic antigens of serogroup 55 of Escherichia coli was centrifuged. 1 ml. of the bacterial free supernatant was mixed with 1 ml. of a 5 per cent suspension of red blood cells and the mixture was incubated for 30 minutes at 37°C. Following incubation the modified red blood cells were washed, centrifuged and reconstituted in 1 ml. volume and added to 1 ml. of antiserum in a Wasserman tube in the presence of 0.5 ml. guinea pig complement (1/10). Haemolysis was read after incubation at 37°C. for 30 minutes. The tube showed haemolysis. Using the same method the red blood cells were modified by the antigen but this time in presence of 150 mgms. of albumen fraction in 1 ml. volume. It was shown that haemolysis did not occur. Thus the mould albumen fraction inhibited the haemolysis caused by the antigen-antibody complex of Escherichia coli under the condition of the experiment. It is suggested that this inhibitory effect was due to the action of the mould on the antigen, either through interference with the adsorption of the antigen by the red blood cells or through its influence on the reactivity of antigen with homologous antibodies; however, a third possibility exists, i.e., that the mould in itself could have anticomplementary action.

Experiment 4

This experiment was done to find out what part of the mould albumen fraction might account for the detoxifying effect and to

determine whether or not the same radicals responsible for toxicity of the mould product were also responsible for its toxin neutralizing effect. The albumen fraction was inactivated:

(a) by heating at 75°C.

(b) by reaction at 37°C. for 1 hour with staphylococcal alpha haemolysin (75 mgms. of albumen fraction in 1 ml. of saline solution mixed with 1 ml. of toxin containing 1600 Burnett units).

(c) by fresh guinea pig serum (150 mgms. of albumen fraction in 1 ml. of saline solution incubated at 37°C. for 30 minutes with 1 ml. of the serum.)

Controls were run by the conventional antihaemolytic titration to be sure that the albumen fraction was inactivated. Complete inactivation was achieved in each instance. In (b) toxin was also destroyed. Mice were then inoculated with amounts of (a), (b) and (c), designed to provide 15 mgms. on a dry weight basis of the albumen fraction. (A lethal dose).

Result: All the mice died except that group inoculated with albumen fraction neutralized by the toxin. That is in a combination between toxin and the albumen fraction as the detoxifying activity is destroyed, the toxicity of the crude albumen fraction for mice also disappears. At first glance it might be concluded that the portionof the crude albumen fraction responsible for neutralization of bacterial toxin is identical with the radical responsible for animal toxicity. However, in a later stage of the work outlined in the section of purification and property of the toxin neutralizing substance, it is seen that the toxin

neutralizing principle and the toxic factor are really two distinct substances. The toxic fraction may be removed from the albumen complex by precipitation with copper sulphate leaving the toxin neutralizing portion intact. It is interesting, however, to find that when the whole albumen fraction is added to toxin the toxic mould factor is apparently "bound-up" or involved in some way in the reaction between two other substances such that it is no longer toxic.

SECTION C

DISCUSSION

DISCUSSION

From the early 1890s when Metchikoff first demonstrated that Bacillus mesentericus and Bacillus substilis were able to produce substances which neutralized diphtheria toxin, tetanus toxin and snake venom "in vitro", many attempts have been made to find substances which might be effective in the therapy of bacterial toxaemias. The interest in detoxifying agents was stimulated later on by Carpenter and Barbou (1938) who stated that sulphanilamide destroyed the toxicity of gonococcal endotoxins and the toxins of Staphylococcus aureus and Clostridium welchii. Since the advent of the antibiotics, some research has been done to find out whether they might inhibit the production of toxins from bacteria, while carrying on their specific activity against the casual agent of the infection. Controversial results have been recorded in the medical literature and no agreement has been reached with respect to their possible therapeutic use.

Ramon and Richou (1945) caused a new wave of interest in the field of the antibiotics in general and of the detoxifying agents in particular with a series of experiments on the filtrates of Penicillium notatum, Streptomyces griseus, and Bacillus subtilis. They demonstrated in the filtrates potent substances totally different from the antibiotics which these filtrate contain, which were able to detoxify certain bacterial toxins. Since the work of Ramon and Richou, experiments have been performed to confirm their findings by many others, in particular by Smolens et al. (1947), Del Vecchio et al. (1947) and Cavalli (1947).

While these experiments were being carried on with strains of Penicillium notatum, Streptomyces griseus and Bacillus subtilis, a mould was isolated in our laboratory by D.G.Denton (1947) which was found to produce a potent neutralizing substance which was secreted into the medium during the growth of the mould in fluid cultures. In the light of this finding and since other than the specific antitoxin no toxin neutralizing substance has ever been found which has proven really effective in the treatment of bacterial toxaemias, it was felt that an attempt should be made to isolate and characterize the active principle and to investigate its spectrum of toxin neutralizing activity.

The mould was sent to the Centralbureau for Schimmelcultures of Baarn (Holland) for identification where it was designated Penicillium cyaneo-fulvum Biourge, NRRL 837.

In regard to the question as to whether this organism should be considered a distinct species, Raper and Thom (1949) stated that although mutants of Penicillium notatum have been observed which approximate this species, since the general type of cultures have been obtained from widely separated natural sources and since Biourge's strains have in general retained their characters for several years in cultures, it seems best to recognize such species as P. Cyaneo-fulvum. This fact is particularly important as to differentiate our detoxifying principle from that described by Ramon and Richou in 1945 and subsequent years. In addition to the fact that the substances come from different sources, other differences are clearly recognizable: Ramon and Richou (1947) claimed that concomitant with neutralization of the toxin by P. notatum's principle there is a loss of immunizing and combining

power of treated staphylococcal toxin, while on the other hand staphylococcal alpha toxin completely neutralized by our P. cyaneo-fulvum principle is still capable of stimulating the production of antitoxin in the rabbit and shows no loss of combining power. This, however, is in line with observations of Del Vecchio et al. (1947) that the toxin neutralizing agent isolated from P. notatum did not destroy the flocculating power of diphtheria toxin. Ramon and Richou (1947) claimed that the activity of the detoxifying agent did not begin to diminish until a temperature of 75°C. was reached. Our principle, however, shows over 70 per cent inactivation at 60°C. in 30 minutes. This is in line with the findings of Cavalli (1947) who noted the destruction of a detoxifying substance from Actinomyces griseus at 60°C. in 30 minutes. Ramon and Richou (1947) noted that their principle did not inactivate appreciably staphylococcal beta-toxin even after 6 hours of incubation at 37°C., while 128 Haemolytic units of staphylococcal B-toxin were neutralized by 150 mgms. of our albumen fraction after 1 hour incubation at 37°C. Ramon and Richou (1948) claimed that their active principle from P. notatum was not dialyzable. This has been our finding. However, Smolens in 1947 claimed that the detoxifying agent from P. notatum was dialysable.

The McGill strain of Penicillium cyaneo-fulvum may be subcultured on Sabouraud dextrose and malt extract slopes and it is upon the latter medium that stock and seed inocoulum cultures are maintained. Of various media tested as Glucose beef infusion broth, Peptone broth, Malt extract, Raulin's medium, Czapex-Dox's

medium, Czapex-Dox Del Vecchio medium and a casein hydrolysate medium, the Glucose beef infusion broth has been shown to be the best medium for the production of the detoxifying substance whereas Peptone broth, Malt extract and Casein hydrolysate media are less suitable. With Raulin's medium, Czapex-Dox and Czapex-Dox-Del Vecchio media no production of the detoxifying agent occurred. The data indicate that glucose and peptone are essential factors for the production of the toxin neutralizing agent which cannot be synthesized by the mould from inorganic substances, tartaric acid and sugar even if all the required mineral ions are present in the medium. As has been shown, several problems arise for the most efficient production and purification of the active principle. In fact, best production of the detoxifying agent and purification through precipitation and other more specialized procedures are closely related problems; a culture medium from which the purified active principle has to be prepared should be as free as possible of any substances that are difficult to separate from it during the purification. A synthetic medium composed of inorganic and simple organic substances is the ideal but in our experiments, Raulin's and Czapex-Dox's media were shown to be useless because while allowing a good growth of the organism, they did not allow any appreciable production of the toxin neutralizing factor. it was not possible under the conditions of our experiments to obtain high yields of the active principle except on complex media containing non dialysable constituents.

In regard to the procedure of purification, fractional

precipitation with ammonium sulphate to a concentration of 48 per cent to precipitate the inactive globulin fraction followed by saturation of the supernatant fluid with ammonium sulphate to precipitate the albumen fraction has proven to be effective with the Glucose beef infusion broth medium; it is less effective with other media as for example Malt extract medium although this latter medium is the best for the conservation of seed cultures and gives a good yield of detoxifying substance in the crude filtrate. When one attempts to precipitate the active principle from a culture grown in this medium, a large part of the active principle is lost. (38 per cent). Precipitation with ammonium sulphate to a concentration of 33 per cent according the method of Del Vecchio et al. (1948) was not successful and only yielded small white granular precipitate of the type of our inactive globulin fraction which was not effective in neutralizing staphylococcal alpha toxin. Concentration of the active detoxifying agent in the albumen fraction by fractional precipitation of a glucose beef infusion broth culture of Penicillium cyaneo-fulvum with ammonium sulphate has thus become the standard procedure. The albumen fraction obtained is a crude material largely of proteinaceous nature which gives positive reactions with the xanthoproteic and the ninhidrin tests. It has a dry weight of 130 - 20 mgms. /ml. of the fluid recovered from the dialysis bag and of 1560 ± 360 / 100 ml. of the original crude filtrate. It is relatively toxic for mice and guinea pigs.

