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IMMUNITY AND POLIOMYELITIS IN MAN: STUDIES USING AGAR ZONE ELECTROPHORESIS AND IMMUNO-ELECTROPHORESIS

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I. INTRODUCTION

In man and experimental animals, the immune mechanisms associated with viral infections appear to differ from those associated with bacterial infections.

It is recognized, at the onset, that to group all viral infections together is probably erroneous. It is the same as assuming that the mechanisms underlying disease produced by the pneumococcus are the same as those produced by the tubercle bacillus. That there does appear to be a difference between groups of viruses is well exemplified by the end-results of disease produced by the Enterovirus groups.

Poliovirus infections have a potentially lethal end-result. Coxsackie and Echovirus infections have invariably a benign end-result. This difference, within a group of viruses possessing almost identical physical and biological characteristics, appears of paramount importance. It suggests that the difference is more closely allied to the immune mechanisms of the host rather than to temporal changes occurring in the infecting viruses. Additional reasons for this statement will be given later on in this text.

Furthermore, it is known that specific antibody can combine with virus and neutralize its infectivity. This can be demonstrated with relative ease by both *in vitro* tests and *in vivo* tests using mainly laboratory animals. Equal importance is placed on such tests when considering infection caused by any of the three groups of Enteroviruses. However, such an assumption may be quite misleading for it is well established that man is the only natural host for the Poliovirus types but that some animals are natural hosts for many Echovirus and the majority of Coxsackie virus types.

Antibody is something tangible and can be conveniently measured. This, together with the fact that it combines with, and neutralizes the infectivity of, a specific virus have been the reasons why humoral factors have been considered the predominant mechanism responsible for viral immunity. That this may not be so, has been impressed upon us for the following reasons: -

(1) The failure to demonstrate circulating antibodies in some individuals following a natural or artificial virus infection, or following injection of killed virus vaccines. Such individuals appear to be no more susceptible to virus infections than those possessing circulating antibodies. These individuals have normal levels of gamma-globulin; can produce antibodies following bacterial infections; and show normal resistance to such bacterial infections. Examples of such have been seen amongst our own laboratory technicians, nurses and aides at the Municipal Hospital, Winnipeg, during two poliomyelitis epidemics, and the author's own immune status in regard to circulating antibodies while working with active cases of infection during two poliomyelitis epidemics.

(2) Individuals suffering from diseases in which dysproteinaemia is a feature often lose their ability to produce a detectable level of both bacterial and viral antibodies. Such individuals appear to be more susceptible to bacterial and fungal infections. Examples of dysproteinaemia in which this defect has been commonly reported include multiple myeloma, macroglobulinemia, cryoglobulinemia, or

combinations of the three.[1]

(3) A case of Landry-Guillane-Barre disease investigated in our laboratory.[2] Following previous adequate antigenic stimulation with Salk vaccine, the patient, a ten year old boy, responded with only a low titre of circulating antibodies to type 1 Poliovirus.

An Echovirus type 6 was isolated from a sample of his cerebrospinal fluid and was considered a contributory factor in the etiology of the syndrome. Only a very low titre of Echo 6 antibodies were detected in the boy's convalescent serum specimen.

Paper electrophoresis revealed an essentially normal gamma-globulin level. The α-1 fraction was missing, but this finding was later shown to be possibly an artefact. Although paralyzed this boy eventually recovered completely and while possessing an essentially normal gamma-globulin level obviously suffered from some immunological defect, as yet undetermined.

(4) Experimental infections of human volunteers with vaccine strains of Poliovirus show no relationship between the ability to subsequently re-infect these individuals, and the level of circulating antibodies they obtain following infection.[3]

(5) Cases of hypogammaglobulinaemia, who possess marked gamma-globulin deficiency, show an increased susceptibility to bacterial infections, particularly those caused by gram-positive cocci. Yet these cases are said to show normal viral resistance, can be vaccinated, exhibit a normal take, but do not develop circulating antibodies following such vaccination.[4]

(6) The variety of responses obtained to vaccination by the

Vaccinia virus under certain defined conditions.[5,6] These are as follows: -

A) <u>Reactions Exhibited by Hypogammaglobulinaemic Patients</u> - may result in -

i) A normal take but a failure to develop detectable circulating antibodies.

ii) A normal take when given vaccinia-immune gamma-globulin prior to vaccination.

B) <u>Reactions in Individuals with Normal Gamma-globulin Levels</u> -

i) The occurrence of generalized vaccinia in children with no abnormal skin lesions who slowly develop circulating antibodies and who eventually recover.

ii) The occurrence of generalized vaccinia in children who have eczema - shown by two types of reaction: -

(a) development of antibody with either recovery or death

(b) no development of antibody and when given gamma-globulin passively may either recover or die.

C) <u>Experimental Vaccinia Infections in Animals</u> -

i) Corneal tissue, taken from rabbits immune to vaccinia and possessing circulating antibody, when grown in tissue culture has been shown to be just as susceptible to vaccinia infection as corneal tissue taken from a non-immune rabbit. This suggests that circulating antibody is the predominant mechanism of protection in this instance.[7]

ii) Following administration of Vaccinia virus animals may develop complement-fixing, agglutinating or precipitating antibodies yet fail to produce any neutralizing antibodies.[8]

(7) Hypogammaglobulinaemic patients appear to possess immunological deficiencies as demonstrated by challenge with various bacterial antigens, yet they do not seem to have an unusual susceptibility to tuberculosis.

However, patients with Hodgkin's Disease often exhibit rapidly progressive tuberculosis. Furthermore, patients with Hodgkin's Disease often fail to exhibit allergic reactions of the delayed type.

The generalized response of the body to viral infections includes certain histological features similar to those seen in diseases possessing a delayed allergy component, for example, tuberculosis. These features appear to be the body's response to tissue necrosis or destruction. These facts are interesting, in as much as they suggest that delayed allergy may be a fundamental defence mechanism which is independent of, or may precede, the production of circulating antibody.[9]

(8) The results of Poliovirus antibody surveys in the literature support the contention that there is no consistent or proportional relationship between infection, or resistance to infection, and the level of detectable circulating neutralizing antibody.[3,10,11]

It remains that measurable levels of neutralizing antibody and the actual immune status of an individual are often at variance. This is best seen in the phenomena associated with the various facets of Poliovirus infections in man. It was, therefore, felt that an investigation into this matter, using Poliovirus infections in humans as a model, would be advantageous for the following reasons: -

(1) The potentially lethal end-result of Poliovirus infection in children and adults is an ever-present problem of the first magnitude to all groups in our society.

(2) Oral live attenuated Poliovirus vaccine was being made available, and actually being administered to, children in Winnipeg. This advent provided an ideal opportunity to investigate actual human infection with a live virus. This represents an example of a virus infecting its natural host. This eliminates possible false assumptions which may arise due to the necessity of transposing results obtained from the experimental infection of animals, to that of humans.

(3) The fact that these children were all vaccinated with killed virus vaccine prior to oral vaccine feeding provided a further opportunity to compare in a limited manner the different manifestations, if any, between two different methods of Poliovirus immunization.

(4) From our own work in the laboratory, and a perusal of the literature, it appeared that the key to the basic mechanisms of immunity could very well be found in the differences seen between resistance to virus diseases and bacterial diseases in general. In addition, the differences seen between the potentially serious, or even lethal, end-result of Poliovirus infections on the one hand, and the invariably benign end-result of infections caused by the biologically similar Echo and Coxsackie groups of viruses on the other hand, provide an even more subtle distinction worthy of investigation.

(5) The laboratory tests used in Poliovirus antibody estimations

measure the ability of antibody to neutralize actual virus infectivity. This eliminates the inconsistencies known to occur between neutralizing, complement-fixing, precipitating, and agglutinating antibodies. This proposed study was thought, also, to provide an opportunity to investigate the relationship of these different types of antibody to one another.

This present study arose as a consequence of the above considerations.

II. REVIEW OF THE PERTINENT LITERATURE -- PART I -- THE SUGGESTED MECHANISM OF POLIOVIRUS INFECTION IN MAN

A consideration of the known facts concerning the suggested mechanism of Poliovirus infection in man, together with a comparison of the immune phenomena associated with natural infection, artificial infection with attenuated live vaccine virus, and the administration of killed virus vaccine, may unravel some of the apparent inconsistencies of the role of circulating antibody in Poliovirus immunity.

This is a complex problem. The experimental and epidemiological evidence used to support the facts stated in the following mechanism are of considerable magnitude, but it is felt that a proper understanding of the problems involved cannot be obtained unless these are mentioned. It is the pious hope, rather than belief, that this will not be boring to the reader but may even be of some interest.

In an attempt to simplify the problem a schematic diagram of the suggested mechanism of Poliovirus infection is shown in Figure 1.

The experimental, epidemiological and histopathological studies resulting in the statements made concerning this mechanism will be enumerated and stress will be laid upon points about which there is some contention.

Much of the fundamental experimental work has been done by A. B. Sabin. From necessity, a considerable proportion of this work had to be confined to higher simian animals, and almost every point suggested by Sabin has been questioned at one time or another by other reliable workers. It is now clear that much of the disputed material has been the result of the use of different animals, by different workers, in their experiments. Due to the recent widespread administration of live oral virus to humans, the position has now been somewhat clarified, most of Sabin's original work has been vindicated, and the following facts now appear to be established.[3,12]

(1) The pharyngeal mucosa of the chimpanzee is more susceptible to infection by Poliovirus types than its intestinal mucosa.

(2) The intestinal mucosa of the chimpanzee is more susceptible to infection than that of cynomolgous monkeys.

(3) The intestinal mucosa of man is more susceptible to infection than that of the chimpanzee.

(4) In both monkeys and chimpanzees the neurones of the brain stem are more resistant to infection than the neurones of the spinal cord.

(5) The neurones of the chimpanzee are more resistant to infection than those of the monkey.

These facts are important because a considerable amount of the evidence used to describe the probable sequence of events following Poliovirus infection in man has been transposed from animal experimentation.

Figure 1 shows the suggested mechanism of natural infection by the Poliovirus in humans. The usual portal of entry of the virus is the mouth. All workers seem to be in general agreement with this statement. It appears that the virus does not multiply in the epithelium of the buccal mucosa, gums, tongue or nasal mucosa but readily multiplies in the epithelium of the throat.[3,12]

It is of interest that pharyngeal multiplication cannot be produced in volunteers unless at least one million tissue culture infective doses of the virus are given.[3,13] Such a high dose can rarely be transmitted during natural infection, and yet in 80 per cent of Poliovirus infections the virus can be isolated from the throat during the first week of the illness. It is contended that this may be due to the ability possessed by some strains of virus to infect the pharyngeal tissues more easily than others. There is no absolute proof that this is so, but it does, however, remain a definite possibility.

During the first week after the onset of symptoms an individual is considered to be capable of transmitting the virus to others. Because of this, virus of pharyngeal origin is thought to be the main cause of natural transmission. However, studies with volunteers who were fed attenuated virus show that natural transmission can occur quite effectively, even when the source of the virus is not pharyngeal.[14,15] These studies have also shown that orally administered virus may reproduce well in the intestinal epithelium but not at all in the pharynx, in persons possessing a high homotypic antibody level.[3] This suggests that the surface cells of the pharynx are protected by antibody present in pharyngeal secretions much in the same manner as it has been shown that pharyngeal secretions containing antibody can protect against influenzal infection.

Twenty-four to forty-eight hours after ingestion of the virus, it starts to multiply in the intestines. The cells in which this multiplication takes place are said to be those of the lymphoid tissue, which is present in considerable quantity along the whole length of the intestines.[16] Sabin, however, says that the virus multiplies first in the superficial cells of the mucosa and then may further replicate in the regional lymphoid tissue.[12] This again is a contentious point and from the experimental evidence has certainly not been proven beyond question.

Following intestinal multiplication the virus may then spread, via the blood stream, to the central nervous system. This appears to be well supported by experimental evidence which involves the detection of viraemia at appropriate times during the infectious cycle. It has been well established that spread from the gut to the central nervous system can be prevented by an adequate level of circulating antibody. (This stage of the infection may be a crucial point in many individuals) The final quantity of virus reaching the central nervous system might possibly be influenced by one or both, of two factors; (a) the amount of virus present in the blood during the viraemic stage, and/or (b) the speed with which the infected individual can produce an adequate level of circulating neutralizing antibody.

