AN INVESTIGATION ON CERTAIN PHYSICAL AND CHEMICAL PROPERTIES OF CHLAMYDIAL AGENTS

A Thesis

Presented to

the Department of Medical Microbiology

Faculty of Medicine

University of Manitoba

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

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January 1972

ACKNOWLEDGEMENTS

The author is very grateful to Dr. H.I. Sayed for his invaluable supervision and guidance during their association and to Dr. J.C. Wilt and Dr. A.M. Wallbank for their valuable suggestion and criticism in the course of the study. The author also wishes to thank the staff of Department of Medical Microbiology, especially Mr. C.D. Grant and Miss M. Mandel for their technical assistance.

ABSTRACT

A trachoma-inclusion conjunctivitis agent was adapted to grow in the allantoic membrane of fertile chicken eggs. The agent was partially purified from allantoic fluid by differential centrifugation and density gradient centrifugation. The preparation was further purified and characterized by isoelectrofocusing. Recovery of the purified agent was almost complete as determined by electron microscopy and a high yield of infectivity was retained after treatment.

Two chlamydial species, characterized by this technique, were found to possess different isoelectric points (pIs). The pI of the IC-Cal-3 agent was 4.1, and that of the psittacosis DD agent was 4.9. Hence isoelectrofocusing might be useful in the identification of different chlamydial species.

The same two chlamydial agents were mechanically disrupted by ultrasonic vibrations, and the products obtained from each agent were analysed spectrophotometrically. The absorption spectra of the products of the two agents were similar though more proteinaceous materials were liberated from the psittacosis DD agent than from the IC-Cal-3 agent.

Isoelectrofocusing was applied to separate the sonicated suspension into individual fractions for chemical and biological analyses. The protein, carbohydrate, and nucleic acid contents of the eleven fractions obtained were determined. Individual fractions were also assayed by the macrophage-spreading-inhibition (MSI) test. The optimal time for performing the MSI test was determined by the

kinetics of MSI test experiment, and was observed to be 14 days post sensitization of the laboratory mice.

Three fractions were found to be good MSI antigens, and apparently sonication did not denature completely the antigenicity of the MSI antigen(s). These three fractions were analysed for amino acid content qualitatively and quantitatively. The amino acids detected were reviewed. The MSI antigen(s) is speculated to reside in a nucleic acid-protein-carbohydrate complex.

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INTRODUCTION

The chlamydial microorganisms are distributed widely among birds and mammals. They are obligate intracellular parasites, and are transmitted directly from one host to another without biological vectors --- some by means of contaminated droplets, and others by direct contact, exposure, or by sexual intercourse.

The interest in these agents developed in the 1930's when an epidemic broke out across different parts of the world due to the export of psittacine birds from South American places. With a relatively pure suspension of agents, investigations on these agents were carried out, and the general properties were studied.

The physiochemical properties of the chlamydial agents have been investigated through two different approaches. The first approach was to study the morphological appearances of the agents by light and electron microscopy; the structural forms in the replication process; the host-agent interactions, and the chemical compositions of the agents. The second approach centered around the studies of the physical and chemical nature of the detected antigens, and attempts to produce an effective vaccine against these agents.

The present study attempted to elucidate certain physiochemical properties of the chlamydial agents by an approach qualitatively different from those applied by previous researchers. The success of a study of the properties of an agent depends on the purity of the agent pool, and the present project attempted to produce a preparation of agents of

the highest possible degree of purity. After obtaining such a pure preparation, the agents are fractionated and the fractions analysed for their physiochemical, antigenic and immunogenic properties.

The physiochemical properties to be investigated in this project includes the following: (1) the physical properties of the agent obtained from certain purification processes, (2) the effect of sonication on the agents and analyses of the chemical nature of the substances released in the process, and (3) the study of the chemical properties of certain antigens isolated from the disrupted agent suspension.

When the physiochemical properties of chlamydial agents are understood, it would allow for designing the proper experimental methods to isolate and purify desirable antigens and may also shed light on host-agent interactions.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

VIRUS OR BACTERIA ?

The Chlamydia genus of microorganisms includes many different agents causing a variety of diseases. The various agents that cause psittacosis, ornithosis, pneumonitis, lymphogranuloma venereum trachoma, and inclusion conjunctivitis are considered to be separate species in the chlamydial group of microorganisms.

Chlamydial agents were originally classified as viruses because they : (a) pass through Berkfeld filters (having a filterable stage), (b) are obligate intracellular parasites, (c) appear within infected cells as inclusion bodies, resembling the pox-viruses, and (d) have the ability to induce the production of interferon (Hanna, 1966).

Apart from these viral characteristics, Moulder (1962 and 1966) demonstrated that the psittacosis species also possesses a number of properties which are characteristic of bacteria : (a) divide by binary fission with possible participation of mesosomes in cross-wall formation; (b) cell wall is comparable to those of the Gram-negative bacteria, containing muramic acid mucopeptides, (c) DNA containing nucleoids with no membrane between them and the cytoplasm, (d) synthesis of foliates by sulfonamide-susceptible agents, and (e) the presence of procaryotic ribosomes.

The chlamydial agents seem to possess both viral and bacterial characteristics, and their position in classification scheme remains to be settled. For the present study, they will be referred to as the chlamydial agents.

STRUCTURE AND COMPOSITION :

1. Morphology :

Various Chlamydiae are indistinguishable from one another on a morphological basis. They resemble Rickettsiae but are slightly smaller, being just visible under the light microscope. At various stages of development, they range from 0.2 to 0.7 micron in diameter. They possess cell walls similar to the Gram-negative bacteria (Schaechter 1957; Salton, 1960 ; Perkins, 1963), and stain readily with basic dyes. They appear red with the Macchiavello's stain, purple with Giemsa's, and deep blue with the Castaneda's method.

Two morphologically different forms, with a continuous gradation of intermediates between them were recognized. One of these two forms is a small particle about 0.3 micron in diameter, with an electron-dense nucleoid, and is usually known as the " elementary body" (E.B.). The other form is a large particle of 0.5 to 1.0 micron in diameter, with no electron-dense centre, and is usually known as the "reticular body " (R.B.). Table I reports the differences between the elementary body and the reticular body forms.

2. Cell wall :

Both the large and small forms of the chlamydial agents are surrounded by limiting membranes. Erlandson and Allen (1964) described a single unit membrane in meningopneumonitis agents, while Mitsui et al., (1964) reported a double membrane around the small form (E.B.) only.

TABLE I

SOME REPORTED DIFFERENCES BETWEEN THE E.B. & R.B. FORMS

	E.B.	R.B.
Size	0.2 - 0.3 u.	1 - 2 u.
Surface	rigid "cell wall"	delicate membrane
Muramic acid	yes	no
Phospholipids	high	low
Infectivity	yes	?
Toxicity	yes	no
Penicillin sensitivity	yes	no
DNA/RNA ratio	1 : 1	1 : 4
Relative density	high	low

Armstrong and Reed (1964) observed that large lymphogranuloma venereum agent particles were bound by an intact bilaminar envelope reminiscent of the combined cell wall and cytoplasmic membrane of bacterial cells. Anderson et al. (1965) then concluded that an ornithosis agent had a true cell wall beneath which was a cytoplasmic membrane that was poorly seen. Other evidence indicated that the envelope is similar to the cell wall of Gram-negative bacteria.

Muramic acid, a unique constituent of bacterial cell wall mucopeptides, has been identified in four different members of the chlamydial agents by Perkins and Allison (1963). Envelopes of the meningopneumonitis agent were sensitive to muramidase although the intact particle was not.

The meningopneumonitis cell wall contains traces of nucleic acids, small amount of carbohydrates, large quantity of lipids, and all of the amino acids of the whole agent. Manire(1966) investigated the isolated cell wall of this agent and reported that it consists of at least two layers; the inner of which is composed of highly ordered arrays of hexagonally packed units. Such structures have been seen in the cell walls of several different bacterial species.

These observations may be interpreted as that the limiting envelope of the chlamydial agents contain a mucopeptide layer similar to that of bacterial cell walls. Other than this, much remains to be clarified.

3. Nucleic acids :

Both DNA and RNA are found in the E.B. and R.B. forms. The DNA of meningopneumonitis agent had been extracted from both E.B. and R.B. populations by methods generally applied to the pox-viruses for DNA extraction . The properties of the extracted DNA indicated it to be a double-stranded molecule. Density gradient centrifugation and determination of thermal denaturation temperature indicated that the DNA from psittacosis agent had a guanosine plus cytosine content of 39%, and that of the trachoma agent was reported to be 44%. It therefore appears that different chlamydial species have different guanosine plus cytosine contents.

The DNA of the R.B. form probably occurs in the form of twisted filaments distributed in irregular areas throughout the cytoplasm. Whereas, in the E.B. form, the DNA filaments are concentrated in the ribosomes which may be seen in the cytoplasm of both the E.B. and R.B. particles.

The infectious E.B. particles are relatively rich in DNA and stain green with acridine orange during the developmental cycle. As the cycle proceeds, the population shifts to the R.B. forms which are relatively rich in RNA, and stain red. Finally, at the end of the cycle, the population again becomes predominantly of the E.B. forms, rich in DNA, and stain green again.

The existence of two types or forms of nucleic acids is one of the main characteristics used to classify chlamydial agents as bacteria rather than as viruses.

4. Lipids :

The chlamydial agents contain large quantities of lipids which are not characterized as yet. About one quarter of the total phosphorus of the meningopneumonitis agent was found to be phospholipids. The same agent contains lecithin and neutral fats but no cholesterol. Cytidine diphosphate choline was also suggested to be present.

REPLICATION :

The chlamydial agents are believed to multiply by binary fission within a living host cell. Bedson introduced the term "developmental cycle" to describe the sequence of events involved in the reproduction of chlamydial agents in the infected cells.

There appears to be no significant differences in morphology or developmental cycle among the members of the chalmydial agents, and a generalized scheme seems to apply to them all. The following concept of the cycle was suggested by Bedson (1932), modified by Litwin (1959, 1961), then clarified by the investigation of Armstrong and Reed (1964).

The developmental cycle may be regarded as an orderly alternation of the small and large particle forms. It is initiated by the highly infectious small form (E.B.) which is taken into the host cell by phagocytosis. The engulfed small particle retains its morphological integrity in vacuoles bound by membranes derived from the surface of the host cell, and there is no eclipse. Without loss of individuality, the small particle is re-organized into a large form (R.B.), and

within the membrane-bound vacuole, the large particle grows in size and multiplies by repeated binary fissions. The developmental cycle is completed by the reorganization of most of the large particles into small ones which are then available for infection of new host cells. The time required for completion of a cycle varies from 24 to 48 hours, depending on the particular host and agent involved.

A one-step growth curve corresponding to a single developmental cycle had been obtained for a number of psittacosis agents growing in the yolk sacs of the chick embryos or in cell cultures. The lag-phase lasts approximately 20 hours, and the logarithmic-phase 5 hours. Large particles begin to differentiate into small particles towards the logarithmic-phase and continues differentiating in the stationary-phase until the host cell bursts.

PROPAGATION AND ADAPTATION :

Chlamydial agents have been propagated in different host cells such as the yolk sac and the allantoic membranes of chick embryos, in different tissue culture cell-lines, and in living mammalian animals.

The chick embryo is the most frequently used host system for propagation. Cox(1939) described a method for the propagation of several Rickettsiae in embryonated chicken eggs. The method by Cox was applied to chlamydial agents by Hilleman (1945) and has come into general use since then. In this method, yolk sacs of seven day old chick embryos were inoculated with 1 ml. 10% suspension of infected mouse brains or yolk sac materials. The infected embryos were incubated at 37° for three to five days, and the yolk sac membranes were harvested by aseptic technique.

Weiss and Dressler (1960) used the yolk sac membrane explant method to cultivate the agent in the explant using centrifugal force to deposit the infectious material onto the explant culture.

Tarizzo and Nabli (1963) and Vali (1968) reported successful adaptation of certain chlamydial agents and four strains of TRIC agents respectively to grow on the allantoic membrane of chick embryos.

Apart from the chick embryo, chlamydial agents have also been propagated in the lungs and spleens of mice (Bedson 1933). Many strains of chlamydial agents can be propagated in mice by inoculating the infectious material via the intracerebral, intranasal, or intraperitoneal routes. Barwell, (1952) inoculated a suspension of psittacosis agents into albino mice by the intraperitoneal route and the agents were harvested from the macerated spleens from mice which died between the second and fourth days.

Chlamydial agents have also been sucessfully propagated in a number of tissue culture cell-lines which include the following : Chang's human liver cells (Litwin et al., 1961), mouse L cells, (Gaylord, 1954; Morgan and Beta, 1957), HeLa cells (Furness et al., 1960), human diploid cell-lines (Pearson et al., 1959), and in a number of primary cell cultures : chicken embryo fibroblast primaries (Morgan and Wiseman, 1946; Piraino and Abell, 1964), chicken chorioallantoic cell-primaries and rabbit kidney cell-primaries (Fraser,1966).

The host system used in the present project for the propagation of the IC-Cal-3 agent was the allantoic membrane of the chick embryo because tissue cultures require the correct combination of certain

cell-line and the agent concerned. A particular chlamydial agent is incapable of infecting any cell-line, and to find out the correct combination of cell-line and agent is by far less economic.

The allantoic membrane was chosen over the yolk sac because previous experience in this laboratory indicated that the yolk sac method involves a greater danger of bacterial contamination. It is very difficult to harvest all the yolk sac membranes efficiently and the purification of the agent from a pool of infected yolk sac membranes is not feasible. The harvesting of infected allantoic fluid is much easier and subsequent application of purification techniques to the allantoic fluid gives a better recovery of agent than from the yolk sac materials.

PURIFICATION OF THE AGENT :

Chlamydial agents propagated in the allantoic cavity have been purified by cycles of low and high speed centrifugation known as differential centrifugation. In Jenkin's modification (1960) of the method of Colon and Moulder (1958), chlamydial agents were separated from the host materials by four cycles of differential centrifugation at $0^{o}C$. This method retained most of the infectivity of the agent, and electron micrographs of the purified preparation revealed that the agent particles were in a relatively pure state.

Fraser and Borman (1965) attempted to purify chlamydial agents from egg materials by fluorocarbon treatments. In this method, trifluoro-trichloro-ethane (Genetron 113) was added to the homogenized egg materials, and the agents were recovered from the aqueous phase of

the supernatant after centrifugation at 2500 XG for 15 minutes. However, the agents after this treatment were markedly inactivated.

The most detailed study of the purification techniques was performed by Wang and Grayston (1967) in which they used density gradient centrifugation, ion-exchanger chromatography using DEAE-Sephadex, and enzyme digestion of host materials followed by removal of host contaminating substances.

The present project had combined some of these described purification methods for application to the IC-Cal-3 agent. The harvested allantoic fluid was partially purified by differential centrifugation, then by density gradient centrifugation, followed by isoelectrofocusing. Isoelectrofocusing had not been applied to purify or characterize any chlamydial agent before. Because of the fact that the isoelectrofocusing technique is capable of separating very minute quantities of dissimilar proteins on the basis of different ionic charges, the chlamydial agents when subjected to such a field of natural pH gradient, will be separated from the contaminating host proteins, and a pure suspension of agents could be obtained. At the same time, the ionic charge on the surface of the agent particle could also be determined.

FRACTIONATION OF THE AGENT :

Disruption of the chlamydial agent into different separable fractions can be achieved in two ways. The first one is to rupture the agent particle chemically or enzymatically, and the other approach is by physical and/or mechanical means.

1. Disruption by chemicals or enzymes :

A method for fractionating rickettsial agents was first described

by Schaechter (1957), and was applied to the chlamydial agents by Jenkin in 1960. In this method, purified particles of agents suspended in phosphate buffer were treated with a 1% sodium deoxycholate solution, and the mixture was shaken at 45°C for four hours. A complement-fixing antigen was detected in the suspension, and the reactivity of the antigen was destroyed if deoxycholate treatment was prolonged for more than four hours. This observation indicated the destructive effect of chemicals on the antigenic determinants of certain antigens.

Perkins and Allison (1963) treated psittacosis P-4 agents by deoxycholate as described by Schaechter (1957) and in addition, used lysozyme as well as thylene-diamine-tetra-acetate in further treatment of the agent suspension. They reported lysozyme was capable of digesting the whole agent completely, and this is another indication of the drastic effect of enzymes on the agent itself. Such enzymatic action may also destroy the antigen that is being sought.

2. Disruption by physical means :

Ross and Gogolak (1957) attempted to rupture the psittacosis 6-BC agent by cycles of rapid freezing and thawing. They treated the agent with 40 cycles of freezing and thawing, but found that the elementary body forms were still intact after such prolonged treatment, however, no reticular body forms were detected. It was then concluded that cycles of freezing and thawing would not disrupt the elementary body but the reticular body forms.

3. Disruption by mechanical means :

The least drastic mode of disruption used was sonic vibration as

applied by Ross and Gogolak (1957). They sonicated the psittacosis 6-BC agent with 9 KC vibration energy at 5°C for 3 hours, and examined the sonicated suspension by electron microscopy. It was found that all particles were disrupted, and no intact agent was detected. Vedros (1967) added glass beads (type 116 from 3M Co.) during the sonication treatment which was carried out with 10 KC vibration energy for 15 to 20 minutes. In this method, a hemeagglutinogen was detected in the sonicated suspension. The same suspension was further sonicated for another 25 minutes, and another hemeagglutinogen with species specificity was detected. The latter antigen was found to be heat labile at 56°C for 1 hour.

From this observation, it was suggested that chlamydial agents are susceptible to mechanical disruption by sonic vibration which will probably release into the suspending medium certain antigens suitable for physiochemical and immunological analyses.

In the present project, the pattern as well as effect of ultrasonic vibrations as applied to two chlamydial agents were investigated.

THE PROBLEM & THE APPROACH

The objective behind the study of the antigenicity and immunogenicity of the chlamydial agents is mainly an effort to isolate specific antigens for diagnostic and/or epidemiological purposes.

Chlamydial antigens have been detected in various preparations of whole agents, cell wall, and somatic material preparations. This indicates that the many different antigens are not confined to specific areas of the agent particle, but located indiscriminately throughout various regions of the particle itself.

So far, no attempt had been made to fractionate these agents in order to study the various fractions independently for their physiochemical, antigenic and immunogenic properties. Such a study will furnish valuable information for designing future projects in the study of the chlamydial agents.

The validity of the results from analyses of the fractions obtained by disintegration of the agent particle depends on two parameters. The first parameter is the purity of the agent pool, and the second one is the suitable methodology applied to the fractionation procedure .