On account of the bulk of the dry material and the toxicity of this albumen fraction several attempts were made to separate

the active principle from the albumen fraction by such methods as adsorption on kaolin, animal charcoal, activated charcoal, by chromatography and electrophoresis (see below) and by precipitation with butanol and acetone. Such methods were unsuccessful. In experiments designed to overcome the toxicity of the albumen fraction, precipitation of the impurities with $CuSO_4$ was finally found to be the answer. Some antibiotic substances like kojic and aspergillic acids were shown by Yabuta (1912) and Tobie and Alverson (1947) to form an insoluble salt with copper sulphate. Such substances were highly toxic per os and intraperitoneally for mice. On the basis of such observation and in consideration of the fact that we were dealing with a mould product an attempt was made to precipitate out of the solution of the rather toxic mould albumen fraction toxic pigments which might resemble aspergillic acid for example. Following precipitation with 1 per cent copper sulphate a voluminous precipitate appears. The copper sulphate precipitation yield a dark green inactive substance which goes into solution with difficulty. 67.4 mgms. upon intraperitoneal inoculation in mice kills the animals within 1 hour. This precipitate is discarded as it is toxic and non active and the supernatant residue is dialysed against saline for 48 hours at -6 mm. of Hg. The final volume of the fluid recovered from the bag is 14 ml. for each 100 ml. of the original crude filtrate. The dry weight of the copper precipitated residue is 15.2 mgms. /ml. Thus the final yield of the purified material is 212.8 mgms. for each 100 ml. of original crude filtrate. This precipitation brings the dry weight of the detoxifying agent from 130 ± 20 mgms. /ml.

to 15.2 mgms. /ml. with a loss of 88.4 per cent of the original dry weight while the detoxifying activity remains at the same level. From the data (in Section IV) it is shown that 1 mgm. of the $CuSO_4$ purified product has a toxin neutralizing ability equal to that of 9.8 mgms. of the crude ammonium sulphate precipitated albumen fraction; that is a concentration of some 10 times more potency on a unit weight basis.

The effectiveness of the copper sulphate precipitation may also be shown with a comparison between the toxicity of the semi-purified albumen fraction and that of copper precipitated residue; it may be shown that 15 mgms. of albumen fraction which is toxic for mice only inactivates in vitro 320 Burnett Units of staphylococcal alpha toxin. On the other hand 15.2 mgms. of copper precipitated substance, a dose non-toxic for animals, inactivates in vitro 3200 Burnett Units of the same toxin. Thus for purposes of therapeutic administration the purified material is more than 10 times less toxic than the crude albumen fraction.

Paper chromatography and microelectrophoresis were unsuccessful as methods to purify the mould albumen fraction and the copper sulphate residue in spite of several tests made with different detecting methods as ultraviolet examination, staining reactions and antihaemolytic titrations. Several reasons have been given for the failure of these techniques in bringing any separation of the toxin neutralizing factors into different components: (1) The detoxifying substance may be totally adsorbed to the filter paper because of the small amount placed at the origin (0.2 ml. containing 30 mgms. of the dry albumen fraction),

(2) The detoxifying substance might have been only partially adsorbed and the active portion which remained might not be sufficient to be detected with staining reactions nor to neutralize even the small amount of staphylococcal alpha toxin used in the antihaemolytic titrations. (3) It is possible that the mould albumen fraction does not move appreciably from the origin because of a very high molecular weight. (4) It is possible that in the travelling from the origin those groups of the molecule responsible for the staining reactions and for the detoxifying activity might be covered by other groups present in the semi-purified substance. Paper chromatography was slightly more efficient than microelectrophoresis in causing the tested substance to move from the origin, but in neither case was the toxin neutralizing substance found in any appreciable amount.

With respect to the properties of the detoxifying agent it has been shown that the active principle is thermolabile being destroyed at 60°C. in 30 minutes. The semi-purified albumen fraction gives a positive reaction with the xantoproteic and the ninhydrin tests; nitrogen determinations show 22.16 mgms. of nitrogen per 150 mgms.of albumen fraction. These data together with the non dialyzability, the precipitation at full saturation, the sensibility to pH and its best production starting from material of high molecular weight like peptone suggest a proteinaceous nature of the active detoxifying agent. On the other hand the low molecular density which brings the substance toward the surface during centrifugation and the inhibition by serum

albumin point to a possible combination of the proteinaceous material with lipids to form a lipoproteic compound stable at room temperature and resistant to some extent to denaturating agents.

In the discussion of the therapeutic implication of toxin neutralizing substances from microorganisms in the "Historical Review" of this paper we did mention the fact that such substances to become useful therapeutic agents must possess certain definite properties, i.e., that the substance must have little toxicity for the intact animal body, it should not produce pathological change when administered in quantity and frequently for prolonged periods, it should act in low concentration so as to render practicable its posology, it should not be antigenic because of the danger of producing allergy and further if antibodies developed repeated doses of the substance would become less and less effective, and finally it should be active in the presence of normal and pathological body fluids. Because of the possible therapeutic value of our mould product it is important to see how the above conditions are fulfilled from the detoxifying agent. From the experiments it is indicated that the procedure of copper sulphate precipitation removes virtually all of the inactive impurities which are toxic to animal body so that at the level of a therapeutic dose the detoxifying agent is non toxic. Furthermore, the copper precipitated residue containing 15.2 mgms. of dry material / ml. and a potency to neutralize 3200 Burnett Units of staphylococcal alpha toxin does not produce any pathological changes in the animal body after prolonged treatment. The antitoxic principle was tested

for antigenicity and was found to be non-antigenic. One more point of interest is the study of the activity of the substance in the presence of normal and pathological body fluids. Many substances which are highly effective in ordinary media are inactivated by body fluids. The experimental data show that the mould active principle, after being tested against normal, filtered and heated guinea pig, horse and rabbit sera is inhibited and loses some 90 to 100 per cent of its detoxifying activity. Furthermore, it has been shown that it is the albumen fraction of serum which is responsible for the inactivating property. It is of interest to note, in this respect, that Davis (1947) reported that the binding property of albumen depends upon the native undenaturated configuration of the albumin molecule and that its special property implies the presence on the surface of the molecule of regions where the specific configuration of the amino-acids residues leads to an interaction with other substances. According to Davis (1949) the chemical forces responsible for the formation of complexes between albumin or serum on one side and antibiotic and similar substances on the other, include electrostaic attraction between oppositely charged groups, hydrogen bonds and Van der Waal's forces. The fact that the active principle of Penicillium cyaneo-fulvum is neutralized by serum or albumin "in vitro" as most of the drugs, seems to point with difficulty to its possible therapeutic use unless an adequate concentration of the active material can be maintained in the body. It is interesting to note that if the

serum is placed in the "in vitro" experiments 15 minutes after that the mixtures between the mould albumen fraction and staphylococcal alpha toxin has taken place, no inactivation of the detoxifying agent occurs and the haemolytic activity of the toxin is inhibited. This condition, of course, cannot be attained in the animal experiments but if high concentrations of the detoxifying agent can be maintained in the body with repeated injections or with the help of adjuvants, it might be possible for the detoxifying agent to counteract the effect of toxin as soon as it is released from the infecting bacterial cells and before all of the toxin neutralizing substance is inactivated.

The whole culture filtrate, the semi-purified albumen fraction and the more highly purified residue following copper sulphate precipitation have been tested against a large number of toxins for toxin neutralizing activity. Most of the experimental work has been done with staphylococcal alpha toxin and its haemolytic, dermonecrotic and lethal effects. Antihaemolytic titration shows that the albumen fraction has an average potency of 17 Provisional units of staphylococcal alpha antitoxin per 150 mgms. of dry material. As can be seen from Table 5, the mould albumen fraction has a varied neutralizing capacity depending on the batch. The reason for the rather wide variation in the potency of different batches has not been determined as yet but several factors have been shown to influence the optimum of production as choice of a properly stabilized stock culture for the inoculation of the liquid medium, prevention of denaturation during the

manipulation in the preparation of the purified product and care that it is not lost by adsorption to filter paper and other materials with which it comes in contact.

