In Vitro Production of The Soluble Toxin By Avirulent Strain of *Bacillus anthracis*.

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IN VITRO PRODUCTION OF THE SOLUBLE TOXIN BY AVIRULENT STRAIN OF *Bacillus anthracis*.

THESIS

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C E R T I F I C A T E

This is to certify that the Thesis entitled " IN VITRO PRODUCTION OF THE SOLUBLE TOXIN BY AVIRULENT STRAIN OF B. anthracis" submitted for the degree of Master of Science (Vet.) in Bacteriology to the Magadh University by Sri Sidheshwar Prasad embodies the results carried out by him under my supervision and guidance.

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C O N T E N T S

SECTION-A

INTRODUCTION

I N T R O D U C T I O N

Anthrax is a disease known from antiquity. The disease became known to primitive men when they took to the profession of domesticating various species of animals and adopted an agrarian mode of life. Though anthrax was recognised as one of the earliest diseases affecting animals and man, the study on this disease remained very much sketchy, confounded by a mass of misconceptions and superstitions. It was not until the microscope was made available in the early 17th century that the study of microbiology and indeed of anthrax was brought into the arena of rigorous microbiological research.

With the advent of microscope the science of bacteriology was placed on the direct evidence of microscopic observation. During the early 19th century, the historical back-ground of anthrax was very much enriched due to ingenious researches of Robert Kock and Louis Pasteur. While the former concerned himself with the investigation on the morphology and cultivation of the causative agent in vitro, the latter was interested in the study of immunity of this formidable disease.

The study of anthrax led to the discovery of several epoch-making findings in the history of microbiology and unravelled many intriguing and hitherto not-fully-understood problems of infectious diseases. (1) It was the first disease of man and animals shown to be caused by a micro-organism and proved to be contagious in nature;

(2) the second bacterial entity in which the principle of immunity was better understood and the prophylactic use of bacterial vaccine was reported to be effective and practical; (3) *in vitro* cultivation of the etiological agent of disease was successfully accomplished for the first time in the study of anthrax; (4) anthrax bacillus was the first micro-organism in which the presence of resistant spore was demonstrated; and (5) it was this disease in which the original work in microbiology was initiated and done which led to the establishment of many well known principles of microbiology.

Anthrax affects a wide variety of animals and man Herbivorous animals are more commonly affected, but the infection is not infrequent in pigs, minks, dogs, elephants, deer, birds and the carnivorous animals kept in zoo. Even frogs and fishes have been known to be susceptible to infection experimentally. Although Algerian sheep are stated to be naturally resistant to anthrax, Gayot (1952) found these sheep normally susceptible.

The incidence of anthrax shows world-wise distribution. In India, it is known to occur in all the states showing seasonal and regional distribution. In Bihar, the disease is reported to be endemic in hilly and afforested areas, particularly in the southern part of the state (such as Ranchi, Hazaribagh, Palamau, Santhalpargana, Shahabad and Gaya districts). The majority of outbreaks occur during the months between May to September, the peak

of the incidence being encountered with the onset of monsoon (June & July).

The annual figure of anthrax infection in man all over the world is estimated approximately to the tune of 20,000 to 1,00000 (Glassman, 1958). In India, the knowledge regarding the incidence of this disease in man is meagre though the majority of the cases are reported to occur in rural areas (Mohan & Ali, 1948; Mohiyuddin & Krishna Rao, 1958).

A complete knowledge regarding host-parasite relationship, pathogenesis and development of immunity is pre-requisite for a clear understanding of any branch of biological science, such as is the microbiology. During recent years, numerous investigators engaged on these aspects of the problem have endeavoured to explain the mechanism of infection, pathogenesis and immunity in anthrax on the basis of rigorous chemical analysis of the etiological principle and immunogenic agent. However, there still remains a considerable gap in our concept of some of these vexed problems.

Until recently, workers had held the view that the death in anthrax was brought about due to massive bacteraemia which resulted in blocking of capillaries and consequent deficiency of essential nutrients in the host.

However, the theory of bacteraemia as the only cause of death in anthrax did not find support of numerous recent investigators, who demonstrated very convincingly

the presence of toxin in the metabolic products of experimentally anthrax infected guinea-pigs (Keppie et al.,1953; Smith et al.,1955). Subsequent researches directed to explain the chemical basis of virulence and immunity enabled the workers to isolate in anthrax culture three different soluble antigens,namely protective factor,oedema factor and lethal factor (Harris-Smith et al.,1958; Thorne et al.,1960; Stanley & Smith,1961 and Smith & Stanley,1962).

In the present study,an attempt has been made to produce in vitro the soluble toxin of B.anthracis with particular reference to the oedema and the lethal factors in non-protein Casamino acids medium. In order to find out the critical factors necessary for the elaboration of the toxin, various amino-acids, salts and nucleic acids were incorporated in the medium and used under different cultural conditions. The biological activity of the toxin was tested in laboratory animals. An attempt was also made to devise an easy and quick method for identifying the soluble antigen in the culture. For this purpose, haemagglutinating activity of the toxic principle was examined.

S E C T I O N - B

REVIEW OF LITERATURE

REVIEW OF LITERATURE

A. HISTORICAL:

Anthrax is one of the earliest diseases known to man. The earliest account of anthrax dates back nearly 6000 to 7000 years in early Mesopotamia and Egypt, where agriculture had then established (Wells,1949).

The first record of this disease is available in the Bible in Exodus, chapter 7 to 9. About 1491 B.C.,Moses had warned Pharaoh, "The hand of the Lord will fall on your livestock in the fields, on horses, asses, camels,herds and flocks with a very severe murrain". The wrath of God struck the land of Egypt and all the cattle died of the disease which was indeed anthrax (Stein,1955).

There are records of various cattle epidemics,inclusive of anthrax, in Hindu literature. The Indian had classified these diseases as early as 500 B.C. In early Greece,anthrax was recognised by Hippocrates as early as 400 B.C. The Romans described anthrax in man and animals in 70-19 B.C. In the fifth century B.C., several major plagues had struck Rome beginning with one of anthrax which reduced the entire human and animal population to half.

In the 16th century, Thomas Blundeville, who was probably the first important veterinary writer in English language,described the disease and recognised relationship between swine and outbreak of anthrax (Smithcors,1957). Despite these observations, it was not until the 18th century that anthrax was clinically defined and was placed on the firm basis

of scientific criticism.

M. Delafond was the first man to have seen the rods of B.anthracis in anthrax blood at the Alfort Veterinary School in France as far back as 1838. He,however, could not understand the significance of such observation (Vallery - Radot, 1923).

The credit of such observation therefore went to Davaine who in a frenzy of scientific effort,unfolded many then unsolved mysteries of this disease. In 1850, he saw the anthrax bacillus under the microscope and in 1863, he discovered that only blood of sick animals contained the organisms. Further, in 1868, Davaine had shown that the anthrax rods could transmit the disease and in 1873, he also reported that the blood when filtered through clay was innocuous (Merchant,1953; Stein,1955). Thus,from his above masterpiece observations,Davaine had shown the identity and the contagious nature of the causative agent of the disease.

Meantime, Pasteur further confirmed the etiological role of anthrax bacilli. He found that when the culture was filtered through membrane fine enough to retain the bacilli, the filtrate could not reproduce a copy of the disease in the susceptible host,while the sediment was highly infective. Thus, not only did he convincingly elucidate the etiology of anthrax but he also established the germ theory of disease (Dubos,1950).

In 1878, Kock in his classical research on anthrax, worked out the complete life cycle of the bacillus and

propounded his 'postulates' which are still regarded as the surest criteria for establishing the relationship between the causal agent and the disease it produces. The famous Kock's postulates thus set the first milestone in the progress of bacteriological research which brought him to be acclaimed as the father of bacteriology.

B. ANTHRAX VACCINES:

In 1880, Chauveau reported that an animal which survived inoculation with anthrax bacilli became more resistant to further infection. In the same year, Toussaint successfully vaccinated sheep with defibrinated anthrax blood that had been inactivated by exposure to 55°C for ten minutes (Merchant, 1953).

Pasteur (1881) convincingly demonstrated the effectiveness and practical use of vaccine. The vaccine consisted of attenuated strain of anthrax bacillus which he obtained by maintaining it at 42°C in artificial culture - a method too well known to be commented upon.

In his famous field experiment done at Rossignol's farm in Pouilly le Fort, Pasteur inoculated cattle, sheep, goats and cows with 5 drops of attenuated culture - the 'Premier vaccin'. Thereafter, he reinoculated these animals with a more virulent culture - the 'Deuxieme vaccin' after a period of 12 days. After a fortnight following the 2nd inoculation, he challenged the animals with a virulent strain of B. anthracis along with an equal numbers of non-immunised control animals. All the control animals died or suffered from anthrax while the vaccinated ones survived the

challenge infection.

Zenskowskii (1887), Kind (1922) and Bekker (1930) reported the successful use of glycerine in a concentration of 50 to 60 % as an ideal vehicle for the suspension of anthrax spores. This helped in maintaining their viability and inhibiting the growth of contaminants. It had further advantage of potentiating the immunogenicity of the spores by providing continuous and prolonged antigenic stimulus at the site of inoculation and preventing them from being phagocytosed by the reticulo-endothelial cells.

Besredka (1921) confirmed the previous finding of Pasteur and further claimed that guinea-pigs and rabbits could be rendered solidly immune when inoculated intradermally with Pasteur's Ist and IInd vaccines. He however,failed to demonstrate passive immunity in other group of animals with 'immune sera'.

The above observations,however,could not be confirmed by Cordier (1925), when he used Pasteur's vaccines for immunising guinea-pigs.

Schilling (1926) succeeded in producing a safer vaccine than that of Pasteur's by incorporating 5% sodium chloride in the culture media. The culture when washed off with normal saline and treated with 0.5% carbolic acid constituted the vaccine. He showed that immunity in sheep with this vaccine lasted for 5 months.

Sobernheim (1931) for the first time introduced the 'serum simultaneous method' of vaccination which is still

occasionally used (Sterne, 1959). He devised this method with a view to eliminate the hazards of vaccination inherent with different types of attenuated vaccines.

Mazucchi (1931) reported that saponin in a concentration of 2-5 % was a safe and potent vehicle for suspending anthrax spores. This induced a violent local necro-inflammatory reaction which helped in localising the organisms in vaccinated subjects. Later on, Anreiter (1932) also reported favourably on the use of 'Carbozoo' vaccine of Mazucchi (op.cit.).

Ramon & Staub (1936, 1937) in an attempt to evolve a simple and a single dose vaccine, attenuated the culture of B.anthracis to a degree of equal to that of Pasteur's Ist vaccine. The attenuated spores were suspended in (i) lanolin, or (ii) a mixture of gelatin and alum. A single dose of these vaccines conferred solid and lasting immunity in sheep when inoculated subcutaneously. This was proved by extensive field trials in sheep. They further modified the vaccine by suspending the culture in 0.2% agar and 1-3% alum. A single dose of 0.25 ml. of such vaccine was found to produce immunity in sheep within 5 days of inoculation. The immunity thus produced lasted for eight months.

Stamatin (1937, 1938) reported success in immunising animals with a variant of B.anthracis. The results of this vaccine compared favourably with other types of vaccine in vogue.