Bodian has stated that the virus spreads directly through damaged blood vessels in the intestine or throat and thus reaches the central nervous system directly, and that the main function of circulating antibody is to prevent inter-neuronal spread.[17] This, however, does not seem likely because during the Francis Field Trials with Salk vaccine and pooled gamma-globulin, the administration of gamma-globulin at an optimal time should have resulted in an all-or-none phenomenon in regard to central nervous system involvement and subsequent paralysis. The field trials did not show this.[18] No difference was found in the incidence of non-paralytic poliomyelitis between tests and controls. There was, however, significantly less permanent paralysis and significantly more minor and transitory paralysis in the treated cases than in the untreated controls. It has been assumed, therefore, that the ability of an infecting virus strain to produce a high level of viraemia is of great importance. If the virus reaches the central nervous system in sufficient quantity it may cause destruction of many neurones and subsequently produce permanent paralysis. Attenuated strains of virus are known to exist which can multiply extensively in the alimentary tract, but produce no significant viraemia.[19] The amount of virus reaching the central nervous system would appear, therefore, to be extremely important. Although Sabin and other workers tend to incriminate a difference in virus strains as being responsible for extensive viraemia, this point is highly contentious and will be considered later when the characteristics of infecting virus strains will be discussed.

Circulating neutralizing antibody in the blood appears to be responsible only in part for the immunity resulting from a natural infection. The other is most likely a cellular immunity. In some instances the appearance of circulating antibody is extremely rapid, being detectable one to two days after the onset of symptoms.[20] This may be important, and may be a reflection of the host's immune mechanism rather than a reflection of the virus characteristics. During, or immediately following, the viraemic stage there is considerable belief that tissues other than the gut and central nervous system can support the secondary reproduction of infecting virus.[21] This point is very contentious. Obviously during the viraemic stage, virus is present in all vascular tissues in large quantities. However, it has been shown by animal experimentation using monkeys, that following intra-muscular or subcutaneous injection of live virus, secondary viral reproduction appears to take place in the regional lymph nodes. This has been determined by serial time estimation of the virus titre in these tissues. This is contentious. It does not confirm virus reproduction in the nodes, as reproduction may occur at the primary site of inoculation, but because of drainage to regional nodes appear there is higher concentration.

Spread to the central nervous system proper appears to be via regional nerve ganglia. This, again, has been shown mainly by animal experimentation.[21] This is used to explain the apparently greater frequency of paralysis occurring in the same limb in which an incompletely inactivated virus vaccine has been injected.

If the virus reaches the central nervous system in sufficient quantities then neurones can be destroyed. The extent of this infection, and the number of neurones destroyed, determines the end-result of the infection. If a large number are destroyed severe permanent paralysis or even death may result. If a fewer number of neurones are destroyed a single muscle group may develop permanent weakness but the patient recovers and is left with only slight or moderate disability. If only a few individual neurones are destroyed then their corresponding muscle fibres will degenerate but may be so few in number as to cause undetectable clinical manifestations. It is at this point in the infectious cycle that Bodian feels circulating antibody has its greatest effect. In other words, if sufficient antibody is present it will stop inter-neuronal spread.

A broad summary of the process may be construed as follows: -

(1) Virus enters the host cell through the mouth.

(2) Virus replication may then take place in the tissues of the alimentary tract. If this occurs, it does so invariably in the intestinal tissue and often in the pharyngeal tissue. The actual cells in which the virus replicates have not, as yet, been adequately determined. There is little doubt that free existing circulating antibody can modify pharyngeal infection. There is some, but less definite, evidence that such antibody can modify intestinal infection. There is much to suggest that intestinal resistance to viral replication is due to a factor or factors other than antibody and as such represents a form of cellular immunity.

(3) Following intestinal multiplication the virus may enter the blood stream. Circulating antibody may exert its greatest protective effect at this stage.

(4) The virus then may, or may not, reach and infect central nervous system cells. Whether or not it causes minimal, moderate or wide-spread destruction of neurones again appears to depend on the presence of, and the level of, neutralizing antibodies in the blood and interstitial tissue fluids.

The above is the most likely and the most usual mechanism of Poliovirus infection in man. Other mechanisms may exist but are probably uncommon. An example of such may be those due to initial introduction of the virus parenterally into tissues other than the alimentary tract.

It may be seen that following natural infection both antibody and tissue resistance of the alimentary tract appear to be concerned with the resulting immunity, and that circulating antibody is mainly effective in preventing spread from the site of primary infection to the central nervous system and that it may have some effect in modifying a subsequent re-infection with the same virus type.

In other words, the presence of circulating antibody can influence the progress of the infection once it has been established or can influence the progress of a subsequent re-infection, but appears to play very little or no part in the prevention of the initial infection.

With these facts in mind it may now be advisable to see how various facets of the process may be modified by (a) prior natural infection, (b) artifical vaccination both with live and dead virus, (c) variations in factors concerning the host, and (d) variations in factors concerning the infecting virus.

III. FACTORS POSSIBLY INFLUENCING THE COURSE OF THE INFECTIOUS PROCESS

In general it may be considered that for a particular infectious agent its ability to cause disease, in its natural host, is dependent mainly on variations in the following: -

(1) The characteristics of the infecting agent.

(2) The extent and mode of the communicability of this agent.

(3) The characteristics concerning the host which are both general and specific.

Virulence is concerned mainly, but not entirely, with the characteristics of the virus itself. Poliovirus virulence, for the purposes of this discussion, may be effectively defined as the ability of the virus to produce disease in the central nervous system of man.

Factors concerning the characteristics and communicability of the infecting virus, and factors concerning the defence mechanisms of the infected host, will now be considered.

Factors Appertaining to the Virus -

It has been shown that there are great variations in naturally occurring strains of each type of Poliovirus. This has been shown experimentally[22] by Sabin, on epidemiological grounds[23] by Howe, and on the results of studies involving the recent mass feeding with oral live vaccines.[3] The more virulent strains (as demonstrated by animal inoculation, and the paralytic rate in human epidemics) do not produce paralysis in all individuals infected. There is no definite evidence that new antigenic strains of virus have emerged over the last few years. A highly neurotropic virus strain can be avirulent after oral administration because it cannot reproduce in the intestinal tract.

Some strains fed orally multiply easily in the gut, but if injected parenterally fail to multiply in regional lymph nodes. Naturally occurring strains differ in their capacity to produce diseases, and there is poor correlation between monkey neuro-virulence tests of these strains and their apparent virulence in man.[3,13]

Virus strains from humans with inapparent infections usually are of low monkey neuro-virulence. Yet, strains from severe epidemics show no consistent difference in regard to monkey neuro-virulence from those strains isolated from patients with inapparent infections. This was shown by the fact that chimpanzees inoculated intra-muscularly did not develop viraemia, and did not excrete virus in the faeces. It was considered that, as this virus was unable to reach the central nervous system, it was, therefore, probably harmless.

A similar phenomenon was shown by Evans who used one highly virulent strain obtained from the Chesterfield Inlet outbreak of epidemic poliomyelitis, and other strains, which were avirulent epidemiologically, and which were obtained from healthy Louisiana families.[24] By inoculating the skin of monkeys it was shown that the Chesterfield Inlet strain readily multiplied and spread to the rest of the body. One of the Louisiana strains, although avirulent epidemiologically, also multiplied and spread readily.

In an attempt to correlate neuro-virulence for monkeys and humans with <u>in vitro</u> characteristics many different types of "marker" tests have been developed. These markers consist of laboratory tests that reveal certain characteristics which apparently are a genetically determined function of different virus strains. These markers are as follows: -

(1) "D" Marker[25] - This was the earliest perfected marker. A Poliovirus strain of low virulence (L.Sc. - an attenuated strain derived from its virulent parent Mahoney strain) failed to produce, or only produced small and slowly developing plaques of degeneration in cell cultures under an agar overlay. This was considered to be a function of the bicarbonate concentration of the nutrient media. Since this time, however, some work done by the Russians has shown that the sodium ion is the real factor and the "D" marker can be reduplicated by varying only the sodium chloride concentration.[3] It is considered to be useful as a genetic marker of stability. Tests on natural strains show that those producing paralytic disease are usually "D" positive. However, strains from silent infections, i.e. clinically symptomless, can be either "D" positive or "D" negative.[3,26] There is no indication that epidemiologically mild strains are different from naturally occurring virulent strains in regard to the "D" marker.

(2) "M.S." Marker[27] - This is a marker which is determined on a stable continuous line of monkey kidney cells and is shown by whether the virus will or will not grow on these cells. Whether they grow or not, differentiates the virulent parent strains from their laboratory sub-strains, which have been attenuated by selection and propagation in the laboratory.

The "M.S." marker also does not show any difference between naturally occurring strains isolated from paralytic cases and those obtained from symptomless carriers in communities free from clinical poliomyelitis.

(3) <u>"T" Marker</u>[28] - Strains able to multiply rapidly and produce high virus yields at 40°C were virulent when injected into monkeys. These are designated "T" positive. Those which could not grow rapidly at 40°C were usually of low virulence. These are designated "T" negative.

(4) <u>"E" Marker</u>[3] - This is a marker which differentiates virulent from attenuated strains by differential absorption and elution of these strains on cellulose columns.

(5) <u>The Antigenic Marker</u>[29] - Within some Poliovirus types, strains exist which differ in the rate at which they are neutralized by homologous and heterologous antisera. A modified form of this test has been devised by Wecker and consists simply of incorporating specific antiserum in an agar overlay following infection of an underlying monolayer of monkey kidney cells by the virus strain under test.[30] The average diameters of the plaques of cytopathogenic effect produced are taken as the indication as to whether the virus is virulent or not.

Numerous other markers have been described in the literature but these have not been adequately investigated and at the moment are not yet accepted. A few of these are as follows: -

(1) Absorption, at an acid pH, and elution at an alkaline pH, of virus strains on aluminum hydroxide gel.[3]

(2) Virus strains shown to have a cystine dependence and others which are inhibited by the presence of cystine.[3]

(3) Bovine and equine sera contain certain inhibitors of virus infectivity. The difference between various strains of virus in regard to their ability to infect, in the presence of these serum

inhibitors, apparently gives some reasonable differentiation.[3]

(4) A human marker has been described which shows that certain strains produce lower plaque counts on human tissues than on fresh monkey kidney tissues.[3]

It can be considered, however, that no individual _in vitro_ laboratory marker by itself is a good criterion of virulence. The best criterion of virulence is still the monkey virulence test. From this it does not seem unreasonable to assume that the initial characteristics of an infecting strain of Poliovirus, as determined by animal and _in vitro_ laboratory markers, show no regular or consistent correlation with the ability of the strain to produce, or not produce, paralytic disease.

Therefore, the properties of the virus itself initially cannot account for the major differences between those persons who suffer paralysis following infection and those who do not.

A further possibility is that disease of the central nervous system occurs only in persons in whom the virus rapidly mutates to greater neuro-virulence early in the stage of intestinal infection.

This is considered unlikely for the following reasons:[24]-

(1) Virus obtained from the central nervous system of monkeys possesses no greater neuro-virulence than virus obtained from the faeces of monkeys.

(2) Infections of cynomolgous monkeys caused by feeding them infected brain tissue fail to cause paralysis.

(3) Many physiological states and pathological conditions appear to increase the susceptibility of infected persons to paralytic

disease. These same conditions have no effect on mutation, as far as we know. Examples of these are tonsillectomy, pregnancy, fatigue, and parenteral injections.