The neutralization of the dermonecrotic effect of the staphylococcal alpha toxin shows that the semi-purified albumen fraction has a neutralizing capacity equivalent to that of 16 Provisional International Units of staphylococcal alpha antitoxin per 150 mgms. of dry material. This result correlates very well with the inactivation of the haemolytic activity of the toxin. The neutralization of the lethal effect as demonstrated in animal experiments follows quantitatively the inactivation of the haemolytic and dermonecrotic effect of the staphylococcal alpha toxin. In summary, from the foregoing experiments it is seen that the mould derivative is able to neutralize "in vitro" the haemolytic, dermonecrotic and lethal effects of staphylococcal alpha toxin and that the relationship between the toxin and the detoxifying agent is the same in the neutralization of the three different activities of the toxin, the inactivation of one effect correlating very well with the inactivation of the others.

It is interesting to note in this regard that there is much contention as to whether the alpha toxin as a single molecule has haemolytic, dermonecrotizing and lethal effect or whether the alpha toxin is a complex substance each of these effects being due to a different toxic molecule. In this respect, the fact that the mould derivative is able to neutralize all three toxic effects simultaneously lends support to the more accepted theory that the exotoxin is simply one substance having different effects on different tissues.

The results have been shown to be reproducible and the inactivation of staphylococcal alpha toxin is a definite conclusion which arises from these experiments, provided that the two substances are mixed in a test tube and incubated for a period of time usually from half one hour to one hour, although on several occasions it was found that even after immediate combination the toxin was effectively neutralized by the albumen fraction.

Other "in vitro" experiments have been performed with a variety of toxins. In this respect it has been shown that the albumen fraction is capable of bringing inactivation of staphylococcal beta toxin, streptolysin O, streptolysin S, Clostridium septicum haemolysin, Clostridium welchii alpha toxin, Clostridium histolyticum haemolysin, and tetanolysin. Different amounts of dry albumen fraction are necessary to neutralize 1 haemolytic unit of each of the different toxins. From Table 12 it can be seen that staphylococcal alpha toxin is the toxin most readily neutralized; streptolysin S, Cl. welchii, lecithinase C, staphylococcal beta toxin, tetanolysin, Clostridium histolyticum haemolysin, Clostridium septicum haemolysin, and streptolysin O require in that order more of the albumen fraction per haemolytic unit. It is noted that more of the albumen fraction is required to neutralize the oxygen labile haemolysins streptolysin O and tetanolysin than the oxygen stable haemolysins, i.e., staphylococcal alpha toxin, staphylococcal beta haemolysin, lecithinase C and streptolysin S. Clostridium histolyticum and Clostridium septicum haemolysins are intermediary in this respect.

The only bacterial product which was found not to be inactivated by the mould albumen fraction was staphylococagulase. Staphylocoagulase is a non-antigenic, heat stable substance that causes plasma to clot by precipitating fibrinogen as fibrin. In this case, however, we do not deal with a real exotoxin, because staphylocoagulase is not a lethal agent in the sense of the classical exotoxins, although it contributes to the virulence and pathogenicity of the bacterial cell.

The "in vitro" experiments show no real correlation with the "in vivo" results. The lethal effect of staphylococcal alpha toxin was not inhibited by the mould albumen fraction and by the copper precipitated residue in experiments done with mice. Animals injected with an LD 50 of staphylococcal alpha toxin and the mould derivatives died in the same proportion as the animals treated only with the toxin and in some cases the figures of the mortality rate were higher in the animals injected with toxin and detoxifying agent. Better results were obtained by injecting albumen fraction and copper precipitated residue in guinea pigs infected with a culture of C. diphtheriae which regularly killed the control animals in 36 hours after subcutaneous injection. The mould albumen fraction saved two out of five treated animals in one experiment and offered no protection in a second experiment, while the copper purified substance protected two out of four guinea pigs in one test and three out of four in second experiment. In summary, it appears that the mould derivatives, although possessing neutralizing activity "in vitro", did not offer a great protection in the

"in vivo" tests. It is possible that staphylococcal alpha toxin, having a great affinity for its substrate in the living body, is immediately bound to tissues where it does its damage and does not offer any opportunity for the detoxifying agent to act upon it. In the experiments with C. diphtheriae it may be said that due to the gradual release of the toxin from the bacterial cells, the detoxifying agent may become free from the complex formed with the body fluids or with the tissue enzymes and partially act on the toxin while it is released by the bacteria and before it attacks the substrate. Moreover, in the experiment with C. diphtheriae the protection given by the mould albumen fraction could also be explained as an antibiotic effect against the bacterial cell although this does seem most unlikely in that no bacterial effect of the toxin neutralizing substance has been demonstrated "in vitro".

One point more is worthy of attention and that is the remarkable activity of the mould derivatives, i.e., the toxoiding property of the detoxifying agent. This process with which the toxins lose their toxicity while retaining their ability to stimulate the production of antibodies is still not understood. Of the several substances which have been proved to accelerate the process, formaldehyde is the most widely used. According to Pappenheimer (1942) the free epsilon amino-groups of lysine residues and the hydroxyl groups of tyrosine residues disappear during toxoiding, which suggests that these groups are essential for the toxicity of the toxin molecule. Pillemer et al. (1948) working with tetanus toxin showed that the toxin tends to be unstable and is spontaneously converted to a highly antigenic

toxoid on standing at 0°C. It appears from ultracentrifugal studies that the toxoid is a dimer with sedimentation constant at 7 S compared with 4.5 S for the toxin. Therefore, Pillemer et al. suggest that the toxin molecules condense through their toxic groups. This process takes place instantaneously in the presence of formaldehyde and because of the very low concentration of formaldehyde it cannot be assumed that the change in the sedimentation constant of the toxoid is due to the quantity of formaldehyde added; the only acceptable suggestion is that the condensation of the toxin molecules is catalysed by formaldehyde (Van Heyningen, 1950). According to this hypothesis the toxic free epsilon amino group of lysin residues and the hydroxil groups of tyrosine residues could be covered and disappear during the toxoiding process, but no conclusive evidence has been reached as yet that these groups are essential for the activity of the toxin.

The neutralization of the toxicity of the molecule without affecting its antigenicity is naturally of foremost importance in the use of the toxoid for immunizing purposes. The ability of the mould derivative to bring a quick toxoiding process without causing a loss in antigenicity was tested in two ways. In the "in vivo" experiment a rabbit immunized with increasing doses of a mixture of staphylococcal alpha toxin and albumen fraction in which the toxin had been completely inactivated, developed in three weeks antibodies to the toxin to the extent that the serum contained the equivalent of 1 Provisional International Unit of antitoxin per ml. Two weeks after the

booster dose the rabbit was challenged with 1.2 lethal doses of staphylococcal alpha toxin and remained alive and well during the period of observation of 10 days while the control died within 1 hour. The "in vitro" experiments designed to test the actual antigenicity of a staphylococcal alpha toxin of a known potency before admixture with the mould albumen fraction and after, showed that the flocculating power of staphylococcal alpha toxin was the same as the flocculating power of the detoxified staphylococcal toxin thus demonstrating the ability of the mould derivative to bring complete inactivation of staphylococcal alpha toxin in 1 hour at 37°C., without affecting the antigenicity of the toxin. This procedure thus yields a toxoid more rapidly and with less disturbance than does formaldehyde.

With respect to the mode of action of the toxin neutralizing substance, a study was undertaken to determine on which part of the system, i.e., toxin or substrate, the mould derivative acts. The mould product might act directly on the toxin molecule, and presumably only on the toxic group since antigenicity is retained, or it may act on the substrate in such a way as to block contact of the toxin with the substrate. Experiments were set up to rule out either one or the other of the two hypotheses. In the first instance the fact that red blood cells, after combination with the detoxifying agent, are haemolyzed by staphylococcal alpha toxin if the mould derivative is centrifuged and washed out, proves that no irreversible combination occurs between the toxin neutralizing substance and the substrate. A further type of experiment was devised to exclude any combination with the substrate

by taking advantage of the fact that once the red blood cells of various animals are modified by somatic antigens of Escherichia coli they can be agglutinated by homologous bacterial antibodies, while in the presence of complement such sensitized erytrocytes are lysed by the antiserum in high dilution. Neter et al. (1953) have outlined the conditions that must be met if lysis of the erythrocytes is to take place: (i) A suitable bacterial antigen must be present in appropriate state and quantity and in a favorable environment to be adsorbed by red blood cells. (ii) The red blood cells must possess reactive groupings (receptors) on the surface to be capable of adsorbing the bacterial antigen. (iii) The adsorbed antigen on the red blood cells must be able to react with the homologous bacterial antibodies. It was further shown that certain material as lecithin and cholesterol inhibit the haemagglutination and the haemolysis. The authors suggested that the inhibitory effect of such agents is largely due to the actions of the agents on the bacterial antigen in such a way as to interfere with the adsorption of the antigen by the erytrocytes.

In our experiments in this respect, the mould albumen fraction was used and haemolysis of the erytrocytes was inhibited, while lysis of the cells occurred in the controls. On the basis of this experiment, it is suggested that the inhibitory effect of the mould is due to its action on the antigen either through interference with the adsorption of the antigen or by blocking the reaction of the antigen with homologous antibodies. However, a third possibility existed, i.e., that the mould in itself

could have anti-complementary activity, although it was shown in a previous experiment that the mould derivative has only a very slight anti-complementary effect.