Sterne (1937a, 1937b) made a noteworthy contribution

in our concept of anthrax immunisation. He demonstrated that the virulent anthrax strain threw a non-encapsulated avirulent variant on growing the culture in 50% serum-agar media in an atmosphere of 10 to 30% carbon-dioxide for one to two days. He found that the avirulent strain could be safely used for the preparation of an efficient immunising agent against anthrax in cattle,sheep and horses.

In 1942, Ramon *et al.* introduced their "anavirulent vaccine" which was prepared from a highly virulent strain. A broth medium containing a small quantity of agar was sown with a heavy inoculum of virulent strain. After incubating the culture at 37°C for 48 hours, the growth was formolised and re-incubated at 41-42°C for 4 days and then tested for sterility. 0.2% of alum was later incorporated in the culture. It was found that the immunity response provoked by the dead organisms was further reinforced by the agar and alum present in the medium.

In India, the credit goes to Rajagopalan and Israil (1952) who, on the lines of Sterne (1937), isolated from a virulent strain of *B.anthracis*, a non-capsulated avirulent strain and attempted to evolve a suitable vaccine for the successful immunisation of cattle,sheep,goats and other species of animals. The vaccine made from the local strain compared favourably with the Sterne's vaccine. Both these vaccines were observed to be safe for use in sheep,cattle, goats,elephants,camel,horses and mules. When a relatively concentrated vaccine was used, it was found that degree of

immunity produced was not appreciably inferior to that resulting from a vaccine containing saponin. As the proportion of animals, which developed abscesses from vaccines containing 0.4 - 0.5% saponin, was large under Indian conditions, they preferred to use a vaccine with no saponin. A solid immunity was established in animals protected with one million spores, within 3-6 days and remained upto one year. They found that in sheep and goats there was severe reaction with glycerinated vaccine and advised to dilute the vaccine three times to the original volume, which reduced the local reaction.

C. PATHOGENESIS:

In the past, the mechanism and the basic principle of virulence and pathogenicity in anthrax remained a much debated question for a considerable length of time. Preisz (1909) suggested that the capsular substance had the property of neutralising the *in vitro* anthracidal activity of normal sera. However, Bail & Weil (1911) showed that organisms were aggressive only when growing in animal tissues and that the 'aggressin' secreted by organisms was responsible for neutralising the anthracidal mechanism. It was not related to the capsular substance.

At one time the workers had held the view that death in anthrax was caused due to massive bacteraemia which resulted in thrombosis of capillaries by bacilli (Peter, 1939) and consequent deficiency of oxygen and essential nutrients to the host. Subsequent investigations have, however, shown that apoplectic death in anthrax can occur in the absence of

noteworthy bacteraemia and therefore the mechanical hypothesis has now been abandoned.

In an attempt to demonstrate the association of intracellular substance of B.anthracis with the pathogenicity of the disease, King & Stein (1950) cultivated the organisms both in simple and in complex media, aerobically and anaerobically. The intracellular product was obtained through mechanical disintegration of the cells and the suspension thus obtained was inoculated into mice. The material however, proved to be non-toxic for these animals.

These authors indicated that the failure to isolate the toxic principles in B.anthracis in vitro may well be due to lack of some special cultural conditions which are not yet determined.

Keppie et al. (1953) harvested the in vivo growth products of B.anthracis from infected guinea-pigs. These workers reported that even large quantities of intra and extracellular products were non-toxic when injected intraperitoneally but it was shown to promote infection with sublethal doses of B.anthracis and to interfere with phagocytosis. Thus they believed that the product was aggressin. Further, they also indicated that extracellular but not intracellular product produced active immunity to anthrax in guinea-pigs. It was suggested that aggressin and the protective antigen may be the same substance.

Keppie et al. (1955a) recorded an interesting observation that bacteraemia alone could not be ascribed as the cause of

death in anthrax. They observed that when streptomycin was used to remove an experimental infection of guinea-pigs with B.anthracis, their life could be saved only if at the time of treatment, the bacteraemia had not increased beyond about 3×10^6 chains per ml. of blood i.e. approximately 1/300th of the degree of bacterial proliferation which had occurred at the time of death in untreated animals. Beyond that critical level, death was encountered despite the fact that no active infection had persisted.

Keppie et al.(1955b) found that distribution of organisms during the final phase of anthrax in guinea-pigs was in the spleen and blood, the percentage of their isolation in these organs being approximately 80% and 20% respectively. This suggested that blood and spleen may perhaps be the possible sites for the elaboration of harmful products.

Smith and his coleagues (1955) carried out extensive studies on the blood chemistry, physiology and histopathology of guinea-pigs dying of anthrax. They found reduced blood volumes, reduced bleeding time,reduced blood pressure, increased clotting time,haemoconcentration, a fall in body temperature, interference in carbon-dioxide and phosphatase metabolism, loss of alkaline phosphatase in kidney and acute renal failure. From these observations,they concluded that a state of secondary shock was a major cause of death in anthrax.

Smith & Gallop (1956) obtained purified fraction from the thoracic and peritoneal exudate of guinea-pigs

dying of anthrax. This substance was found to be an immunising antigen and had marked antiphagocytic properties. This immunising aggressin was a lipoprotein, containing a small amount of carbohydrate residues. It was found to be different from immunising antigen of B. anthracis produced in vitro. This immunising aggressin was termed as factor 'Y'.

The above findings of these workers on the much debated question on the pathogenesis of anthrax opened a new approach of investigation which led to the demonstration of various fractions of toxic factors of B.anthracis which will be described elsewhere.

D. ANTHRAX TOXIN : A RETROSPECT

With the recognition of secondary shock as the cause of fatal syndrome in anthrax, workers renewed their interest into the problem of pathogenicity and immunity in anthrax and this paved the way for the chemical study of the complex problem resulting in the characterisation of three different soluble antigens in anthrax bacilli.

(i) PROTECTIVE FACTOR :

Gladstone (1946) for the first time succeeded in isolating in vitro, a protective antigen from the cell-free metabolic product of B.anthracis. He used sheep serum culture medium. The cell-free filtrate was highly immunogenic. When injected thrice at weekly intervals subcutaneously in rabbit, it protected 100% of these animals against the challenge dose of 100 A.L.D. virulent spores. He demonstrated the presence of the protective factor in 18 hours culture; but on further

incubation to 48 hours, the antigen was completely lost. The loss of antigen was attributed due to the presence of some proteolytic enzyme produced towards the end of the growth when the bacilli were autolysing and were forming spores.

While the problem was engaging the attention of the workers to eliminate the production of proteolytic enzyme in anthrax culture, Rogers (1948), while working on Staphylococcal hyaluronidase, reported the advantages of using cellophane sac to provide continuous nutrients to the organisms.

Influenced by the observation of Roger (op.cit.), Gladstone (1948) devised an apparatus for the production of soluble protective antigen in a cellophane sac which was continuously supplied with sterile broth and oxygen. This device yielded twenty five - fold increase of the antigen than in his previous static culture medium. The antigen thus obtained conferred solid immunity in rabbits. The hyper-immune serum recovered from these animals was capable of conferring passive immunity, thus dispensing with the need of living organisms in producing effective antisera.

Heckley & Goldwasser (1949) used a simplified medium consisting of denatured sheep serum-albumin and ox serum ultrafiltrate for antigen production. The authors,while harvesting the antigen from such media, observed that the morphology of the anthrax bacilli was different from that grown on ordinary medium. The organisms were much shorter and broader with square corners and irregularly stained cytoplasm, the cells appearing dead and partially autolysed, though the

total cell count indicated that all cells were viable.

Wright et al.(1954) obtained protective antigen in sterile culture filtrates after 20-hour static growth of a non-proteolytic mutant strain of B.anthracis. They used a chemically defined non-protein medium consisting of amino-acids and inorganic salts. This antigen was highly immunogenic for rabbits and monkeys. They discussed the growth requirements of B.anthracis and pointed out that antigen production is probably associated with a particular type of metabolic activity of the organisms.

Belton and Strange (1954) successfully isolated a non-toxic component of protective antigen both from virulent and avirulent strains of B.anthracis, when grown in casein-synthetic medium. This antigen effectively immunised rabbits and monkeys. The hyper-immune serum prepared with such antigen in horses was equally potent when compared with antisera prepared with living spore vaccine.

Strange & Belton (1954) in a detailed chemical analysis of the filtrate of B.anthracis grown in non-protein medium isolated a mixture of growth products which contained a protein and polysaccharide. The protein was concentrated in crude antigen by dialysis and lyophilisation which solidly protected rabbits against a challenge dose of 250 A.L.D. of virulent spores.

The effect of alterations in the cultural conditions and the compositions of medium on in vitro elaboration of protective antigen was further investigated by Puziss & Wright (1954). These workers employed a number of amino-acids and salts

and stressed their relative importance for the growth and production of protective antigen of B.anthracis. They found that sodium bicarbonate,calcium ion, leucine,isoleucine, methionine, proline, phenylalanine and histidine were critical factors in the elaboration of antigen.

The above work was further extended by Wright et al. (1954) and brought out experimental evidences that alum-precipitated protective antigen was non-toxic but produced significant immunity in rabbits,guinea-pigs and monkeys. The immunity persisted for more than three months in rabbits and fourteen months in monkeys. The antigen when injected into man, was well tolerated without producing any serious local reaction.

While working on the immunological heterogeneity of various strains of B.anthracis obtained from different sources, Auerbach & Wright (1955) showed that no major immunogenic types existed among these organisms. A non-proteolytic,non-encapsulated mutant of Vollum strain of B.anthracis effectively immunised rabbits against ~~th~~ challenge with 35 strains of organisms of varied origins. However,the antigens produced by two of their strains were no more effective in immunising rabbits against homologous challenge, than was the antigen elaborated by the Vollum mutant.

By this time a number of workers directed their investigations ~~for~~ improving upon the medium and concentrating the antigen through precipitation with ethanol at low temperature, ammonium or sodium sulphide and through dialysis and freeze drying. They observed that the cell-free antigen thus obtained protected monkeys against 10,000 m.l.ds. of

virulent anthrax spores (Boor, 1955; Boor & Tresselt, 1955a and Tresselt & Boor, 1955).

Boor & Tresselt (1955b) further produced a stable protective antigen in culture medium, containing yeast extract and serum-albumin. They successfully immunised sheep with the cell-free antigen either by a course of 3 intradermal injections of an aqueous solution or one subcutaneous injection of antigen adsorbed on colloidal aluminium hydroxide which acted as stabiliser or adjuvant.

A significant contribution was made by Thorne & Belton (1957) who devised agar-diffusion method for titrating anthrax immunising antigen in culture filtrate as well as the anthrax immune sera. They observed excellent correlation between the titres of the culture filtrates and their immunising activities in rabbits. Based on the technique of Ouchterlony (1953), they demonstrated marked improvement in antigen production in modified non-protein medium.

Wright & Puziss (1957) demonstrated the production of protective antigen in modified anaerobic culture medium. The medium described by Puziss & Wright (loc.cit.) was modified by adding adenosine, glycine & l-alanine, reducing the concentration of ferrous sulphate and omitting cystéine. The final culture medium was adjusted to pH 8 before filtration. They considered anaerobic cultivation a type of simplification for large scale production of antigen. It was further added that under anaerobic condition the antigen production was at least equal to that obtained from aerobic culture, though the growth was somewhat slower.