(4) Certain undetermined environmental conditions increase the incidence of cases of paralytic poliomyelitis. Examples of some of these are as follows: -

A) Paralytic poliomyelitis occurs with a higher incidence amongst adults living in households containing young children, than amongst adults living in households with an equivalent number of adults.[31,32,33,34]

B) One type of Poliovirus causes a high paralytic rate in one part of a community, spreads just as effectively in another part, and yet here causes little paralysis. This was based on a study of an epidemic that occurred in a collection of identically housed individuals. There was a high paralytic attack rate in one area and a much lower rate in another, yet similar, area. Apparently, the difference in paralytic rate was not due to prior antibody status.[35]

C) Paralytic attack rates tend to be higher in relatively small and sharply defined populations. This is unlikely to be due to a genetically determined susceptibility. For example, the measles epidemic in Fiji in 1875 killed one-quarter of the population. It would be expected that subsequent severe epidemics of measles would occur until selection for measles resistance had occurred. Apparently, this did not happen.[24]

<u>Factors Appertaining to the Host</u> -

These may be divided into two main groups, cellular and/or humoral.

(1) <u>Cellular Resistance</u> - This can be general or local.

A) <u>General</u> -

i) Age and Sex

ii) Nutritional status

iii) General health - i.e. factors affecting, or predisposing to, disease; or the presence of coincidental disease; or a change in physiological status, e.g. pregnancy.

iv) Racial origin

v) Temperature of environment

B) <u>Local</u> -

i) Site and mode of initial infection

ii) Type of cellular histology and the physiological state of the infected cells locally. Presence or absence of specific receptors, etc. on these cells.

iii) The status of circulating white blood cells

(2) <u>Humoral Resistance</u> - This may be divided into two parts, specific and non-specific, and may be defined as follows: -

A) Specific humoral protective substances are specific protective (neutralizing) antibody.

B) Non-specific humoral protective substances are substances which are not specific antibody. They represent part of the various non-specific mechanisms which constitute the first line of defence in immunologically inexperienced animals. These substances may enhance the action of a specific antibody or act entirely on their own.

It may be considered that the inherent resistance of an animal is almost entirely dependent on the ability of its non-specific defences, both cellular and humoral, to combat parasitic invasion until such time as it can produce specific protective antibody.

It appears that the crux of this whole problem, as applied to the disease of poliomyelitis in man, lies in the above statement. A comparison of the differences between the immune reactions occurring in man following natural infection, artificial infection with live attenuated vaccine and immunization with dead Polioviruses further accentuates the importance of the problem. This aspect will now be considered.

IV. THE IMMUNE RESPONSE RESULTING FROM DIFFERENT METHODS OF ANTIGENIC STIMULUS

This is presented in tabular form. (See Table 1). Much of the evidence from which it has been compiled has been discussed previously. The remaining evidence has been obtained from reports of experimental studies using the live oral vaccine. These are described in full in the Proceedings of the Second Conference on Live Poliovirus Vaccine, held in Washington, D.C., in June, 1960.

Table I demonstrates -

(a) that the antibody level following antigenic stimulus may be high, low, or absent

(b) that the antibody produced by killed virus does not usually protect a person from primary infection of the intestine

(c) that antibody produced by live virus may only be a reflection of an associated cellular resistance and, by itself, is unable to prevent intestinal infection

(d) that in the majority of instances, live virus infection results in life-long immunity, yet many of these cases show no detectable levels of antibody.

Why is it then that some individuals develop serious disease following an intestinal infection and others do not? Antibody levels are no reflection of this difference. Yet, a certain level of circulating antibody is known to modify the degree of central nervous system involvement and can prevent paralysis.

These discrepancies may be explained in a number of ways. The fact that a quantity of inadequately neutralized virus may appear in the blood may theoretically result from any of the following: -

(1) A defect in the speed of production of qualitatively normal antibody

(2) A defect in the quality of antibody produced

(3) An unusually large amount of virus rapidly entering the blood stream. This may be the result of either -

(a) a large number of intestinal cells initially infected by a large number of virus particles

(b) a moderate number of intestinal cells initially infected by a moderate number of viral particles but which are capable of rapidly replicating and infecting adjacent cells

(4) The absence of an, as yet, hypothetical factor or factors capable of restraining virus spread from the appropriate intestinal cells, until such time as antibody can reach an adequate neutralizing level. This factor or factors may be humoral, completely within the challenged intestinal cell, be a secretory product of the cell, or be one of its products of disintegration.

The mechanism is unlikely to be a defect in the speed of production of qualitatively normal antibody. This is because numerous cases have been reported of paralysis occurring in individuals who have been fully Salk vaccinated, and who have possessed a pre-disease antibody titre of considerable magnitude. Any subsequent antigenic stimulus should result in an accelerated immune response and consequent protection.

Furthermore, volunteers who were fed attenuated virus have given evidence of intestinal infection, as shown by excretion of virus in considerable quantities and for some weeks, and yet have produced no detectable antibodies.

That a large number of cells may be infected and produce a large number of virus particles is hardly likely to be the explanation. This state of affairs is often produced by the administration of oral live vaccine to volunteers, and yet often causes a more adequate and more rapid antibody response in these individuals.

The existence of some cellular factor involved in resistance is a definite possibility. A defect in the quality of the antibody produced also is a definite possibility.

Can these two possibilities be consistent with one another? Any suggested mechanism incorporating these two should be able to explain the other discrepancies already noted. This might be possible if the following hypothetical situation existed: - "The presence of a substance at the cellular level, which is capable of combining with the virus and inhibiting its further infectivity for a short period of time." This substance might be expected to have the following characteristics: -

(1) It is most likely produced by the cells infected

(2) It should be present in an amount directly proportional to the amount of virus reproducing in those cells

(3) It should be specific in its reactivity only for the types of virus which are capable of infecting that particular cell, under conditions of natural infection.

(4) Appertaining to (3) it should possess a protective ability which is most marked for, or exclusively for, the histological

type of cell from which it came.

(5) It should be of such a size and possess such physical characteristics which will enable it to appear in detectable quantities in the circulating blood.

(6) Its action should be that of combining loosely with the virus following reproduction of the virus initially in cells of the alimentary tract. It should be detectable for such a period of time during which the normal circulating neutralizing antibodies have been formed to a detectable level.

The possible nature of this substance can only be a conjecture. It could well be a cell surface receptor substance and as such may be either lipoid, polysaccharide, or protein in nature. It may or may not be heat labile. If one is to hazard a guess it might be suggested that this substance is heat stable. This is so because, if this factor is in any way associated with the qualitative differences often seen between neutralizing antibodies in the laboratory, the fact that such sera are almost invariably inactivated at 56°C for half an hour, would favour its being heat stable.

The reader's first impression of this hypothesis may be that it is hypothetical to the point of being ridiculous. This may, indeed, be so. However, a consideration of the following points may engender some glimmer of faint hope for the writer's redemption.

It has been observed that -

(1) Neutralizing antibodies appear to vary in their ability to inhibit infectivity of virus. This statement is based on observations that antibodies first produced following infection with live

virus produce a virus-antibody complex which is very easily reversible.[43,44,45,46] This has been observed from the use of two different kinds of neutralization tests on cell culture.

In the cytopathogenic-inhibition (C.P.I.) test, neutralization of the infectivity of a virus can often be seen to occur for two to three days. This neutralisation may then disappear completely at the four to six day reading. Similarly, neutralization by the serum may be evidenced by an initially high titre seen on the second day reading and yet be very greatly reduced by the fourth to sixth day reading. This is taking into consideration the fact that the virus titre itself may fall and that non-infective viral particles may utilize neutralizing antibody.

This "low-avidity" type of antibody shows up most easily in the metabolic-inhibition, or pH test. This test utilizes the fact that an actively metabolising, undamaged cell in culture will produce an acid pH as shown by the colour of an indicator incorporated in the media. A cell damaged or killed by virus action produces a less acid media.

Using this test it can be shown that these low avidity antibodies appear first following an infection and that during the convalescent period they may remain at the same level or fall, at the same time "high-avidity" neutralizing antibody has started to rise.[47]

Attenuated strains of test virus show low-avidity antibody the best because these strains multiply poorly in an acid pH.[25]

These points are of fundamental importance when the following facts are considered: -

(a) Most surveys of neutralizing antibody levels utilize the C.P.I. test.[48]

(b) Some adults possess low-avidity type antibody alone which can be detected only by the pH test.

(c) Persons possessing antibodies prior to a subsequent contact with virus antigen show a rise only in the high-avidity type antibodies.[3]

(d) A comparison of the types of antibody produced following vaccination of non-immune persons with either live virus or inactivated virus shows that in non-immune individuals the first type of antibody produced cannot combine irreversibly with the virus. It also shows that the low-avidity antibodies formed in non-immunes are heterotypic and can react with all three types of Poliovirus - a fact not demonstrated so completely by the high-avidity type antibody.[3] This latter point is of some importance as it shows that the detection of these different antibodies is not merely a reflection of the sensitivity of the two tests. If this were so sera containing high titres of high avidity antibody should always show some level of low-avidity antibody following a natural infection. This is not so.

(2) Other laboratory tests capable of visualizing the Poliovirus antibody combination are the complement-fixation and the precipitation tests. This latter can be directly visualized or can be detected by agar diffusion methods.[49] Neither of these tests have shown a consistent reliability in regard to the estimation of antibody levels.

In the complement-fixation test, in most instances, quite marked crossing has occurred between all three types of Poliovirus and also considerable crossing between and among the Coxsackie and Echovirus groups.

The antigen most often used in this test is the supernatant fluid from infected human or monkey kidney cells which have subsequently been disrupted by some physical means. This suggests that at least part of the antigen thus produced contains a product of cell disintegration, and that this may be responsible for its heterotypic action.

Furthermore, it has been shown that the degree of complement-fixing antibody response in volunteers who were fed the live oral virus is related fairly constantly to the titre of virus excreted by that person, and therefore presumably to the number of intestinal cells infected.[50] This is not so with the neutralization tests. This is also not so with antibody which has been produced by Salk vaccination in non-immunes.[51] It has always been considered that complement-fixing antibodies rise and fall early following disease and that neutralizing antibodies rise slowly later in the convalescent period and then remain for some considerable time. There appears to be some justification for this statement in the case of Poliovirus infections, but the literature is still extremely confused on this point.

(3) It has been shown that the products obtained from disruption of HeLa and monkey kidney cells, both of which were susceptible to Poliovirus infection, were capable of inactivating Poliovirus infectivity, in much the same manner as it is inactivated when it is absorbed onto intact susceptible cells. Disruption of cells insusceptible

to Poliovirus infection produces no such factor.[52]

This factor was shown to have the following characteristics: -

(a) It was insoluble.

(b) It appeared to be unassociated with cell nuclei.

(c) It was inactivated by fat solvents, trypsin, and by heat at 37°C for 24 hours and at 56°C for half an hour.

(d) It was specifically antigenic, i.e. the antiserum to this factor, when added to cells prior to the virus being added, prevented the virus from being absorbed. If applied afterwards, however, it did not prevent virus infectivity.

(e) Lipase and the receptor-destroying enzyme of vibrio cholera did not affect the factor.

This adds support to the contention that the product of a disrupted cell can neutralize the absorption of a virus onto other cells. These and other facts suggest that this factor is cytoplasmic in origin and is a lipo-protein, possibly in the nature of a cell surface receptor.

That this has been shown to be insoluble by experimental study does not detract from the possibility that it may be a factor in virus neutralization following an initial infection, particularly an infection of the intestine.

(4) The existence of another interesting group of substances, intimately concerned with viral immunity at a cellular level. These are the interferons. They are a group of substances which have been shown to protect tissue culture cells against a wide variety of viruses. They are important and will be considered at some length.

The first of these substances described was produced by adding inactivated Influenza virus to whole-chick chorioallantoic membranes suspended in simple balanced salt solutions.[53] A similar substance has been described which is called "virus inhibitory factor". This appears to possess characteristics almost identical with that of the originally described interferon and is probably the same substance.[54]

It has been known for a long time that cells infected with viruses in tissue culture can reach a state of chronic infection even without antibody being present. This appears to be an almost symbiotic state which, in many instances, confers on the cells complete immunity to subsequent infection by the same virus, and also to primary infection by a large variety of different viruses.