Another experiment was done to find out what part of the mould albumen fraction might account for the detoxifying activity and to determine whether or not the same radical radical responsible for toxicity of the mould derivative also accounts for its toxin neutralizing effect. It was shown that when the detoxifying activity is destroyed the toxicity of the mould derivative disappears. From this experiment, it might be concluded that the toxic group and the detoxifying group are one and the same. However, the fact that the toxic fraction may be removed from the albumen complex by precipitation with copper sulphate leaving the toxin neutralizing portion intact, shows that toxin neutralizing principle and toxic factor are really two distinct substances. It is interesting, however, to find that when the albumen fraction is added to the toxin, the toxic mould factor is apparently bound up so that it is no longer toxic.

To conclude the discussion of the mode of action of the detoxifying agents, it must be said that while it is obvious that the mould derivative combines with the toxin and not with the substrate, it is not known at the present time what reaction occurs between the toxin and the mould derivative and which particular groupings are involved in the toxoiding process since the entire field of the mode of action of the toxins is not understood, with the exception of <u>Clostridium welchii</u> alpha toxin

which is known to be lecithinase C.

Van Heyningen (1950) claims that sometimes the substrate of a toxin can be defined anatomically, like blood or nervous system, but more often its location can only be described as wide-spread and that, in general, if a toxin does not attack a specialized tissue, it can be assumed that it is dermonecrotic. Since a dermonecrotic effect is shown by several toxins, it follows that the skin contains the substrate of many toxins and that the same substrate is also found in most of the other tissues of the body.

The study of the identity of the substrate is of considerable importance. This is particularly true since some of the experimental data suggests that the detoxifying principle reacts with the toxin competitively to the substrate, possibly because of some similarity in the structure of the two substances. It is possible that the reaction between toxin and mould derivative may be due to electrostatic attraction between oppositely charged groups on the surface of the molecules.

With regard to the structure of the mould derivative and of the substrate on which the toxin acts, no experimental data are available although we do know that the specific substrate of Cl. welchii alpha toxin is lecithin C against which the mould product is active. An hypothesis may be made to explain some of the facts. The low molecular density of the albumen fraction which brings the substance toward the surface during centrifugation and the inhibition by serum albumin point to a possible lipoprotein nature of the detoxifying agent. The lipoprotein

molecule was studied by Onkley (1953) and found to contain cholesterol, cholestserol esters, lecithin, phospholipid, amino acid residues and other groupings. He found that in some lipoprotein molecules although there is more lipid than peptide, the solubility properties of the molecules are those of a typical euglobulin molecule suggesting that the lipids, aminoacids residues and water are arranged in such a way that the protein moiety and perhaps the charged phospholipid residues are largely exposed at the surface. The study of the chemistry of the lipoproteins offers a possible interpretation of our experimental results. Lecithin C is known to be the substrate of Clostridium welchii alpha toxin, and it was found from Neter (1953) to inhibit haemagglutination and haemolysis of the erytrocytes sensitized by the Escherichia coli somatic antigens in the presence of the homologous antiserum. Cholesterol is a constituent of most of the tissues, like skin, blood serum, stroma of the red blood cells and others. Furthermore, it is a known antihaemolytic compound active against the oxygen labile toxins and it is suggested that its presence in the red blood cells is to prevent their haemolysis. From this data it may be suggested that the lecithin and the cholesterol present in the molecule of the lipoprotein may act competetively to the lecithin or the cholesterol of the toxin substrate. In this case the inhibition of the detoxifying agent by serum albumin could be explained as a reaction between the albumin and the lipid residue of the lipoprotein molecule.

Experiments being carried out at the present time with the

neurotoxin of Clostridium tetani may throw some light on this question because it is necessary to imagine a different substrate for it, since the neurotoxins are definitely not dermonecrotic and their mode of action may involve different mechanisms.

From a general point of view the problem of the mode of action of the toxin neutralizing substance is an important one in that it may help to explain the nature and the mode of action of the bacterial toxins. The fact that toxins of different origin and apparently different activity can be treated "in vitro" and sometimes "in vivo" with a single substance which can neutralize their toxicity, suggests that the bacterial toxins may have much in common. Cavalli (1948) offers the hypothesis of a possible activity of the microbiol detoxifying agent on the epsilon amino-group of diphtheria toxin which is responsible for its toxicity according to Pappenheimer (1942) and draws some analogies between the epsilon amino-groups of diphtheria toxin and related groups in the other toxins.

To conclude, it may be seen that the study of the toxin neutralizing principle from Penicillium cyaneo-fulvum opens many interesting fields of study because of the therapeutic possibilities of the detoxifying agent and since its nature and activities may provide a tool for the investigation of the structure of the toxins, their substrates and their mode of action.

SECTION D

SUMMARY

SUMMARY

1. A strain of Penicillium cyaneo-fulvum Biourge has been isolated in this laboratory which produces in a suitable medium, a substance capable of inactivating a wide range of bacterial toxins. Of various media tested, Glucose beef infusion broth has given the best yields of this substance. The detoxifying principle may be obtained in concentrate form by fractional precipitation of the culture filtrate with ammonium sulphate to a concentration of 48 per cent of saturation to precipitate the globulin fraction which is inactive, followed by full saturation of the supernatant fluid with ammonium sulphate to precipitate the albumen fraction. All of the detoxifying activity resides in the albumen fraction which is reconstituted in saline to 1/10 of the original volume of crude filtrate and dialyzed against saline for 48 hours at -6 mm. of Hg. followed by precipitation with copper sulphate in a concentration of 1 per cent. The copper sulphate precipitate is toxic for animals and possesses no toxin neutralizing principle is dialysed against saline for 48 hours at 5°C. at -6 mm. of Hg. to eliminate the copper sulphate and lyophilized to obtain the active principle in a stable, dry, concentrated form. The final product possesses a high degree of toxin neutralizing activity and is non-toxic to animals.

2. The active principle is thermolabile being destroyed at a temperature of 60°C. in 30 minutes. It is not antigenic. The

final product contains a high concentration of protein in its make-up, but it is not known as yet whether or not the active principle is protein in nature. It has not been possible to purify the detoxifying agent by adsorption in kaolin, animal charcoal and activated charcoal, nor have paper chromatography and microelectrophoresis proven of value in its isolation. It is inactivated by the blood serum of the guinea pig, rabbit, horse, and human; the albumen fraction of the serum is the factor responsible for inhibition of the detoxifying agent.

3. The mould product has a wide range of antitoxic activity. It readily inactivates "in vitro" the haemolytic, dermonecrotic and lethal effect of staphylococcal alpha toxin. 10 mgms. of the final product has a neutralizing capacity equivalent to that of 5 to 40 Provisional International Units of staphylococcal alpha antitoxin. The neutralization of one effect correlates well with the inactivation of the others. The active principle also inactivates "in vitro" staphylococcal beta toxin, streptolysin S, streptolysin O, Clostridium welchii alpha toxin, Clostridium septicum haemolysin, Clostridium histolyticum haemolysin and tetanolysin. The mould product is more active against some toxins than against other different toxins. "In vitro" experiments with staphylococcal alpha toxin and C.diphtheriae show that the mould derivative does not offer a high degree of protection. The reason for the lack of correlation between "in vitro" results and "in vivo" results has not been ascertained as yet, although neutralization of the mould

product by serum albumen is a possible factor.

4. The crude albumen fraction of the mould culture filtrate has a low toxicity for rabbits and guinea pigs, but mice are rather highly sensitive to it. The toxic symptoms caused by the albumen fraction may be counteracted by treatment of the animals with an antihistaminic drug. The precipitation with copper sulphate yields a more highly purified product which is non-toxic is those doses that are therapeutically effective against the toxins. In regard to therapeutic value the copper precipitated residue is some ten times more potent than the albumen fraction on a unit weight basis.

5. The experimental data indicates that the active principle acts directly on the toxin rather than the toxin substrate. It appears to act upon the toxic radical of the toxin molecule leaving the radical responsible for antigenicity intact. The toxin inactivating factor has been shown to be a useful toxoiding agent in that it brings complete detoxification of staphylococcal alpha toxin after 1 hour at 37°C. without destruction of immunizing and combining power.

SECTION E

BIBLIOGRAPHY

BIBLIOGRAPHY

Adams, M.H., Hendee, E.D.; Jour. Immun., 51: 249, 1945

Adams, M.H., Hendee, E.D. and Pappenheimer, A.M.; Jour. Expe. Med., 85: 701, 1947

Anderson, W.H. and Booderson, T.; Proc. Soc. Expe. Bio. Med., 70: 322, 1949

Andrews, W.F.; Monograph on diphtheria; Med. Res. Coun. London. 1923, Page 275.