In order to produce the best degree of purity of the agent pool,the purification techniques as described by previous researchers must be re-examined. The problem arises here is to apply an appropriate technique for each of these re-examinations.

The second problem is to develope a suitable fractionation technique to disintegrate the agent particle into separate fractions for further experimental analyses. The technique must not be too drastic so that antigenic structures of individual antigens isolated by the fractionation process would not be denatured.

Having identified our objective as : the production of an extremely pure suspension of chlamydial agents, the separation of the agent into fractions, and the analysis of the physiochemical, antigenic, and immunogenic properties of these fractions, our approach was conceived under four main steps :

1. Propagation of the agent,
2. Purification of the agent,
3. Fractionation of the agent,

and 4. Characterization of the fractions.

The techniques involved in these studies are discussed in the next section " Materials & Methods".

MATERIALS & METHODS

MATERIALS & METHODS

MICROORGANISMS :

The yolk sac adapted strain of inclusion conjunctivitis IC-Cal-3 agent was obtained from Dr. Wang of the University of Washington. This agent was adapted to the allantoic membrane of chick embryo in the present project.

The psittacosis agent, DD-34 strain was provided by Dr. Winn of Markham Laboratory, Chicago.

The IC-Cal-3 and the psittacosis DD-34 agents were used in the present study as representative chlamydial agents.

PROPAGATION & ADAPTATION :

1. Method of inoculation :

The eggs were placed with the round end up in the paper cartons and the air sacs were located by candling. The area opposite to the embryo and away from major blood vessels was marked with a circle, approximately 1/8th of an inch in diameter. The whole marked area was then wiped with 70% alcohol followed by iodine solution. An electric vibrator was then used to create an indentation inside the the marked circle without cracking the shell. The circle was marked as near to the rim of the air sac as possible so that the needle, when inserted vertically into the allantoic cavity, would not injure the embryo. The hole made by the needle was sealed by sterile 50% mixture of paraffin and wax. The inoculated eggs were then incubated at 37°C in an electric incubator.

The eggs were candled daily, and those eggs which died on and between the third and ninth day after inoculation were harvested. The dead eggs were immediately transferred to a 4°C cold room as soon as they were detected dead, and was kept overnight in the cold room. Harvest of the allantoic fluid was performed on the following day.

2. Method of harvest :

The air sac area was wiped with 70% alcohol, followed by iodine solution. A small portion of the sterilized shell, about half an inch in diameter, was cracked and removed with sterilized forceps. An 18 gauge needle attached to a 5 ml. disposable plastic syringe was inserted through this opening into the allantoic cavity and the infected fluid removed by aspiration. The collected fluid was pooled together in a disposable plastic 250 ml. tissue culture bottle. The pooled fluid was then subjected to various methods of purifications.

3. Primary inoculation :

The yolk sac materials containing the IC-Cal-3 agents in the ampulla was serially diluted to the 1/10 and 1/100 dilutions with sterile phosphate buffered saline (PBS) pH 7.2. Inocula were prepared by mixing equal volumes of each dilution with sterile full strength tryptose phosphate broth (TPB) pH 7.2, containing streptomycin sulphate solution (60 mg/ml.), giving a concentration of 2.4 mg/0.2 ml. per egg. Each dilution was inoculated into two dozens eggs via the allantoic route. The harvested fluid from each dilution was pooled together and stored at -70°C for re-inoculation or passage.

4. Propagation & passage :

The harvested allantoic fluid from primary inoculation was used as the source of infectious agents for the first passage. The infectious allantoic fluid from each passage was used as the source of infectious agent for the subsequent passage.

The passage inocula were prepared by mixing 10 ml. of infectious allantoic fluid from the previous passage with 6 ml. full strength tryptose phosphate broth (TPB) and 4 ml. of streptomycin sulphate solution (60 mg/ml.). 0.2 ml. of this suspension was inoculated into each egg, and the same procedure as described above was applied to every passage.

Sterility tests were performed on the harvested allantoic fluid before each subsequent passage by inoculating two drops of the fluid into tubes of thioglycollate broth, and the tubes were incubated at 37°C for 4 days. Smears, prepared from the inoculated thioglycollate broths, were prepared and stained with the Gram-stain. Possible bacterial contamination was examined by light microscopy.

ANALYSIS OF DATA :

1. Calculation of percentage death :

The number of dead eggs, for each day after the inoculation of the infectious agents, was recorded, and the percentage death values of injected eggs were calculated for each individual day after the injection.

Eggs which were found dead within 48 to 72 hours after inoculation were discarded because truma rather than infection might be involved.

The total number of eggs in the inoculated population used for calculating the percentage death values was the difference between the number of eggs injected on day zero and the number of eggs discarded on day 3. The percentage death values for each day post inoculation were calculated by dividing the cumulative number of dead eggs by the total number of eggs in the inoculated population. It is as follows:

$$\text{Percentage death for day X} = \frac{\text{Cumulative death}}{\text{No. of eggs in population}} \times 100\%$$

Using this formual, the percentage death values for each day post inoculation in individual passages were obtained.

2. Calculation of Mean percentage death :

For each passage, a mean percentage death value could be obtained by dividing the sum of all percentage death values of a single passage by the number of days post inoculation in that particular passage. The calculations , including standard deviations were performed by a programmed IBM computer.

3. Calculation of ELD_{50} for each passage :

The ELD_{50} value for any infectious agent is usually expressed in particle concentration. For the IC-Cal-3 agents, the ELD_{50} value was defined as the time required to cause 50% death in an inoculated population of chick embryos using identical inocula size throughout. The unit of ELD_{50} would be either in terms of days or reciprocal days. The latter was calculated by taking the reciprocal of each day and

corrected to one decimal place, e.g., day 1 would be 1.0, and day 5 would be 0.2 and so on.

The ELD_{50} values were obtained by a graphical method. The percentage death for each day post inoculation was plotted against the number of reciprocal days post inoculation. From this plot, the point at which the graph intercepted the 50% death would indicate the ELD_{50} in terms of reciprocal days.

In order to obtain the correct statistical values for the ELD_{50s}, the plot of percentage death against reciprocal days was made on Probit analysis papers. This statistical analysis would allow for the formation of a straight line graph, in which the percentage death values are plotted on the Y axis, and the reciprocal days are plotted on the X axis. The ELD_{50} values could be read off directly from the graph by taking the value on the X axis where Y = 50% death. The ELD_{50} values for different passages were obtained by this Probit analysis.

4. Calculation of mathematical relationship :

For any set of data, there usually exists a mathematical relationship among the variables involved. The most common type of relationship between two variables is either a linear or non-linear type. In order to establish the type of mathematical relationship existing between two variables; percentage death and number of days post inoculation, the data were analysed by the " Two variables simple linear regression and correlation " statistical test which was programmed into an IBM computer. This test gives the coefficient of correlation between the two variables concerned.

The coefficient varies theoretically between zero and 1. A coefficient of zero or approaching zero indicates no linear relationship, while a coefficient of 1 or near 1 indicates a linear type of relationship. The coefficient can also be either positive or negative. A positive value means a directly proportional phenomenon and a negative value means an inversely proportional phenomenon.

Using the percentage death values as the X variables, and the reciprocal days as the Y variables, the coefficient of correlation for each passage was calculated.

PURIFICATION AND CHARACTERIZATION :

Since chlamydial agents are propagated in living host tissues, the problem here is to separate the agent particles from the host materials. A portion of the total infectious particles is normally released into the yolk sac , allantoic fluid or tissue culture fluid depending on the host system involved. The quantity of chlamydial agent recovered can be markedly enhanced by disrupting the host cells by shearing forces. This may be accomplished by subjecting tissues to homogenization or alternate cycles of freezing and thawing. In our present study, we used a Sorvall homogenizer for such purpose.

1. Purification by differential centrifugation :

A schematic representation of the procedure is shown in Figure 1. The infected allantoic fluid was centrifuged at 30,000 XG for 1 hour to sediment all the agent particles. The pellet was resuspended in sterile PBS pH 7.2 The suspension was then subjected to 3 cycles of freezing and thawing followed by homogenization with the

PURIFICATION BY DIFFERENTIAL CENTRIFUGATION

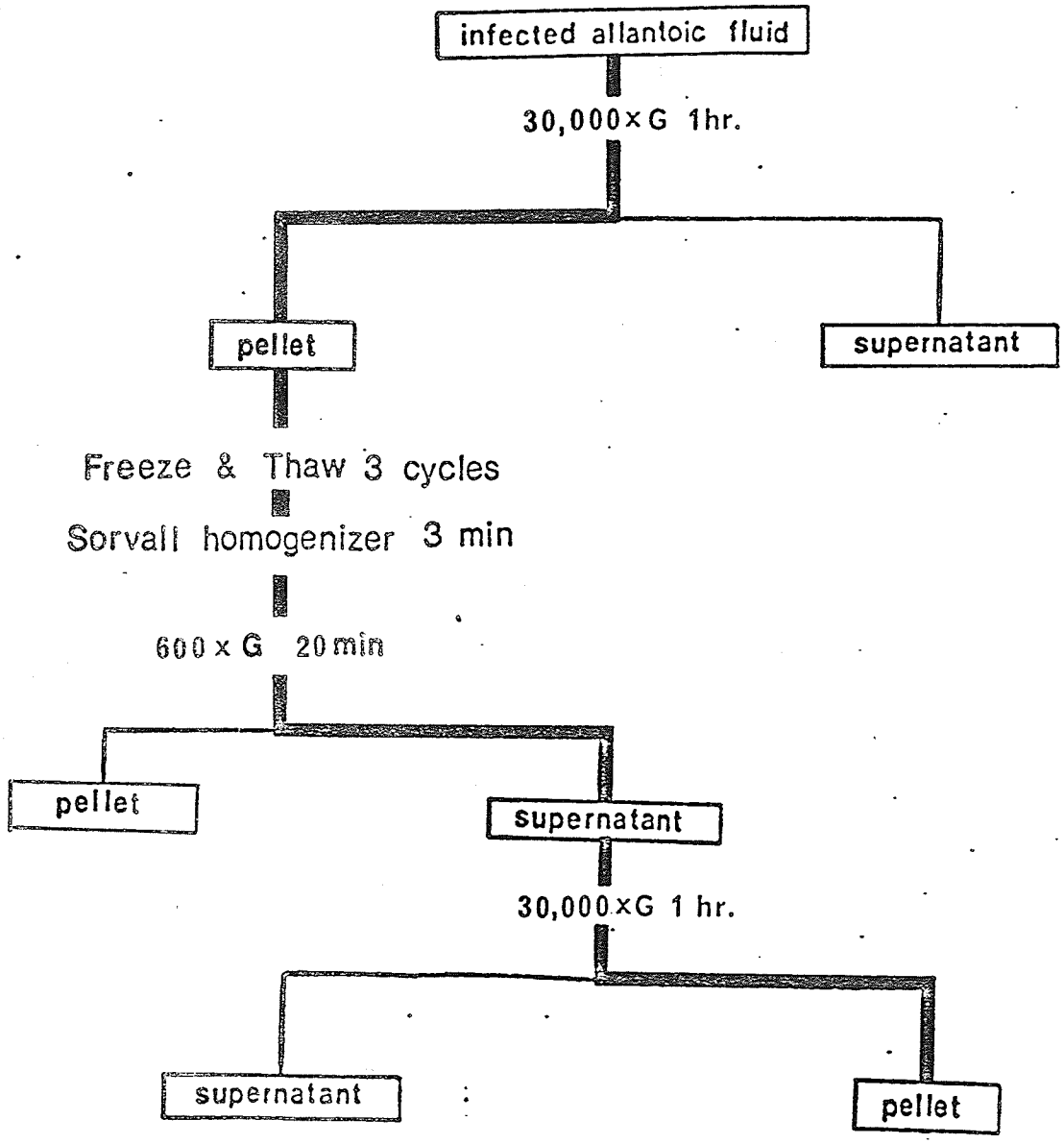


FIGURE 1 - A DIAGRAMMATIC REPRESENTATION OF THE METHOD OF DIFFERENTIAL CENTRIFUGATION.

The thick line represents the flow direction, and the cycle is repeated after the step of freezing and thawing and homogenization.

Sorvall homogenizer for 3 minutes. The homogenized suspension was centrifuged at 600 XG for 20 minutes. The pellet from this low speed centrifugation was discarded because it contained host tissues. The supernatant was then centrifuged at 30,000 XG for 1 hour. The pellet from this high speed centrifugation contained the agents while the supernatant contained contaminating soluble host materials. The pellet was resuspended in sterile PBS pH 7.2, and the suspension centrifuged again at 600 XG for 20 minutes. The resulting pellet was also discarded, and the supernatant was centrifuged at 30,000 XG for 1 hour to sediment the partially purified agent particles. The cycle of low and high speed centrifugation was repeated three times.

2. Purification by density gradient centrifugation :

Density gradient centrifugation technique was applied to the suspension of agents partially purified by differential centrifugation in order to study the degree of purity obtainable by this method.

A continuous density gradient was set up in a centrifuge tube using sucrose solution. Controlled temperature in the proceee was essential because thermal convections would disturb the positioned zones, thus affecting the degree of separation.

One ml. of a suspension of IC-Cal-3 agents, partially purified by differential centrifugation and containing 18 mg/ml. protein, was layered on top of a sucrose density gradient in a 38 ml. cellulose nitrate centrifuge tube. The density gradient was set up by a LKB 8121 density gradient mixer. The solutions for setting up the gradient were 18 ml. of 40% sucrose and 18 ml. of 1 M KCl solutions, giving a density gradient between 1.0 to 1.17. The cellulose nitrate

tube was then centrifuged in an IEC swing-head type centrifuge at 1500 XG for 2 hours at 6°C. One ml. fractions were then collected from the bottom of the gradient, and each fraction was diluted to a final volume of 3 ml. by PBS pH 7.2, followed by absorbance measurement at 280 mu. Different fractions were examined by electron microscopy for the presence of agent particles.

3. Purification & characterization by isoelectrofocusing :

(a) Purification by isoelectrofocusing :

The basic principle of isoelectrofocusing involves the phenomenon of electrophoresis in a natural and continuous pH gradient.

It is known that every protein or charged particle possesses an isoelectric point (pI) at which the particle or molecule is stationary in an electric field. Such a charged particle, if immersed in a solution having a pH dissimilar to its own pI, will acquire a new electrostatical charge, and will migrate towards the area where it is isoelectric.

In electrophoresis, the charge of the particle is determined by the pHs of the two buffers used as electrodes. Such a discontinuous pH gradient, as set up by acid and alkali, can only provide two different pH regions with a large pH gradient in between. In isoelectrofocusing, a whole range of ampholytes (poly-amino-poly-carboxylic-acid) of different pHs are introduced between the acid and alkali electrodes, providing a continuous pH gradient and different regions of isoelectric points. A suspension of chlamydial agents, together with contaminating host materials, if electrophoresed in this isoelectrofocusing system, will be separated from the contaminating materials because of different electrostatical charges, and theoretically

this is the best method of purification. At the same time, the electrostatic charges on the agent particle surface can also be studied.

The suspension of agents obtained from certain zone from the density gradient centrifugation experiment was put into a dialysis bag and dialysed against PBS pH 7.2 at 4°C for 24 hours. The dialysate was then centrifuged at 30,000 xG for 1 hour to sediment the agents which were then resuspended in sterile distilled water. The protein content of this suspension, determined spectrophotometrically, was 24 mg/ml. 0.3 ml. of this suspension was analysed by an isoelectrofocusing experiment.

The LKB electrofocusing columns LKB 8108 and 8102, and LKB ampholyte solutions were used. Glycerol instead of sucrose was used to set up the density gardient for the purpose of preventing bacterial contamination and to avoid hydrolysis of sucrose by sulphuric acid giving false positive absorbances at low pHs.

The cathode on top of column system was used in the experiment. The light electrode, containing sodium hydroxide solution constituted the cathode, and the dense electrode, containing the sulphuric acid solution was the anode. The dense solution was made up by a 46.6% glycerol solution, and the light solution contained distilled water only. Ampholytes of different pHs at 2% final concentration was used, ¾ of the required amount of ampholytes were added to the dense solution and ¼ to the light solution. The dense and light solutions were put into the LKB density gradient mixer and the electrofocusing column was filled by a peristaltic pump at a filling rate of approximately 4 ml. per minute.

Electrophoresis was carried out at 300 volts for 60 hours, and the temperature of the column was maintained around 10°C by running tap water. At the end of the electrophoresis period, fractions of 3 ml. each were collected by a LKB fraction collector at a rate of approximately 1 ml. per minute. The pHs and the absorbances of each fraction were measured. Different fractions were examined by electron microscopy.

(b) Characterization by isoelectrofocusing :

In addition to the IC-Cal-3 agent, another chlamydial species, the DD-34 strain of psittacosis agent was also characterized by the isoelectrofocusing technique. Similar materials and methods as used in the experiment for the IC-Cal-3 agent were applied. The results from these two experiments were compared.

4. Effect of isoelectrofocusing on infectivity :

A duplicate run of the isoelectrofocusing experiment using all sterile apparatus and materials was performed as described in a previous section. Extreme precautions were taken to ensure that the IC-Cal-3 agents were not contaminated, and sterility tests were performed for equipments and materials used in the experiment.

The IC-Cal-3 agents, after electrofocusing treatment, were sedimented by centrifugation. The pellet containing the treated agents was resuspended in sterile PBS pH 7.2. This suspension was was then prepared into inocula as described previously and were inoculated into the allantoic cavities of 4 dozens chick embryos. The injected eggs were candled daily and the percentage death values were calculated by the method described in a previous section.

FRACTIONATION AND ANALYSIS :

1. Fractionation of agent particles :

The method chosen for disruption of the agent particles was ultrasonication using the Bronwill Biosonik III system. The mechanism of disruption could be explained briefly as follows :

An ultrasonic wave consists of alternate cycles of compression and rarefraction. If this cycle is applied to a liquid medium, microscopic bubbles will be created. Gaseous bubbles will be formed at the compression region and travels at high speed to the rarefraction region. The resulting flow of bubbles and liquid causes rings of high intensities, and the friction between these rings and the suspended agent particle will rupture the agent by mechanical shearing forces. The point of disruption is randomly distributed among the weak bonding regions, and because of structural differences in various linkages and bondings, certain specificity in disintegration will be expected to occur at the weakiest binding sites. Initially, a portion of the agents will be disrupted, releasing the intracellular contents, but some agents will remain intact. If the time of the sonication process is prolonged, practically all the agent particles will be disintegrated. The time of sonication can be controlled, and therefore the degree of disintegration is entirely reproducible.