It has been shown that interferon is produced at a late stage of virus infection, using <u>in vitro</u> experiments in cell culture with the vesicular stomatitis virus, tick-borne Encephalitis viruses, the Parainfluenza viruses, Poliovirus and some Coxsackie viruses.[55,56,57] The same has been shown by <u>in vivo</u> experiments with Vaccinia virus in rabbits,[58] with Influenza virus in fertile hens' eggs,[59] and with Influenza virus in live mice.[60]

Of immediate interest to us is the interferon produced by the Coxsackie and Polioviruses.

The pertinent characteristics of the interferons will now be described.[61,62,63]

A) <u>Production</u> -

i) They can be produced by either live or inactivated virus.

ii) They can only be produced by living cells left in contact with a virus for a period of time.

iii) They occur only at a late stage in the infection of cells. This has been shown by the differential titre obtained between virus and interferon, in regard to the rate at which each is produced by infected allantoic cells of chick embryos.

B) Activities -

i) They will protect cells against a small dose of infecting virus, either in culture or in living animals, if given prior to, or at the same time as, the infecting virus.

ii) They will protect cells against a larger dose of infecting virus, either in cell culture or in living animals, only if given prior to the infecting virus.

iii) They will not combine with the virus, in any way, *in vitro*.

iv) They will best protect cells against infecting virus if these challenged cells are of the same animal origin as those from which the interferon itself was produced. They will protect other cells but not to such an extent.

v) Certain viruses are more resistant than others to the action of the interferons. In general the small Encephalitis viruses are very sensitive, the Influenza and Pox viruses are moderately sensitive, but Fowl Plague and the Herpes Simplex virus are relatively resistant to its action.

vi) An interferon is not a self-replicating particle but once it enters a cell it protects that cell for most of the cell's life, unless the cell is caused to reproduce quite rapidly. This suggests that the interferon is "diluted out" over many cell generations.

C) Properties -

i) They appear to be proteins as shown by the following: -

(a) Pepsin destroys their activity rapidly.

(b) Trypsin partly destroys their activity.

(c) They are precipitated by saturated ammonium sulphate solution.

(d) Their activity is destroyed by shaking with amyl-alcohol-chloroform mixtures.

(e) They absorb to bentonite completely, but only 25% can be eluted from bentonite at a pH of 9 or above.

(f) They show no ultraviolet absorption sprectrum between 260 and 290 millimicrons - this suggests that they do not contain tryptophan, tyrosine, phenylalanine or nucleic acid constituents.

(g) They resist treatment with 0.001 molar sodium periodate - a reaction which tests for carbohydrates.

ii) Size, by gradacol membrane filtration, gives an individual particle a diameter of approximately 48 millimicrons, and it is found to be not dialisable. Both these factors suggest that the interferon is a protein the size of other biologically active macro-molecules. They are known to diffuse freely in and out of cells in much the same way as antibody.

iii) Interferons produced by the Influenza viruses are different from the inactivated viruses themselves as shown by the following: -

(a) They are not absorbed onto chick red cells - virus is absorbed.

(b) They are sedimented by 100,000 g for 4 hours, whereas Influenza virus can be sedimented by only 25,000 g in 30 minutes.

(c) Their activity is not affected by ribonuclease, desoxyribonuclease, papain, or the receptor-destroying enzyme of vibrio cholera.

(d) They can be differentiated from Influenza virus by their heat lability - they are destroyed at 60°C for 1 hour.

(e) Antisera against the inactivated virus used to produce the interferon does not neutralize the effects of the interferon.

iv) They retain their activity over a pH range of from 2 to 11.

v) They are non-antigenic even when mixed with an adjuvant.

vi) They do not combine with the virus _in vitro_.

D) _Site of Action_ -

From the above it appears that interferons are protein products of the cells. The site of their action appears to be intracellular rather than at the cell surface. This means that the inhibitory action occurs at the stage of virus repdoduction within the cell and not by preventing virus adsorption to the cell. These facts have been shown by the following:[64] -

i) The infectivity of a non-infectious mixture of interferon and virus is completely restored by simply diluting the mixture.

ii) It can be shown that interferons react with host cells. This was shown by measuring the rate at which interferons adsorb onto chick fibroblast cells. It was found that the rate of development of resistence of these cells to infection with Eastern Equine Encephalitis virus paralleled closely the absorption of interferon. The longer interferon was allowed to remain in contact with the cells the more resistant to infection they became and the more interferon was absorbed from the fluid. Prolonged and severe washing following this stage does not restore susceptibility of the cells. Therefore, the interferon must enter the cell. Freezing, thawing and disruption of the cells do not free interferon. Therefore, it is either metabolized or goes into a stage similar to virus elipse.

iii) Interferon does not prevent virus infection by altering the absorption virus-cell phase - this has been shown by the fact that Eastern Equine Encephalitis will absorb onto susceptible cells in the same manner and in the same quantity as it absorbs onto interferon treated cells.

iv) Interferon acts by inhibiting intracellular activities of the virus. This is shown by comparing curves of virus grown on susceptible and interferon-resistant cells. It was found that resistance depends on duration of contact of the interferon with the cells, and showed that most virus had penetrated resistant cells one hour after infection; because treatment with immune serum at this time did not effectively reduce the number of infective areas obtainable in a culture.

In respect to these interferons, it is quite remarkable how similar are their characteristics to the so-called "transfer factor or factors" described.[65] This factor, or factors, are present in the leucocytes of hypersensitive individuals and are responsible for the transfer of delayed hypersensitivity from that individual to a non-sensitive recipient.

A stimulating and fascinating account of the transfer factors can be read in the Ciba Foundation Symposium on Cellular Aspects of Immunity.[66] The similarity of the two groups of substances in many instances is quite remarkable and is certainly worthy of consideration. The actions of the two appear to be completely opposite, i.e. hyper-sensitivity is usually deleterious to an individual whereas the presence of interferon would be apparently advantageous. Nevertheless, if one considers that the delayed hypersensitivity which occurs in tuberculous infections may be nothing more than a very basic immune mechanism, the differences between the two substances is not quite so extreme.

This long, and perhaps somewhat tedious, review has revealed that certain substances have been reported to modify virus infections at the cell level. At this point, the pertinent facts that have been disclosed will be reviewed and an interpretation given of them.

V. INTERPRETATION OF PART I OF THE REVIEW OF THE LITERATURE AND SUMMARY

It appears that there is good evidence to make the following statements: -

(1) Circulating neutralizing antibodies to Polioviruses appear to have some influence on the progress of an established infection.

(2) Circulating neutralizing antibodies to Polioviruses appear to modify the host response arising from a subsequent Poliovirus re-infection.

(3) Circulating neutralizing antibodies to Polioviruses play very little or no part in the process of initial infection.

(4) Circulating antibody levels bear no consistent or direct relationship to any of the methods of immunity either in degree or type.

(5) Immunity to subsequent re-infection in humans is longer-lasting and more adequate following natural infection, or artificaal infection with orally administered live attenuated viruses, than following immunization with dead virus.

(6) Immunization with dead Poliovirus vaccine lasts only as long as circulating antibody remains. Although this type of immunity protects against central nervous system spread of the virus with its sequelae of paralysis, it has little or no influence on subsequent or initial intestinal infection. It can, however, protect the pharynx from such infection if the antibody level is high enough to be present in pharyngeal secretions.

(7) There is considerable evidence that the quality of neutralizing antibodies may not be uniform. The quality of the antibody

initially produced after infection of the alimentary tract with live virus differs from that produced by parenterally administered dead virus. The quality of first formed antibody, following any type of antigenic stimulus, also appears to depend on whether or not there has been a previous experience with this antigen.

(8) The virulence of a particular Poliovirus strain is more closely allied to host defence mechanisms than to its own inherent characteristics.

(9) Laboratory _in vivo_ tests in monkeys and _in vitro_ tests in cell culture systems bear no constant relationship to virulence. However, certain, but not all, characteristics of a particular strain of virus may be constant enough, even after many serial passages in cells, to label the strain and allow its subsequent identification.

(10) Strains of attenuated virus fed orally to volunteers, and which spread most readily to intimate contacts, are also the strains which produce high levels of serum antibody.

(11) By inference some non-specific defence mechanism at the cellular level appears to restrain virus activity shortly after initial infection and may render the tissues affected more resistant to subsequent challenge by the same or similar virus. The interferons may be an example of such a mechanism.

(12) The actual cell type in which the Poliovirus first multiplies in the gastro-intestinal tract has not yet been established with absolute certainty.

It is considered that three of the foregoing points are important - namely,

(1) That circulating neutralizing antibodies play little or no part in modifying an initial active infection with the Poliovirus.

(2) That the level of circulating neutralizing antibodies bears no direct or constant relationship to any of the methods of immunity either in degree or type.

(3) That there is strong evidence that a qualitative difference exists between neutralizing antibodies and that this difference is best seen between active immunity due to infection, and active immunity due to the administration of dead virus parenterally.

On the evidence available the latter observation appears to be the only manner in which there is any constant association between the level and type of neutralizing antibodies, and the two main methods of producing immunity which are (a) active infection produced naturally or by artifical feeding, and (b) administration of dead virus parenterally.

This aspect of the problem brings us directly to the investigation contemplated. The existing work appertaining to this aspect only will now be reviewed.

VI. REVIEW OF THE PERTINENT LITERATURE - PART II - HUMORAL SUBSTANCES ASSOCIATED WITH POLIOVIRUS INFECTIONS

The subject matter consists of the humoral mechanisms of defence of man against Poliovirus infection. It is thought that these mechanisms will be reflected in some way by the presence or absence in the circulating blood of one or more substances. These substances may be directly responsible for an anti-viral activity or may just reflect an immune mechanism which is predominantly cellular.

The following may be considered as an illustration of this point. Lysozyme,[67] a cellular secretion, has a direct bactericidal action in blood and body secretions. As far as is known, this is its only function and its activity is confined to the serum or body secretions in question. On the other hand, amylase, a pancreatic or salivary gland secretion has a primary action on carbohydrate digestion in the mouth and the small intestine. Yet, in tissue destruction, involving either the salivary glands or pancreas, a very high level of amylase may appear in the blood but, as far as is known, has no physiological action normally in this situation.

So may it be in human Poliovirus infections. The presence or absence of a specific substance in the blood may directly or indirectly reflect the workings of an immune mechanism. Hence the review of this subject must of necessity involve both humoral factors having a direct action on the Poliovirus and humoral factors having no direct effect.

Classification of Humoral Substances -

These can be either -

(1) Non-specific protective substances, other than protein

antibodies

(2) Specific neutralizing proteins, i.e. antibodies.

(3) Substances having no direct action on the virus, but which are present or absent in varying degrees, and are incidental to virus-cell interaction elsewhere.

(4) Substances, the presence or absence of which, is a reflection of the genetic make-up of the individual in question. These have no direct action on the virus itself, but may be associated with a genetic susceptibility or resistance to Poliovirus infections.

A) Non-Specific Protective Substances -

These will be considered only in relation to human virus infections with particular stress being laid on the Enterovirus group. These substances can either -

(1) Neutralize the infectivity of the virus, or

(2) Inhibit some other virus activity, which for all practical purposes is confined only to the phemonenon of hemagglutination. The Enterovirus group are not active hemagglutinators, therefore this facet is not of immediate concern.

These substances can further be divided into those which are heat stagle - i.e. will withstand heating at 50°C to 60°C for one half hour and still retain their activity, and those which are heat labile. Unfortunately, the majority of these inhibitors have been detected in animal sera other than human. Nevertheless, they still assume some importance and consist of the following: -

Heat Stable Inhibitors -

(a) Lipid-Lecithin-like substances found in human sera which inhibit the infectivity of the Myxoviruses and Psittacosis.[68,69]

(b) Lipid inhibitors found in the sera of mice, hamsters, rabbits and horses which inhibit the infectivity of the Arbor B. group (e.g. St. Louis, Japanese B, and Russian Far East) of viruses.[70]

Heat Labile Inhibitors - These have been reported to inhibit infectivity of the following viruses -

(a) Vaccinia[71]

(b) Variola[71]

(c) Rous sarcoma virus[72]

(d) Western Equine Encephalitis[73]

(e) Mumps[74]

(f) Influenza A and B[75]

(g) Newcastle Disease virus[76]

(h) Herpes Simplex[76]

(i) Dengue[77]

(j) Measles[78]

The nature of most of these non-specific protective substances has been poorly defined. Ginsberg[79] feels that from the limited investigations carried out on these substances, they all appear to be similar to the substance called Properdin.[80] This assumption seems to the writer to be somewhat premature, particularly in view of the fact that there is serious doubt that the substance Properdin exists as a distinctly separate entity. The Enteroviruses and Adenoviruses are not inactivated or their infectivity inhibited by this substance. They are, therefore, of no immediate concern, yet the substance Properdin is contentious enough to be discussed further.