Berliner, F. and Schoenheimer, R.; Jou. Biol. Chem., 124: 525, 1938

Bernheimer, A.W.; Jour. Expe. Med., 80: 309, 1944

Bernheimer, A.W.; Jour. Expe. Med., 80: 321, 1944

Bernheimer, A.W.; Jour. Exper. Med., 80: 333, 1944

Bernheimer, A.W.; Jour. Gene. Phys., 30: 337, 1947

Blair, J.E. and Halman, F.A.; Jour. Infe. Dis., 72: 246, 1943

Blair, J.E., Carr, M. and Buchanan, J.; Jour. Imm. 52: 281, 1946

Boor, A.K. and Miller, C.P.; Science, 102: 427, 1945

Boor, A.K., Miller, C.P. and Hawk, W.D.; Fed. Proc., 6: 240, 1947

Broom, W.A. quoted by Florey, H.W. et al; London, Oxford University Press, Pg. 281, 1949

Burnett, F.M.; Jour. Path. Bact., 32: 717, 1929

Burnett, F.M.; Jour. Path. Bact., 33: 1, 1930

Burnett, F.M.; Jour. Path. Bact., 34: 471, 1931

Buxbaum, L., Nenner, N. and Dolgopolo, V.B.; Jour. Bact., 53: 507, 1947

Calmette; cited by Nelis, P.; Rev. Bel. Sci. Med. 16: 375, 1945

Carpenter, C.M. and Barbou, G.M.; Proc. Soc. Eq. Biol. and Med. 41: 354, 1939

Cavalli, L.L.; Boll. Ita. Siero., 26: 149, 1947

Chain, E. and Iennings, M.A.; Brit. Jour. Exper. Path. 23: 202, 1942

Checcacci, L.; Boll. Isti. Siero. Mila., 30: 113, 1951

Cohen, B., Perkin, M.E. and Putermann, S.; Jour. Bact., 39: 59, 1940

Cruikshank, I.C. and Freeman, G.S.; Lancet 233, 567, 1937

Davis, B.D.; "Evaluation of Themo Therapeutic Agent", Columbi University Press, Pg. 44-61, 1949

Davis, B.D. and Dubos, R.I.; Jour. Exp. Med., 86; 215, 1947

Del Vecchio, G. and Argentano; Boll. Soc. Ital. Biol. Sper., 23: 1016, 1018, 1019, 1021, 1947

Del Vecchio, G., Del Vecchio and Argentano; Boll. Soc. Ita. Biol. Sper., 24: fasc. 3, 1948

Dolman, C.E. and Kitching, J.S.; Jour. Path. Bact., 41: 137, 1935

Dolman, C.E.; Can. Pub. Heal. Jour., 23: 125, 1932

Dowling, H.F. and Hirsch, H.L.; Proc. Soc. Exp. Biol., 63: 167, 1946

Eagle, H.; Jour. Expe. Med., 85: 141, 163, 175, 1947

Ehremberg, L., Fisher, L. and Lofgreen, N.; Svensk. Kem., 58: 85, 1946

Emmerish, R. and Lowe, O. cited by Florey et al.; "Antibiotic", London Oxford University Press. Pg.539,1949

Ercoli, N., Lewis, M.N. and Moench, L.I.; Jour. Phar., 84: 120,1945

Fellin, E. and Ghione, M.; Gior. Batte, Immun., 38: 433, 1948

Follensby, E.M. and Hooker, S.B.; Jour. Immu., 31: 141, 1946

Foster, J.W.; "Chemical Activity of Fungi", New York Academy Press Inc., Pg. 87, 1949

Franklin, A.E. and Quastel, J.H.; Science, 110: 447, 1949

Fraser, D.T. and Halpern, K.C.; Jour. Imm. 33: 323, 1947

Gaddum, J.H. quoted by Boyd, W.C. "Fundaments of Immunology" Intescience Publishers, New York 11 ed. Page 453, 1947

Glenny, A.T. and Stevens, M.F.; Jour. Path. Bact., 40: 201,1935

Hartley, P. and Llewellin Smith, M.; Quarterly Bulletin of Health Org., January 1935: 68

Hauduroy and Rosset; Rev. Tuber. 10: 5, 1946

Hewitt, L.F. and Todd, E.W.; Jour. Path. Bact.; 49: 45, 1939

Hopkins, J.G.; Lancet, ii: 631, 1943

Hunter, F.R.; Proc. Soc. Exper. Biol. and Med., 74: 697, 1950

Imbriano, A.E.; An. Soc. Cien. Argen., 154: 47, 1952

Kalz, F. Telner, P., Quastel, J.H. and Van Straten, A.M.A.; Arch. Derma. and Syph., 68: 167, 1953

Kerrin, J.C.; Brit. Jour. Expe. Path., 11: 153, 1930

Kitching, J.S. and Farrel, L.N.; Jour. Immun. 34: 1, 1938

Krebs, H.A.; Bioc. Jour., 38: xxlx, 1944

Lawrence, C.A.; Science, 98: 413, 1943

Lawrence, C.A.; Science, 99: 15, 1944

Lawrence, C.A.; Jour. Bact., 49: 47,1945

Loeb, L.; quoted by Topley, W.W.C. and Wilson, G.S. "Principles of Bacteriology and Immunity" London, Edward Arnold 111 ed. Pg. 617, 1948

Loeffler; Centr. Bakte., 2: 105, 1887

Manzullo; quoted by Imbriano, A.E.

McFarlane, M.G.; Biorch, Jour., 111: 36, 1942

McFarlane, M.G. and Knight, B.C.J.G.; Bioch. Jour. 35: 884, 1941

McFarren, E.F., quoted by Block, R.J., Lestrange, R. and Zweig, G. "Paper Chromatography", New York, Academy Press Inc. Pg. 58, 1952

Menk; cited by Bernheimer, A.W., Jour. Exp. Med. 80: 321, 1944

Metchnikoff, E.; Ann. Insti. Pasteur, 11: 801, 1897

Miller, C.P. and Boor, A.K.; Proc. Soc. Expr.Biol., 61: 18, 1946

Miller, C.P., Hawk, W.D. and Boor, A.K.; Science, 107: 118, 1948

Minett, F.C.; Jour. Path. Bact., 42: 247, 1936

Much, H.; quoted by Topley, W.W.C. and Wilson, G.S. "Principles of Bacteriology and Immunity", London, Edward Arnold ed. 111, Pg. 617, 1948

Neill, J.M.; Jour. Expe. Medi., 44: 227, 1926

Neisser, M. and Wechsberg, F.; quoted by Topley W.W. and Wilson, G.S. "Principles of Bacteriology and Immunity" London, Edward Arnold, 111 ed. Pg. 614,1948

Nelis, P.; Rev. Belg. Sc. Med., 16: 375, 1945

Neter, E.; Jour. Infec. Disea., 76: 20, 1945

Neter, E. and Will; Jour. Bact. 48: 253, 1944

Neter, E., Bertram, L.F. and Arbesman, C.E.; Proc. Soc. Expe. Biol. and Med., 79: 255, 1952

Neter, E., Bertram, L.F., Zak, D.A., Murdock, M.R. and Arbesman, C.E.; Jour. Expr. Med., 96: 1, 1952

Nicolle, M.; Ann. Inst. Pasteur, 21: 613, 1907

Onkley, J.L.; "Some Conjugated Proteins" A symposium, Rutgers Univer. Press.1953

Pappenheimer, A.M. JR.; Jour. Bact., 43: 273, 1942

Pappenheimer, A.M. and Shaskan, E.; Jour. Biol. Chem. 155, 265, 1944

Parish, H.J. and Clark, W.H.M.; Jour. Path. Bact., 35: 251, 1932

Pillemer, L., Whittler, R.G., Burrel, J.I. and Grossberg, D.B.; Jour. Expe. Med. 88: 205, 1948

Putzer, B. and Grubb, T.C.; Jour. Inf. Disea., 78: 251, 1946

Ramon, G. and Richou, R.; Rev. Immu., 9: 189, 1944

Ramon, G. and Richou, R.; Rev. Immu., 10: 9, 1946

Ramon, G. and Richou, R.; Rev. Immu., 11: 5, 1947

Ramon, G. and Richou, R.; C.R. Acad. Scie., 225: 472, 1947

Ramon, G. and Richou, R.; C.R. Acad. Scie., 224: 1407, 1947

Ramon, G. and Richou, R.; C.R. Acad. Scie., 225: 344, 1947

Ramon, G. and Richou, R.; C.R. Acad. Scie., 224: 1007, 1947

Ramon, G. and Richou, R.;, C.R. Acad. Scie., 223: 1078, 1946

Ramon, G. and Richou, R.; C.R. Acad. Scie., 224: 1605, 1947

Ramon, G. and Richou, R.; C.R. Acad. Scie. 224: 1680, 1947

Ramon, G. and Richou, R.; C.R. Acad. Scie., 224: 509, 1947

Raper, K.B. and Thom, C.; "Manual of the Penicillia" Pg. 371, 1949. Baltimore, The William and Wilkins Co.