Tremendous amount of heat energy will be generated during the sonication process and might denature the antigens released, therefore the experiments were conducted in a 4°C cold room and the apparatus water-cooled before and during the process.

The agents purified by isoelectrofocusing were fractionated by ultrasonic vibrations. A volume of twenty ml. of a suspension of purified agents was placed into a sterile disposable type plastic centrifuge tube with a capacity of 40 ml. The centrifuge tube was water-cooled constantly and maintained at approximately 6°C. The sonicating probe (Bronwill titanium intermediate tip BP-111-12T) was inserted through the top of the tube into the suspension of agents as deep as possible without touching the side or the bottom of the tube. Sonication was carried out at 30% intensity of 20 KC for intervals of one, two, and three hours.

2. Analysis of effect of sonication :

The effects of sonication on the agent particle were analysed by three different approaches. (1) Examination of the physical appearance of sonicated agents by electron microscopy. (2) Examination of sonicated suspension by spectrophotometry. (3) Examination of fractions of the suspension by biological and chemical tests. These three approaches are described separately in the following :

(a) Analysis by electron microscopy :

After the three hour period of sonication, a portion of the suspension was centrifuged at 60,000 XG for 1 hour to sediment the various fragments from the disintegrated agent particles. The pellet was resuspended in distill water and was examined by electron microscopy.

(b) Analysis by spectrophotometry :

Spectrophotometric analysis is based on two formalized

laws of optics, namely the Lambert's and the Beer's laws. The absorption spectrum obtained from quantitative spectrophotometric measurements is characteristics for a particular compound or class of compounds. The location of maxima and minima is therefore commonly used in identifying different classes of compounds, and the intensity of absorption is used for quantitative estimation of the concentration of the compound. In the present spectrophotometric analyses, both of these two applications as derived from the Lambert's and the Beer's laws are applied.

(i) Absorption spectra of agents in sonication :

The absorption spectra, defined as the plotting of the absorbance readings against given wavelengths at given times, of two chlamydial species, the trachoma inclusion conjunctivitis strain IC-Cal-3 and psittacosis strain DD-34 agents were investigated at different intervals of sonication.

The absorbance readings of the suspension of agents purified by the isoelectrofocusing technique were measured at wavelengths of 200, 220, 240, 260, 280, 300, 320, 340, 360, 380, 400, 500, and 600 mu. before and at hourly intervals during the sonication process.

In order to maintain a more or less constant volume of the agent suspension in the sonication experiment, ten-fold dilutions were prepared from each hourly intervals and measured at the wavelengths previously mentioned. The ten-fold dilutions were prepared by withdrawing 0.3 ml. of the sonicated suspension after each hour

of sonication and diluted by distilled water to a final volume of 3 ml. for measurement of absorbances at the designated wavelengths. Thus the data for the study of the absorption spectrum for each of the two chlamydial agent would be obtained.

(ii) Protein denaturation analysis by spectrophotometry :

In general, proteins absorb ultra-violet lights in the region of wavelengths above 250 mu., with a maxima near or around 280 mu, where absorption is mainly due to the presence of aromatic amino acid residues. In many proteins, following denaturation, a change of absorbance will be observed. These changes are useful in evaluating alterations in the enviroment of the aromatic residues and in conformational structures of the proteins. Alterations in the state of the protein and in its chromophores are frequently studied by recording differences in the absorption spectra, i.e. direct plotting of the differences in absorption between the protein under different conditions, as in the present experiment against different sonication intervals. By comparing the absorbances at 280 mu. of different sonication intervals, the percentage of protein denaturation in respective intervals could be calculated.

(iii) Qualitative analysis by spectrophotometry :

Spectrophotometric analysis may be used to detect the properties or classes of compounds of certain macromolecules that absorb ultra-violet radiations as described previously. Measurement of the absorption ratio of 280/260 mu. is frequently used to detect the presence of nucleic acids, which possess absorption maxima at

260 mu. in contrast to proteins which possess a maxima near 280 mu. The values of the ratio of 280/260 mu. are specific for different types of chemical substances. For proteins, glyco-proteins, and nucleic acids, they all have a specific ratio. By calculating the 280/260 ratios for different intervals of sonication and comparing them to the standard values, the types of compounds released by sonication could be studied.

CHARACTERIZATION OF FRACTIONS :

The suspension of agents which was subjected to 3 hours of ultrasonic vibrations was separated by isoelectrofocusing into fractions according to different pHs. These fractions were then analysed and compared.

The materials and methods used in this isoelectrofocusing experiment were similar to that described in the previous section on isoelectrofocusing. The pH range of the ampholytes varied from 3 to 10, and glycerol was used to set up the density gradient. The column used was the LKB 8102 with a capacity of 440 ml. Electrophoresis was carried out under the same conditions for 72 hours, and at the end, fractions of 3 ml. each were collected. The pHs and absorbance readings of these fractions were measured.

The fractions collected and measured were plotted against their corresponding pHs and absorbances. From this graph, different peaks obtained were characterized by the following tests :

1. Biological test :

(a) Macrophage-spreading-inhibition (MSI) test :

This technique was applied to the chlamydial agents by Sayed et al., (1971) in which they found a positive correlation between the in vivo skin-reaction and the in vitro inhibition of spreading of sensitized macrophages. They also demonstrated that the in vitro inhibition of macrophages was not mediated by cytophilic antibodies but by a macrophage inhibiting factor (MIF).

In the present experiment, the MSI test was used to analyse the antigenicity and immunogenicity of the fractions obtained from isoelectrofocusing of the sonicated IC-Cal-3 agents.

(i) Sensitization of animals :

Both foot-pads of inbred CF-1 mice were inoculated with approximately 0.05 ml. of purified IC-Cal-3 agents (18 mg protein/ml.) which were thoroughly mixed with equal volume of Freund's complete adjuvant.

(ii) Kinetics of the MSI test :

In order to find out the optimal time of carrying out the MSI test after sensitization of the animals, the test was applied to a sensitized population at intervals of 1,2,3,4,5, and 6 weeks after sensitization. The MSI indices for each week were plotted against time so as to give the relationship between the MSI indices and time in the course of sensitization. The test antigen used was a suspension of whole IC-Cal-3 agents.

(iii) The MSI procedure :

Two weeks after sensitization (time determined by the kinetics of MSI test), the animals were sacrificed. The peritoneal cells were collected by injecting into the peritoneum 0.5 ml. medium 199 followed by massaging the abdomen gently, and the exudate was then withdrawn with a 20 gauge needle. The peritoneal cells were collected in a test tube which was centrifuged at room temperature for 5 minutes at 600 X G in an IEC centrifuge. The pellet containing the macrophage were then resuspended in 0.6 ml. of medium 199. This suspension was then divided into two equal portions of 0.3 ml. each. To one portion, one drop of test antigen suspension was added, and the tube was incubated at 37°C for 30 minutes. The remaining portion was not treated with any test antigen but was also incubated at 37°C for the same period. After this incubation period, one drop of the portion treated with the test antigen was transferred to one of the two grided wells of a Hellige Tru-Count counting chamber microscope slide, and one drop of the untreated portion was transferred to the other grided well on the same slide. Then the whole slide was incubated in a moist chamber at 37°C for 30 minutes. After this incubation period, the slide was examined by phase contrast microscopy (Zeiss photomicroscope, lens 16/0.40). The number of both spreading and non-spreading macrophages in the two wells were counted and recorded. The percentage of spreading macrophages was determined by the ratio of spreading cells to non-spreading cells. The mathematical formula for such calculation is described as follows;

for portion treated with test antigen ;

$$\text{\% cell spreading in presence of antigen} = \frac{\text{no. of spreading cells + antigen}}{\text{no. of non-spreading cells + antigen}}$$

for portion untreated with test antigen ;

$$\text{\% cell spreading in absence of antigen} = \frac{\text{no. of spreading cells - antigen}}{\text{no. of non-spreading cells - antigen}}$$

After obtaining the % cells spreading for both portions with and without test antigen treatment, the MSI index could be calculated by the following formula :

$$\text{MSI index} = \frac{\text{\% cells spreading in presence of antigen}}{\text{\% cells spreading in absence of antigen}} \times 100\%$$

(b) Analysis of fraction from sonication by the MSI test :

The fractions obtained from isoelectrofocusing of the sonicated IC-Cal-3 agents were used individually as the test antigen suspension in the MSI tests performed for these fractions, and an MSI index was obtained for each of these fractions.

2. Chemical tests :

(a) Lowry test for protein content determination :

Proteins may be estimated by colorimetric measurement of the blue colour produced by the addition of the Folin-Ciocalteu phenol reagent to an alkaline solution of the protein. The precision, around 3 to 5% is adequate for many purposes. The analysis can be performed directly on a protein solution without preliminary digestion. By plotting the absorbances against standard protein concentrations, a linear relationship

is obtained, and the colorimetric value per ug protein can be calculated and used as a factor to convert optical density to protein content.

Procedure :

(i) Reagents : i) 2% Na_2CO_3 in 0.1 N NaOH,

ii) 0.5% $CuSO_4:5H_2O$ in 1% Na or K tartrate,

iii) 50 ml. of i) and 1 ml. of ii),

iv) Folin reagent diluted to 1 N by distilled water.

(ii) Preparation of standard curve :

10 mg of bovine serum albumin was dissolved in 10 ml. distilled water and a 1/10 dilution of this solution was prepared. From this 1/10 dilution, 1 ml., 0.8 ml., 0.6 ml., 0.4 ml., and 0.2 ml. aliquots were pipetted into five test tubes and distilled water was added into each tube to give a final volume of 1 ml. The blank tube contained 1 ml. of distilled water only. Then 5 ml. of reagent iii) were added into each tube, mixed and stood for 10 minutes at room temperature. Then 0.5 ml. reagent iv) was added and mixed immediately. The tubes were allowed to stand at room temperature for 30 minutes, then were read at 750 mu. By plotting the absorbances against protein concentrations, the standard curve was obtained.

(iii) Preparation of samples :

The sample solution to be examined for protein content could be diluted in 10 fold or half fold dilutions, and 1 ml. of each sample was treated the same way as described in preparation of standard curve. Using the standard curve, the protein content of individual samples were determined graphically.

(b) Dubois test for carbohydrate content determination :

Carbohydrates form either furfuraldehyde or its homologs with strong acids. These derivatives produce colored compounds by polymerization or condensation with aromatic phenols, and this was the basis of the Molisch reaction. Dubois et al. (1956) elaborated the phenol-sulfuric acid procedure which is the most sensitive among these types of reactions. In this test, concentrated sulphuric acid was used to hydrolyse the carbohydrates and 5% phenol solution provided the aromatic compounds for condensation with the derivatives of the hydrolysis.

Procedure :

(i) Reagents : i) 5% phenol crystals dissolved in distilled water,

ii) 90% concentrated sulphuric acid,

iii) Dextrin as carbohydrate standard solution.

(ii) Preparation of standard curve :

Dextrin which has been dried in vacuum desiccator was prepared into a stock solution containing 200 ug polysaccharide per ml. Aliquots of 0.1, 0.2, 0.3, 0.4, and 0.5 ml. were pipetted into five test tubes thus containing 20, 40, 60, 80, and 100 ug of polysaccharide respectively. The final volumes of each tube was adjusted to 0.5 ml. by addition of distilled water. The blank tube contained 0.5 ml. distilled water only. The test tubes were chilled for 15 minutes in an ice bath, and 0.5 ml. of 5% phenol solution was added. Then 2.5 ml. of concentrated sulphuric acid were added to each tube and thoroughly mixed. The tubes were then transferred to a boiling water bath for exactly 15 minutes, and returned to the ice bath immediately. The absorbance was read at 490 mu. one hour later. A standard curve could be obtained by plotting the

absorbances against polysaccharide concentration.

(iii) Preparation of samples :

The sample of solution to be examined was diluted in 10-fold dilutions and 0.5 ml. aliquots were treated by the method and materials as described previously in " preparation of standard curve ".

(c) Amino acid content analysis :

Certain fractions from the sonicated suspension of IC-Cal-3 agents were selected for amino acid content analysis. The analysis was kindly performed by Mr. P. Young of the Department of pharmacology University of Manitoba using a Technicon Auto analyser.

The procedure, briefly, involved the hydrolysis of the samples by 6 N HCl, and the acid was removed by flash-evaporation. The non-amino acid materials were separated from the amino acids by passing the hydrolysed preparation through different columns. The amino acids were then analysed by the Technicon Auto Analyser (Technicon Corp., Ardsley, New York) specially designed single column ion-exchange chromatographic apparatus for amino acid analysis. The chromatograms obtained showed the number of amino acid by dots. These dots were counted for each individual amino acid, and were then calculated by a written programme fed into the computer of the Health Sciences Department, University of Manitoba. The types and quantities of amino acids were then obtained in terms of micromoles.

The sample volume used for fractions 1, 6, and 9 were all 0.5 ml. containing approximately 118 ug, 324.5 ug, and 1 ug respectively as determined by the Lowry test for protein content approximation.

EXPERIMENTAL RESULTS

EXPERIMENTAL RESULTS

PROPAGATION AND PASSAGE :

1. Calculation of percentage death :

For each passage, the number of eggs injected on day zero, the number of eggs died before the third day post inoculation, the number of eggs in the population, and the cumulative death values for each day after the inoculation are shown in Table II.

Substituting the cumulative death values and the number of eggs in the population into the mathematical formula described previously, the percentage death values for each day in individual passages were obtained. The percentage death values for these passages are shown in Table III.

2. Calculation of Mean percentage death :

For each passage, a mean percentage death value was obtained. The results are shown in Table IV.

The relationship bewteen the percentage death and the course of passage was investigated by plotting the mean percentage death values of individual passages against the number of passages, and the plot is shown in Figure 2.

From Figure 2, it could be observed that the mean percentage death values increased in direct proportion with the first few passages, but the increase more or less reached a plateau beyond passage number 10. This indicated that the percentage of dead eggs increased with the first

TABLE II

CUMULATIVE DEATHS FOR RESPECTIVE PASSAGES

	Passage no.								
Day	3rd	4th	5th	6th	8th	15th	16th	17th	18th
3	1	10	10	18	17	6	18	41	47
4	5	21	17	36	38	15	36	85	97
5	8	35	26	50	67	60	61	136	151
6	11	48	32	64	90	33	74	166	184
7	12	55	36	75					
8	15	58	42	83					
9	15	77	46	93					
10	19	84	51	102					
11	23	90	56	107					
12	27	98	60	117					
13	30	108	60	124					
14	30	108	60	124					
15	30	108	60	124					
Injected	33	128	82	146	142	53	96	202	212
48 hrs.D.	3	15	12	18	22	14	15	23	18
Total	30	113	70	128	120	39	81	179	194

Day..... = no. of days post inoculation.

Injected = no. of eggs injected on day zero.

48 hrs.D.= no. of eggs died before the 3rd day post inoculation.

Total ...= no. of eggs in the population.

TABLE III

PERCENTAGE DEATHS OF RESPECTIVE PASSAGES

Day	Passage no.								
	3rd	4th	5th	6th	8th	15th	16th	17th	18th
3	3.3	8.8	12.8	14.0	14.1	15.3	22.2	22.9	24.2
4	16.6	18.5	24.2	28.1	31.6	38.4	44.4	47.4	50.0
5	26.6	31.0	37.1	39.0	55.9	74.1	75.3	76.0	77.8
6	36.6	42.4	45.7	50.0	75.0	90.0	91.3	92.7	94.8
7	40.5	49.0	54.0	58.5					
8	50.0	54.8	60.0	64.0					
9	50.0	68.1	65.7	72.7					
10	63.3	74.3	72.8	79.7					
11	76.6	79.7	80.0	83.6					
12	90.0	86.7	85.7	92.1					
13	100.0	95.5	94.2	96.6					
14	100.0	95.5	94.2	96.6					
15	100.0	95.5	94.2	96.6					

Day..... = no. of days post inoculation

TABLE IV

MEAN PERCENTAGE DEATHS OF RESPECTIVE PASSAGES

Passage No.	Mean % death	Standard deviations
3	35.8	19.6
4	43.3	22.9
5	43.8	20.8
6	50.7	22.5
8	44.1	26.7
15	54.4	33.8
16	58.3	30.9
17	59.7	30.8
18	61.7	31.0

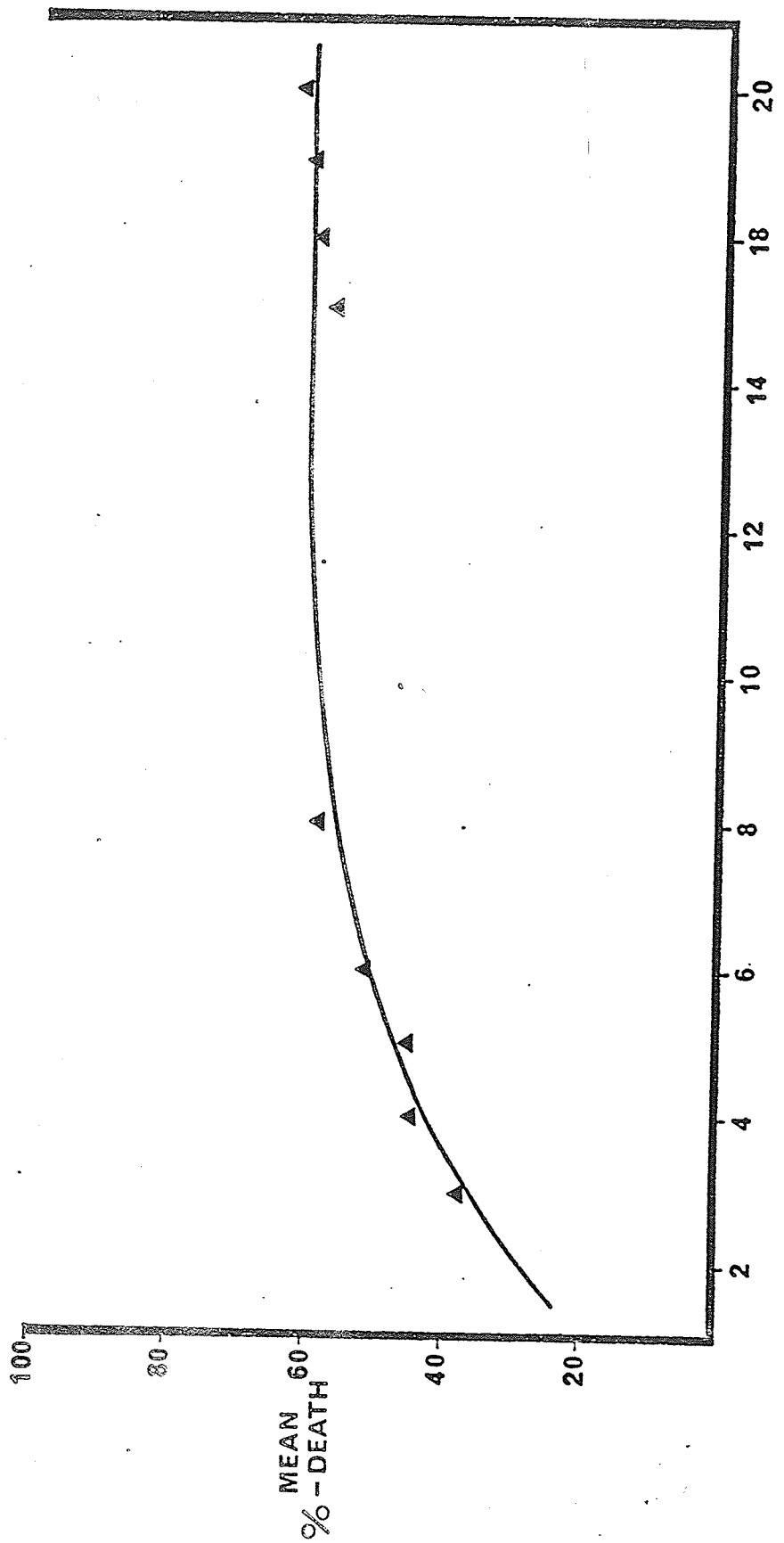


FIGURE 2 - RELATIONSHIP BETWEEN THE MEAN % DEATH VALUES AND THE NO. OF PASSAGES IN ADAPTATION & PROPAGATION OF THE IC-CAL-3 AGENTS.

few passages to a maximum and remained there without further increase in spite of increases in passage numbers. The increase in the first few passages is likely due to adaptation of the agents to the allantoic membrane, which was considered to be a new host tissue.