In 1958, Smith further added to our concept of antitoxic immunity in anthrax. He isolated *in vitro* two virulence factors from anthrax culture, namely the toxin and the capsular polyglutamic acid; of these, the former was immunogenic and formed the prime basis in anthrax immunity. Smith's toxin consisted of two factors which acted synergistically. It lost toxicity very easily while still retaining the antigenicity. Smith thus advanced for the first time, the idea for the existence of multiple fractions in anthrax toxin in artificial culture and this enabled him to prepare *in vitro* an excellent non-toxic immunogenic vaccine against anthrax.

Strange & Thorne (1958) isolated protective antigen from an avirulent strain of *B.anthracis* in a high state of purity in a semi-synthetic medium. The purified antigen was protein which protected rabbits against the challenge infection of anthrax spores. The antigen gave a distinct precipitation line with homologous antisera on Ouchterlony plate.

In the following year a potent almunium hydroxide gel vaccine against anthrax was developed in Boor's medium containing sheep serum albumin and yeast extract. This vaccine protected sheep for as long as 7 months after vaccination against virulent strains of *B.anthracis* (Molnar, 1959).

Wright *et al.* (1962) in an effort to improve growth and elaboration of protective antigen, used chemically defined medium (599 medium) developed earlier by Puziss & Wright (1954) with a few modifications in respect of certain amino-acids.

They found that under altered cultural conditions, it was possible to obtain a five-fold increase in the production of protective antigen from a non-proteolytic and non-capsulated avirulent mutant of B.anthracis.

(ii) TOXIC FACTORS:

Using B.anthracis, Smith & co-workers (Smith, Keppie & Stanley, 1953a, 1953b) for the first time succeeded in harvesting a large quantity of organisms and their products grown in vivo in experimental guinea-pigs. This enabled them to subject the cells and their extracellular product to a detailed chemical analysis which could reveal the mechanismx of chemical basis of virulence of B.anthracis.

These workers substantiated the widely held view that there was sharp differentiation between the organisms grown in vivo and those grown in broth, in respect of their morphological characters and their susceptibility to phagocytosis. They also observed that organisms harvested from infected guinea-pigs showed rapid lysis in ammonium carbonate solution. This was, however, not apparent in any of the organism grown under a variety of conditions in vitro. This observationx served to underline the chemical difference between these organisms.

Until the year 1954, there was no experimental evidence in the literature to suggest that B. anthracis was able to produce a well defined toxin in vitro. It was Evans & Shoesmith (1954) who obtained cell-free filtrate from an old culture medium which on precipitation with saturated solution of ammonium sulphate, proved toxic to rabbit when injected

intradermally. The lesions produced in these animals were strikingly similar to those seen in natural cases of cutaneous anthrax. However, the specificity of such reaction elicited by Evan's toxin remained unconfirmed.

Smith & Keppie (1954) can however, be enlisted among the first of those workers who proved beyond doubts, the specificity of lethal and oedema factors obtained from the guinea-pigs plasma dying of anthrax. They injected intradermally 0.2 ml. of filtered heparinised plasma into test guinea-pig and observed an extensive area of oedema and congestion. The tissue damaging activity of the plasma increased regularly with increase in the number of organisms in the blood. Anthrax antiserum produced against 'Weybridge' spore vaccine neutralised the skin reaction completely.

When a mixture of plasma and peritoneal exudate was similarly used, the reaction was minimal. They explained that that the failure in producing the skin lesions in the past may have been due to the fact that the previous workers had largely used the exudate. They also showed specific lethal effect of the undiluted plasma in mice and guinea-pigs when inoculated intravenously and intraperitoneally. The anthrax antiserum also removed the lethal effect of toxic plasma. Thus specificity of lethal and oedema factors was for the first time determined with certainty.

In the year 1955, Smith et al. further confirmed the earlier observations of Smith & Keppie (1954) and suggested that both the oedema production and the lethal activity were caused by the same substance.

Later the toxic plasma of guinea-pig dying of anthrax was shown to contain two components designated as F_1 & F_2 which acted synergistically; in as much as the two factors, when combined, were capable of producing oedema and lethal effect (Smith et al., 1956). F_1 was quickly deposited by ultracentrifugation leaving the other (F_2) in the supernatant. The former proved to be an immunising antigen and an aggressin. The immunising factor 'Y' isolated by Smith & Gallop (1956) was considered to be F_1, toxoided by extraction process. The F_2 component of the toxin had shown the same biological properties as that of the purified protective factor obtained in vitro by Strange & Belton (1954), but it was not found to be identical with it.

However, the above workers failed to obtain the toxin in vitro when B. anthracis (strain NP) was grown in tryptic meat broth and in the medium used by Belton & Strange (loc. cit).

Concomitant with the above observation, Belton & Henderson (1956) announced that sterile plasma from guinea-pigs dying of anthrax, produced a characteristic skin lesion in rabbits, which was neutralised with anthrax antiserum. This finding led to the basis of carrying out skin test for the titration of anthrax antigen produced in vitro.

Tempest & Smith (1957) while testing the effect of several metabolite analogues in vivo on the growth of B. anthracis and their ability to synthesise toxin during the terminal stage of bacteraemia, concluded that hypoxanthine, adenine, methionine, alanine, phenylalanine and tryptophan are involved in growth of the organisms whereas pyrimidines and

nicotinic acid were necessary for the synthesis of toxin in guinea-pigs.

Harris-Smith & Co-workers (1958) isolated the lethal and oedema producing toxin from the serum culture of both attenuated immunogenic 'Sterne' strain and virulent NP strain of B.anthracis. The toxin was found to be identical with the one originally recognised in vivo.

In course of their work, they noted that the concentration of toxin was maximal only in the early culture. It disappeared from the culture when the organisms were grown for beyond seven hours. They pointed out that in the past the failure to recognise the toxin in culture was mainly due to its early appearance and rapid disappearance under ordinary growth conditions. The destruction of toxin in old culture was attributed to be due to the elaboration of an intracellular enzyme which was demonstrable in the later phase of the growth. They failed to maintain the toxin concentration or to increase it by the addition of various nutrilites.With continuous culture, however, it was possible to maintain the organisms in the toxigenic growth phase.

The factor I & II (F_1 & F_2) of B.anthracis reported earlier by Smith et al.(1956) were further isolated and purified by Stanley et al.(1960) by fractionation method which involved minimum of inactivation. While the final preparations of F_1 & F_2 were toxic when injected together, they were inocuous when administered separately.

Thorne et al. (1960)isolated toxin of B.anthracis when grown in Casamino acids medium of Thorne & Belton(1957).

They separated the toxin into two components by filtering through fritted glass filter. One component present in the filtrate was the protective antigen. The other component referred to as filter factor and which was toxic, was adsorbed on the glass filter but was elutable with carbonate buffer at pH 9.7. Each of these two factors was non-toxic when tested alone. When added together however, they formed a toxic mixture as evidenced by skin reaction in guinea-pigs and lethality in mice. The toxin was specifically neutralised by B. anthracis antiserum.

Stanley & Smith (1961) further added to our concept of different factors of anthrax toxin produced in vitro. They used Casamino acids medium of Thorne et al. (loc.cit.) and isolated three different factors separately, when the original culture was passed through the column of diethylaminoethyl-cellulose (DEAE-C) and subsequently eluted with phosphate buffer. The factor I and III were toxic and produced cutaneous oedema in rabbits and killed mice respectively, when mixed with factor II which was the protective antigen. The newly recognised lethal factor (Factor III) was serologically different from Factor I & II and was also found to be present in anthrax toxin produced in vivo.

Smith et al. (1961) further extended their work in order to ascertain the purity of antigenic factors of B. anthracis. They showed that gel-precipitation procedure was superior to the conventional method of analytical electrophoresis and ultra-centrifugation due to large efforts

involved and sufficient preparations required in the latter procedure. They suggested that agar-diffusion method was a practical working rule for assessing the purity of such preparations.

Beall et al.(1962) confirmed the observation of Stanley & Smith (1961) and demonstrated three factors of anthrax toxin. The culture supernatant was filtered through a fritted-glass filter and the filter was subsequently eluted with carbonate buffer. They pointed out that rats were more susceptible to lethal effect than the mice and guinea-pigs. The synergistic activity of toxic factors and protective antigen was also confirmed.

Smith & Stanley (1962) further purified factor III of anthrax toxin which increased the lethality of mixture I & II for mice and decreased their capacity to produce cutaneous oedema in rabbits. Following the procedure of Thorne et al. (1960), they obtained crude anthrax toxin which yielded purified factor I & III with the help of chromatography on DEAE-C column. The final preparation of factor III showed a single peak in ultra-centrifuge and a single band on paper electrophoresis. It was protein in nature.

SECTION - C

MATERIALS AND METHODS

MATERIALS AND METHODS

Strain:

Non-capsulated avirulent " Weybridge" strain (34 F/2) of B.anthracis was used throughout the experiment. The original freezedried anthrax spore sealed in vacuo was received through the Research Officer (Biological Product) Livestock Research Station,Bihar,Patna who obtained it from the Central Veterinary Laboratory, Weybridge, England.

Hyperimmune anthrax anti-serum (H533):

This was obtained from Dr. H.Smith, Microbiological Research Laboratory, Porton, England.

Preparation of anthrax seed suspension :

This was prepared as follows:

(i) Anthrax spore suspension:

First of all,spores were seeded on nutrient agar slant and incubated at 37°C for 24 hours. The growth was washed in about 3 ml. of sterile physiological saline solution and examined microscopically and culturally for purity.

The Roux flask containing casein digest medium at pH 7.4 was inoculated with 3 ml. of saline suspension of the organisms. The flask was incubated at 37°C for 72 hours and then left at room temperature (22-24°C) for a further period of 24 hours. The surface growth was removed with the help of sterile glass beads in 10 ml. of sterile normal saline solution. The spore suspension was collected aseptically in sterile test tubes and examined microscopically and

culturally for purity. The growth suspension showed anthrax spores almost to the extent of over approximately 99%.

(ii) Spore count :

The spore suspension was heated to 80°C for 20 minutes in water bath in order to destroy the residual vegetative bacilli and was then subjected to ten-fold dilutions in sterile distilled water. The final dilution made was 10^{-8}. 0.1 ml. each from the last three diluted suspensions was spread over the surface of the nutrient agar plates with the help of a sterilised glass spreader. The number of bacterial colonies which developed after overnight incubation at 37°C was counted and average number of spores present in the original saline suspension per ml. calculated. Thus, the average spore count was found to be 14×10^{9}/ml.

(iii) Stock anthrax spore suspension :

The original saline spore suspension containing 14×10^{9} spores/ml. was further diluted in sterile normal saline solution so that the final stock suspension contained 1×10^{8}/ml. of spores. This was stored in the refrigerator ($5^{\circ}C \pm 1^{\circ}C$) throughout the period of the study. It was checked periodically for purity by microscopic and cultural examination.

Media :

In order to study the metabolic requirement and critical factors necessary for the growth and toxin production in vitro of B.anthracis, several modifications of Casamino acids medium described by Belton & Strange (1954) were used in the present work. The following four groups of media were

included in this study.

Medium No. I:

This consisted of the medium used by Belton and Strange (loc.cit.) for the isolation of protective antigen excepting that the solution 'B' (which contained salts, nucliec acids etc.) used by them was omitted. Thus, it comprised of the following four different solutions. Glass redistilled water was used throughout for the preparation of the medium.

Solution 'A':

Casamino acids (Difco)	...	90 gm.
L- Tryptophan (B.D.H.)	...	1.3 gm.
Glycine (B.D.H.) ...	...	0.375 gm.
L- Cystine (E. Merck)	...	0.30 gm.