Reed, L.J. and Munch, H.; Am. Jour. Hyg., 27: 493, 1938

Richou, R. and Gerbaux, C.; Can. Jour. Comp. Med., 16: 311, 1952

Robertson; Jour. Path. Bact., 20: N. 3, 1910

Roy, T.E.; Jour. Immu., 33: 437, 1937

Segalove, M. and Hite, K.E.; Proc. Soc. Expe. Biol., 64: 218, 1947

Smith, M.L. and Price, S.A.; Jour. Path. Bact., 47: 361, 1948

Smith, W., and Hale, J.H.; Brith. Jour. Expr. Path., 25: 101, 1944

Smolens, J. McAleer, D.S. and Laren, M.; Proc. Soc. Exp. Biol. Med. 65: 172, 1947

Smythe, C.V. and Harris, T.N.; Jour. Imm. 38: 283, 1940

Stansfield, J.M., Francis, A.E. and Stuart Harris, C.H.; Lancet, ii: 370, 1944

Stewart, S.E.; Jour. Bact., 35: 13, 1938

Tobie, W.C. and Alverson, C.; Jour. Bact. 54: 543, 1947

Todd, E.W.; Jour. Path. Bact., 39: 299, 1934

Todd, E.W.; Jour. Path. Bact., 47: 423, 1938

Todd, E.W.; Jour. Hyg. 39: 1, 1939

Thompsett, R.; Jour. Bact., 53: 581, 1947

Van Heyningen, W.E.; "Bacterial Toxins" Charles. Thomas Publisher, Springfield, Ill. 1950

Villa Anna Maria; Mycopath. et Myc., 5: 1, 1950

Waldbaum, L.E. and Reymann, C.B.; Jour. Path. Bact. 36: 469, 1933

Wadsworth, A.B.; "Standard Method" Page 222. The William and Wilkins Co., 1947

Yabuta, T.; quoted from Florey, H.W. et al. "Antibiotic", Oxford University Press, Page 288, 1949

SECTION F

APPENDIX

APPENDIX 1

1. Peptone broth.

Finely minced beef heart, freed of fat is added to distilled water. The mixture is heated at 75°C. to 80°C. for one and a half hours. The fluid extract is siphoned off and filtered through paper pulp in a Buchner funnel, under slight suction.

The following ingredients are added to the filtered beef heart extract: proteose peptone (Difco), one per cent; and one per cent of the stock solution made of sodium chloride, twenty-five per cent; potassium chloride, two per cent; and calcium chloride one per cent.

The pH of the mixture is adjusted to pH 8.4 using 10 N NaOH. Phenol red is the indicator solution employed for this procedure. The mixture is heated at 120°C. for twenty minutes, and the precipitated phosphates are then removed by filtration through paper pulp.

Following filtration the pH is again adjusted to pH 7.2, using 1 N HCL and phenol red as the indicator. The solution is again heated at 120°C. for 20 minutes.

Precipitated phosphates are again removed by filtration through paper pulp, using slight suction.

The clear filtrates is adjusted to pH 7.2; the medium is poured in 500 ml. flasks (100 ml. per flask) and the bottles sterilized by autoclaving at 120°C. for twenty minutes.

2. Glucose beef infusion broth.

To peptone broth at pH 7.2 add 1 per cent of dry dextrose. The medium is poured in 500 ml. flasks (100 ml. per flask) and the bottles are sterilized by autoclaving at 120°C. for 20 minutes.

3. Sabouraud's slopes.

Proteose peptone (Difco)	1.0 per cent
Agar	1.8 per cent
Maltose	4 per cent
Tap water	

Wash agar shreds by soaking for 1 hour in water; squeeze as dry as possible in muslin. Dissolve the agar in water with peptone in the autoclave. Filter through paper pulp in a Buchner funnel under slight suction. Add 4 per cent maltose. Adjust the pH to 6 . Dispense into 6 x 5/8" tubes with about 5.0 ml.of medium and plug. Autoclave at 120°C. for twenty minutes, and slope for hardening.

4. Sabouraud dextrose slopes.

Sabouraud dextrose agar (Difco)	65 mgs.
Distilled water	1000 ml.

Suspend 65 mgs. of Sabouraud dextorse agar in 1000 ml. of cold freshly distilled water and heat to boiling to dissolve the medium completely. Dispense into 6 x 5/8" tubes, 7 ml. per tube. Plug. Sterilize in autoclave at 120°C. for 20 minutes. (Inasmuch as this medium has an acid reaction, final pH 5.6, care should be taken to avoid overheating which will result in a softer medium.)

5. Malt extract medium.

Distilled water	1000 ml.
Malt extract	20 gms.
Neopeptone	1 gm.
Cerelose	20 gms.

Dissolve the constituents of the medium; filter through Whatman No. 2 filter paper. Sterilize at 120°C. for 20 minutes.

6. Malt extract slopes.

Malt extract	20.00 gms.
Neopeptone	1.00 gms.
Cerelose	20.00 gms.
Agar	20.00 gms.
Distilled water	1000 ml.

The various constituents of the medium are brought into solution, and the agar is then added and dissolved, by autoclaving if necessary. Dispense into 6 x 5/8" tubes, plug and autoclave at 120°C. for twenty minutes; slope for hardening.

7. Czapek-Dox-Thom medium.

Sucrose	30.00 grams.
Sodium nitrate	2.00 grams
Dipotassium phosphate	1.00 gram
Magnesium sulphate (crystals)	0.50 gram
Potassium chloride	0.50 gram
Ferrous sulphate	0.01 gram
Tap water	1.000 ml.

Dispense in 500 ml. flasks (100 ml. of medium per flask). Sterilize by autoclaving at 120°C. for twenty minutes.

8. Czapex-Dox modified by Del Vecchio.

To the normal constituents of the medium 40 gms. of lactose and 20.00 gms. of peptone are added. The medium is sterilized and poured as above.

9. Raulin's liquid medium.

Dist. water	1500 ml.
Sugar candy	70.00 gms.
Tartaric acid	4.00 gms.
Ammonium nitrate	4.00 gms.
Potassium carbonate	0.6 gm.
Magnesium carbonate	0.4 gm.
Ammonium phosphate	0.6 gm.
Ammonium sulphate	0.25 gm.

10. Casein hydrolysate medium.

Bacto-casitone (Difco)	30.00 gms.
Distilled water	1000.00 ml.

Dissolve bactocasitone into the distilled water. The medium is poured in 500 ml. flasks (100 ml. per flask) and the bottles are sterilized by autoclaving at 120°C. for 20 minutes.

11. Peptone agar.

Finely minced beef heart, freed of fat, is added to distillated water. Five hundred grams of minced heart are required for each liter of distilled water. The mixture is heated at 75°C. to 80°C. for one and a half hours. The fluid extract is filtered through paper pulp, under slight suction.

Agar in a concentration of 1.5 per cent is dissolved, by autoclaving if necessary, in one fifth the volume of the meat

infusion, to which is added proteose peptone and the stock solution of salts to make one per cent in the final volume. Add the remaining four-fifths of the infusion, still at a temperature of 75°C. to 80°C., stir well and set aside in the cold, overnight to gel. The following day the gel is melted at 100°C. and the reaction is adjusted to pH 8.5 with 10 N NaOH. Heat the bulk to 120°C. in the autoclave for twenty minutes. A precipitate should be produced. Filter through a paper pulp filter under slight suction. Adjust the reaction to pH 7.2 with 1 N HCL. Check the pH of the bulk after adding the calculated amount of reagent. This is very important as the physical character of the agar alters the conditions of neutralization so that more than the calculated amount is usually necessary. Bottle and sterilize at 120°C. for twenty minutes.

(a) Peptone agar slopes: dispense about 5.0 ml. of the peptone agar medium into 6 x 5/8" tubes, plug, autoclave at 120°C. for twenty minutes, and slope for hardening.

(b) Blood agar plates: add 4 per cent of citrated human blood to Peptone agar cooled to about 55°C. and pour plates. (about 20 ml. per plate). Incubate 24 hours for sterility.

12. Loeffler's medium.

3 parts ox-serum

1 part 1 per cent dextrose broth (add 1 per cent dextrose to ordinary peptone broth).

Adjust to pH 8.0 to phenol red using N. NaOH Tube in 6 x 3/4" tubes about 7 ml. to a tube. Bake in the autoclave

in sloping position.

Baking: Turn off all taps of the autoclave to shut in air. Use full steam until temperature of 120°C. is reached. After 20 minutes open lower rear tap slowly, keeping pressure up. When tap is fully opened and pressure is constant, open top tap slowly. When temperature has reached 120°C. with taps open, leave for 20 minutes. Wax plugs or screw caps.

13. Robertson's meat mash

Use 500 gms. of chopped beef. Drop into 500 ml. of boiling N/20 NaOH. Boil slowly for 20 minutes. Add enough distilled water to well cover the meat and keep the fat on the surface. Cool and remove the fat. Strain through muslin, squeezing as dry as possible. Allow to dry slightly on a tray. Place about 1-1/4 inches of meat in a 6x3/4" tube. Fill with peptone broth pH 7.6 to about 1 inch above the meat. Plug. Steam for 1/2 hour in the autoclave. Autoclave at 120°C. for 20 minutes.