3. Calculation of ELD_{50} for each passage :

The ELD_{50} values were obtained by Probit analysis method. The percentage death for each day post inoculation was plotted against the number of reciprocal days post inoculation. From this plot, the point at which the graph intercepted the 50% death would indicate the ELD_{50} in terms of reciprocal days. An example of the graph which would give the ELD_{50} values of any passage is shown in Figure 4.

In Figure 4, only 3 passages were selectively plotted. Each passage was represented by a separate symbol. The three lines in the graph passed through corresponding points which were closest to a straight line and were within the 50% region of percentage death. Such a straight line is statistically valid, and is commonly used in bio-assay because the most accurate portion of the Probit analysis is the region around the 50% value. The ELD_{50s} for passage numbers 5, 8, and 17 were 8.2, 4.8, and 4.0 days respectively.

Applying Probit analysis to the data collected, the ELD_{50} values for different passages were obtained, and the results are shown in Table V.

The relationship between the ELD_{50} and the course of passage was investigated by plotting the ELD_{50s} against the number of passages. Such a plot is shown in Figure 5, and from this graph, it could be observed that the ELD_{50s} decreased as the course of passage progressed. A decrease in ELD_{50} indicated a decrease in time requirement for the same inoculum

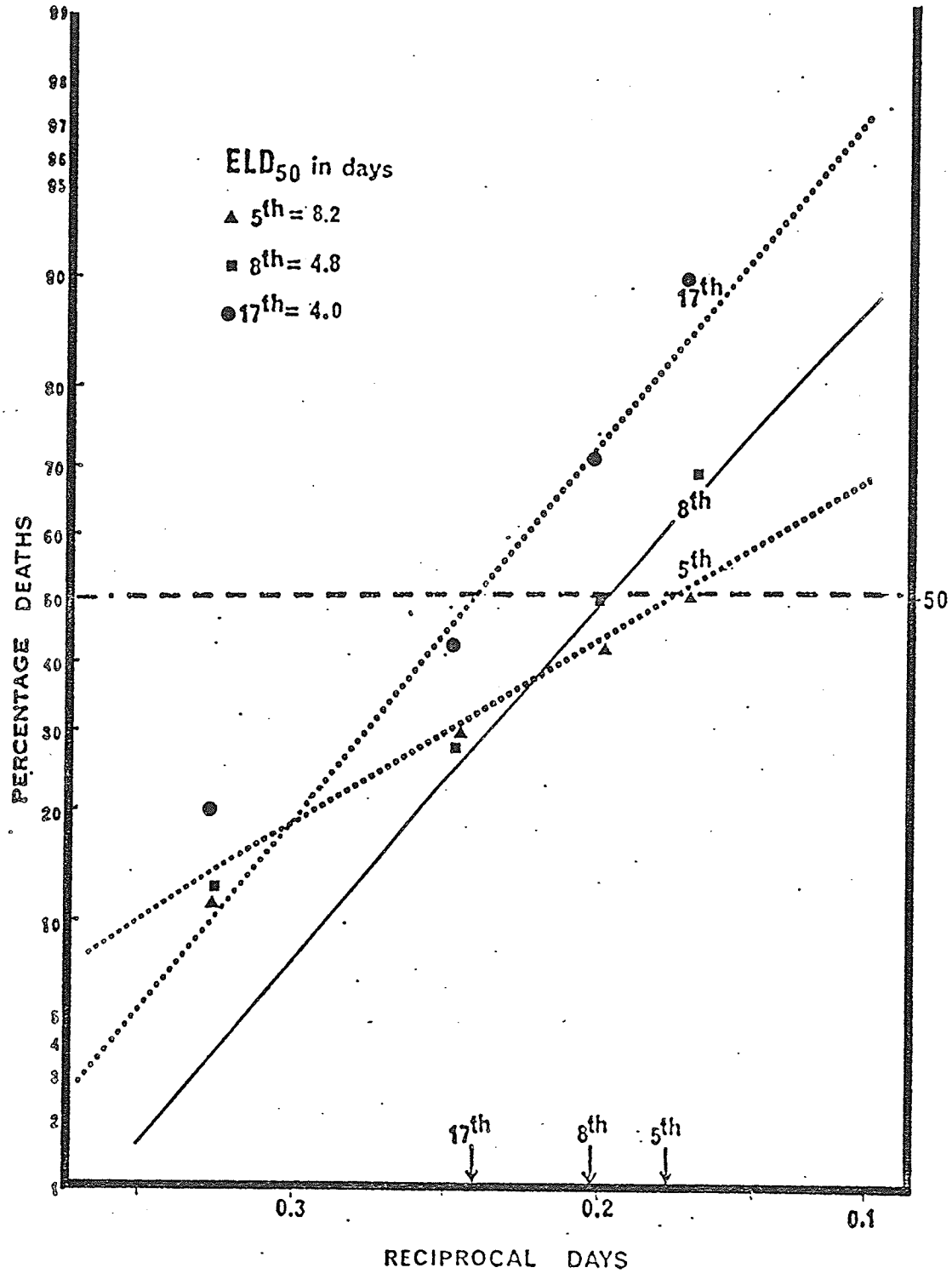


FIGURE 3 - PROBIT ANALYSIS FOR OBTAINING THE ELD_{50} VALUES OF INDIVIDUAL PASSAGES.

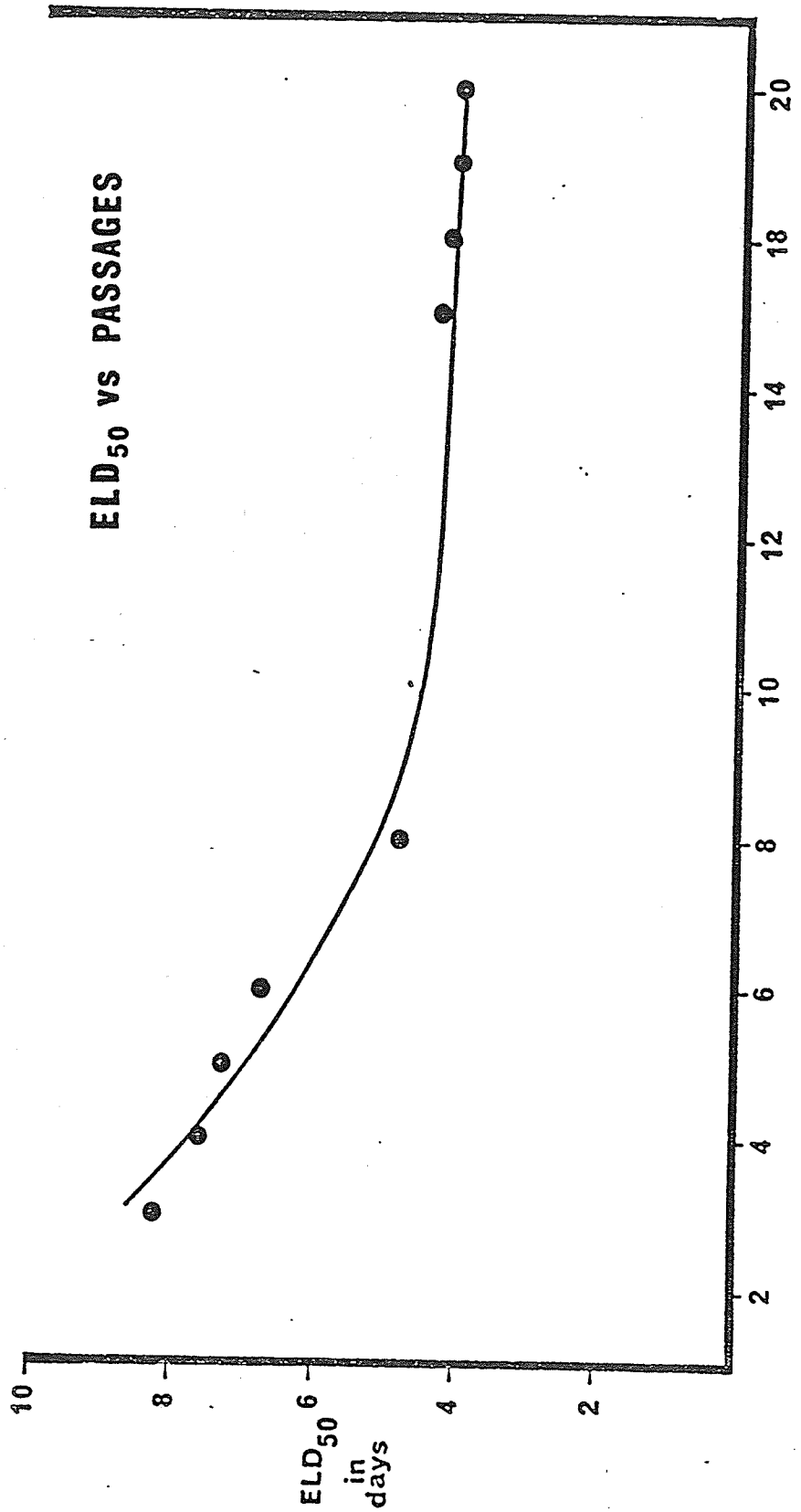


FIGURE 4 - RELATIONSHIP BETWEEN THE ELD_{50} VALUES AND THE NO. OF PASSAGES IN ADAPTATION & PROPAGATION OF THE IC-CAL-3 AGENTS.

size of infectious agents to cause death to 50% of the inoculated population of chick embryos. This decrease in time requirement could be regarded as evidence of adaptation of the IC-Cal-3 agents to the allantoic membrane of the chick embryos.

With striking similarity to the behaviour of the mean percentage death values as depicted in Figure 3, the ELD_{50} values, after a sharp decrease in the first few passages, also reached a stationary phase, in this case a minimum (in terms of less time required) beyond passage number 10. However, it could be concluded that in the adaptation of the IC-Cal-3 agent to a new host tissue, the infectious agent would only cause death to a certain maximum percentage of chick embryos within certain minimum time requirement.

4. Calculation of mathematical relationship :

Using the percentage death values as the X variables, and the reciprocal days values as the Y variables, the coefficient of correlation for each of the passages were calculated by the statistical test described in a previous corresponding section, and the results are shown in Table V.

The coefficients of correlation for the corresponding passages were all very close to 1, indicating the existence of a linear correlation between the percentage death and reciprocal day values. The negative sign shown was due to the use of reciprocal days instead of actual days in the calculation process, and does not indicate any inversely proportional correlation.

TABLE V

THE ELD_{50}s AND CORRELATION COEFFICIENTS OF RESPECTIVE PASSAGES

Date	Passage No.	ELD_{50}	Correlation coefficient
10-09-70	0	---	-----
28-09-70	1	---	-----
05-10-70	2	---	-----
13-10-70	3	8.2	- 0.972
20-10-70	4	7.6	- 0.956
26-10-70	5	7.3	- 0.975
03-11-70	6	6.8	- 0.975
09-11-70	7	---	-----
16-11-70	8	4.8	- 0.947
30-11-70	9	---	-----
07-12-70	10	---	-----
15-12-70	11	---	-----
22-12-70	12	---	-----
30-12-70	13	---	-----
05-01-71	14	---	-----
12-01-71	15	4.35	- 0.985
19-01-71	16	4.18	- 0.986
26-01-71	17	4.06	- 0.992
02-02-71	18	4.02	- 0.959
08-02-71	19	---	-----
15-02-71	20	---	-----

--- data not available

PURIFICATION AND CHARACTERIZATION :

1. Purification by density gradient centrifugation:

After the two hours centrifugation period, the fractions collected were examined spectrophotometrically. The absorbances were plotted against the density gradient. In Figure 5, the left vertical axis represents the absorbance readings, the right vertical axis represents the density gradient, and the horizontal axis denotes the number of tubes collected. Three fractions were obtained by pooling eluates : (1) from tube no. 6 to 18, (2) from tube no. 19 to 31, and (3) from tube no. 32 to 38.

Fractions (1) and (3) were found to contain high absorbancy materials. All three fractions were centrifuged at 30,000 XG for 1 hour in an attempt to sediment any particulate materials to be examined by electron microscopy.

The first fraction which was visible as an opaque zone at a position about ¾ down the centrifuge tube was found to contain the agent particles, and the density of this fraction was found to be 1.11. An electron micrograph of the agent detected in this fraction is shown in Plate I, in which all the agent particles were observed to be the size of the elementary body forms.

No agent particle was detected in the other two fractions after centrifugation and examination by electron microscopy, indicating that the ultraviolet light absorbing substances in these two fractions were non-agent or contaminating materials, possibly from host origin.

From this density gradient centrifugation experiment, the hydrated density of the IC-Cal-3 agent was determined to be approximately 1.11 at 4 to 6 degrees centigrade.

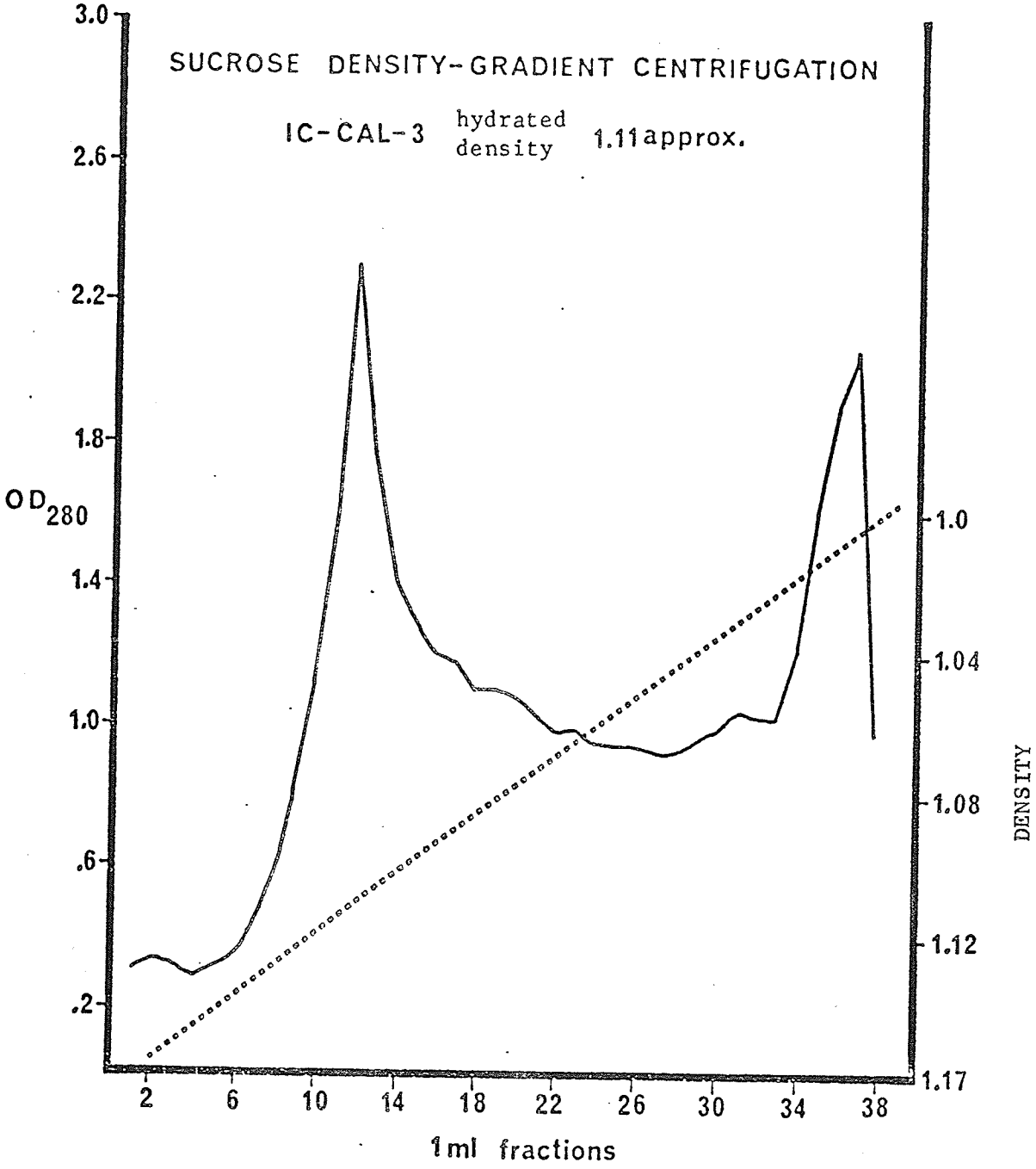


FIGURE 5 - ABSORBANCES OF FRACTIONS OBTAINED FROM SUCROSE DENSITY GRADIENT CENTRIFUGATION.