The tryptophan was dissolved separately in 10 ml. N. HCl by constant stirring with a glass rod and adding more acid in one ml. volume, if further required. The cystine was mixed with 3 ml. of distilled water and concentrated hydrochloric acid (Analar grade) was added drop by drop while stirring until it had completely dissolved. These two solutions were added to the casein and glycine dissolved separately in water and the volume was made up to one litre with distilled water. The pH was approximately 5.0.

Solution 'B' :

Potassium dihydrogen phosphate (KH_2PO_4) (B.D.H.)	...	34 gm.
Potassium hydroxide 5% (V/W) (KOH) (B.D.H.)	...	160 ml.

The phosphate was dissolved in approximately 800 ml. of water and hydroxide solution added until the pH was 6.8. The

volume was made up to one litre with distilled water.

Solution 'C'*:

Sodium bicarbonate (E.Merck) ... 2.5 gm.
($NaHCO_3$)

D- glucose (B.D.H.) ... ... 1 gm.

Glutamine (Swiss) ... ... 0.001 gm.

The solids were dissolved in 90 ml. of water, sterilised by seitz filtration and tubed in 9 ml. volume aseptically. It was stored in refrigerator until use.

Solution 'D'*:

Ferrous sulphate (Analar) ... 2.78 gm.
($FeSO_4$, $7H_2O$)

Manganese sulphate (A.R.) ... 0.0223 gm.
($MnSO_4$,$4H_2O$)

The ferrous sulphate was dissolved in small quantity of water containing 0.3 ml. of concentrated hydrochloric acid. The manganese sulphate was dissolved separately in a small volume of water and mixed with the above solution. The volume was made up to 400 ml. The solution was sterilised by seitz filtration and tubed in 10 ml. volume aseptically.

Basal medium :

This was prepared in Roux flasks , by adding 4 ml. each of the solutions 'A' & 'B' to 81 ml. of water; flasks were plugged with non-absorbent cotton wool. They were sterilised by autoclaving at 5 lbs./sq. inch for 20 minutes followed by at 15 lbs./sq.inch for 20 minutes. The pH of the basal medium after autoclaving was approximately 7.0. The flasks were stored in refrigerator until required.

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* Solutions 'A' & 'B' kept long in the refrigerator but solution 'C' & 'D' deteriorated on keeping; therefore they were prepared fresh & used within 24 hrs.of their preparation.

Final medium :

To complete the medium, 9 ml. of solution 'C' and 2 ml. of solution ~~x~~ 'D' (containing spores inoculum) were added aseptically to each flask containing the basal medium (89 ml.) giving a final volume of 100 ml. The addition of solution 'D' produced collidal opalascence. The pH of the final medium was 7.5 to 7.6.

Medium No. II :

The medium described in No.I was used with the following modifications:

Certain amino-acids considered to be the critical factors for growth and elaboration of antigen were incorporated and added in the medium in quantity as in 599 medium (Puziss & Wright, 1954).

DL- Isoleucine (B.D.H.)	...	0.320 gm.
DL- Methionine (E.Merck)	...	0.150 gm.
L - Proline (B.D.H.)	... ...	0.150 gm.
DL- Phenylalanine (B.D.H.)	...	0.340 gm.
L - Histidine (E. Merck)	...	0.480 gm.
DL- Threonine (B.D.H.)	...	0.300 gm.
L - Glutamic acid (E.Merck)	...	0.840 gm.
L - Leucine (E.Merck)	...	0.640 gm.
Calcium chloride (English) ($CaCl_2$, $2H_2O$)	...	0.075 gm.
Magnesium sulphate (B.D.H.) ($MgSO_4$, $7H_2O$)	...	0.050 gm.

L-Leucine, DL-Phenylalanine and L-Glutamic acid were suspended separately in small quantity of water and N.HCl added drop by drop until dissolved completely. Other

constituents were dissolved separately in water and added to the above solution. The volume was made up to 500 ml. The pH of the final solution was approximately 5.1. The solution was tubed in 10 ml. volume and autoclaved at 15 lbs./sq. inch for 15 minutes. The sterilised solution was added to the final medium at the time of inoculation. Further procedure described earlier in medium No.I for the preparation of " final medium " was followed except that the quantity of distilled water was reduced from 81 ml. to 71 ml. in order to make up the final volume to 100 ml.

A preliminary attempt was made to examine the effect of altering the concentration of sulphates (Ferrous sulphate 0.00015 %, Manganese sulphate 0.0004%) in solution 'D' as suggested by Wright et al. (1962). This alteration, however, did not promote antigen production. Further, it did not produce opalascence in the final medium which was considered desirable for the elaboration of antigen (Belton & Strange, 1954). Therefore, the concentration of sulphates was restored as in medium No.I.

Medium No.III :

This medium agrees more closely to that of Belton & Strange (op.cit.) than the medium described in No.II. It was prepared as described in No.I with the following additions and modifications:

The amino-acid-salt solution used in medium No.II was omitted. The solution 'B' of Belton & Strange (op.cit.) was included except that the two nucleic acids i.e. adenine and guanine could not be incorporated due to their

non-availability. Thus, the solution hereafter termed as 'E' contained the following ingredients:

Solution 'E' :

Calcium chloride	...	...	0.5512 gm.
Magnesium sulphate	...	...	0.250 gm.
Uracil (English)	...	...	0.070 gm.
Thiamine-HCl (E.Merck)		...	0.0025 gm.

Each solid was dissolved separately in small quantity of water and mixed in order given. The solution was made up to one litre with distilled water. This solution was added in 4 ml. volume to the basal medium along with solutions 'A' & 'B' as done in medium No.I before autoclaving.

The concentration of carbonate and glucose mentioned in solution 'C' was increased to 0.8 and 0.2 % respectively. As the amino-acid-salt solution was omitted in this medium, the quantity of distilled water was increased from 71 ml. to 77 ml. to make up the volume to 100 ml. in the final medium. The basal medium was autoclaved at 15 lbs/ sq. inch for 20 minutes as followed by Thorne & Belton(1957). Prolonged autoclaving done previously was discontinued.

Medium NO.IV :

This was the medium described in No.III, except that adenine and guanine were also incorporated in the solution 'E'. Thus, this solution contained the following ingredients:

Calcium chloride	...	...	0.5512 gm.
Magnesium sulphate	...	...	0.250 gm.
Adenine (German)	...	...	0.70 gm.

Guanine (German)	...	...	0.075 gm.
Uracil	....	...	0.070 gm.
Thiamine-HCl	...	...	0.0025 gm.

Guanine was made into paste with 20 drops of concentrated hydrochloric acid and other solids were dissolved separately in water and mixed with it in order given. The pH of the final medium was adjusted at two levels i.e. at pH 7.6 and 8.5.

In the later part of the experiment with medium No.IV, the glass redistilled water was further purified by passing through the columns of Amberlite ion exchange resin. The resins IRC-50 (H) & IR-45(OH) were filled separately in two burettes about 1.5 cm. in diameter. The glass redistilled water was allowed to pass through the columns of the two resins about 12 cm. in length successively under atmospheric pressure. The pH of the water thus obtained was 6.8.

All the solutions used in the medium were prepared with this water with a view to examine its effect on the rate of bacterial growth and antigen production.

TABLE I

Composition of four groups of media used for toxin production.

Constituents	gm/100 ml. of medium			
	Medium I	Medium II	Medium III	Medium IV.
Casamino acids	0.36	0.36	0.36	0.36
L-Tryptophan	0.0052	0.0052	0.0052	0.0052
Glycine	0.0015	0.0015	0.0015	0.0015
Potassium dihydrogen phosphate	0.136	0.136	0.136	0.136
Potassium hydroxide	0.032	0.032	0.032	0.032
Sodium bicarbonate	0.25	0.25	0.80	0.80
D-glucose	0.1	0.1	0.2	0.2
Glutamine	0.0001	0.0001	0.0001	0.0001
Ferrous sulphate	0.0139	0.0139	0.0139	0.0139
Manganese sulphate	0.00011	0.00011	0.00011	0.00011
DL-Isoleucine	-	0.0064	-	-
DL-Methionine	-	0.003	-	-
L- Proline	-	0.003	-	-
DL- Phenylalanine	-	0.0068	-	-
L- Histidine	-	0.0096	-	-
DL-Threonine	-	0.0060	-	-
L- Glutamic acid	-	0.0168	-	-
L- Leucine	-	0.0128	-	-
Calcium chloride	-	0.0022	0.0022	0.0022
Magnesium sulphate	-	0.0010	0.0010	0.0010
Uracil	-	-	0.00028	0.00028
Thiamine-HCl	-	-	0.00001	0.00001
Guanine	-	-	-	0.00003
Adenine	-	-	-	0.0028

Production of anthrax toxin :

On going through the account given elswhere on the pathogenesis and immunity in anthrax, it will appear that the workers during the last decade obtained experimental evidences regarding the role of anthrax toxin in the causation of the disease. They also observed that an extracellular soluble antigen was responsible for conferring immunity against anthrax in experimental animals. They used both synthetic and non-synthetic media of complex chemical composition containing a large number of amino-acids and salts for successful production of the soluble toxin.

Therefore, the principal object of the present study was to examine the possibility of producing anthrax toxin with particular reference to the oedema and lethal factors under altered cultural conditions which could facilitate easy and quick isolation of these factors.

Four groups of Casamino acids media (Table I) differing in their amino-acids content and salt concentration as described earlier were used. 0.1 ml. of the stock anthrax spore suspension containing 10^8 spores/ml. was added to 10 ml. of the sulphate solution 'D'. After thorough mixing, 2 ml. of this solution (2 x 10^6 anthrax spores) were inoculated into Roux flask containing 100 ml. of the final medium. The flask was then incubated aerobically at 37°C.

Screening test for the presence of toxin in the whole culture:

In the beginning of the experiment, the culture flask was incubated for a period of 16 hours aerobically. About 10 ml. of the culture growth was taken out with the

help of a sterile pipette at every two hours interval commencing from the 4th hour and onward up to 16th hour. Due precautions were taken to avoid contamination of the culture flasks during this operation. Each time after taking out the culture fluid from the flask, 0.1 ml. was spread over on to the surface of blood agar plate in order to examine freedome of contamination. On no occasion was the presence of extraneous organisms detected. Ten ml. of the liquid culture thus collected (in total 7 collections were made) at different hours of incubation were immediately chilled at 0°C in the deep-freez chamber of the refrigerator and used for skin test in rabbits and guinea-pigs within an hour of their collection.

Thereafter, similar collections of the culture growth were made from another culture flask grown separately at 37°C for 28 hours. The growth was removed at every two hours as done before beginning at 16th hour of incubation. In this batch, materials from another 7 collections were available which were also subjected to skin test as mentioned above. All the four groups of different media were dealt with similarly at different times.

Isolation of cell-free anthrax toxin :

This was carried out on the lines suggested by Beall et al.(1962) . The foregoing screening test had shown qualitative evidence of positive skin reaction at and after the 16th hour of incubation in the medium No.IV. Therefore, the work was further extended to isolate the soluble toxic factors in this culture medium at four hourly intervals,

commencing from the 16th hour onward ~~upto~~ up to the 28th hour during the course of incubation. This was done as follows:

(i) Preparation of crude supernatant of the toxin :

Roux flasks, five to six in number, each containing 100 ml. of the medium No.IV were inoculated at a time with the stock anthrax spore suspension along with the solution 'D' as reported above and were incubated aerobically. After the desired period of incubation (at 16,20,24 and 28 hours of incubation), the flasks were examined microscopically for contamination. The culture flasks which were found free of contamination, were pooled together and chilled to $0^{o}C$ in the deep-freeze. Usually 500 ml. of the culture growth were available for study. All subsequent processes for isolation of toxin were done in the cold room at $10^{o}C$.