Properties of the Complement-Properdin System - [81,82]

(a) It is a protein.

(b) It migrates with the gamma-1 or β-2 globulins.

(c) It consists of 0.02% of the total serum proteins.

(d) It has a sedimentation coefficient of 18 S. (S = 1 Svedberg unit - a rate of fall of a molecule in an ultra-centrifuge).

(e) It has an isoelectric point of 5.6.

(f) It is antigenic.

(g) It is actively neutralized by antibody.

(h) It is heat labile when heated to a temperatuse of 56°C for one-half hour.

This substance is said to neutralize a wide range of viruses and bacteria, a list of which has been given above and to which can be added the T-bacteriophages of even numbers.[83]

Pillemer's original description showed that the method for estimating properdin was based on the degree to which complement fraction C^13 was inactivated. This is because for neutralization, properdin utilizes all four fractions of complement and needs the presence of a di-valent cation, such as magnesium or manganese. Pillemer's arguments against properdin being a natural antibody present in a low concentration, is based on the fact that zymosan, (a carbo-hydrate extract of the walls of certain types of yeast), when added to serum combines with properdin. Complement 1 (C^11), complement 2 (C^12), and complement 4 (C^14) are needed but are not destroyed, whereas complement 3 (C^13) is inactivated. The reverse is true, in a true antigen-antibody reaction which fixes complement.

Nelson,[81] however, points out that by the usual methods of measuring complement the number of reactive units of $C^{1}3$ in human serum is only 1/15 to 1/20 of the number of reactive units of $C^{1}1$ or $C^{1}4$, so that an equivalent loss of both components would appear as a more significant loss of $C^{1}3$. In addition, loss of 100 to 400 units of $C^{1}1$ and $C^{1}4$ would be masked by the error of measurement (plus or minus 50%) which is inherent in the classic technique by which these substances are titrated. By using an antigen-antibody system composed of bovine albumin and rabbit anti-bovine albumin as a complement-fixing reaction and comparing this with the zymosan-properdin reaction, he found that the same number of units of $C^{1}1$ and $C^{1}4$,as that of $C^{1}3$, were utilized. Nelson feels that properdin is not a single substance but a collection of low-avidity antibodies against a variety of polysaccharide antigens. These require complement to bond antibody to antigen. Properdin has not been completely purified, and the problem will probably not be resolved until this is done.

B) Humoral Substances Apparently Incidental to Virus Infection -

These may represent a quantitative or qualitative change of substances normally present in the serum, or the appearance of a substance not normally present in the serum. A consideration of the substances and changes which occur in serum immediately following the establishment of an infection or a stressful situation might be of interest. These substances and changes are generally called the "acute phase reactions".

Acute Phase Reactions -[84,85]

(a) An increase in the erythrocyte sedimentation rate[86]

(b) An increase in the concentration of fibrinogen[86]

(c) An increase in the concentration of muco-proteins and protein-bound carbohydrates (glyco-proteins)[87]

(d) An increase in the concentration of hyaluronidase inhibitor[88]

(e) A decrease in the concentration of iron-binding proteins[89]

(f) An increase in heparin-precipitable protein[90]

(g) An increase in the concentration of complement[91]

(h) The appearance of an abnormal protein, called the C-reactive protein[92,93]

The nature and properties of the C-reactive protein deserve special attention. These are considered next.

C-reactive Protein - can be found in sera obtained from unwell individuals. It was first discovered in 1930[94] and found while testing sera obtained from patients ill with pneumococcal pneumonia. It reacts with a somatic fraction of the pneumococcus called somatic C-carbohydrate. The reaction first visualized was that of precipitation. This was unexpected, as it occurred only in the serum obtained during the acute phase of the disease. This is unlike an antigen-antibody reaction. Furthermore, the carbohydrate was found to form a precipitate with acute phase sera from patients with infections other than pneumococcal pneumonia. It has been isolated in a relatively pure state by two methods of salt fractionation.[95,96]

Properties - The pneumococcus contains a Forsmann antigen which, unlike S. dyserentii, is not the dominant antigen. (The Forsmann antigen is an antigen which occurs widely in animals and plants. It is not species specific).

The C-polysaccharide is closely related to, if not identical with, the Forsmann antigen of the pneumococcus.

An antibody is formed against this substance in the acute stage of many diseases affecting man. This antibody is called the C-reactive protein (C.R.P.). A similar protein can be induced in rabbits following intensive immunization with foreign proteins. This latter protein is called the Cx-protein (CxP), and will be discussed later.[97,98]

The following facts have been established: -

(1) C.R.P. is never present in the serum of well persons, although relatively minor pathology may stimulate its appearance.

(2) C.R.P. appears in the blood very quickly (a few hours) following any insult causing tissue destruction. It disappears 1-2 days after the provoking cause has been removed.[99]

(3) It appears normally in all diseases with associated dysproteinaemia which have been studied. These are - rheumatic fever, rheumatoid arthritis, hypogammaglobulinaemia, the lymphomas, the leukemias, and Hodgkin's Disease.[100]

(4) In cases of agammaglòbulinaemia, although unable to produce circulating antibodies, the affected individuals can develop refractoriness to bacterial enterotoxins, e.g. injections of typhoid vaccine. The appearance and disappearance of refractoriness is paralleled exactly by the appearance and disappearance of C.R.P.[101]

(5) C.R.P. has been noted to occur occasionally in the last

trimester of pregnancy, and it has been suggested that this may be caused by a foetal-maternal immunological incompatibility.[102]

It is possible that the appearance of C.R.P. reflects a cellular immune mechanism more fundamental than, and probably unassociated with, antibody production.

In the English literature no evidence has been found ascribing any viral neutralizing ability to C.R.P. There is much literature written in Russian on this subject. At the time of writing, this literature and a translation has hot been obtained.

Nature of C-reactive Protein -

(a) It is a protein.

(b) It is antigenically distinct from normal human serum proteins.

(c) It precipitates with somatic C-polysaccharide of the pneumococcus only in the presence of ionizable calcium. The antiserum (A.C.R.P.) test does not require calcium. This fact is used to suggest that the C-polysaccharide - C.R.P. precipitation reaction is not a true antigen-antibody reaction. This, however, is open to marked criticism.[103]

(d) Its presence can be detected using the following phenomena: -

(1) Precipitation[104]

(2) Complement-Fixation[105]

(3) Pneumococcal capsular swelling[106]

(4) Latex Fixation[107]

(5) Agglutination of C-polysaccharide-coated erythrocytes[108]

(e) It is said to contain three antigenic constituents.[109] If serum is absorbed with C-polysaccharide, the components causing precipitation with C-polysaccharide, and hemagglutination are removed. Also, the factor causing precipitation with A.C.R.P. in agar only, is removed. However, the absorbed serum will still show precipitation in the capillary tube with A.C.R.P.

(f) Salt fractionation (ammonium sulphate) of C.R.P. positive sera has shown the following: -[109]

(1) The factor involved in hemagglutination is in the gamma-globulin fraction.

(2) Cx-polysaccharide precipitin is in all fractions

(3) The antiserum reacting component comes down in the fraction precipitated by 60% salt cocentration (this is mainly albumin with some α and β-globulins).

(g) There is no agreement in three different studies as to with which serum fraction C.R.P. migrates during electrophoresis. This is probably due to the three different antigenic constituents having different mobilities.[110,111,112]

(h) There is good evidence to support the contention that the $gamma_x$ line recently described using immuno-electrophoresis, contains C.R.P.

It is generally accepted that the specific neutralizing substances produced by the body following an antigenic stimulus are composed of proteins, that is, they are the antibodies. From the previous discussion it can be seen that non-specific neutralizing substances, or substances produced following a viral infection which may have little or no direct effect on the virus, also have a protein constituent.

If the blood can be considered a mirror of the tissues, and the substances pertinent to this investigation have a protein component, then investigation of the proteins which may appear in the serum of individuals possessing some degree of immunity to poliomyelitis, should be fruitful. At the same time such an investigation would show up any genetic pattern reflected by a change in the serum proteins and which also may be a reflection of the immunological integrity of the individual in question. Such a situation exists in mice. Gowan has shown that a particular strain of mice, which he designated E strain, possessed a particular β-1 globulin. During infections with mouse typhoid this β-1 globulin increased. He found another new protein, an α-1 globulin, to be present only in the serum of a strain of mice which he called the S strain. He also found that differences in concentration of the gamma-globulins were strain-dependant.

This now allows us to amplify somewhat the hypothetical mechanism of poliomyelitis immunity which may occur following infection with the virus in humans. This can be stated briefly as follows: -

In a non-immune individual following active infection with a Poliovirus there is some mechanism which is capable of restraining excessive virus spread until such time as specific neutralizing antibody is present at a sufficient level to permanently neutralize the virus.

This mechanism, to be effective, must either be present at the cellular site of infection prior to the infection, or be produced in a matter of a few hours following infection.

The following substances will appear to satisfy these criteria: -

(1) Substances Acting at a Cellular Level -

(a) The interferons.

(b) The lipo-protein cell receptor substance described by Holland and MacLarin.

(2) Substances which Combine Directly with Viruses in the Serum -

(a) The properdin-like substances, if these, in fact, exist at all.

(3) Substances, A Quantitative Change of Which is Apparently Incidental to Virus Infections -

(a) An increase in muco-proteins and glyco-proteins in the serum.

(b) An increase in complement.

(c) A decrease in the iron-binding proteins of the serum.

(d) An increase in fibrinogen.

(e) The appearance of C-reactive protein - never present normally.

It is known that the neutralizing ability of sera, obtained from individuals following some antigenic stimulus with Polioviruses, does not vary its titre very much following inactivation of its complement by heating for one half hour at 56°C. In view of this, and knowing that complement and the properdin-complement system are destroyed by such heating, these will not be further considered.

When testing for antibodies serum is used and not plasma and it is unlikely that fibrinogen has any great effect in the initial mechanisms of Poliovirus neutralization.

It can be postulated, therefore, that shortly after the onset of an infection there may be either -

(1) A change in the quality or quantity of humoral substances normally present in the serum, or

(2) The appearance of substances not normally present.

From this review it would appear that the following known substances might act in this manner: -

(1) The interferons.

(2) The lipo-protein factor described by MacLarin and Holland.

(3) Serum muco- and glyco-proteins.

(4) The C-reactive protein.

(5) A qualitative change in the substances defined as neutralizing circulating antibodies.

As stated, most of these substances are either pure protein in nature or contain a protein and should, therefore, be detectable by zone electrophoresis or immuno-electrophoresis. These two procedures will now be reviewed and discussed.

VII. ELECTROPHORESIS

In 1930 Tiselius described his now classical method of separating the protein fractions in serum, by utilizing their different rates of migration in an electric field.[114] He did this in a liquid medium and obtained five separate fractions which were mixed at their boundaries but which were quite distinct in between. This method was called, therefore, moving boundary electrophoresis.

Since that time numerous modifications have been introduced, the main one being the use of a solid or semi-solid anti-convection media which enables complete separation of the proteins without the formation of any boundaries composed of a mixture of these proteins. The first, and still the most popular because of its reproducibility, was that which utilized paper as the anti-convection media and was first described in 1937.[115] The use of a stabilizing medium in electrophoresis is now called zone electrophoresis, a term which was first coined in 1953.

Numerous methods and media have been since described, each with disadvantages and advantages depending on the nature of the material ultimately desired. However, electrophoresis using agar as the anti-convection media has many advantages for the purpose we have in mind. These are: -

(1) The rapidity and ease of preparing agar and the simplicity of the apparatus used.