The dotted line represents the density gradient, and the absorbances are represented by the solid line.

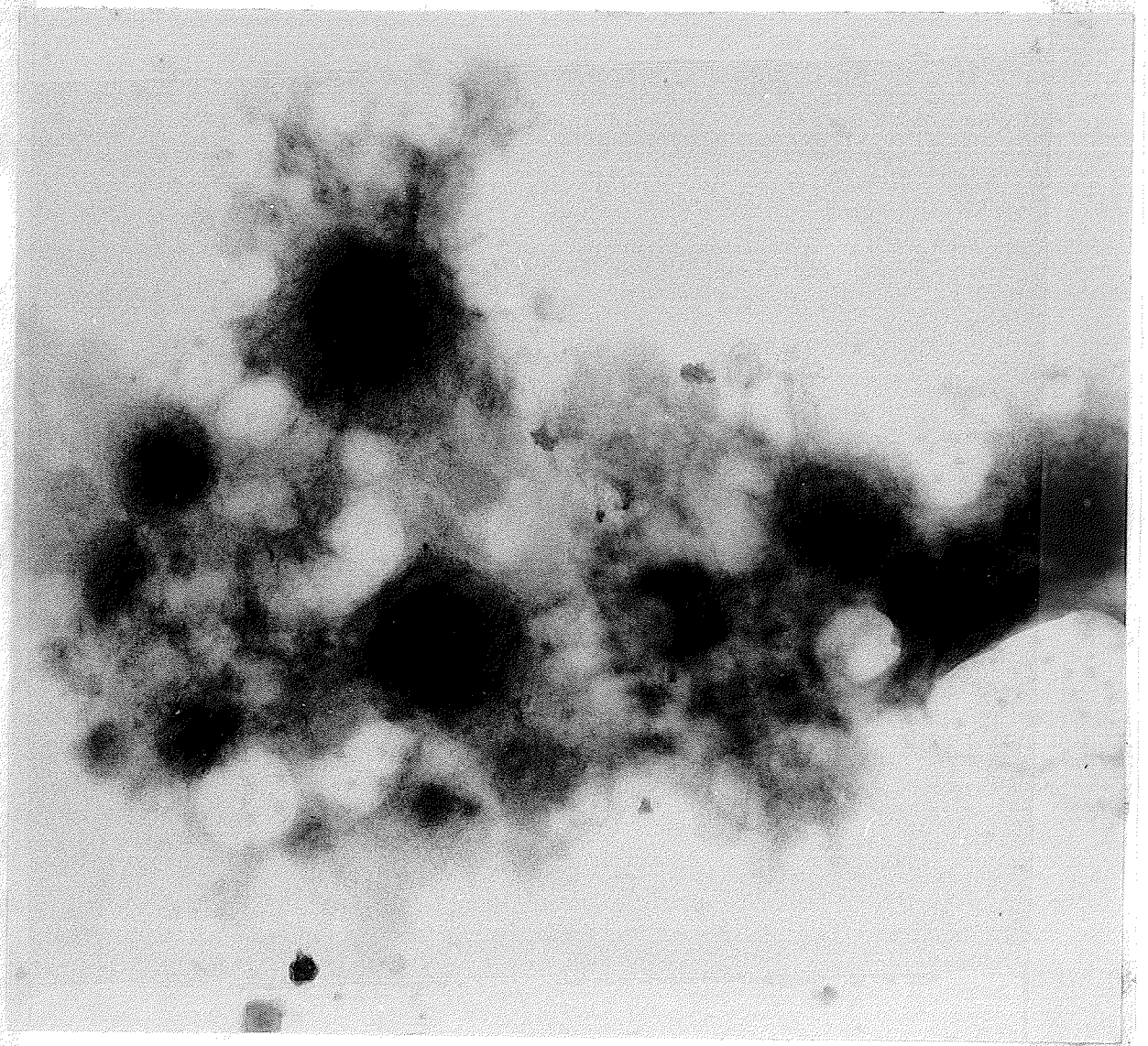


PLATE II - ELECTRON MICROGRAPH OF THE IC-CAL-3 AGENTS PURIFIED BY SUCROSE DENSITY GRADIENT CENTRIFUGATION.

(Magnification : 28,300 X)

2. Purification & characterization by isoelectrofocusing :

(a) Purification of IC-Cal-3 agents :

At the end of the electrophoresis period, fractions of 3 ml. each were collected, and the abosrbances and pHs were measured. The absorbances of these fractions were plotted against their corresponding pH readings, and the results are shown in Figure 6. In this graph, the dotted line represents the pH gradient, and the solid line represents the absorbances of individual fractions.

Three fractions of different absorbances and pHs were observed from Figure 6. The first fraction included tubes no. 10 to 15, the second fraction tubes no. 20 to 22, and the third fraction tubes no. 23 to 26. The isoelectric points of these fractions were 4.1, 7.0, and 8.1 respectively. Similar fractions from separate but similar runs were pooled together and centrifuged at 30,000 XG for 1 hour after dialysis against distilled water for removal of ampholytes and glycerol. After centrifugation, a pellet was obtained from the first fraction (pH 4.1), but no visible pellet was obtained from the other two fractions. The pellet from the first fraction was resuspended in sterile distilled water and was examined by electron microscopy. Although no pellet was obtained from fractions two and three, they were also examined by electron microscopy, and no agent particle was detected in either of them. They were therefore considered to be contaminating materials.

In the first fraction, agent particles of the size of elementary body forms were detected. No reticular body forms were visible. An electron micrograph of the agents purified by isoelectrofocusing is shown in Plate II. From this experiment, the isoelectric point of the IC-Cal-3 agent was determined to be approximately 4.1.

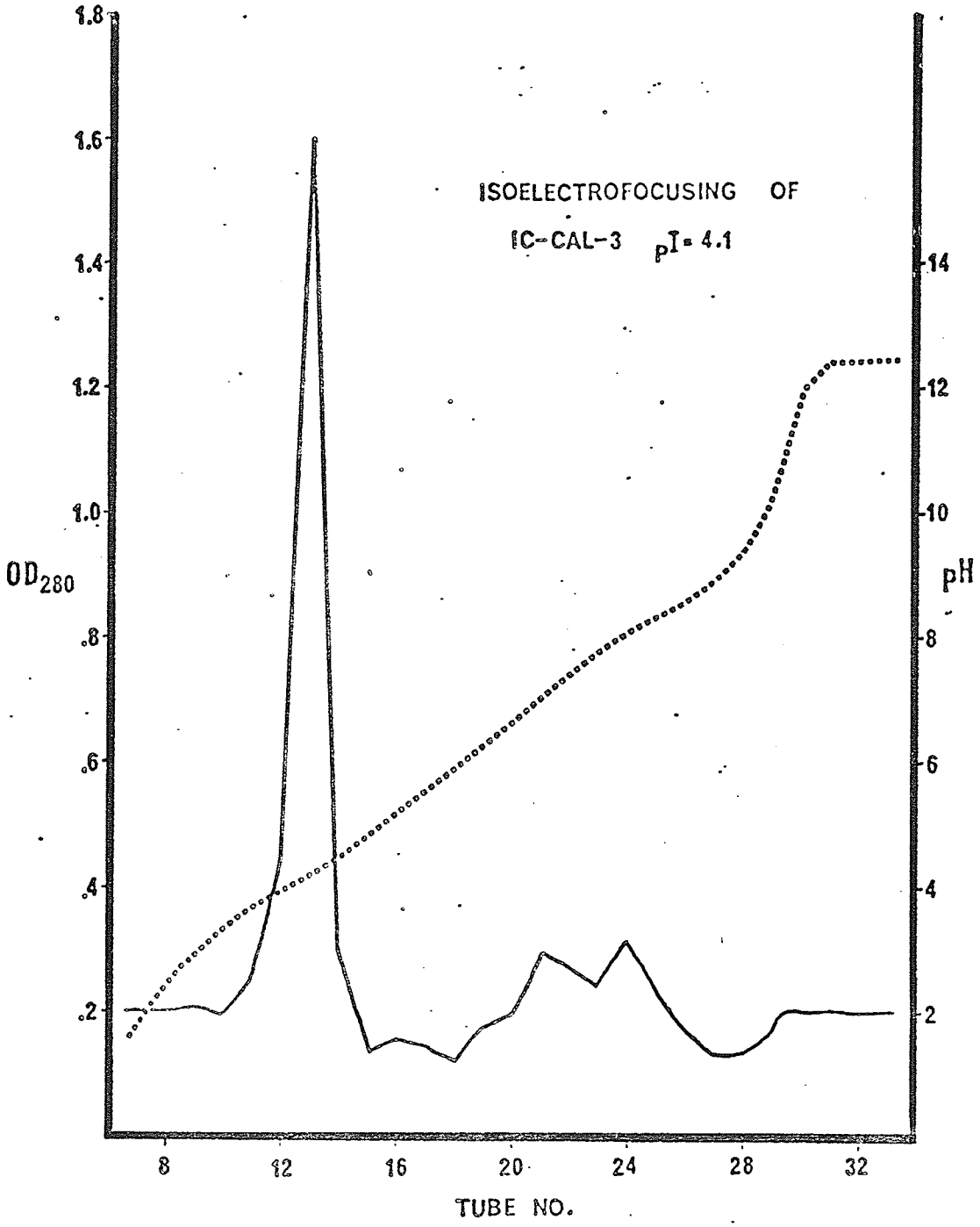


FIGURE 6 - ABSORBANCES OF FRACTIONS OBTAINED FROM THE ISOELECTROFOCUSING EXPERIMENT.
The dotted line represents the pH gradient, and the absorbances are represented by the solid line.

If we recall that the isoelectrofocusing experiment was performed with materials purified by the density gradient centrifugation method, we can observe that isoelectrofocusing was capable of further separating non-agent materials from the agents, producing a degree of purity which was not obtainable by the density gradient centrifugation method. Moreover, results from the isoelectrofocusing experiments are absolutely reproducible and with accuracy.

Purification by the combination of density gradient centrifugation and isoelectrofocusing methods proved to be very effective because the first method separates by molecular sieving, and the latter one by electrostatic interactions.

(b) Characterization by isoelectrofocusing :

In addition to the IC-Cal-3 agent, the isoelectrofocusing technique was applied to another representative chlamydial species, the DD-34 strain of psittacosis agent.

To compare the characterization by isoelectrofocusing of these two chlamydial species, the results of the two individual isoelectrofocusing experiments were mapped together on the same graph. Since the parameters of the two experiments were identical the absorbances of the two separate experiments could be compared. The comparison is plotted in Figure 7.

As in Figure 6, symbols used in Figure 7 are the same : the pH gradient is represented by the dotted line and the absorbances are represented by a solid line for the IC-Cal-3 agent, and by a broken line for the psittacosis DD-34 agent. The initials IC and DD denotes the two species respectively.

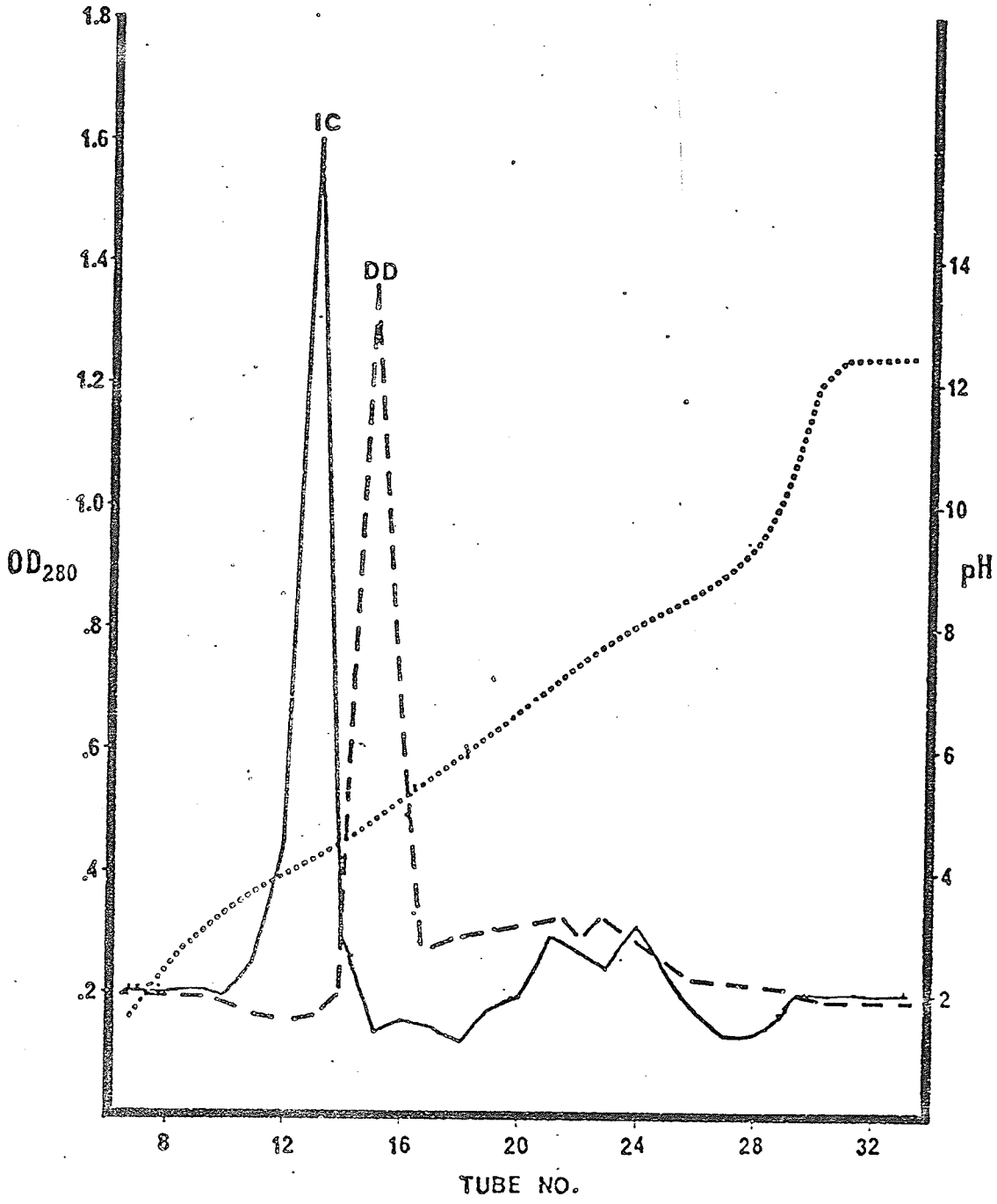


FIGURE 7 - COMPARISON OF CHARACTERIZATION OF TWO CHLAMYDIAL AGENTS BY ISOELECTROFOCUSING TECHNIQUE.

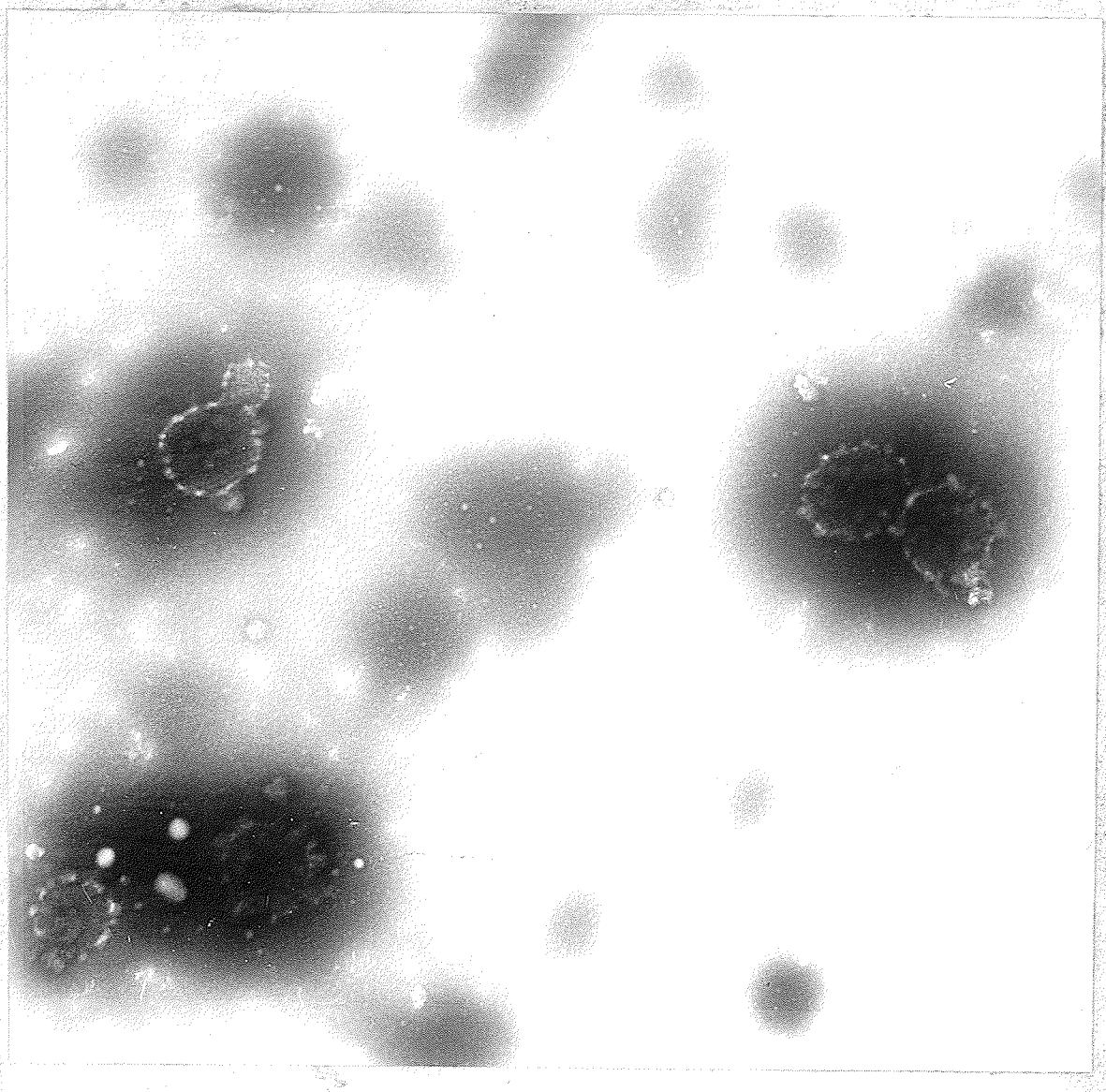


PLATE II - ELECTRON MICROGRAPH OF THE IC-CAL-3 AGENTS PURIFIED BY ISOELECTROFOCUSING.