500 ml. of the growth culture thus obtained, was brought in the cold room (temp. $10^{o}C$) and centrifuged at 2000 r.p.m. for 10 minutes. The supernatant was collected in sterilised test tube and kept in deep-freeze until use for the assay of the toxin. This constituted the test material No.I (T.M.I).

(ii) Separation of cell-free toxin from crude supernatant:

The supernatant liquid (about 500 ml.) was filtered through fritted glass filter of fine porosity (G/4) under normal atmospheric pressure and the filtrate was collected and stored in sterilised test tube in deep-freeze for test (T.M.2).

Thereafter, the glass filter was fitted to another sterilised filtering flask. 0.3 M $NaHCO_3$ - Na_2CO_3 buffer,

pH 9.7 prepared as given below, was used for the purpose of eluting the toxin from the filter disc.

0.3 M solutions of sodium carbonate and sodium bicarbonate were prepared separately in distilled water and the two solutions were mixed in the ratio of 4 ml. and 6 ml. respectively. The pH of the mixture was adjusted to 9.7.

Thus, 10 ml. of the buffer was run over the filter disc and a few drops were allowed to pass through by applying negative pressure. The buffer was then left on the glass filter disc for about half an hour for elution to occur. The eluate was collected in sterilised test tubes and stored in refrigerator for biological assay (T.M.3). The samples of the filtrate and the eluate were also plated on blood agar plates. In each case, the materials were found to be free from micro-organisms.

Biological assay of the toxin :

Anthrax toxin isolated *in vitro* has been shown to cause oedema in the skin of rabbits and guinea-pigs when inoculated intradermally and kill rats and mice when injected intravenously (Thorne *et al.*, 1960; Stanley & Smith, 1961). These biological activities have been attributed due to the presence of two different toxic factors (namely oedema and lethal factors) when combined with the soluble protective antigen. Therefore, an attempt was made to demonstrate the presence of these toxic factors in the materials hitherto collected, in these animals.

As purified protective factor was not available, the filtrate (T.M.2) which is reported to contain the

protective antigen, was substituted for this purpose (Thorne et al.,loc.cit.).

The following materials,as and when they were ready, were used for the skin test :

(1) Culture supernatant (T.M.1)

(2) Culture filtrate .. (T.M.2)

(3) Eluate ... . (T.M.3)

(4) Eluate + Culture filtrate(T.M.4)-in the ratio of 1:4

All the materials were inoculated within three hours of their collection. Besides,the materials collected earlier for screening test were also subjected to skin test.

(i) Skin test :

Skin test for the demonstration of oedema factor in the above mentioned materials was done by inoculating them in doses of 0.2 ml. each intradermally in adult rabbits and guinea-pigs of indigenous breed.

The hairs on either side of the back of the animals were shaved carefully ensuring freedom of cut injury or razor scratch on the skin in the evening preceding inoculation.

0.2 ml. of the material was inoculated intradermally with the help of tuberculin needle attached to a tuberculin syringe previously sterilised. A pea-like swelling developed at the site of inoculation which was taken as indicative of intradermal inoculation. The above swelling disappeared within 5 to 6 hours after the inoculation. Each material was inoculated in pair (one on either side of the back) at a distance of approximately 2.5 cm. from one another. Six

inoculations in rabbits and four in guinea-pigs were made on either side of the back of these animals.

In order to titrate the oedema-producing toxin present in the test materials (T.M.1,T.M.2,T.M.3 & T.M.4), they were diluted to 1:5, 1:10, 1:15, 1:20, 1:25 and 1:30. All these solutions were injected intradermally in doses of 0.2 ml. as usual. The dilutions of T.M.1 and T.M.4 were invariably done in culture filtrate whereas that of T.M.2 and T.M.3 was routinely made in 0.02 M phosphate buffer, pH 8.0, containing 0.5% of gelatin (W/V).

The specificity of the skin reaction elicited by the different dilutions of the above test materials obtained after 24 and 28 hours of incubation was determined by using hyperimmune anthrax anti-serum (H533). For this purpose , 0.1 ml. of anti-serum was mixed with 0.3 ml. of the materials from all the dilutions and mixture was inoculated intradermally in rabbits and guinea-pigs. Besides,0.2 ml. of the 0.3 M carbonate buffer, pH 9.7 used for eluting the toxin from the filter disc, was also injected intradermally at various dilutions such as neat, 1:5, 1:10 and 1:15 in gel-phosphate which acted as control. All the experimental animals were observed for a period of 4 days.

The result of (+) ve or (-) ve skin reaction was recorded after 24 hours as evidenced by the production of cutaneous oedema and redness at the site of inoculation. The degree of oedematous reaction was expressed by assigning (+ + +) for marked oedema, (+ +) for moderate oedema and (+) for slight oedema and (-) for negative reaction.

As mentioned earlier, the screening test did not suggest the production of oedema factor in the first three media. However, it was desired to examine whether it was possible to obtain the toxic components after subjecting the test materials to subsequent processing in course of elution of the filter factor. Therefore, the Ist three media (Media I,II & III) were also dealt with in similar manner as reported above, except that the specificity of the reaction with hyperimmune anthrax anti-serum was not determined.

(ii) Lethal activity of the toxin:

Materials used for conducting skin test (such as T.M.1,T.M.2,T.M.3 & T.M.4) were also subjected to test for demonstrating the lethal activity of the toxin. This was done by inoculating 2 ml. of the undiluted materials intravenously in white rats. The inoculum was injected into the dorsal vein of penis in rats. These animals were observed for a period of one week after the injection.

Direct haemagglutination test :

This was done with the test materials obtained from medium No.IV.

Sheep erythrocytes:

The blood from jugular vein was aseptically collected from two sheep in sterile modified Alsever's solution. The ratio of blood to Alsever's solution was 1:1.2. The blood so collected was allowed to stabilise in the refrigerator for three days. The cells should not haemolyse during washing or when allowed to stand in a

packed form in modified Alsever's solution(Whitman, 1947).

The composition of the Alsever's solution used was as follows:

Dextrose	...	2.05 gm.
Sodium citrate	...	0.80 gm.
Sodium chloride	...	0.42 gm.
Distilled water	...	100 ml.

The pH of the solution was adjusted to 6.1 with 10% citric acid solution.

After 3rd day of collection, the plasma was separated by centrifugation at 3,000 r.p.m. for 10 minutes. The cells were washed thrice with sufficient quantity of buffered saline at pH 7.2 (0.05 M phosphate buffer). After final washing, the cells were packed at 1500 r.p.m. for 10 minutes. The cells were further diluted to make a 2.5 % suspension. This was done by adding 0.25 ml. of packed cells to 9.75 ml. of sterilised buffered saline (pH 7.2). The test was carried out with 0.25% erythrocyte suspension.

Procedure :

Materials such as culture supernatant, culture filtrate and eluate were used for this test. They were diluted separately in buffered saline in serial double-fold dilution in 0.5 ml. volume in multi-whole perspex haemagglutination plates. To each dilution of sample was added 0.5 ml. of 0.25% sheep erythrocytes .

Protocol of the test:

Cup nos.	1	2	3	4	5	6	7	8	9	10	11
Saline(ml.)	-	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	-
Carbonate buffer	-	-	-	-	-	-	-	-	-	-	0.5
Test sample(ml.)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	-	-
	(Serial two-fold dilution)										
Dilution factor	neat	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	-	-
Sheep cells (0.25%)ml.	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

The hole containing 0.5 ml. saline and 0.5 ml.sheep cells formed the control. As the eluate contained carbonate buffer, another control was also set up with 0.5 ml. of the buffer solution and 0.5 ml. of the sheep cells. The whole system was incubated at 37°C for one hour and then left at room temperature for 6 - 12 hours.

The result of the haemagglutination test was recorded according to the pattern of sedimentation of red cells in the hole. Characteristic scattered pattern of sedimentation of cells in the hole of the H.A. plate was recorded as (+) ve whereas a round button-like sedimentation of red cells in the bottom was taken as (-) ve reaction.

S E C T I O N - D

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

A. RESULTS:

In an attempt to isolate the soluble anthrax toxin *in vitro*, a Weybridge strain (34 F/2) of *B.anthracis* was grown aerobically in four groups of Casamino acids media. The presence or absence of the toxin in the various test materials collected at different hours of growth was tested by their ability to produce cutaneous oedema and lethality in laboratory animals. An attempt was also made to examine the possible correlation, if any, between the biological activity and the haemagglutinating activity of the toxin.

Bacterial growth:

While examining the bacterial growth in the test media at different hours of incubation, it was observed that vegetation had not started in the four-hour-old culture in any of the media put to test. By 8th hour, scanty vegetation had occurred as noticed on the microscopic examination and slight increase in the turbidity of the culture. By this time, the bacilli looked shorter in size and had formed small chains of twos or four. They were noticeable only after careful search in several microscopic fields. Between the 12th and 16th hours, the growth was found to be comparatively better but a marked difference was noticed in their yield in different media. In media no.I & II, the chains were readily detectable and occupied almost the entire field of the microscope. The media no.III and IV showed, on the other hand, still better growth as evidenced

by the number and the size of the bacterial chains.

At 20 hours and onward, the growth in media no.III & IV was found to be maximal. The stained preparations showed numerous long interwoven chains of the bacilli which had formed large clumps. In the first two media, however, it was comparatively poor.