(2) A stable and transparent gel can be obtained with a low concentration of agar, if the agar used is very pure.

(3) The gel contains a high proportion of fluid (99%). This

VIII. IMMUNO-ELECTROPHORESIS

If rabbits, horses, or other animals are immunized with human serum they will produce antibodies. These will react by forming precipitation arcs when allowed to diffuse towards one another, in agar. Thus, the human serum in question behaves as an antigen, and the animal antiserum in question behaves as an antibody. If the human serum, as antigen, is electrophoresed in agar and it is separated into its various fractions, and then if allowed to react by precipitation with animal anti-human sera, a large number of arcs will be formed which are considered to represent distinctly different antigenic constituents of the human serum. These different constituents can further be identified by their specific reaction with specific antibodies, sometimes by defining their chemical nature with special stains, and sometimes by demonstrating the presence of enzymatic activity. A substance can only be detected by a specific precipitation reaction if it is an antigen or at least a hapten. The reaction in immuno-electrophoresis is highly specific and highly sensitive, only a few micrograms of the sample are sufficient if micro-immuno-electrophoresis is used.

Principles of Immuno-Electrophoresis -

The substance to be analyzed in solution is separated in an electric field. As soon as a sufficient degree of separation has been obtained, an immune serum containing precipitating antibodies is allowed to diffuse into the gel from a trough cut along the line of axis of the electrophoretic migration. When an antigen meets its specific antibody a precipitate is formed in the shape of an arc. Since each lessens the absorption of proteins to the agar. This is a disadvantage possessed by paper and starch - e.g. in starch macromolecules often fail to migrate at all.

(4) Only lysozyme[116] and fibrinogen[117] have shown anomalies of migration in agar. All other substances tested migrate in the same manner as in a completely liquid medium.

(5) Agar when dried is completely clear and provides a favourable optical density for quantitative studies following staining of the bands.

(6) Antigen-antibody precipitates can take place following electrophoresis.

The disadvantages of using agar are that the preparation of the material itself is difficult to standardize, and the chemical structure of agar is unknown and, in fact, seems to vary from day to day.

By this method the serum proteins can be divided into the five classical fractions with comparative ease. These fractions are albumin, α-1, α-2, β, and gamma-globulins as originally termed by Tiselius. With certain modifications, the β and gamma fractions can be further subdivided and this will be discussed later in the text.

<u>Principles of Electrophoresis</u> -

A protein particle will move in an electric field in proportion to the following: -

(1) Its net surface charge - a factor depending on its inherent nature and the type and pH of the buffer used.

(2) Its size and molecular weight.

(3) Its shape.

(4) The type of anti-convection media used.

(5) The temperature of the immediate environment during electrophoresis.

(6) The stability of the system during electrophoresis, e.g. evaporation of the buffer, gassing at the electrodes, and condensation on the surface of the supporting media.

Using a buffer at pH 8.6 all of the serum proteins behave electrically as anions (i.e. they possess a negative surface charge). The gamma-globulins are those proteins which, by definition, move the least in an electrical field at a pH of 8.6; albumin, by definition, is the protein which possesses the greatest mobility at pH 8.6.

Agar possesses a small positive charge itself, when sodified, and this causes a flow of buffer from the anode side to the cathode side of the system. This phenomenon is in a reverse direction to the flow of electricity and is termed electro-endosmosis. In many systems it is an inconvenience, but in agar electrophoresis it is an added advantage. This factor of electro-endosmosis can be measured by following the path of an uncharged particle such as dextrose.

Buffers of low ionic strength allow a fast rate of migration of the proteins, with little heat production, but cause marked diffusion of the bands.

Buffers of high ionic strength give a small distance of separation, a high heat production, but extremely sharp bands. It has been found by trial and error that a barbital buffer of pH 8.2 - 8.6 and an ionic strength of 0.05 - 0.075μ, is the most satisfactory.

The Various Protein Fractions Obtained by Electrophoresis -

Much confusion has been caused by comparing the results obtained by electrophoretic separation in the various stabilizing media. A physiologically active substance may migrate as a gamma-globulin on paper but as a β-globulin in agar. However, an attempt will be made to review these and to confine the media used to agar. A brief description will be given of the substances known to be present in each band formed in agar.

These substances will be described, in order, from those which migrate the fastest in an electric field to those which migrate the slowest.

(1) The Albumins[118,119,120] -

There are two serum fractions which have a speed of migration faster than that of the main serum albumin fraction. These have been given the name delta-1 and delta-2 proteins. Some workers have called them pre-albumins, but there is some evidence that they are not identical with pure albumin. Delta-1 is a holo-protein (completely protein) which can also be found in cerebral spinal fluid. Delta-2 is a lipo-protein whose mobility may vary from one serum to another; its mobility is known to vary with storage of the sera, particularly at room temperature. The serum albumin itself appears to be reasonably homogenous and is represented by a narrow dense band. Sometimes a muco-protein which is really an acidic α-1 glyco-protein is found very close to the albumin fraction. This protein is considered to have changed its mobility during the course of electrophoresis, specimen preparation, or during storage of the serum. Recently, it has been shown that some individuals may possess two albumin fractions of different antigenicity. This is, apparently, an inherited characteristic unassociated with disease.

(2) The α-Globulins[121,122,123] -

There are at least three different α-1 globulins. The exact identity of these is still unknown.

The α-2 globulins contain a large number of constituents, which by immune electrophoresis have numbered ten. Only four of these have been definitely identified. These four are: - an α-2 macroglobulin, a lipo-prôtein, a hapto-globin (a glyco-protein capable of forming a complex with hemaglobin), and ceruloplasmin (a protein which contains copper and possesses some oxidase activity which is the basis of a specific stain used for its identification.

(3) The β-Globulins[123,124,125,126] -

The proteins having mobilities which coincide with the β-1 globulins comprise at least four constituents. Only one has been identified. This is siderophilin, a protein containing iron and which is thought to be responsible for the transport of iron within the body.

The β-2 globulins show two definite components which are often seen. There is a suggestion that others may be present. The two often seen are called β-2A and β-2M.[127] The β-2A globulin has only been demonstrated by immuno-electrophoresis. Usually, on immuno-electrophoretograms, the arc formed by the β-2A globulin is masked by the gamma-globulins, but can be readily visualized if the antisera has been adsorbed with gamma-globulin and the anti-gamma-globulins have been removed. This β-2A globulin is extremely important because it is rarely present in the newborn child,[128] and it is absent in certain pathological situations. The β-2M globulin can only be detected in normal serum if the analysis is performed on a large volume of the sample. This is because it is present in very low concentration. It may be abundant, however, in certain pathological conditions. It has been identified as a macroglobulin and possesses a molecular weight of 1,000,000.

(4) <u>The Gamma-Globulins</u> -

The gamma-globulins consist of a group of globulins which appear to be antigenically identical but vary in their mobility, extending from the slowest gamma-globulin region to the α-2 globulin region. These gamma-globulins have been considered to be a family of proteins, but it has been shown that there are minor antigenic differences between them. They contain the majority of classical antibodies.

(5) <u>The Lipo-Proteins</u>[129] -

Most of the lipids migrate with the α-2 globulins in agar. They are usually called, however, β-lipoproteins since by using paper electrophoresis they are found in the β-globulin zone. A small portion of the lipo-proteins migrate, as has been previously stated, with the serum albumin and there is the pre-albumin or rapid albumin fraction, delta-2, which is also a lipo-protein.

<u>Abnormalities of the Various Serum Fractions</u>[130] -

(1) <u>Abnormal Serum Albumin</u> -

There is a familiar abnormality associated with serum albumin deficiency. If serum from these individuals is electrophoresed a double arc is obtained with immune serum, one which corresponds to the normal serum albumin and the other which corresponds to an α-1 globulin mobility. The two arcs are confluent and it is thought that the second arc is due to a serum albumin which is only very slightly modified.

(2) The Agammaglobulinaemias (Hypogammaglobulinaemias) -

This anomaly is usually accompanied by a deficiency of both β-2 globulins and sometimes by a deficiency of the delta protein as well. In some cases which show an immunological defect only the β-2 globulins are found to be deficient and the gamma-globulins are present in normal titre. The name, therefore, is unfortunate.

(3) Multiple Myeloma -

In this disease the gamma-globulins and the β-2A globulin are affected. In many cases there is decided increase in the gamma-globulins of a certain mobility. This defect can be shown as an increase in the fast-moving gamma-globulins (i.e. gamma-1 or β-2), shown as a thick band. Or, it can take on the appearance of an excessive amount of slow-moving gamma-globulin which again appears as a thick band in the slow gamma-globulin (gamma-2) region.

There is a third type, in which an additional line can be seen, which by immuno-electrophoresis joins onto the gamma-globulin band. This line is due to the presence of Bence-Jones protein, which is considered to be composed of incomplete gamma-globulins and can appear in the urine. The Bence-Jones proteins are of interest. They represent incomplete gamma-globulins and, due to a difference in their antigenicity, they can be divided into at least three groups. Most immune sera containing antibodies to normal human serum react only with one or other of these groups. This has made it possible to distinguish at least five or six different antigenic groupings in the molecule of the gamma-globulins. In multiple myeloma the β-2A globulin is nearly always either totally absent, or present in increased amounts.

(4) <u>Macroglobulinaemias (Waldenstrom's Syndrome)</u>[131] -

This syndrome gives a characteristic picture of a well-defined band, with one or two arcs on immuno-electrophoresis, appearing in the zone of β-globulin mobilities. This band is typical of the type of band given by the β-2M macroglobulin which is always present in very small quantities in normal serum. No pathological proteins appear in this syndrome but there is an increase in the normally present β-2M and there is often an abnormal mobility of some of the gamma-globulins. Often the β-2A globulin is diminished or absent.

(5) <u>Inflammatory Diseases</u>[132] -

Studies on cases of infectious hepatitis and alcoholic cirrhosis of the liver have revealed an increase in the gamma-globulins, and the β-2A and β-2M globulins. This is often accompanied by a reduction in the amount of β-1 globulins (this does not affect siderophilin). There is no change in the immuno-chemical properties of these proteins. Similar changes have been observed in infectious mononucleosis, the lymphomas, polyarthritis and multiple sclerosis. They appear to correspond to an inflammatory process.

(6) Cryoglobulins[133]

These appear sometimes in cases of myeloma, sometimes in cases of macroglobulinaemia and sometimes with just straight inflammation. When isolated and purified these show immuno-chemical characteristics corresponding to the gamma-globulins. Sometimes, however, they are macroglobulins; these are called cryo-macroglobulins. They appear to contain, or to consist of, β-2M globulin.

(7) Other Pathological Conditions -

Because so little is known about the actual constituents which can be found in normal serum it is very difficult to define the changes which occur in many described pathological conditions. However, the following have been noted: -

(a) Extremely abnormal diagrams found in lipoid nephrosis[134] - shown by a decrease in serum albumin, β-2A globulin, and especially the gamma-globulins. The α-2 globulins are increased.

(b) In cases of acute disseminated lupus erythematosis an increase in the gamma-globulins occurs, but more particularly, a considerable loss of β-2A globulin, which returns to normal during remissions of the disease.[135]

(c) In lymphatic leukemia anomalies of the gamma-globulins may also be observed. In cancer no characteristic changes have been observed so far, except some cases in which the glyco-proteins are increased.[136]

(d) C-reactive protein, which is of particular interest to us, appears in most acute inflammatory and traumatic states. It is said to be a globulin which is always absent from normal serum but which can be detected with the aid of a special immune serum and it is said to migrate with the β-globulins by one worker, but others claim that this protein is in the α-2 globulin region, others that it is in the α-1, and some say that it even migrates with the albumin.

VIII. IMMUNO-ELECTROPHORESIS

If rabbits, horses, or other animals are immunized with human serum they will produce antibodies. These will react by forming precipitation arcs when allowed to diffuse towards one another, in agar. Thus, the human serum in question behaves as an antigen, and the animal antiserum in question behaves as an antibody. If the human serum, as antigen, is electrophoresed in agar and it is separated into its various fractions, and then if allowed to react by precipitation with animal anti-human sera, a large number of arcs will be formed which are considered to represent distinctly different antigenic constituents of the human serum. These different constituents can further be identified by their specific reaction with specific antibodies, sometimes by defining their chemical nature with special stains, and sometimes by demonstrating the presence of enzymatic activity. A substance can only be detected by a specific precipitation reaction if it is an antigen or at least a hapten. The reaction in immuno-electrophoresis is highly specific and highly sensitive, only a few micrograms of the sample are sufficient if micro-immuno-electrophoresis is used.