APPENDIX 11

Media Used To Prepare The Toxins

1. Staphylococcal toxin medium.

Take finely minced fresh beef hearts, freed of fat, 500 gms. to 1 liter of distilled water; heat at 75°C. to 80°C. for 1-1/2 hours. Filter through paper pulp in a Buchner funnel under slight suction. Fibre agar (shreds of original bleached Gelidium spiriforme) sufficient to give a final concentration of 0.3 per cent is washed by soaking for an hour in distilled water and squeezed as dry as possible in muslin.

Melt the agar in that volume of meat infusion which will give a 10 per cent solution of agar, and to which has been added Proteose Peptone (Difco) to make a concentration of 1 per cent in the final volume. Add this to the bulk of the meat infusion.

Set aside in the cold overnight to gel in order to adsorb the accessory growth factors. Next day melt at 100°C. and adjust the pH to 9.0 to phenol phthalein with 10 N NaOH. Heat at 115°C. for 20 minutes to precipitate phosphate. A visible precipitate should be produced. Filter through paper pulp. Adjust the reaction to pH 7.2 to phenol red using N HCL. Heat at 115°C. for 15 minutes. Filter through paper pulp. Adjust the reaction to pH 7.2. Bottle and sterilize at 120°C. for 20 minutes.

2. Todd's broth for Streptococcus haemolysins.

Minced beef (not heart)	1 lb.
Distilled water	1 liter

Place in ice box overnight. Next day heat slowly to 95°C. for 5 hours in a water-bath. Filter through paper pulp discarding the first running, cool to 60°C. Adjust the pH 8.0 to phenol red.

Add:		
	Proteose peptone (Difco)	2.0 per cent
	NaCl	0.2 per cent
	Dextrose	0.2 per cent
	$NaHCO_3$	0.2 per cent
	Na_2HPO_4	0.1 per cent

Readjust to pH 8. Leave in the cold room overnight, sterilize by filtration through a Seitz filter.

3. Brewer's medium for Clostridia.

Any meat infusion	1.000 ml.
Peptone "Thio"	1.0 per cent
NaCl	0.5 per cent
Dextrose	1.0 per cent
Agar	0.05 per cent
Sodium thioglycollate	0.1 per cent
Methylene Blue	0.0002 per cent

Adjust to pH 7.4-7.6, and sterilize in the autoclave at 120°C. for 20 minutes.

4. Special medium for Clostridia.

(Adaptation from Adams M.H. & Hendee E. D., 1945)

Casein hydrolisate (Casitone)	20.00 gms.
Dextrose	10.00 gms.
Tryptophane	0.025 gm.
Nicotinic acid	0.020 gm.
Calcium pentothenate	0.020 gm.

Pyridoxin	0.020 gm.
Thiamine	0.010 gm.
Riboflavin	0.005 gm.
Biotin	0.001 gm.
Distilled water	1.000 ml.

Dissolve the casein hydrolysate into 900 ml. of distilled water and add the other constituents but dextrose. Dissolve the dextrose in 100 ml. of distilled water. Sterilize separately the two solutions by autoclaving at 120°C. for 20 minutes. After cooling down, mix the solutions and mix well.

APPENDIX 111

Apparatus

1. Whatman filter paper.

Genuine Whatman filter paper No. 3; W&R Balston Ltd., Made in England.

2. Dialyzing sacs.

Made from dialysis tubing; wall thickness 0.00072" and width 1-5/8".

3. Dialysis.

Cellophane dialysis tubing was cut into lengths of approximately 30". The tubing was soaked in water and a knot tied about 3" from one end. This was further strengthened by tying cotton string firmly around the tubing, just above the knot. In the other end of the tubing was inserted a rubber cap with a small funnel and a bended glass tube passing through it. The rubber cap was tied strongly to the tubing. Approximately 200 ml. of the solution was poured through the funnel in the cellophane tubing which was then placed in a 20 liter carboy filled with 18 liter of physiological saline. The air was evacuated from within the carboy through the bended glass tube at -6 mm. of Hg. The carboy was then left in the ice-box for 48 hours.

4. Micro-Kjeldahl analysis.

The digestion mixture consisted of concentrated nitrogen-free sulphuric acid and a few grains of a potassium sulphate-selenium mixture (7-1).

The material to be analysed was digested for approximately 6 hours. The Parnas and Wagner apparatus was employed for the distillation using 30 per cent sodium hydrozide to release the ammonia. The excess N/70 hydrocloric acid was titrated with N/70 sodium hydroxide using methyl red as the indicator.

5. Glass wool: Pirex glass wool.

APPENDIX IV

Biochemical Tests

1. Xantoproteic: add a few drops of nitric acid to 1/2 ml. of the solution. A white precipitate forms which, on heating, turns a yellow and finally dissolves, producing a yellow solution. Cool and add ammonium or sodium hydroxide in excess. An orange colour denotes the presence of phenyl groups, tyrosine and tryptophane particularly. Phenylalanine does not respond as the test is usually performed.
2. Ninhydrin: (triketohydrindene hydrate). To an approximately neutral solution add a few drops of the reagent (see below), heat to boiling for one to two minutes and allow to cool. A blue colour results if the test is positive due to the presence of free carboxyl and alpha amino groups. Certain amines also gives the reaction. Reagent :0.1 per cent triketohydrindene hydrate.
3. Sulphur: Add an equal volume of KOH to the solution, and then a few drops of lead acetate solution and boil. Darkening of the solution indicates cystine or cysteine.
4. Benedict test: To 5 ml. of the reagent add 8 drops of the test solution, mix well and place in a boiling water-bath for three minutes and allow to cool. If no glucose is present the solution will remain clear.

Reagent:	Copper sulphate	17.3	gms.
	Sodium citrate	173.0	gms.
	Sodium carbonate	100.0	gms.
	Distilled water	1000.0	ml.

With the aid of heat, dissolve the sodium citrate and carbonate in about 800 ml. of water. Pour this into a glass graduate and make up to 850 ml. Dissolve the copper sulphate in about 100 ml. water. Pour the carbonate citrate solution into a large beaker and add the copper sulphate solution slowly with constant stirring and make up to 1 liter. The mixed solution is ready for use.

5. Fehling reaction.

To about 1 ml. of Fehling's solution in a test tube add 4 ml. of distilled water. This is done to determine whether the solution will, of itself, cause the formation of a precipitate of brownish-red cuprous oxide. If such a precipitate forms the Fehling solution must not be used. If it will not, proceed as follows:

To 1 ml. of the warm Fehling solution add 4 ml. of the test solution a few drops at a time and heat the mixture after each addition. The production of a brownish-red cuprous oxide indicates that reduction has taken place. The test is positive in presence of reducing sugars.

Fehling's solution: Copper sulphate solution - 34.65 gms. of copper sulphate, dissolved in distilled water and made up to 500 ml.

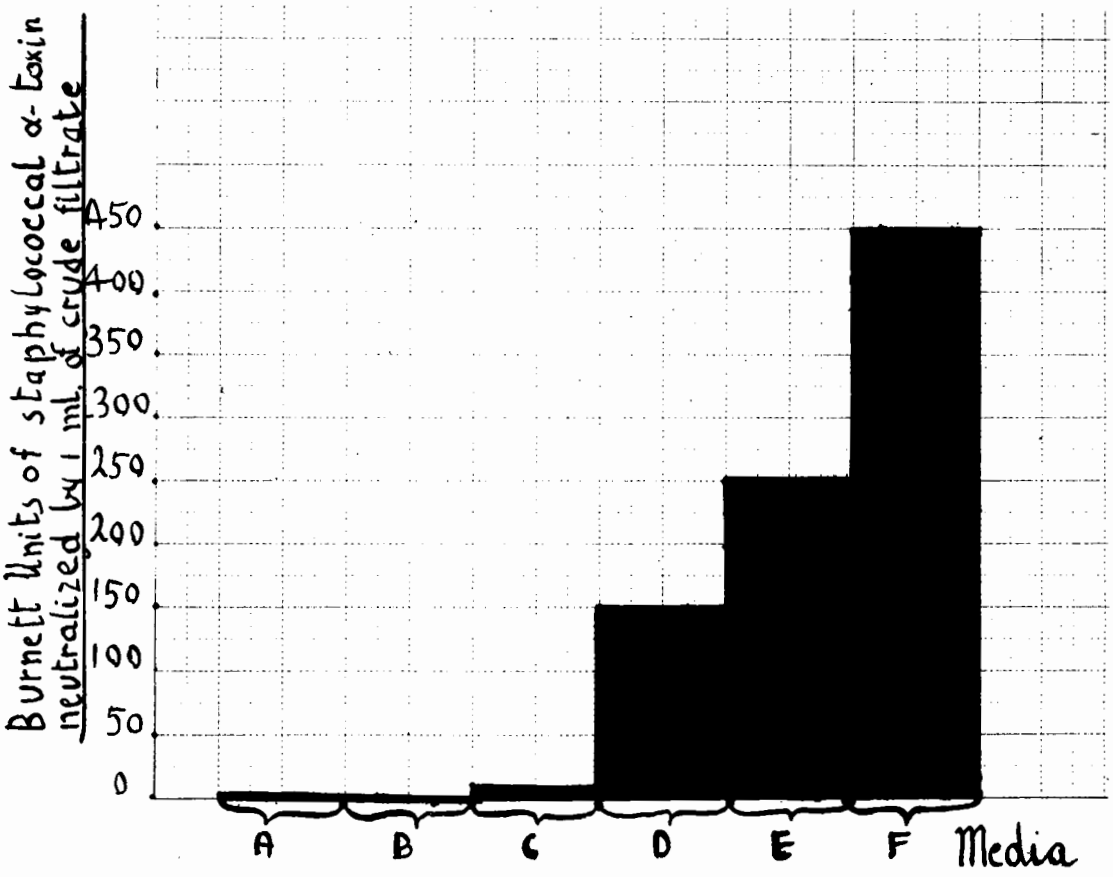
Alkaline tartrate solution - 125 gms. of potassium hydroxide and 173 gms. of Rochelle salt dissolved in distilled water and made up to 500 ml.