Screening test of whole culture:

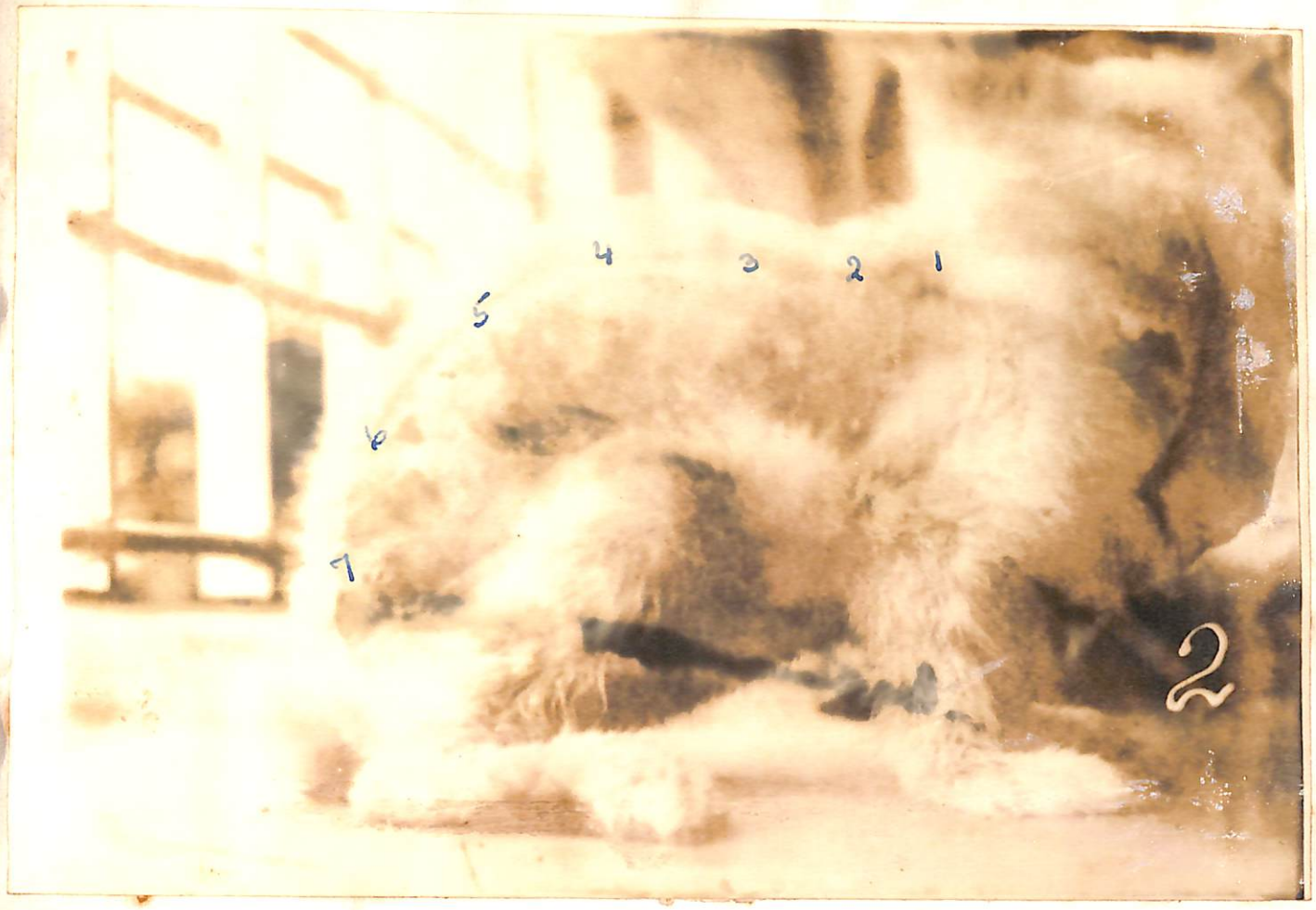
The presence or absence of the oedema producing toxin in the whole culture was screened by intradermal inoculation of the culture growth at two hourly interval. For this purpose, the test strain of B.anthracis was grown aerobically in all the four groups of media (pH 7.6) separately and the whole culture was subjected to skin test in rabbits and guinea-pigs. The media no.IV, however, was also tested at pH 8.5.

In course of the experiment, it was observed that the growth obtained from the first three media up to 28 hrs. of incubation was completely innocuous and did not elicit any skin reaction in rabbits and guinea-pigs when inoculated intradermally. The media no.IV also did not cause cutaneous oedema when tested up to 16 hours of incubation. However, the first evidence of positive skin reaction in rabbits with this medium(pH 7.6) was noted in the 18-hour old culture. The reaction was characterised by a central area of reddening and slight diffuse cutaneous oedema of the size of approximately 4 to 6 mm. at the site of intradermal inoculation. The reaction continued to be evident up to the 28th hour of incubation - the period so far tested.

In the medium No.IV tested at pH 8.5,similar cutaneous reaction was noticed with 18-hour-old culture. Between 20 and 24 hours, the reaction was found to be moderate and well defined and the size of the swelling varied between 6-9 mm. as measured by Vernier callipers. On the other hand, the materials collected between 26 and 28 hours of the growth provoked only slight palpable oedema showing gradual waning of the toxic factor. The above reactions were also noticeable in guinea-pig, but the degree of oedema was comparatively less when compared to that observed in rabbits (Table II).

Biological activity of the culture supernatant and the soluble product of growth of B.anthracis:

As mentioned earlier in the previous section, an attempt was made to obtain the cell-free anthrax toxin in all the four groups of culture media at different hours of growth. As done for the screening test, the pH of the first three media was maintained at 7.6 while that of the fourth medium, it was 8.5. The cultures were grown for 16,20,24 and 28 hours and the growth materials were filtered through fritted glass filter. Subsequent elution of the filter factor from the glass disc was accomplished by carbonate buffer. Thereafter, four different test materials such as culture supernatant, culture filtrate , eluate and eluate-filtrate mixture were subjected to lethal and skin tests in rats and rabbits respectively.



Skin reaction on screening test in rabbit caused by the whole culture of medium No.IV.

1.	Whole	culture	...	16 hr.
2.	"	"	...	18 hr.
3.	"	"	...	20 hr.
4.	"	"	...	22 hr.
5.	"	"	...	24 hr.
6.	"	"	...	26 hr.
7.	"	"	...	28 hr.

TABLE II

Result of screening test for oedema factor

Medium No.	pH	Material	Time interval in hour												
			4	6	8	10	12	14	16	18	20	22	24	26	28
I	7.6	Whole culture	-	-	-	-	-	-	-	-	-	-	-	-	-
II	7.6	"	-	-	-	-	-	-	-	-	-	-	-	-	-
III	7.6	"	-	-	-	-	-	-	-	-	-	-	-	-	-
IV	7.6	"	-	-	-	-	-	-	-	+	+	+	+	+	+
IV	8.5	"	-	-	-	-	-	-	-	+	++	++	++	+	+

The result of these tests showed that all the above test materials were devoid of lethal activity, as none of them caused death in white rats. The skin test also showed uniform negative result with all the test materials obtained from the Ist three culture media.

The materials obtained from the fourth medium, however, showed the presence of oedema factor and this confirmed the earlier observations obtained on the screening test.

Result of skin test in rabbits carried out with various test materials obtained from medium No.IV:

16 hours:- As observed with the first three culture media the growth in the medium No.IV too was found to be non-toxic when tested at 16 hours of incubation. All the four test materials (T.M.1, T.M.2, T.M.3 & T.M.4) obtained from this culture failed to elicit any skin reaction even when used undiluted. T.M.3 & T.M.4, however, caused cutaneous necrosis up to the dilution of 1:5.

20 hours:- The T.M.1 which comprised of culture supernatant caused moderate cutaneous oedema in the dilutions made up to 1:10. Thereafter, the degree of oedematous reaction decreased slightly which was apparent at the dilution of 1:15 and 1:20. Further dilution of the material showed negative response on skin test. T.M.2 and T.M.3 which consisted of culture filtrate and the eluate respectively were also found to be biologically inactive. They did not provoke any skin reaction even with the undiluted material. With the latter, however, an area of necrosis was noticed at the site of inoculation. The necrotic changes were found only up to the dilution of

1:5. T.M.4 (eluate - culture filtrate mixture) also produced cutaneous necrosis when diluted to 1:5. At 1:10 of this material, there was again significant skin reaction but the reaction waned at the dilution of 1:15. Further dilution of T.M.4 produced no reaction in the skin of rabbit.

24 hours:- T.M.1 of 24-hour-old culture showed results similar to those observed with the corresponding material of the preceding batch except that the decrease of oedema had started earlier at the dilution of 1:10 and the reaction continued to be evident up to the dilution of 1:20. Further dilution produced negative reaction on skin test. T.M.2 & 3 again showed results comparable to those observed with corresponding materials of 20-hour-old culture. T.M.4 also showed parallel results except that the production of oedema was not so significant when compared with materials collected at 20 hours after incubation.

28 hours:- Gradual decrease in the degree of skin reaction was noted when the incubation of the culture was prolonged to 28 hours. T.M.1 which caused moderate cutaneous oedema in the undiluted material, produced only slight skin reaction when diluted to 1:5. However, this reaction was observed up to the dilution of 1:15, after which there was no reaction at all. T.M.2 and T.M.3 produced results as seen with the earlier batches of the culture, while T.M.4 showed necrosis in the dilution of 1:5 and slight oedema at 1:10. Further dilution of this material produced no result.

As mentioned earlier, carbonate buffer which was used for eluting the filter factor from the glass filter was also put to skin test in order to compare the results obtained with T.M.3 & T.M.4. The control buffer caused marked cutaneous necrosis up to the dilution of 1:5. Necrotic changes were not seen on further dilution to 1:10 and above (Table III).

In the fourth group of medium, amberlite resin-treated water was used for preparing all the solutions and the medium. A comparable set of dilution of all the test materials obtained from this medium after 20 and 24 hours of incubation was also used for skin test separately.

It was observed that these materials produced skin reaction which was comparable with those when glass redistilled water was used for this purpose. The reaction was in no way superior to the former.

Specificity test of skin reaction with hyperimmune anthrax anti-serum (H533):-

In order to determine the specificity of the skin reaction, all the test materials collected at 24 and 28 hours of incubation and which had provoked cutaneous reaction in rabbits, were also inoculated in similar dilutions in combinations with hyperimmune anthrax anti-serum. The inoculum consisted of the toxin-antiserum mixture in the ratio of three volumes of the former and one volume of the latter.

It was observed that the skin reaction obtained with T.M.1 and T.M.4 of these two batches of culture was completely

Skin reaction in rabbit caused by different test materials collected at 20 hours from medium No. IV.

1. Culture supernatant (T.M.1)
2. Culture filtrate (T.M.2)
3. Eluate (T.M. 3)
4. Eluate-filtrate mixture (1:10) (T.M.4)
5. Carbonate buffer control.

TABLE III

Titration of test materials obtained from medium No.IV for oedema factor in rabbits.

Time interval	Material	Dilution of test material						
		Neat	1:5	1:10	1:15	1:20	1:25	1:30
16 hrs.	T.M.1	-	-	-	-	-	-	-
	T.M.2	-	-	-	-	-	-	-
	T.M.3	Necrosis	Necrosis	-	-	-	-	-
	T.M.4	Nil	Necrosis	-	-	-	-	-
20 hrs.	T.M.1	++	++	++	+	+	-	-
	T.M.2	-	-	-	-	-	-	-
	T.M.3	Necrosis	Necrosis	-	-	-	-	-
	T.M.4	Nil	Necrosis	++	+	-	-	-
24 hrs.	T.M.1	++	++	+	+	+	-	-
	T.M.2	-	-	-	-	-	-	-
	T.M.3	Necrosis	Necrosis	-	-	-	-	-
	T.M.4	Nil	Necrosis	+	+	-	-	-
28 hrs.	T.M.1	++	+	+	+	-	-	-
	T.M.2	-	-	-	-	-	-	-
	T.M.3	Necrosis	Necrosis	-	-	-	-	-
	T.M.4	Nil	Necrosis	+	-	-	-	-
Control	Carbonate buffer	Necrosis	Necrosis	-	-	*	*	*

TABLE III

Titration of test materials obtained from medium No.IV for oedema factor in rabbits.