Principles of Immuno-Electrophoresis -

The substance to be analyzed in solution is separated in an electric field. As soon as a sufficient degree of separation has been obtained, an immune serum containing precipitating antibodies is allowed to diffuse into the gel from a trough cut along the line of axis of the electrophoretic migration. When an antigen meets its specific antibody a precipitate is formed in the shape of an arc. Since each antigen forms a separate band it is possible to determine the number of constituents in the mixture. Particularly when dried, agar is transparent and thus the arcs or bands of specific precipitation are visible and can be recorded.

Electrophoresis should be long enough to cause distinct separation of each protein fraction. Precipitation is allowed to occur in wet chambers maintained at a constant temperature. The time that elapses before the precipitates appear and the degree of curvature of the bands depend on a number of factors: -

(1) The amount of antibody present in the immune serum.

(2) The size and shape of the antigen molecule.

(3) The temperature

(4) The relative quantities of antigen and antibody present.

(5) The distance between the antigen and the immune serum.

As a rule, the first precipitate appears within 24 hours and is completely developed in four to five days.

<u>The Advantages and Disadvantages of this Method</u> -

The principal disadvantages are: - (a) the use of agar, a reagent which is difficult to standardize; (b) the immune serum used may not contain antibodies against all the constituents of the mixture studied; (c) only soluble substances can be identified.

The advantages that immuno-electrophoresis possesses over straight agar electrophoresis and paper electrophoresis are as follows: - (a) several constituents with similar or identical mobilities can still be identified because they produce different specific precipitation arcs; (b) a mixture can be examined without any preliminary treatment; (c) complete analysis can be performed on a very small sample, e.g. 0.01 ml. with the micro-method; (d) each constituent is characterized by two different criteria, i.e. its mobility and its antigenic specificity.

IX. LOCATION OF ANTIBODIES

This is most confused. The use of different anti-convection media, the use of different methods of separation, the use of different terminology, and a failure to define the conditions under which serum samples were stored, have all added to the present chaotic state of the literature. An attempt will be made to clarify this somewhat.

For the purposes of this discussion the following terms are defined: -

(1) Electrophoresis - That method using agar as the anti-convection media, i.e. agar zone electrophoresis.

(2) Protein Fractions - Those which can be resolved normally by the use of agar electrophoresis. In general, they are defined by using the original criteria of Tiselius.

These are, in descending order of mobility, using a barbital buffer of pH 8.6 -

(a) The Albumins - (they possess the greatest mobility)

(b) The α-1 and α-2 globulins.

(c) The β-1 and β-2 globulins. The β-2 region is that region lying between the gamma-globulin peak and the main classical β region (β-1). This is not identical with the β-2 region described by Laurel,[137] which is a slower moving β-band produced by adding calcium ions to the buffer used in paper electrophoresis methods. The β-lipoprotein, which by paper electrophoresis is in the β-2 band, is not present in the β-2 region obtained by agar electrophoresis. Other names given to this band have been "gamma-1", "T", "M", and "X".

(d) The gamma-globulins - (they possess the least mobility).

These will include all proteins in human sera which are antigenically similar. Their mobility, alone, is not used in this definition. They can be demonstrated, by immunological methods, to extend from the slow α-region (α-2) to the slow gamma-region. In this respect, the "gamma-globulins" is really a misnomer.

(3) Antibodies - Serum proteins which show a specific active biological reactivity with substances antigenic to humans. This reaction is usually of a protective nature but may sometimes be harmful, i.e. auto-immune antibodies, anti-insulin antibodies, incompatible iso-antibodies, etc. These proteins usually have the following characteristics: -

(a) Their specific activity is not significantly influenced by heating at 56°C for one half hour.

(b) Their activities can be visualized by one or more of the following methods -

i) Neutralization of infectivity of the homologous infective antigen.

ii) The fixation of complement following union with homologous antigen.

iii) Precipitation with homologous antigen.

iv) Agglutination of homologous antigen.

v) Inhibition of agglutination and/or lysis of erythrocytes by antigen (this action may be active or passive, on the part of the antigen).

vi) Producing or augmenting lysis of particulate organisms.

vii) Augmenting phagocytosis, of particulate antigen, by viable leukocytes.

viii) Other methods which enable detection of antigen-antibody reactions considered biologically specific. e.g. fluorescent tagging, radioactive isotope tagging, conglutination, etc.

(c) The activities listed in (b) will occur over a wide range of temperature (4^oC - 40^oC) but are usually optimal at 37^oC.

(4) Conditions of Storage and Treatment of Serum Prior to Electrophoresis - Often these are not defined. They are important. Artefactual bands can be formed and misinterpreted. Studies of these factors are an essential prerequisite to adequate interpretation of results. This will be discussed later.

(5) Specific Proteins - Holo-protein is composed entirely of protein. Conjugated proteins are those chemically united with a non-protein substance (presthetic group). They include the following: -

(a) Nucleoproteins - protein plus nucleic acid.

(b) Glycoproteins and mucoproteins - protein plus carbohydrate. Glycoproteins contain less than 4% carbohydrate, mucoproteins more than 4%.

(c) Lipoproteins - protein plus lipid.

(d) Phosphoproteins - protein plus phosphorous radical.

Bearing in mind the above criteria, there is now sufficient evidence to say that the various bands contain the following antibodies: -

(1) Albumin - No antibodies definitely found.

(2) α-1 Globulin - No antibodies definitely found.

(3) α-2 Globulin - Antibodies found but these proteins possess

an antigenic specificity characteristic of fast gamma-globulins.

(4) β-1 Globulins - Contain complement.[138] No satisfactory evidence that they contain specific antibodies.

(5) β-2 Globulins - (Designated by some as the gamma-1 globulins) - β-2 globulins contain antibody activity (work not yet completed).[139] β-2A globulins contain the following antibodies:[139,140] cold agglutinins, saline-type RH agglutinins (anti D_1C factors), isohemagglutinins, the "rheumatoid factor", lupus erythematosis factor (only anti-nuclear factor), Wasserman reagin and heterophile antibodies. It has been said that the β-2M globulins carry antibodies especially capable of reacting with red blood cells.

(6) Gamma-Globulins (gamma-2 globulins) - Contain most of the classical antibodies, and includes viral antibodies.

From the above it can be seen that most, if not all, antibodies are contained in the gamma-1, β-2A, and β-2M globulins. The name "immunoglobulins" has recently been given to these.[139]

In general, they all increase together in chronic infections. Diseases associated with dysproteinaemia show a fall in one or more of the immunoglobulins, e.g. primary and secondary agammaglobulinemia. Diseases associated with overactivity of cells derived from the reticulo-endothelial system (reticulo-sarcoma, macroglobulinemia, the leukemias and multiple myeloma) give rise to "paraproteins", which appear to be complexes formed from one, or even two, of the immunoglobulins. In these diseases, the immunoglobulins showing no "complex" formation are usually reduced in quantity.[139]

The establishment of this base-line for the methods used is absolutely necessary. Without it, misinterpretation of results is likely and artefacts may be considered to be due to pathological changes.

The basis of this study has now been reviewed. It now remains to formulate an experimental approach. This is as follows: -

(1) To study sera of children, under two years of age, who have previously been immunized with Poliovaccine, either live oral, dead parenteral, or both.

(2) To estimate the level of neutralizing antibodies in these sera, by various methods, before and after immunization.

(3) To subject these sera to agar zone electrophoresis and immuno-electrophoresis using micro-methods.

(4) To correlate the changes, if any, found between the various serological methods used in the investigation.

(5) To study the C-reactive protein and its relation to Poliovirus immunity.

By the above means it is hoped to unravel some of the inconsistencies found to exist in humans following a Poliovirus antigenic stimulus and the subsequent immune status produced.

X. MATERIALS AND METHODS

Materials -

All the personnel studied were children of both sexes. These children had all been given dead (Salk-type) Poliovaccine, containing Poliovirus types 1, 2, and 3. The schedule of immunization was as follows: -

(1) A total of four injections was given to all the children (1cc. subcutaneously).

(2) The first injection was usually given when each child was between three and six months of age. A small percentage (approximately 16%) was older, but no child commencing the schedule was over twelve months of age.

(3) The first three injections were given at one month intervals, and the fourth was given at, or later than, seven months following the third injection.

(4) Blood was collected from each child at least three weeks after the fourth injection was given.

(5) The serum thus obtained was screened for neutralizing antibodies to Poliovirus types 1 and 3 only.

(6) By the above means the children were divided into three groups for the purposes of this investigation. These three groups were as follows: -

(a) Those who possessed neutralizing antibodies to both types of Poliovirus lower than a level of 1:10 dilution of serum. (Double negative children)

(b) Those who possessed neutralizing antibodies to only one type of Poliovirus lower than a level of 1:10 dilution of serum. (Single negative children)

(c) Those who possessed neutralizing antibodies to both types of Poliovirus higher than a level of 1:10 dilution of serum. (Double positive children)

To the double negative children, orally administered live attenuated Poliovirus (oral vaccine) of both types was given. To the single negative children oral vaccine of an appropriate type was given. To a few of the double positive children Type 1 or Type 3 oral vaccine was given alternately, as controls.

Ten days following the administration of oral vaccine a rectal swab was taken from each child and an attempt made to isolate the fed virus on tissue culture.

At an interval of six weeks or more following the administration of the oral vaccine a second blood sample was taken. The second blood, and its counterpart first blood, were then titrated in parallel to estimate the Poliovirus Type 1 and Type 3 antibodies present. This was done by two-fold dilutions, form a 1:4 dilution (when the quantity of serum was adequate) to a final dilution of 1:1024, for each serum tested.

Methods -

The tests carried out on the sera obtained from each group of children are as follows: -

(1) Cytopathogenic-inhibition test (See Appendix 1 and 2) on monkey kidney cell culture. This was the only procedure used in the original screening test. Two and four-day readings were recorded.

(2) The Complement-fixation test. (See Appendix 1 and 2) This was done on a selected number of sera.

(3) Agar zone electrophoresis - by a micro-method. (See Appendix 1 and 2)

(4) Immuno-electrophoresis - by a micro-method. (See Appendix 1 and 2)

(5) The capillary tube C-reactive protein test. (See Appendix 1 and 2)

(6) An immuno-electrophoretic method of C-reactive protein estimation. (See Appendix 1 and 2)

The role of the C-reactive protein in its reactivity with Polioviruses was investigated.

Commercial C-reactive protein and C-reactive protein prepared by an ether-dialysis fractionation method from 14 human sera was used. The human sera varied from a 1+ to 3+ C.R.P. positive as shown by the capillary tube method. Tests were done as follows: -

(1) Electrophoretic homogeneity and its comparison with serum protein bands normally obtained, was estimated.

(2) Neutralization tests and complement-fixation tests were performed on C.R.P. - Poliovirus mixtures.

(3) Immuno-electrophoretic analysis was carried out.

For the sake of clarity, all test materials and methods are listed in Appendix 1 and Appendix 2. Appendix 1 lists and defines the source of materials used in the preceding tests. Appendix 2 describes the methods used in performing the various tests.

The number of sera tested in each of the three groups of children are as follows: -

The total tested, following four injections of Salk-type vaccine (See Appendix 1) was 207. Of these, 22 (10.6%) were negative at a serum dilution of 1:10 for both types of Poliovirus antibodies. 82 (39.6%) were similarly negative for one or both types of Poliovirus antibodies. 56 of these latter were fed the appropriate type or types of live vaccine. Of these 56 a Poliovirus type was isolated in 46 (82%) from a rectal swab taken on the tenth day after feeding.

For the purposes of this study, those children who were negative to both types of Poliovirus antibody, who were fed oral vaccine, and who obtained a good antibody rise to both types of virus, constituted the first group.