(Magnification : 26,240 X)

From Figure 7, it could be observed that these two chlamydial species are both negatively charged particles having different individual isoelectric points. The IC-Cal-3 agent has an isoelectric point of 4.1, and the psittacosis DD-34 agent has an isoelectric point of 4.9. The variation of isoelectric points among the two chlamydial species suggested that the surface structures or components of these two agents are different although they appear morphologically similar when examined microscopically.

3. Effect of isoelectrofocusing on infectivity :

The effect of isoelectrofocusing on the infectiousness of the so treated IC-Cal-3 agents was investigated by observing the mortality rate of the inoculated chick embryos. The response expected was an all or none response. The percentage deaths for each day post inoculation were calculated by the method described previously, and the results are shown in Table VI. Probit analysis was carried out by plotting the percentage death of the treated agents against time post inoculation as shown in Figure 8. Isoelectrofocusing obviously did not completely inactivate the infectious agents, but the infectivity of the agent was definitely affected. This kind of partial inactivation is commonly observed as in the case of inactivation of viruses by pHs significantly different from its usual enviroment.

TABLE VI

PERCENTAGE DEATH OF EGGS AFTER TREATMENT OF AGENTS BY ISOELECTROFOCUSING

Day	Cumulative death	No. of eggs	% death
0	0	48	0.0
1	5	"	10.4
2	8	"	16.6
3	1	40	2.5
4	8	"	20.0
5	20	"	50.0
6	23	"	57.1

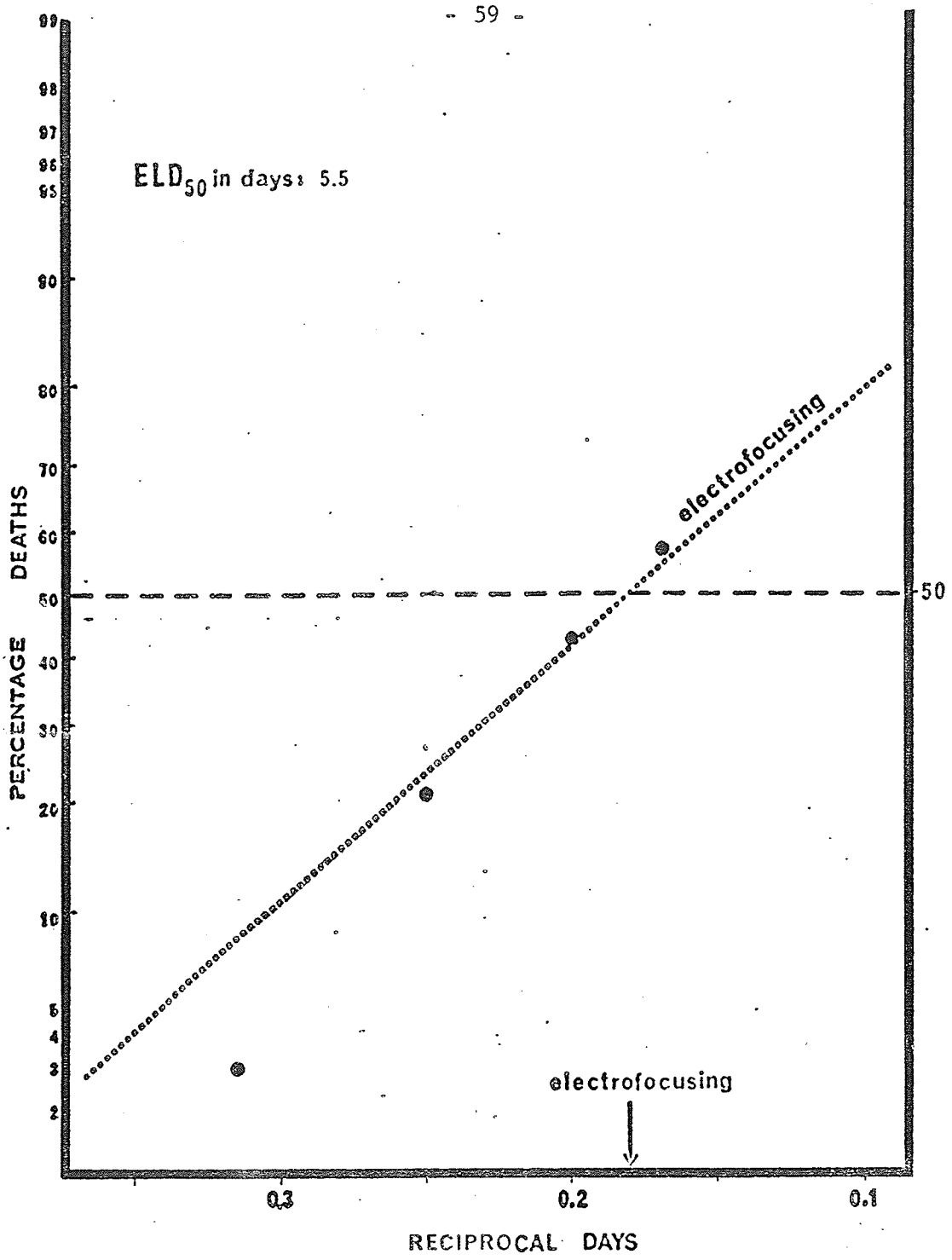


FIGURE 8 - PROBIT ANALYSIS OF ISOELECTROFOCUSING TREATED IC-CAL-3 AGENTS.

FRACTIONATION AND ANLYSIS :

1. Fractionation of agent particles :

Analysis by electron microscopy :

When the suspension of agents which have been sonicated for three hours was centrifuged at 60,000 xG for 1 hour, a very small whitish yellow pellet was obtained. The pellet, when examined by electron microscopy as shown in Plate III revealed no intact agent particles. Also no ghost-cell structure devoid of intracellular materials was detected. In fact pieces of stain-absorbing fragments which could not be positively identified as part of the agent particle were observed in the micrograph. This indicated that ultrasonic vibration was capable of rupturing the agent, releasing the intracellular materials into the surroundings. Although the exact site or pattern of disruption could not be determined, it could be observed that sonication was not only capable of disrupting the agent, but also capable of disintegrating the particulate cell wall like structure into small fragments, and the absence of ghost cell like structure confirmed this observation. The degree of disruption as revealed in the electron micrograph, was reproducible.

2. Analysis of effects of sonication :

(a) Analysis by spectrophotometry :

Spectrophotometric analysis was carried out in three ways : (1) an analysis of the absorption spectra of the two chlamydial agents, (2) an analysis of the denaturation of proteins, and (3) a qualitative analysis

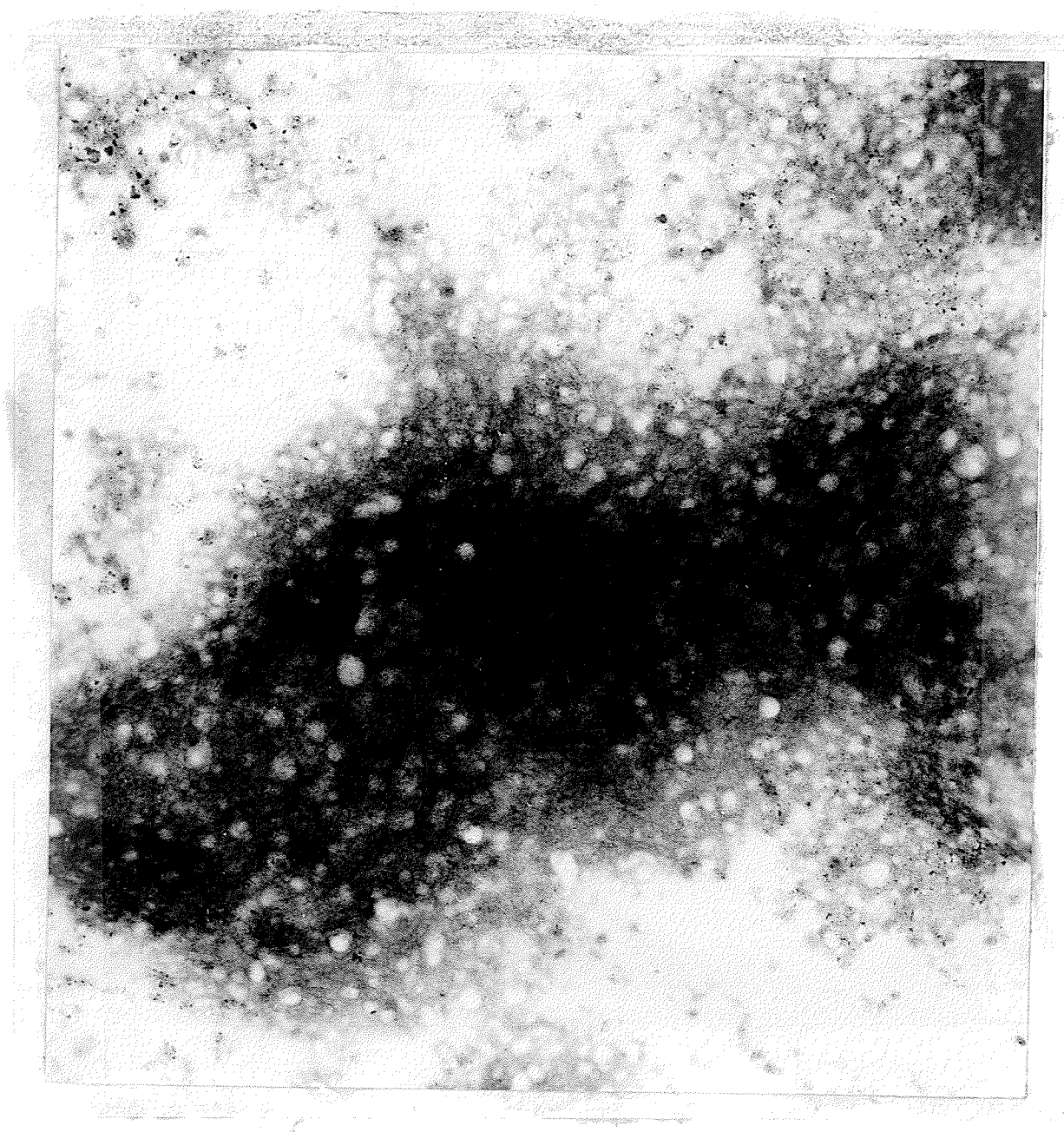


PLATE III - ELECTRON MICROGRAPH OF THE PELLET FROM THE SONICATED SUSPENSION OF IC-CAL-3 AGENTS.

The pellet was obtained by centrifugation at 60,000 xG for 1 hr. of the IC-Cal-3 agents after 3 hrs. of sonication. Note the complete absence of the intact particle form or any ghost-cell and any recognizable fragment of the agent. (Magnification : 26,600X).

on the products of disintegration. The results of these analyses are discussed separately in the following :

(i) Absorption spectra of two chlamydial agents :

The results of the absorption spectra of the IC-Cal-3 and the psittacosis DD-34 agents are shown in Tables VII and VIII respectively. In these tables, the sonication intervals are expressed by the subscripts 0, 60, 120, and 180 meaning before sonication, at 60, at 120, and at 180 minutes after sonication respectively. The different spectra for each interval of sonication were plotted in Figure 9 for the IC-Cal-3, and in Figure 10 for the psittacosis DD agents. The absorbances for both agents decreased with increase of wavelengths, and the decrease is especially prominent within the ultra-violet radiations region, i.e., between 200 and 400 mu. For individual agents, a comparison between spectra of different sonication intervals would indicate the relationship between the time factor of sonication and the disruption of the agent particle. A similarity existed among the two agents in that disintegration appeared to be most intense within the first 60 minutes of sonication. Further increase in sonication time did not produce a more significant increase in the alteration of the absorption spectrum. The effective time of mechanical disruption of agent by sonication was therefore concluded to be around 60 to 120 minutes at 20 KC sonicating force. Repeated experiments have shown a reproducibility of the results presented here.

(ii) Protein denaturation analysis :

The absorbances at 280 mu. for both IC-Cal-3 and psittacosis DD-34 agents at different sonication intervals were measured and plotted in

TABLE VII

ABSORPTION SPECTRUM OF IC-CAL-3 AGENTS

Wavelengths	Sonication time in minutes			
	t_0	t_{60}	t_{120}	t_{180}
200	1.31	1.76	1.95	2.31
220	1.08	1.52	1.49	1.37
240	0.94	1.17	1.09	1.02
260	0.83	0.88	0.78	0.74
280	0.76	0.73	0.65	0.61
300	0.70	0.60	0.53	0.49
320	0.64	0.50	0.44	0.41
340	0.60	0.44	0.39	0.36
360	0.55	0.38	0.34	0.32
380	0.52	0.33	0.29	0.28
400	0.48	0.29	0.26	0.25
500	0.36	0.18	0.15	0.12
600	0.29	0.12	0.10	0.08

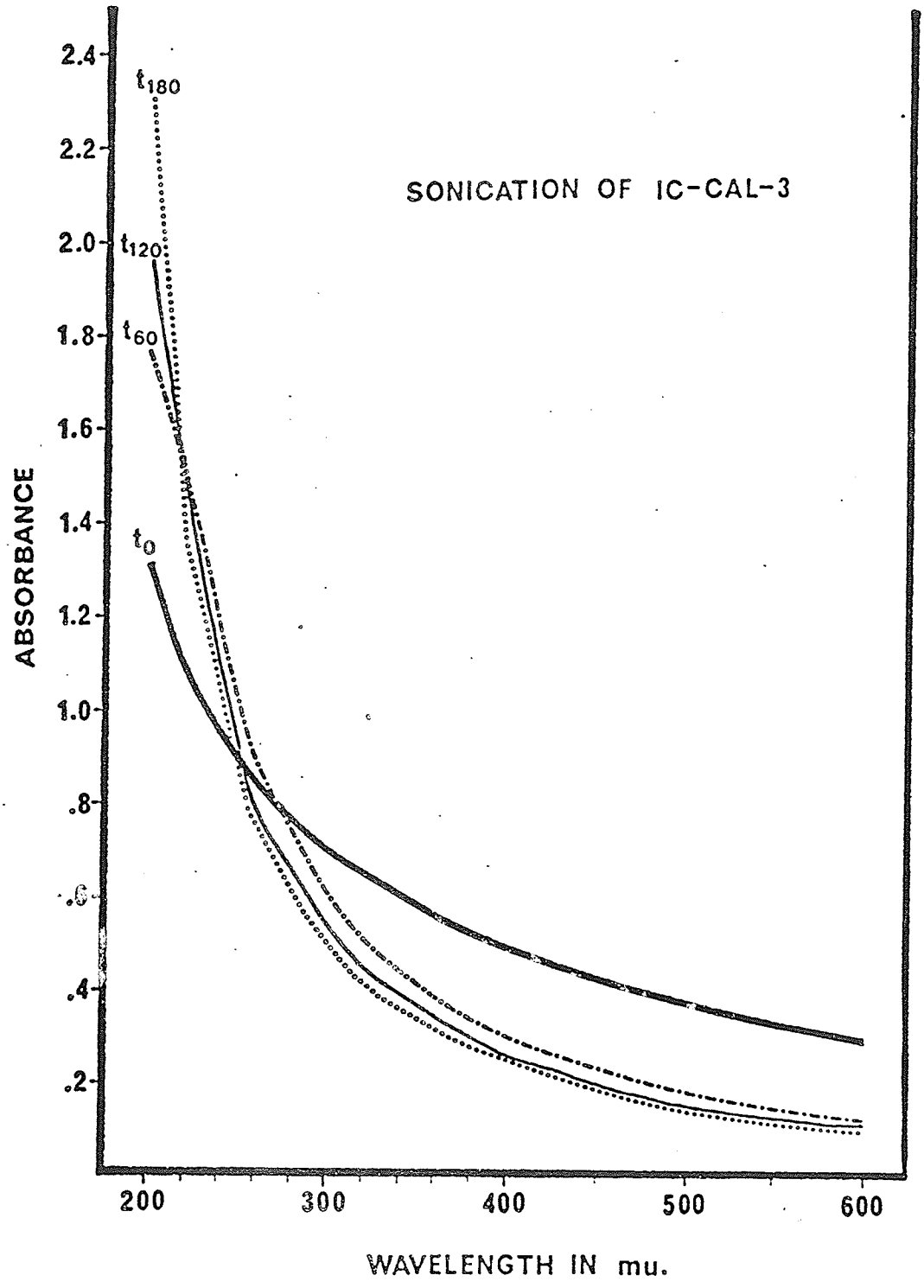


FIGURE 9 - ABSORPTION SPECTRA OF THE IC-CAL-3 AGENTS. Absorption spectra were obtained from different intervals of sonication. Each line represents an individual spectrum. The subscripts represent the interval of sonication in minutes.