Time interval	Material	Dilution of test material						
		Neat	1:5	1:10	1:15	1:20	1:25	1:30
16 hrs.	T.M.1	-	-	-	-	-	-	-
	T.M.2	-	-	-	-	-	-	-
	T.M.3	Necrosis	Necrosis	-	-	-	-	-
	T.M.4	Nil	Necrosis	-	-	-	-	-
20 hrs.	T.M.1	++	++	++	+	+	-	-
	T.M.2	-	-	-	-	-	-	-
	T.M.3	Necrosis	Necrosis	-	-	-	-	-
	T.M.4	Nil	Necrosis	++	+	-	-	-
24 hrs.	T.M.1	++	++	+	+	+	-	-
	T.M.2	-	-	-	-	-	-	-
	T.M.3	Necrosis	Necrosis	-	-	-	-	-
	T.M.4	Nil	Necrosis	+	+	-	-	-
28 hrs.	T.M.1	++	+	+	+	-	-	-
	T.M.2	-	-	-	-	-	-	-
	T.M.3	Necrosis	Necrosis	-	-	-	-	-
	T.M.4	Nil	Necrosis	+	-	-	-	-
Control	Carbonate buffer	Necrosis	Necrosis	-	-	-	-	-

TABLE IV

Result of skin test with various test materials with and without specific anthrax anti-serum

Hour of incubation	Test materials	Dilution of test materials													
		Neat		1:5		1:10		1:15		1:20		1:25		1:30	
		T.M.	T.M. + anti-serum	T.M.	T.M. + anti-serum	T.M.	T.M.+ anti-serum	T.M.	T.M.+ anti-serum	T.M.	T.M. + anti ser.	T.M	T.M + a.s	T.M	T.M + a.s
24	T.M.1	++	-	++	-	++	-	+	-	+	-	-	-	-	-
	T.M.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	T.M.3	Necrosis	Necrosis	Necrosis	Necrosis	-	-	-	-	-	-	-	-	-	-
	T.M.4	Necrosis	Necrosis	Necrosis	Necrosis	+	-	+	-	-	-	-	-	-	-
28	T.M.1	++	-	++	-	+	-	+	-	-	-	-	-	-	-
	T.M.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	T.M.3	Necrosis	Necrosis	Necrosis	Necrosis	-	-	-	-	-	-	-	-	-	-
	T.M.4	Necrosis	Necrosis	Necrosis	Necrosis	+	-	-	-	-	-	-	-	-	-

TABLE V

Result of haemagglutination test with various test materials of medium No.IV.

Incubation in hours.	Test materials	Dilution factor									Controls	
		Neat	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	Saline	Carbonate buffer
16	T.M.1	+	+	+	+	-	-	-	-	-	-	x
	T.M.2	+	+	+	-	-	-	-	-	-	-	x
	T.M.3	Hemolysis	Hemolysis	-	-	-	-	-	-	-	x	Hemolysis
20	T.M.1	+	+	+	+	+	+	-	-	-	-	x
	T.M.2	+	+	+	+	+	-	-	-	-	-	x
	T.M.3	Hemolysis	Hemolysis	-	-	-	-	-	-	-	x	Hemolysis
24	T.M.1	+	+	+	+	+	+	+	-	-	-	x
	T.M.2	+	+	+	+	-	-	-	-	-	-	x
	T.M.3	Hemolysis	Hemolysis	-	-	-	-	-	-	-	x	Hemolysis
28	T.M.1	+	+	+	+	+	+	-	-	-	-	x
	T.M.2	+	+	+	+	+	+	+	-	-	-	x
	T.M.3	Hemolysis	Hemolysis	-	-	-	-	-	-	-	x	Hemolysis

B. DISCUSSION :

Bacterial growth:

The rate of growth of the "Weybridge" strain of B.anthracis differed considerably depending upon the cultural conditions provided in the different groups of media.

The medium No.I contained Casamino acids and only a few of the growth factors and salts, while medium No.II had in addition an array of amino-acids (Table I). In spite of the modifications in respect to these growth factors, the first two media showed only moderate growth which was comparable with each other. On the other hand, the third medium from which a number of amino-acids were omitted, contained uracil and thiamine while the fourth one had in addition adenine and guanine. When compared with the first two media, the growth in the latter two groups was more pronounced particularly after 20 hours of incubation. Further, a close parallelism was observed in the rate of growth of the organism in the last two groups.

During recent years several workers have examined the metabolic requirements necessary for promoting growth and antigen production of B. anthracis. They contended that sodium bicarbonate, ferrous sulphate and glucose were essential for the excellent growth of the bacilli(Puziss & Wright, 1954; Thorne & Belton, 1957).

They also observed that with the increased concentration of bicarbonate, a vegetative inoculum was preferable , because spore inoculum gave only poor yield of the organism under such condition.

In the present study, salts of calcium and magnesium ion were incorporated in all the groups of media except in No.I. From the results, it seems apparently difficult to assess the role of these salts in the metabolic pathway of the organism. Moderate growth response in medium No.II but optimum in No.III & IV in the presence of these two salts may perhaps be attributed to the nutritional antagonism of the amino-acids in the former and the synergistic activity of uracil and thiamine in the latter two groups of media. Adenine and guanine did not seem apparently to be associated with enhancing the growth of the test organism.

The concentration of sodium bicarbonate and glucose in the last two media was increased from 0.25% to 0.8% and 0.1% to 0.2% respectively. These two constituents seem to have enhanced the growth appreciably by virtue of their buffering action and added nutrients in the media. A comparison between the rate of growth of the organism in different media with and without ferrous sulphate was not made. However, it was felt that under the cultural conditions provided in the present investigation, the relative deficiency in the growth rate noticed in the first two media was overcome by the presence of other critical growth factors, provided in others.

Considering further the results obtained in the present study, it also appeared that the amino-acids provided in the medium No.II were not necessary for promoting the growth because the yield of the organisms grown in the presence of these amino-acids could be favourably compared with that obtained in medium No.I which had the basal medium containing Casamino acids but was deprived of these factors.

Of the cultural requirements in general, optimum pH of the media is one of the important factors, which conditions the metabolic activity of any micro-organism. The medium No.IV was put to test at two pH levels i.e. 7.6 & 8.5. As there was no significant difference in the growth of the organisms at these two pH levels, it was felt that the requirement of hydrogen-ion concentration between these levels in the growth media was not very critical.

Antigen production:

(i) Oedema factor:- During recent years, much attention has been paid on the critical factors essential for the elaboration of soluble antigens by B. anthracis. It has been maintained that a high alkaline pH of the medium is conducive for the production of antigen.

Thorne & Belton (1957) increased the concentration of sodium bicarbonate in Casamino acids medium from 0.25% to 0.75% and obtained about four-fold increase in the yield of the antigen. When tris-buffer(Tri-hydroxymethyl-aminomethane) was substituted for the carbonate at the

pH value of 8.7 - 9.5, it was possible to obtain the antigen though in comparatively lower titre. Thus, it appears that the carbonate is not indispensable for the antigen production. It helps antigen synthesis by maintaining a high alkaline pH of the medium at which it is stable.

Calcium chloride has been held to influence the synthesis of protective antigen. Without this, a marked reduction in the elaboration of the antigen has been observed. Besides, the adverse effect of omitting purines and pyrimidines and other related compounds on growth and production of antigen has also been reported by Puziss & Wright (1954). The authors found that B. anthracis grown in absence of these agents not only showed slow growth but also gave poor yield of the antigen. Although NP-A strain has been adopted to grow in media devoid of these factors, the production of antigen was inhibited with such adapted strain. Glucose has been reported to be another important constituent of the medium for the synthesis of antigen. Glucose, when autoclaved with other constituents of the medium, results in the inhibition of antigen production. This is ascribed to the presence of some inhibitory factor which forms in combination with other substances in the medium,— probably the cysteine, at the time of autoclaving. In the present experiment, glucose was sterilised separately by filtration. Therefore, the possible role of any such inhibitory agent in the production of antigen was avoided.

In the present experiment, sodium bicarbonate (0.25%) and glucose (0.1%) were added in equal quantity in media No.I & II. In addition, calcium chloride, magnesium sulphate and eight more amino-acids such as isoleucine,methionine,proline,phenylalanine,histidine, threonine,glutamic acid and leucine were also included in the latter. Subsequent modifications in the composition of the last two media were made by omitting all the eight amino-acids provided in the second group, increasing the concentration of glucose to 0.2% and bicarbonate to 0.8%. Uracil and thiamine were added in the third, and adenine & guanine were further included in the fourth medium.

The results set out in the table II & III clearly show that the amino-acids provided in the medium II were unnecessary not only for the optimum growth of B.anthracis but also for the elaboration of the oedema factor. Similarly magnesium sulphate and calcium chloride did not play any role in the synthesis of this antigen, though the latter has been thought to be essential for the elaboration of protective antigen. A higher concentration of glucose and carbonate was found to be desirable for obtaining optimum growth, but it was not considered indispensable for the production of oedema producing factor. Carbonate in higher concentration,indeed provided a suitable pH environment to the media which is essential for antigen synthesis; but it can be substituted with other suitable buffer without much detriment to antigen production. On comparison of the results of skin test with

materials obtained from media No.III & IV,it will appear that medium No.IV which contained adenine and guanine produced oedema factor while the same medium but without adenine and guanine failed to produce this toxin. Therefore, it is evident that of all the constituents, adenine and guanine were the most critical factors which were directly associated with the antigen production. Which of the two factors was responsible for the elaboration of the antigen in the present study could not be elucidated. Further investigation aimed at determining the relative importance of these agents by testing the effect of adenine and guanine separately in the Casamino acids medium might throw further light to the question.

In the past, the optimum hour of antgien production has varied considerably under different hands depending upon the cultural conditions and other technical details pertaining to its isolation. The majority of the workers reported that the average hour of antigen elaboration lay between 23 & 27 hours (Thorne et al.,1960; Beall et al.,1962). Belton and Strange (1954) on the other hand,recorded the average period for the production of protective antigen to fall between 18 to 36 hours with the optimum of 22 hours. Further, rapid disappearance of toxin in the serum culture incubated beyond seven hours has also been reported due to the appearance of intra-cellular enzyme at the later phase of the growth(Harris-Smith et al.,1958). In the present work, oedema producing toxin

was evident in the 18-hour-old culture and continued to be formed up to 28 hours of incubation. The maximum period up to which the toxin could form was not determined. However, it was clear that the maximum production of toxin occurred between 20 & 24 hours of growth (Table II & III).

It has been reported earlier that the difference in the hydrogen-ion concentration of the media between the pH 7.6 and 8.5 did not affect markedly the rate of growth of the organisms. Neverthiless, the present experiment has shown pronounced effect of these two pH levels on the rate of antigen production (Table II & III). Under both the conditions, the test strain produced oedema factor; but at high alkaline pH, the degree of skin reaction was more pronounced. It can, therefore, be concluded that although the high pH of the media may not be primarily responsible for influencing the growth of the organisms, it is indispensable for better yield of the antigen. This observation, therefore, further emphasises the need of higher concentration of carbonate in the media.

The synergistic activity of the protective antigen and other toxic factors has been amply confirmed by various workers. The protective antigen has been found to be present in the culture filtrate, whereas the lethal and oedema factors are preferentially adsorbed on to the glass filter disc. The latter are elutable with carbonate buffer at pH 9.7. Thorne *et al.* (*loc. cit.*) compared the biological activity of the crude culture filtrate with that of the purified protective antigen and confirmed the presence of

the active component in the filtrate. In their experiment, they also substituted culture filtrate for protective antigen and recorded similar result. In the present work, culture filtrate was used as the source of protective antigen. The skin test done with different test materials has shown that the culture filtrates and the eluates were inactive, when inoculated alone. On the other hand, the culture supernatant and eluate-filtrate mixture produced varying degree of cutaneous oedema in the skin of rabbit. This further confirms the synergistic activity of the protective antigen and the oedema factor.