These solutions should be preserved separately in rubber-stoppered bottles and mixed

in equal volumes when needed for use.
This is done to prevent deterioration.

FIGURE 1

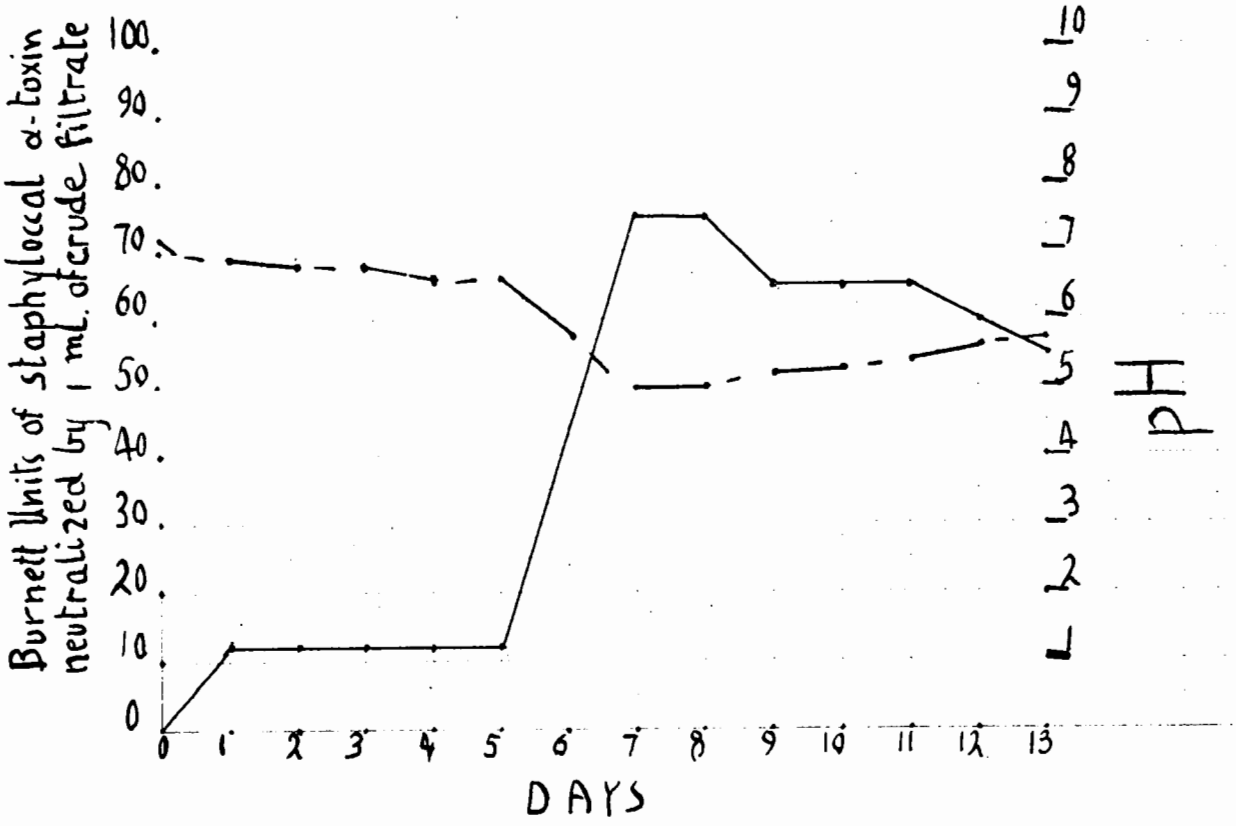
YIELD OF MOULD NEUTRALIZING SUBSTANCE IN CRUDE FILTRATE OF DIFFERENT MEDIA



A - CZAPEX-DOX MEDIUM
B - RAULIN'S MEDIUM
C - CASEIN HYDROLYSATE MEDIUM
D - MALT EXTRACT MEDIUM
E - PEPTONE BROTH
F - GLUCOSE BEEF INFUSION BROTH

FIGURE 2

THE EFFECT OF AGE OF CULTURE ON THE PRODUCTION OF TOXIN NEUTRALIZING SUBSTANCE AND PH OF THE MEDIUM

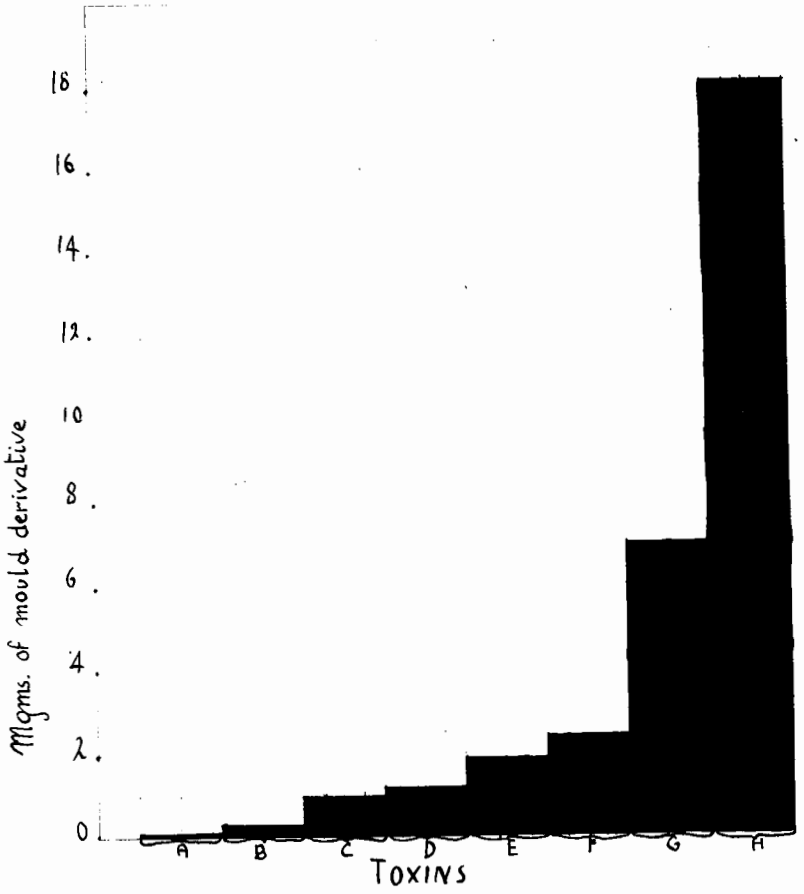


_____ - PH OF THE MEDIUM

– – – – – – – – – – - BURNETT UNITS OF STAPHYLOCOCCAL ALPHA TOXIN NEUTRALIZED BY 1 ML. OF CRUDE FILTRATE

FIGURE 3

TOXIN INACTIVATION OF THE SEMI-PURIFIED ALBUMEN FRACTION FROM PENICILLIUM CYANEO FULVUM AGAINST VARIOUS BACTERIAL TOXIN



A - STAPHYLOCOCCAL ALPHA HAEMOLYSIN
B - STREPTOLYSIN S
C - Cl. WELCHII ALPHA TOXIN
D - STAPHYLOCOCCAL BETA HAEMOLYSIN
E - TETANOLYSIN
F - Cl. HISTOLYTICUM HAEMOLYSIN
G - Cl. SEPTICUM HAEMOLYSIN
H - STREPTOLYSIN O

FIGURE 4

EFFECT OF TEMPERATURE ON A SALINE SOLUTION OF LYOPHYLIZED MOULD DERIVATIVE, USING 150 MGMS/ML. AGAINST 3200 BURNETT UNITS OF STAPHYLOCOCCAL ALPHA TOXIN AFTER HEATING AT THE DESIGNATED TEMPERATURE FOR 30 MINUTES