TABLE VIII

ABSORPTION SPECTRUM OF PSITTACOSIS DD AGENTS

Wavelengths	Sonication time in minutes			
	t_0	t_{60}	t_{120}	t_{180}
200	2.23	2.30	2.24	2.21
220	1.20	1.19	1.17	1.12
240	0.76	0.53	0.46	0.43
260	0.56	0.34	0.28	0.26
280	0.49	0.29	0.24	0.22
300	0.42	0.22	0.18	0.17
320	0.37	0.19	0.15	0.15
340	0.33	0.16	0.13	0.14
360	0.29	0.15	0.12	0.13
380	0.27	0.13	0.11	0.11
400	0.24	0.11	0.10	0.10
500	0.16	0.07	0.07	0.08
600	0.12	0.05	0.05	0.07

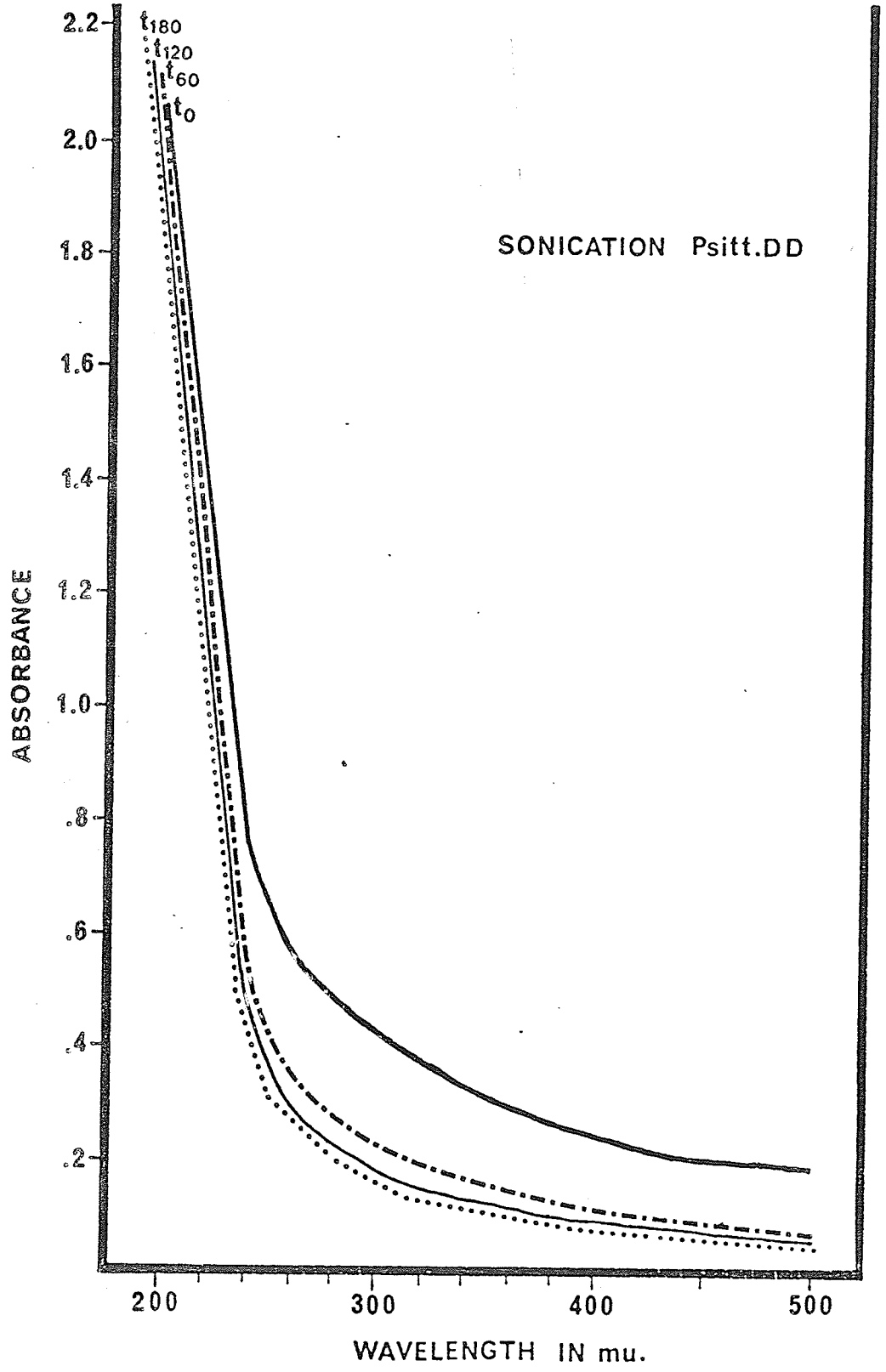


FIGURE 10 - ABSORPTION SPECTRA OF THE PSITTACOSIS DD AGENTS. Absorption spectra were obtained from different intervals of sonication. Each line represents an individual spectrum. The subscripts represent the interval of sonication in minutes.

Figure 11 with OD_{280} readings against sonication times.

In comparison to the reading at t_0, a decrease in absorbances were observed for both agents, and the decrease was more pronounce in the psittacosis DD agent. This decrease in absorbance indicated that the chromophores responsible for ultra-violet light absorption were denatured, and the denaturation would have been occured in the protein molecule containing the chromophores. The effect of ultrasonic vibrations would therefore seems to reside partly in altering the structure or conformation of the protein moieties.

The percentage of protein denatured by ultrasonic vibration could be calculated from the ratio of the OD_{280} reading at time X of sonication to the OD_{280} reading at time 0 (before sonication). The mathematical formula is shown as follows :

$$\% \text{ denaturation} = \frac{OD_{280} \text{ at } t_X}{OD_{280} \text{ at } t_0} \times 100\%$$

The percentage of denaturation of proteins at different sonication intervals for both IC-Cal-3 and psittacosis DD-34 agents are shown in Figure 12. There is an observed difference in the percentage of protein denaturation between the IC-Cal-3 and the psittacosis DD-34 agents at similar intervals of sonication. The denaturation of protein was more extensive in the psittacosis agent in comparison to the IC-Cal-3 agent indicating that there are structural or conformational differences between the two chlamydial species. These experimental results were reproducile with high degree of accuracy.

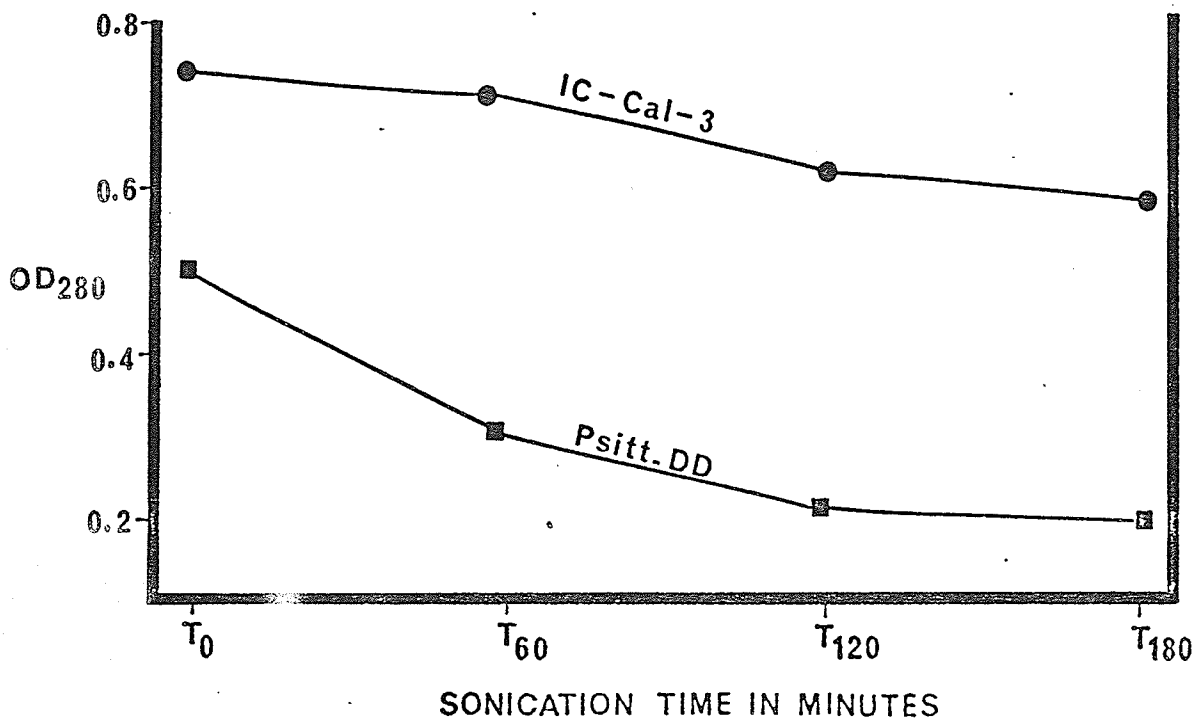


FIGURE 11 - ABSORBANCES AT 280 MU OF THE IC-CAL-3 & PSITTACOSIS DD AGENTS AT DIFFERENT INTERVALS OF SONICATION.

$$\frac{OD_{280}\ t_x}{OD_{280}\ t_o} = \%$$

total % denaturation of protein

IC-Cal-3	
t_0	—
t_{60}	3.4 %
t_{120}	13.6 %
t_{180}	19.8 %

Psitt.DD	
t_0	—
t_{60}	41.9 %
t_{120}	51.1 %
t_{180}	55.2 %

FIGURE 12 - PERCENTAGE DENATURATION OF PROTEINS OF BOTH IC-CAL-3 AND PSITTACOSIS DD AGENTS AT DIFFERENT INTERVALS OF SONICATION.

(iii) Qualitative analysis by spectrophotometry :

There are specific ratios for different types of biochemical substances for the ratio of absorbances at OD_{280} to OD_{260}. For the nucleic acids, the ratio is 0.5, for carbohydrate-protein complexes, e.g. glyco-proteins or muco-proteins, the ratio is 0.8, and for the proteins, the ratio is 1.75. Qualitative spectrophotometric analysis was performed by comparing the observed ratios to the standard ones as described previously.

The 280/260 ratios for both the IC-Cal-3 and psittacosis DD-34 agents at similar intervals of sonication together with the standard ratios are shown in Figure 13. In this figure, for the IC-Cal-3 agent, the 280/260 ratios at 60, 120, and 180 minutes of sonication were all observed to be around 0.8, and the same was observed for the psittacosis DD-34 agent. The results here might indicate either of two possibilities : first one is that the materials released by the process of sonication were likely to be carbohydrate-protein complexes, resembling the nature of glyco-proteins or muco-proteins. The second explanation is that ultrasonic vibrations have certain limitation which was the incapability of further disintegrating the carbohydrate-protein complexes released in the first sixty minutes of sonication since the ratios for both agents remained unchanged in spite of prolonged period of sonication. The latter explanation is in confirmation with the absorption spectra studies in which, for both agents, disintegration of the agent particles occured most significantly within the first hour sonication. After this period, an increase in the sonication time did not result in significant alterations in the absorbance readings.

	ratio $\frac{280}{260}$
nucleic acid	0.50
mucoprotein glycoprotein	0.80
protein	1.75

IC-Cal-3

t_0	0.910
t_{60}	0.827
t_{120}	0.830
t_{180}	0.810

Psitt.DD

t_0	0.875
t_{60}	0.852
t_{120}	0.857
t_{180}	0.846

FIGURE 13 - SPECTROPHOTOMETRIC ANALYSIS OF SONICATED SUSPENSIONS OF CHLAMYDIAL AGENTS.

The analysis was performed by comparing the 280/260 mu. ratios of both the IC-Cal-3 and psittacosis DD agents to the standard ratios for nucleic acids, proteins, and muco- or glyco-proteins.

CHARACTERIZATION OF FRACTIONS :

The suspension of IC-Cal-3 agents which had been subjected to three hours of sonication was fractionated by the isoelectrofocusing technique. The absorbances and the pHs are plotted in Figure 14, and from this graph, different peaks obtained were characterized by the following tests :

1. Biological analysis :

(a) Kinetics of the MSI test :

Using the formula as described previously for the calculation of the MSI index, the MSI indices for the six weeks of experimentation were obtained. The MSI indices of different time intervals post sensitization of the animals were plotted against time in weeks, and the graph is shown in Figure 15.

From this graph, it could be observed that for mice, the optimal time for performing the test is approximately 14 days after sensitization of the experimental animals. For each week, four mice were sacrificed, and the average of the MSI indices was taken to be the MSI index for that particular reading.

(b) MSI test for fractions from sonication :

Since the optimal time for performing the MSI test was determined to be at 14 days post sensitization, and in order to maintain homogeneity for the condition of all the tests, the eleven fractions obtained from sonication of the IC-Cal-3 agent were characterized by the MSI test on the same 14th day after sensitization. Due to limited facilities, only two mice were sacrificed for each fraction, and the average of these

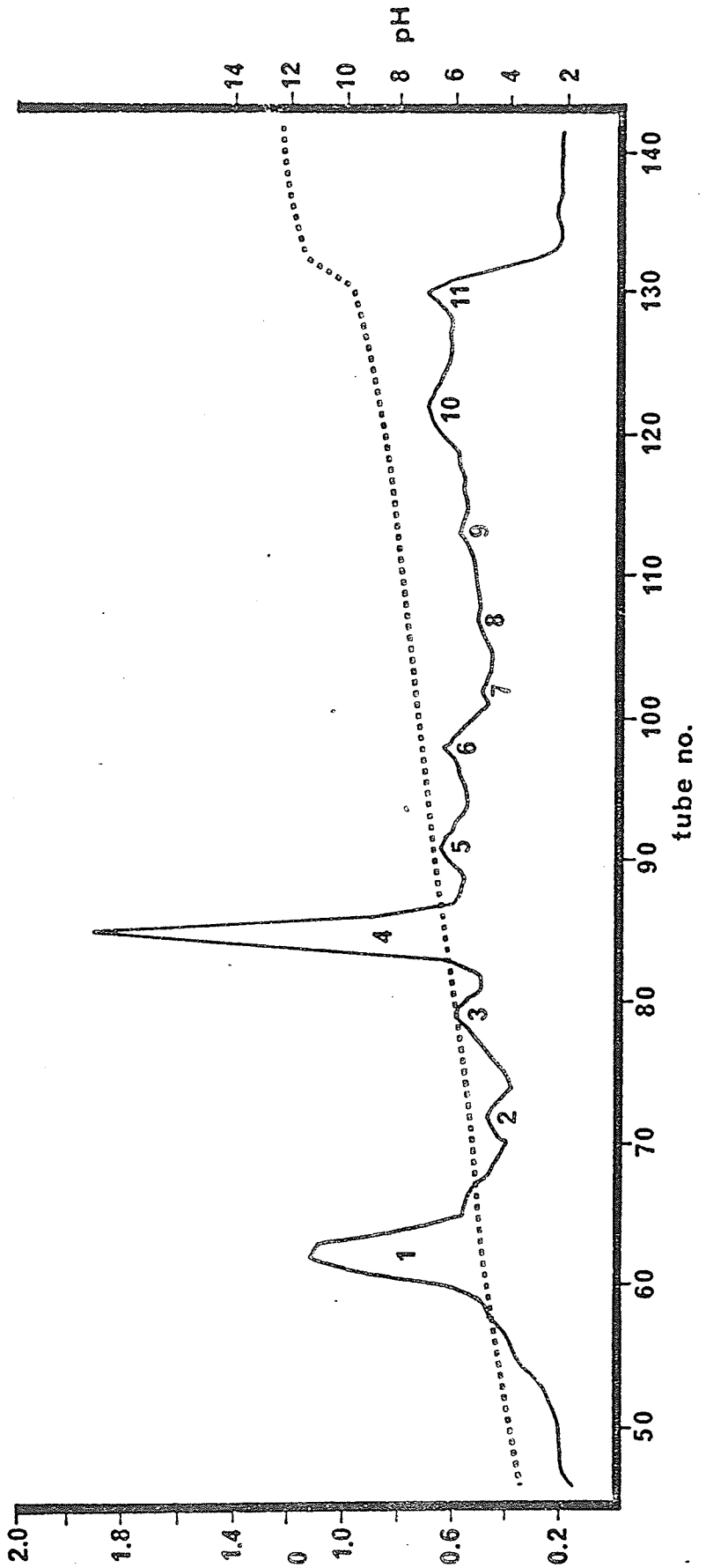


FIGURE 14 - ABSORBANCES OF FRACTIONS OBTAINED BY ISOELECTROFOCUSING OF SONICATED IC-CAL-3 AGENTS.

The dotted line represents the pH gradient, and the absorbances of the fractions are represented by the solid line. The various fractions are the numerials within each peak.

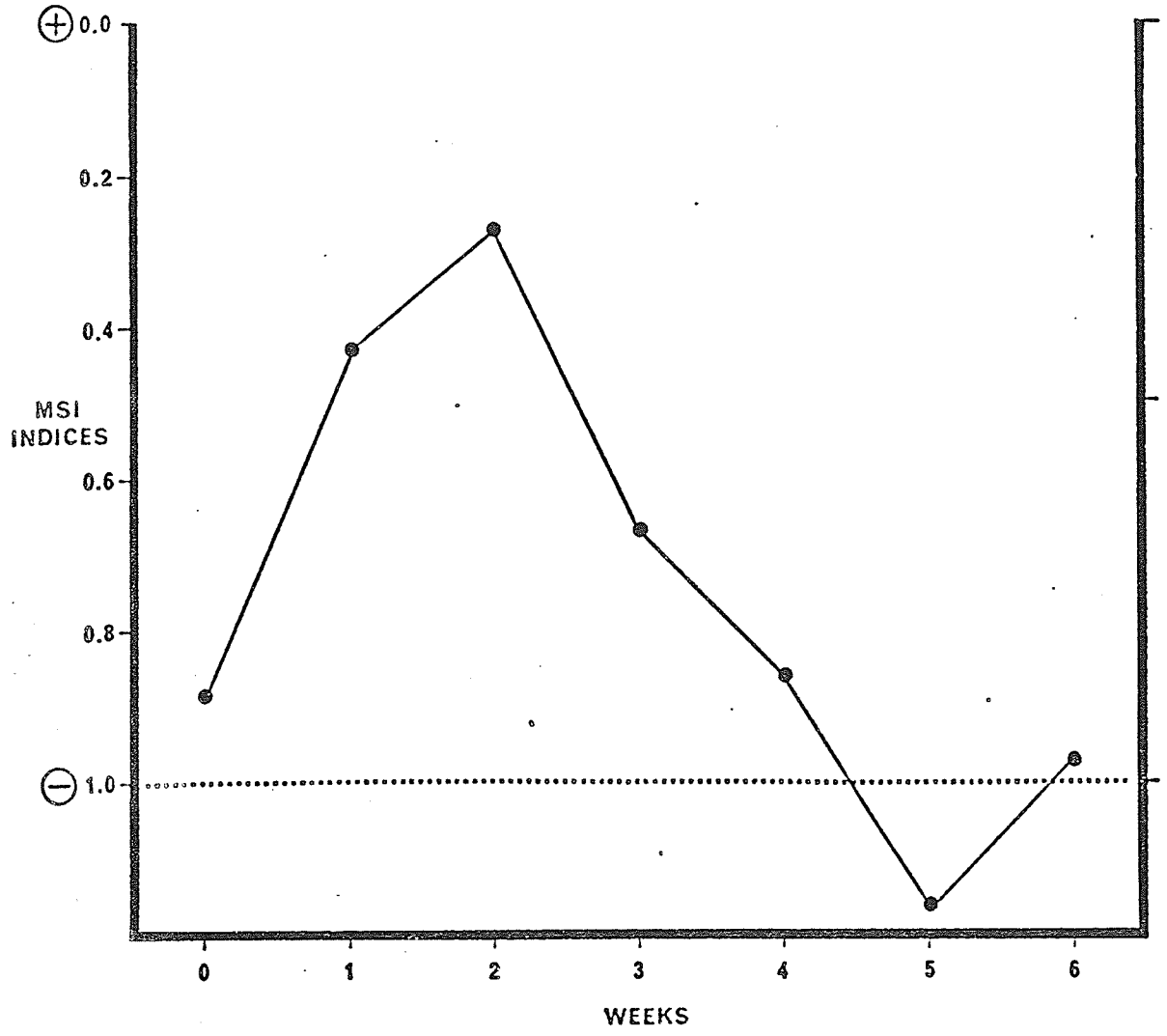


FIGURE 15 - KINETICS OF THE MSI TEST.