An attempt was made to titrate the potency of the oedema producing toxin present in the different test materials. It will appear from table III that although T.M.1 & T.M.4 produced cutaneous reaction in the dose of 0.2 ml. intradermally, the potency of the toxin was not of a very high order. Even at the optimum period of antigen production i.e. at 20 and 24 hours, T.M.1 showed positive skin reaction only up to the dilution of 1:20, while T.M.4 was toxic only when diluted up to 1:15. Further dilution of these materials rendered them biologically inert. In the later phase of the growth, the materials were still poorer in their toxic content. Further, T.M.3 & T.M.4 produced necrosis at the point of intradermal inoculation up to the dilution of 1:5. This can be attributed to the presence of the carbonate present in the eluate as the skin test carried out with the carbonate control also produced similar reaction.

The contention that skin reaction may be provoked by the product of bacterial disintegration of an ageing culture is not illogical. Evans and Shoesmith (1954) reported to have produced cutaneous reaction in rabbit with soluble toxin of B.anthracis precipitated by ammonium sulphate. Although this was the first experimental evidence to show the toxic nature of the skin factor, Evan's toxin could not merit much attention of the workers due to the lack of testimony regarding the specificity of its reaction.In order to determine the specific nature of the skin reaction elicited by the different test materials in the present experiment, the materials were inoculated into rabbits in combination with the hyperimmune anthrax anti-serum. It was observed that the materials which provoked typical skin response in rabbits became non-toxic when mixed with the specific anti-serum. Thus, the biological specificity of the reaction recorded in this investigation was confirmed (Table IV).

Several workers reported the use of distilled water purified by passing through the column of amberlite resin for the purpose of preparing media and isolated a high titred antigen from B. anthracis (Belton & Strange, 1954; Thorne et al., 1957; Thorne et.al., 1960). On the contrary, Puziss & Wright (1954) and Wright et al. (1962) found glass redistilled water to be as effective for antigen production. In our experiment resin-treated water

was also used separately for the preparation of medium No.IV. The result of skin test carried out with the test materials of this medium did not show any advantage of the resin treated water over the glass redistilled water because the reactions under both these conditions were comparable.

(ii) Lethal factor:

As reported earlier, all the test materials obtained under the different cultural conditions in the present experiment proved to be devoid of lethal factor, in as much as none of them killed white rats when administered in the doses of 2 ml. intravenously.

During the last decade, production of soluble lethal factor of B. anthracis in Casamino acids medium has been demonstrated by a large group of workers (Thorne et al., 1960; Beall et al., 1962; Smith & Stanley, 1962). They fractionated this factor by various chemical methods and the purified fraction proved highly toxic for rats and mice.

In the present investigation, the negative finding on the lethal test of test materials apparently seems to be disappointing. Whether it is attributable to the low level of toxin production or to the complete absence of the lethal factor in the culture is difficult to say. Nevertheless, it is important, firstly, because it clearly shows that the two components of the toxin, i.e. the oedema and lethal factors, are biologically distinct. Secondly, the literatures so far available have repeatedly

indicated that both these components appear in the culture as well as in the eluate simultaneously. The *in vitro* production or elution of oedema factor alone have not so far been reported. It has always been found to occur in association with the lethal toxin. Although more works are needed to explain the cause of failure of demonstrating the lethality of our materials, the experimental evidences hitherto reported hold out promising line of approach for isolating the soluble oedema-producing toxin and avoiding the concomitant appearance of lethal factor simultaneously in the non-protein culture media. These results, when confirmed, will overcome the difficulty encountered in separating the different soluble factors of anthrax toxin and render their isolation easy. Besides, it would also throw light on the mechanism of antigen production by the bacilli *in vivo* and add further to our present concept of the complex pathogenesis of anthrax in animals.

There are various factors which determine the *in vitro* elaboration of soluble toxin by *B.anthracis*. Firsly, the oedema-producing activity of the culture and the eluate depends much on the optimum elaboration of the antigen. The potency of these materials is governed not only by the rate of synthesis of the toxin by the organisms but also by such factors as the rate at which it is released into the medium, its rate of destruction in the culture and the filtrability and stability of the

toxin in the eluate. In addition, this is also related to the optimum activity of the protective antigen which is necessary to bring out the classical toxic reaction by virtue of its synergistic effect. In our experiment, the antigenicity and the potency of the filtrate could not be determined, though the result of the skin test obtained has disclosed beyond doubt the active nature of this agent. However, it is felt that the potency of the protective antigen in the culture filtrate may not have been to the degree necessary to provoke significantly marked cutaneous reaction and to demonstrate high titre of the oedema factor. Alternatively, it might as well be that the toxic component in the eluate itself may not have been potent enough to show up the high titres of the antigen. Such discrepancies may as well affect the lethal activity of the toxin (Thorne et al. loc.cit.)

Anthrax toxin contained in the filter factor is known to be very fragile and non-dialysable. It is destroyed at 4°C or -20°C . In the present experiment, the processing of the whole culture was done at 10°C. The skin inoculation of different processed materials was made within three hours of their collection while the screening test of the whole culture was carried out immediately after the desired period of incubation. In course of the skin test, it was noted that the cutaneous oedema caused by the whole culture at the time of screening test was more marked than that found with the processed

materials. Similarly, the T.M.4 (eluate - filtrate mixture) had shown reduced titre of the oedema factor when compared with that obtained with T.M.1 i.e.culture supernatant. The low titre of the oedema-producing toxin in general and the difference in the degree of cutaneous reaction with different test materials in particular may be explained due to the unstable nature of the toxin at 10°C to which it was exposed. This may also perhaps ~~fmx~~ account for the negative response on the lethal test of our test materials.

For the <u>in vitro</u> study on the metabolism of micro-organism, the purity, grade and the keeping quality of the analytical reagents bear a direct impact on the precision of the result. For the present work, all the chemicals and reagents were obtained through the commercial firms. How far the grade of chemicals employed in the test was responsible to contribute to the present result is difficult to say. Only repeated tests directed to confirm the above observations under similar conditions might answer the question.

The responsiveness of the test animals is of paramount importance for the study on the activity of any biological material. The relative susceptibility or resistance of animals controls in a large major the result of such test. The majority of workers employed animals of known line, breed and susceptibility for the study of the biological activity of anthrax toxin. Even within such

strain of animals, one species has been found to be more susceptible to the toxin than the others. In our experiment, rabbits, guinea-pigs and white rats of indigenous line were employed for the biological test, and it is not unlikely that these animals may have shown partial or complete refractoriness to one or the other factor of the anthrax toxin. Therefore, the results recorded in the present experiment are to a large extent subject to the various limitations described above.

Haemagglutination test:

The mechanism of haemagglutination reaction has been a subject of extensive research in virology. Such studies have shown a close correlation between the identity of infective virus particles and the haemagglutinin. This has been particularly noticed in the myxo-group of viruses. The nature of haemagglutinin differs with the type of viruses. It is muco-protein, lipo-protein or phospholipid in nature. Our knowledge regarding the scope and usefullness of haemagglutination test in Bacteriology is very much limited. Recently, Carter and Rappay (1963) described an indirect haemagglutination test for the detection and measurement of antibody against specific lipo-polysaccharides of P. multocida. They found the reaction to be specific and recommended its use as an important epidemiological tool for the study of pasteurellosis.

In course of investigation, the culture supernatant, culture filtrate and the eluate were subjected to direct

haemagglutination test. The first two materials showed haemagglutinating activity between the 16 & 28 hours of growth, the maximum titre recorded being 1:64. On the other hand, the eluate showed uniformly negative result (Table V). It may be recalled here that in the present work the earliest presence of oedema factor was demonstrated in the 18-hour-old culture. This factor continued to be formed in the culture up to the period of 28 hours of incubation. In no instance, the materials showed the evidence of toxin in the 16-hour-old culture. Therefore, the haemagglutination reaction observed with the materials collected at 16 hour does not bear correlation with the toxicity of the product. This is also evident from the fact that the eluate which contained the oedema-producing toxin did not show H.A. reaction. Therefore, it is contended that the H.A. activity was associated with the protective antigen present in the filtrate and not with any other component of the toxin. This agrees with the observation of other workers who demonstrated the appearance of protective antigen in their culture as early as 16 hours after incubation (Belton & Strain, 1954; Boor & Tresselt, 1955b). Thus, the author ~~fiii~~ feels that this test may prove to be an easy serological procedure for the detection of the soluble protective antigen. On chemical analysis, the filtrate has been shown to contain a mixture of protein and polysaccharide. On dialysis , the protective antigen was concentrated in the crude

protein alone (Strange & Belton, 1954). Whether the H.A. activity of the filtrate was the reaction of similar substance present in the filtrate or was the interaction of some hitherto unrecognised antigen is difficult to answerr. Only future investigation directed to isolate the different chemical fractions of the filtrate and the eluate may explain the mechanism and the specificity of such reaction.

S U M M A R Y

S U M M A R Y

A critical consideration of some past and recent studies bearing on the *in vitro* production of soluble toxin by *B.anthracis* was attempted and the literature, published prior to January,1963, formed the basis of the present review. Several series of experiments were designed to examine the critical factors essential for the elaboration of anthrax toxin *in vitro*.

In the present investigation, avirulent "Weybridge" strain of *B.anthracis* was studied for its ability to produce soluble toxin in various modifications of Casamino acids media containing amino-acids, salts and other growth factors separately and in combination under different cultural conditions. The appearance of the lethal and oedema-producing toxin in the culture was tested during the different phases of growth in white rats, and rabbits and guinea-pigs. An effort was also made to detect and measure the soluble factors of the toxin by the conventional method of direct haemagglutination test.

The results hitherto obtained have shown that under the cultural conditions provided in the present experiment, the oedema factor forms in the culture between 18 & 20 hours of cultivation. The maximum production of this factor occurred between 20 & 24 hours of incubation.

The factor was elutable with carbonate buffer but the culture filtrate was biologically inert. The toxic eluate when mixed with the filtrate caused characteristic cutaneous reaction in rabbits characterised by diffuse palpable oedema with central area of reddening. The eluate or filtrate did not provoke any reaction when used alone. The skin reaction was specifically inhibited by the hyperimmune anthrax anti-serum. It was also observed that rabbits were better test animals than guinea-pigs for conducting skin test.

The investigation brought out experimental evidences to show that the optimum conditions of growth were not necessarily the optimum conditions for toxin production. The amino-acids did not seem to be necessary either for promoting growth of the organism or for the elaboration of the toxin. On the other hand, the presence of adenine and guanine in the medium was considered to be indispensable for toxin synthesis.

On comparing the result of biological activity of the toxin collected at pH 7.6 and 8.5, it was observed that a high alkaline pH was preferable for the better yield of the oedema factor. Therefore, the importance of using a high concentration of bicarbonate in the medium was stressed.

The experiment has further revealed that the conditions for the synthesis of oedema and lethal factors are different. In the present experiment the test materials

which had showed the oedema-producing activity was devoid of lethality for white rats. This suggests that the two factors are biologically distinct - an observation which is consistent with the views of earlier workers. The above result has borne out further promises of producing the oedema factor alone without the concomitant production of lethal factor *in vitro*.

The result of the haemagglutination test carried out with the different soluble factors of the ~~tx~~ toxin has disclosed that the test can be profitably employed as an easy and quick serological procedure for the detection of the soluble protective antigen in the culture. This is the first report on the application of haemagglutination reaction for the measurement of protective antigen. This test, however, has not been found useful for the detection of oedema-producing toxin.

The role of various limiting factors which determine the *in vitro* production of soluble anthrax toxin has been discussed and further suggestions offered to extend the scope of such investigation under the cultural conditions described.

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