An MSI index of 0.0 indicates maximum inhibition of spreading of macrophage, while an index of 1.0 indicates no inhibition. Indices above 0.5 are considered to be positive inhibition in the test.

TABLE IX

MSI TESTS OF FRACTIONS FROM SONICATED IC-CAL-3 AGENTS

Fraction no.	+Ag		-Ag		Indices	Average index
	Sp	Nsp	Sp	Nsp		
1	4	6	7	1	0.457	0.445
	4	7	9	2	0.444	
2	4	6	14	2	0.457	0.460
	4	7	10	3	0.472	
3	6	1	7	1	0.979	0.840
	5	6	6	3	0.681	
4	4	3	9	1	0.634	0.638
	8	6	8	1	0.642	
5	4	3	7	1	0.651	0.638
	3	3	8	2	0.625	
6	3	3	7	1	0.638	0.530
	3	4	12	2	0.489	

TABLE IX continued.

Fraction no.	+Ag		-Ag		Indices	Average index
	Sp	Nsp	Sp	Nsp		
7	6	4	10	1	0.660	0.660
	5	4	10	2	0.667	
8	6	8	14	5	0.805	0.767
	7	6	14	4	0.730	
9	3	4	6	1	0.500	0.500
	2	3	12	2	0.498	
10	6	1	8	1	0.964	0.803
	3	3	7	2	0.642	
11	6	2	9	2	0.917	0.829
	4	2	9	1	0.741	

two indices were taken to be the MSI index for that particular fraction. The results are shown in Table IX. Three fractions with a MSI index at or below 0.5 were considered to be reactive in the test and were further analysed for their amino acid contents.

2. Chemical tests :

Spectrophotometric analysis of fractions :

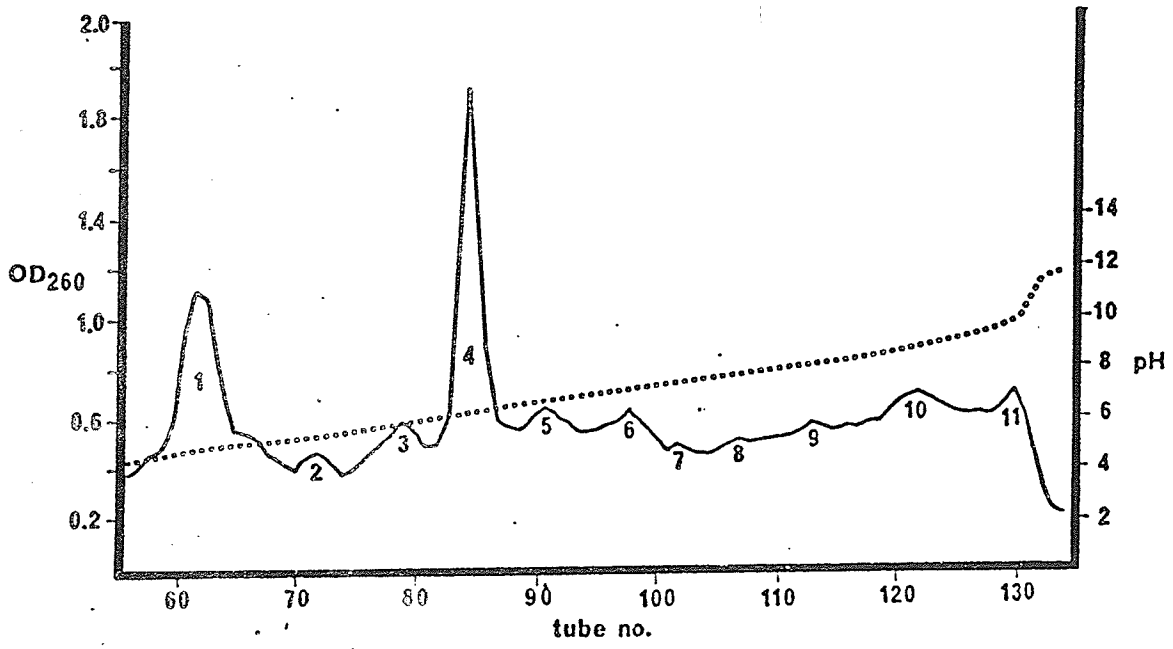
The ratios of 280/260 mu. were calculated for each of the fractions obtained from isoelectrofocusing of the sonicated IC-Cal-3 agents. These ratios are shown in Figure 16, in which fractions or peaks number 1 and 4 are considered to be carbohydrate-protein complexes, and fraction number 5 is likely to be nucleic acids. The biochemical natures of fractions number 2,3,6,7,8,9,10, and 11 are difficult to be elucidated because of deviations of these ratios from the standard ones. Further investigations were carried out to elucidate or confirm their biochemical natures.

(a) Lowry Test for protein content determination :

Using the standard curve obtained, the protein contents of the 11 fractions were determined. The results are shown in Figure 16. Fractions number 1,4,6,7,10, and 11 were of high protein contents, while fractions number 5,8 and 9 have very little protein in them.

(b) Dubois test for carbohydrate content determination :

Using the standard curve obtained, the carbohydrate content of the 11 fractions were determined. The results are shown in Figure 16. The amount of carbohydrate in most of the fractions were very small except for fractions 6, 10, and 11. The low quantities of carbohydrates detected in fractions 5,8, and 9 confirmed the accuracy of the spectrophotometric determination of the biochemical nature of these fractions.



PEAKS	1	2	3	4	5	6	7	8	9	10	11
pH	4.7	5.3	5.8	6.2	6.6	7.0	7.3	7.6	8.1	8.7	9.8
280/260	.72	.38	.32	.73	.48	.39	.30	.28	.28	.30	.25
Lowry ug/ml	236	11	9	236	1	649	88	1	1	816	588
Dubois ug/ml	12	10	11	20	13	132	37	13	18	138	112
MSI	.44	.46	.84	.63	.63	.57	.66	.73	.50	0.8	.82

FIGURE 16 - CHEMICAL & BIOLOGICAL PROPERTIES OF FRACTIONS OBTAINED FROM ISOELECTROFOCUSING OF SONICATED IC-CAL-3 AGENT SUSPENSION.

(c) Amino acid content analysis :

Three fractions, number 1, 6, and 9 showing high MSI reactivities were selected for analysis of amino acid contents because they contained different proportions of proteins, carbohydrates, and were of different pHs. These three fractions can be taken as representatives of the other fractions obtained from the sonicated agents, and an investigation of amino acid contents was thought to shed light on the biochemical relationship between the various fractions. The results of the analysis are shown in Table X.

A general observation from the analysis is that although the pHs of the three fractions were quite different, they somehow contained more or less the same types of amino acids except for one or two. The sulphur containing amino acids, cystein and methionine were absent in all of the fractions, and this is the same for the amino acid proline. Serine, glycine and ornithine were found in comparatively large amount in fraction 1, and a large quantity of lysine was detected in fraction 6. In fraction 9, no single amino acid was detected in unequally large quantity.

The ratios of individual amino acids varied among the three fractions, and no general pattern such as absence of certain particular amino acid in one fraction or its presence in large quantity in another fraction was observed.

TABLE X

RATIOS OF AMINO ACIDS IN MICROMOLES

	Fraction 1	Fraction 6	Fraction 9
Aspartate	8	9	2
Threonine	5	5	1
Serine	20	9	3
Glutamate	4	8	1
Proline	0	0	0
Cysteine	0	0	0
Methionine	0	0	0
Glycine	15	9	3
Alanine	9	10	2
Valine	6	5	0
Isoleucine	5	4	1
Leucine	5	6	1
Tyrosine	2	0	0
Phenylalanine	4	2	0
Lysine	3	23	2
Histidine	3	2	1
Arginine	2	4	0
Ornithine	11	6	4

DISCUSSION

DISCUSSION

Isolation and propagation of the trachoma agents were initiated by T'ang et al. in 1957 using the yolk sac membrane of chick embryos as the host tissue. The adaptation of four trachoma strains (Vali 1968) to the allantoic cavity of the same host introduced another cell-line for propagation of the TRIC agents. The present report confirmed the availability of the allantoic membrane as host tissue for cultivation of the IC-Cal-3 strain of trachoma agents. The evidence supporting such a confirmation can be found in Tables II, III, and IV on pages 40, 41, and 42 respectively.

The successful adaptation of the IC-Cal-3 agent to the allantoic cavity introduced a more efficient propagation method for obtaining large quantities of this strain of agents. It is known that the recovery of the agents from infected allantoic fluid is much easier than that from infected yolk sac materials. Smears for microscopic examinations are easily prepared from allantoic fluid in contrast to the difficulties involved in preparing such from harvested yolk sac materials.

From the presented data on the process of propagation, it could be observed that both the ELD_{50} and the mean percentage death values behaved in a somewhat similar pattern.

The mean percentage death values increased sharply during the first few passages, then approached its maximum after the tenth passage. In spite of an increase in passages, the percentage of chick embryos died from the infection remained constant. This indicated that with a given inoculum size, a certain maximum percentage of embryos will die from the infection. In the present study, this maximum percentage had

never been observed to be 100%. In fact, the maximum percentage of dead embryos for any of the twenty passages performed was close to 60% of the inoculated population. Evidence supporting this observation can be located in Figure 2 on page 43.

In a similar pattern, the ELD_{50} values decreased sharply in the first few passages and the decrease terminated after the tenth passage. In spite of an increase in passages, the ELD_{50} values remained constant, indicating that there was a minimum time requirement for a certain inoculum size of infectious agent to cause death to the infected embryos. The evidence supporting such a conclusion can be observed from Figure 4 on page 46. The minimum time requirement was independent of the number of passages as long as the size of the inoculum was kept constant. The minimum time requirement observed in our study was around four days after the inoculation of infectious agents.

Future propagation project can be designed with consideration of these two observed phenomena.

The mechanism of adaptation of the chlamydial agent to a new host tissue remains unclear. Three possible explanations seem appropriate. One of them is the alteration of the ratio of E.B./R.B. particles. This alteration brings about an increase in the number of infectious E.B. forms, leading to more host cells infected, thus causing an earlier death and/or a larger death percentage of the inoculated population. The inadequacy of this explanation lies in the fact that all E.B. particles are derived from the R.B. forms. An increase in the E.B. population might be a direct consequent to an increase in the R.B. population (Bedson, 1936). The only situation that this explanation is valid is when individual R.B. particles produce more E.B. particles than they used to produce in

the former host tissue, and consequently, an increase of the E.B./R.B. ratio occurs. Whether there is a fixed number of E.B. particles that can be produced by one R.B. or not is unknown at present, and the validity of this explanation depends on the validity of the developmental cycle concept.

Another possible explanation is that the R.B. forms, which usually have a much lower infectivity than the E.B. forms (Litwin, 1959), becomes more infectious. The consequence of this increased infectiousness of the R.B. forms could also bring about the observed pattern of adaptation of the agents to the allantoic membrane. However, this explanation needs further studies on the infectivity of the R.B. forms.

Another likely cause of adaptation of the IC-Cal-3 agents to the allantoic membrane is an increase in the degree of virulence of the infectious E.B. particles in the mixed E.B. and R.B. population. The number of infectious particles would be increased in this population resulting in increased efficiency of infection by the same number of physical particles in a fixed size of inoculum.

In order to explain the observed phenomenon that the ELD_{50} values have a minimum time requirement for causing death to 50% of the injected population, we have to look at the phenomenon of adaptation from the following perspective : For any number of infectious E.B. particles, there is a finite number of host cells in the allantoic membrane available for infection. An increase in the number of E.B. particles would bring about an earlier death of the chick embryo since more host cells are infected and consequently destroyed. Because of the limited number of host cells in the allantoic membrane, the more host cells

killed in the process, the earlier the embryo will die, resulting in a decrease in time requirement for bringing death to the infected embryo. However, if all of the host cells are infected, an increase in the number of infectious E.B. particles cannot infect any more host cells because all of them have been depleted previously by prior infections. The chick embryo dies as a result of this maximum infection at the earliest time after the inoculation of the agents. This earliest time (approximately 4 days in our present observation) appears to be identical to the minimum time requirement of the ELD_{50s} for causing death to 50% of the infected population.

Investigations on the purification techniques applied to the two chlamydial agents have revealed several observations. The process of freezing and thawing and homogenization had ruptured the R.B. forms (Ross and Gogolak, 1957). Combined with differential centrifugation, the ruptured R.B. forms were removed, and only the E.B. particles which could withstand such severe physical treatments were detected in the electron micrographs.

Purification by density gradient centrifugation had not only shown that it is possible to remove contaminating host materials from the agent particles by molecular weight and density gradient separation, but also determined the hydrated density of the IC-Cal-3 agent to be approximately 1.11 at $4^{o}C$. Evidence demonstrating the ability of partial purification by density gradient centrifugation can be observed in Figure 5 on page 50.

Isoelectrofocusing when applied as a purification technique, appears to be the most efficient procedure and had proven its effectiveness over the density gradient centrifugation method which is

conventionally applied. Unlike systems that purify by differences in molecular weights, the isoelectrofocusing technique could produce a higher degree of resolution since it separates according to the net electrostatic charges on the surface of the particle. The degree of purity obtained by isoelectrofocusing was absolutely reproducible. However, the technique seems to have certain adverse effect on the infectivity of the agent resulting in partial inactivation as shown in Figure 8 on page 59.

From the experiments performed in this study, it seems that the two chlamydial agents, IC-Cal-3 and psittacosis DD-34 , both possess a net negative charge on their particle surface (resembling that of Gram-negative bacteria) and different isoelectric points. The evidence which led to this conclusion can be found in Figure 7 on page 55.

The finding that different chlamydial species possess different isoelectric points gives evidence that these agents are not only different in pathogenesis, host range, tissue tropism but also in the surface structure or components as well. Because of different surface charges, the isoelectrofocusing technique may be useful in the identification of different chlamydial species. However, more chlamydial agents have to be examined before a general statement could be made.

From these studies, it appears that chlamydial agents are probably attracted to the host cell membrane by electrostatic interactions resembling that of viruses. A possible explanation of the phenomenon of tissue tropism of these agents might be based on the differences in the electrostatic interactions between different agents and the corresponding specific host cells.

An attempt was made to disintegrate the agent particles and to analyse the isolated fractions for physiochemical and antigenic properties. The effect of sonication on the IC-Cal-3 and psittacosis DD-34 agents seems to follow a similar pattern. The first similarity was found in the studies of absorption spectra of the two agents. It was observed that the effective period of sonication was around one hour in which most likely the bondings or linkages susceptible to the applied sonication energy were all broken, releasing fragments which seem to be, spectrophotometrically speaking, proteins, nucleic acids, and carbohydrate-protein complexes. As to pinpoint the particular bonds or linkages affected by ultrasonic vibrations, the present report can only suggest that the most probable sites of denaturations are associated with the protein moieties within the structural framework of the agent particle. Evidence supporting this suggestion is provided by the increase in the percentage of denaturation of proteinaceous substances (as shown in Figure 12 on page 69), and large quantities of free amino acids detected in the amino acid analysis experiments as shown in Table X on page 80.

The second similarity was that in both agents, (as shown in Figure 12 on page 69) proteins were denatured by the process of sonication. The percentages of protein denaturation however differed between the two investigated chlamydial species. During the first hour of sonication, less than 5% of proteins were denatured in the IC-Cal-3 agents, but for the psittacosis DD-34 agents, close to 50% of the proteins were denatured. This observed difference might suggest that although the two species appear to be morphologically similar and

contain the same group-specific antigen, there are however species-specific structural differences between these two chlamydial agents manifested by the different isoelectric points of the two agents.

The third similarity lies in the observation that although ultrasonic vibrations seem capable of disintegrating chlamydial agents at the protein moieties, the sonic waves seem however incapable of further denaturing the released carbohydrate-protein complexes. This observation is derived from the fact that the ratio of 280/260 mu. for both agents remained practically constant after the first hour of sonication as shown in Figure 13 on page 71.

Therefore, on a theoretical basis, the process of sonication as applied to the two chlamydial agents, could disintegrate the agent particle releasing fragments containing determinant group(s) of certain antigen(s) as long as the structural linkages affected by ultrasonic vibrations donot constitute part of the antigenic determinant. This hypothesis is verified by the successful isolation of three MSI antigenic preparations obtained from sonicated suspension of IC-Cal-3 agents as shown in Figure 16 on page 78.

Using the MSI test, the antigenic and immunogenic properties of fragments released by sonication were investigated. The experiment on the kinetics study of the MSI test established an universal standard for carrying out the test at the optimal time post sensitization of the experimental animals.

The chemical nature of the fractions obtained from fractionation of the agent particles was studied by the Lowry test for protein content determination and by the Dubois test for carbohydrate content determination. Three fractions exhibiting different MSI activities were selected for amino acid content analysis. The results obtained

indicated that the types of amino acids detected in the three MSI antigenic preparations were very similar except for one or two differences. The sulphur containing amino acids cysteine and methionine were however absent in all of the three fractions. The results obtained from these biochemical studies suggested that the MSI antigen(s) is contained within a protein-carbohydrate-nucleic acid complex. Further purification and investigation into the structure of this antigen is suggested.

SUMMARY

A trachoma inclusion conjunctivitis agent was successfully adapted to grow on the allantoic membrane of the chick embryos. The various conventional purification techniques were re-examined and were compared to the isoelectrofocusing technique which was observed to be the best method. Two chlamydial agents were characterized by the same technique for their electrostatic properties. Both agents were found to have a net negative surface charge and different isoelectric points. The effects of sonication on the same two agents were investigated, and certain MSI antigenic preparatiosn were isolated from the sonicated agent particles. The biochemical nature of these antigens were studied and the MSI antigen was postulated to be contained in a protein-carbohydrate-nucleic acid complex.

The present study had made the following contributions:

Propagation by the allantoic cavity method was introduced as a very efficient method of propagation, and a complete set of guidelines for designing future propagation project was provided. Together with a detailed scheme of purification, a new purification and characterization technique; isoelectrofocusing, which had proven to be superior to the other conventional methods was introduced. The effects of sonication was studied, and a set of conditions for reference in designing future fractionation studies with chlamydial agents was provided. Three MSI antigens were isolated and their biochemical nature studied.

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