Those children who were negative to one type of virus antibody, who were fed oral vaccine and who obtained a good antibody response to the type fed, constituted the second group.

Those children who obtained a good antibody rise to both virus types following Salk vaccination constituted the third group.

Five were taken from the first group, two from the second, and three from the third. The sera from these ten children were extensively studied. It was felt that these three groups would reflect most markedly the existence of any difference in the immune response.

XI. RESULTS AND INTERPRETATIONS

Table II lists the ages, in months, at which the various procedures were carried out on the ten children.

Table III sumarizes the results obtained by the various tests.

The following can be deduced from these tables (recognizing the very small number of tests) -

(1) Age alone is not responsible for failure to respond to Salk vaccine as Cases 8, 9 and 10 were of a comparable age to Cases 1 to 5. The mother's antibody level at the time of birth, unfortunately, is not known.

Furthermore, Cases 6 and 7 show response to Salk vaccine of one different type only. In these cases, this suggests that failure to respond is not due to a general immunological defect on the part of the child or a defect in the antigenic potency of the vaccine in question (these children were given vaccine, the same batch of which produced adequate titres of antibody to both Poliovirus types in other children). It may, therefore, be a reflection of the passive antibody levels obtained from the mother.

(2) In general rises in the complement-fixing antibody levels paralleled the rises in neutralizing antibody obtained after feeding the oral vaccine, but to a lesser extent.

In the children who were not given oral vaccine, the complement-fixing antibody levels did not compare with the neutralizing antibody levels. This supports the contention that some factor other than neutralizing antibody is responsible for complement fixation following active cell infection. This might well be a product of cell disruption.

(3) In many instances the 2-day reading of the neutralization tests showed a one-tibe higher neutralization titre than the 4-day reading. This suggests that the 2-day reading may be a more sensitive reflection of a type of neutralizing antibody.

Of particular interest in this respect is the great difference seen between the 2 and 4-day reading for Type 3 virus, in cases 3, 6 and 7 and for Type 1 virus in case no. 6. Cases 3 and 7 also show a major rise in complement-fixing antibodies. Both of these children were fed the appropriate virus.

This adds further to the contention that a product of cell disruption may be responsible, at least in part, for complement-fixation.

The role of C-reactive protein as a participant in any of the above phenomena was investigated. Table III shows that only the first serum sample of Case no. 7 showed a positive capillary tube reaction for C.R.P. Unfortunately, insufficient sera remained to perform complement-fixation tests on the sample.

Tests were, therefore, done to determine if C-reactive protein possessed any ability to neutralize Poliovirus infectivity or to react with Poliovirus antigens and to fix complement.

Commercial C.R.P. was used (C.R.P. - Positest) together with partially purified C.R.P. obtained from pooled positive sera (See Appendix 2). Both types of C.R.P. used separately produced the following results: -

(1) Using the cytopathogenic-inhibition test on monkey kidney cells C.R.P. showed a neutralizing titre to Type 1 Poliovirus of 1:4

on the 2-day reading and of $<$ 1:4 on the 4-day reading.

(2) By the same test C.R.P. showed a neutralizing titre to Type 3 Poliovirus of 1:8 on the 2-day reading and $<$ 1:4 on the 4-day reading.

(3) Using both Poliovirus Types 1 and 3 antigens C.R.P. fixed complement at a dilution of 1:4 in each case.

(4) Furthermore, the metabolic-inhibition test, using monkey kidney cells, showed that C.R.P. at a dilution of 1:20 neutralized the infectivity of Poliovirus Type 3. All controls (sera negative to C.R.P. by the capillary test) showed no neutralization even when used undiluted.

These results are most interesting. It must, however, be borne in mind that the original dilution of the C.R.P. is actually unknown as, unfortunately, quantitation of the precipitate obtained during each fractionation procedure was not measured. Nevertheless, visual inspection of the protein density of the bands obtained by zone electrophoresis appeared to represent considerable dilution in the case of both the commercial product and that produced in this laboratory.

A communication requesting the nature of, and the method of production of, the commercial C.R.P. has remained unanswered. (This is hardly surprising).

Electrophoresis and immuno-electrophoresis with, and without, specific absorbtion reveal both products to contain <u>no</u> detectable gamma-globulin (See Fig. ~~19~~ 15).

This allows at least one assumption to be made, namely: - that there is some factor capable of neutralizing Poliovirus infectivity and also of reacting with Poliovirus antigenic material to fix complement, which is not a gamma-globulin by any definition. This factor may or may not be identical with C.R.P. but appears to be associated with it.

Immuno-electrophoresis is a sensitive method of detecting any antigenic differences that may exist between proteins. Agar zone electrophoresis is capable of separating proteins of similar surface charge by a degree which can be detected. However, there are numerous factors, as seen by the review of the literature, which are artefactual and have caused considerable confusion in regard to interpretation.

Therefore, prior to any qualitative study of sera from children in the three study groups it was considered necessary to establish a base line in regard to the electrophoresis methods. These factors were considered: -

(1) The effects of the routine treatment each serum specimen received in the laboratory prior to testing. This involved the effects of storage, freezing and thawing, and heating at 56°C for one half hour.

(2) The effects of hemolysis.

Five fresh normal sera were studied. The following results are examples of the effects of each variable shown by agar zone electrophoresis and immuno-electrophoresis.

Figures 4 and 5 show the normal bands and arcs obtained by both methods of examination.

Figure 6 shows the effects of heating fresh serum at 56°C for intervals of one-quarter, one-half and one hour as shown by zone electrophoresis and immuno-electrophoresis.

(1) Heating for one-quarter hour has little effect on

either method.

(2) Heating for one-half hour markedly changes the β-2 globulin region as seen by zone electrophoresis, but it will be seen that the arcs obtained by immuno-electrophoresis are unchanged, although much immobile protein can be seen at the application well.

(3) Heating for one hour results in a diminution of the intensity of the albumin and α-globulin arcs, and an apparent lengthening of the gamma-globulin arc.

Figure 7 shows the effects of routine storage (as represented by overnight freezing of fresh serum at -28°C. to -30°C. and thawing at room temp. for from 2-4 hours). This procedure was carried out three times on each serum. The effects are negligible.

Figure 8 shows the effects of hemolysis on serum samples. These are: -

(1) Marked alterations in the β-globulin region as shown by zone electrophoresis, and

(2) Little change in the immuno-electrophoretic pattern.

From the above it can be deduced that: -

(1) Routine storage has little effect on either zone or immuno-electrophoresis.

(2) Heating for one-half hour at 56°C causes marked changes in zone electrophoresis but little change in immuno-electrophoresis.

(3) Heating for one hour changes both.

With this knowledge at hand the sera obtained before and after oral vaccine of cases 1, 2, 3, 4, and 5 were studied.

The results can be seen in Figures 9, 10, 11, 12 and 13.

They are essentially similar and consist of the following: -

(1) By zone electrophoresis, the apparent appearance of an extra band in the gamma-1 and β-2 positions.

(2) A "bowing" of the β-2 arc which appears to be a fusion of a β-1 arc and a β-2 arc. The fusion suggests antigenic identity and as such may represent an increase in one of the "immunoglobulins".

The actual significance of this is not yet known, but from the base line experiments it would not seem to be an artefact because a change appears in the zone electrophoresis bands together with a change, in the same position, of the arcs obtained by immuno-electrophoresis.

Next, sera from children who responded well to both virus types following Salk-type immunization were compared with the sera of those children who did not respond to either virus types. This represents comparing the sera of cases 8, 9 and 10 with the pre-oral vaccine sera of cases 1, 2 and 3.

Figure 14 shows these results. It can be seen that a similar difference exists between these sera as between the sera, before and after, of children who responded well to the oral vaccine. This suggests that <u>no</u> inherent defect accounts for the different response seen between the two groups of children, but that a temporal change in the immune conditions of the children is most likely. This may well be reflected by the titre of passive antibodies each child possessed at the time of Salk-type vaccination.

Immuno-electrophoretic studies on C-reactive protein proved interesting. Using commercial C.R.P. and ether-precipitated C.R.P. certain facts were gleaned from agar-diffusion studies which warrant comment.

Figure 14 shows commercial C.R.P. Positest (believed to be purified C.R.P.) electrophoresed in parallel with ether-fractionated C.R.P. and an albumin control alongside. It can be seen that the commercial product shows a high electrophoretic homogeneity with a mobility similar to that of the α-1 globulins. The ether-precipitated C.R.P. is composed of two fractions, one with a mobility of albumin and one with a mobility of α-1 globulin. Both bands represent low protein concentrations. The electrophoretic pattern of each fraction concentrated by freeze-drying is shown as well. The commercial C.R.P. has a mobility slightly less than the ether fraction.

Figure 14 also shows the immuno-electrophoretic studies of C.R.P.-positive and C.R.P.-negative sera, and commercial C.R.P. - all electrophoresed and allowed to react with anti-C.R.P. rabbit serum.

These show: -

(1) That the rabbit produced A.C.R.P. reacts by a line of identity with the human serum, both C.R.P.-positive and C.R.P.-negative. (Line No. 1). This is a β-1 or α-2 globulin.

(2) That the C.R.P.-positive serum reacts by a faint line (Line No. 2) with A.C.R.P. and this is in the gamma-globulin region.

(3) That the anti-human rabbit serum and the anti-C.R.P. rabbit serum react with each other by a strong line which is situated at the mid-point between the two, (Line No. 3), and that this line is interrupted by bands in the β region of the human serum and fuses with these forming two lines of identity, one of which further bifurcates and fuses with another human arc in the α-globulin region. (Line No. 4)

These observations are interesting in as much as the area where the lines of identity are formed probably represents the area containg C.R.P., but that there is at least one other specific line of reaction with C.R.P. in the fast gamma-globulin area (Line No. 2).

C-reactive protein was shown to be unstable following heating at 56°C for one hour and of variable stability when heated at 56°C for one-half hour.

Pooled commercial gamma-globulin (Connaught) showed a 3+ positive with anti-C.R.P. rabbit serum by the capillary tube method and a strong line of precipitation by agar diffusion.

XII. <u>DISCUSSION AND TENTATIVE CONCLUSIONS</u>

The results obtained in this study cannot, by any criterion, be considered more than preliminary.

The numbers involved are few. The variables are many. It is felt, however, that this work is a start. Each statement of fact, arising from this work, has been the result of an experiment which has been consistently reproduced at least three times.

Hence, it appears that: -

(1) The C-reactive protein, or a substance or substances closely allied to it, may be responsible for some degree of Poliovirus neutralization and complement-fixation as shown by the usual laboratory tests.

(2) Cross-reactions, usually of a minor degree, shown by these and other tests may be due to the presence of such a substance.

(3) At least two precipitation arcs, one in the gamma-globulin region and one in the α-globulin region, are produced by immuno-electrophoresis of C.R.P.-positive human sera against anti-C.R.P. rabbit sera. The presence of a "deviation phenomenon" suggests the presence of a third component.

(4) An ether-precipitation method has been devised for the precipitation of C.R.P. from human sera in an, as yet, incompletely pure state.

(5) Following adequate immunization as shown by the presence of a high level of Poliovirus neutralizing antibodies an extra band, in the gamma-1 or β-2 region appeared in the post-immunization serum sample. This was present in post-immunization serum samples when

either the Salk-type or oral vaccine (of either Poliovirus type) was successfully used.

This tends to eliminate the Diphtheria-Tetanus-Pertussis component of the vaccine from alone being responsible for the appearance of this band. It appeared after oral Poliovirus administration alone.

(6) The live-oral Poliovirus vaccine appears to be safe and extremely effective. It is simpler to administer and eminently more effective than the dead virus type (Salk).

Future Work Recommended -

The entire work described herein should be repeated using larger numbers of test specimens. More use should be made of the elegant and very sensitive technique of immuno-electrophoretic analysis.

C-reactive protein is a substance of importance in serological diagnostic work. Its exact nature should be fully determined.

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APPENDIX 1 - MATERIALS USED AND SOURCE

1. Monkey kidney cells - kindly supplied by Connaught Medical Research Laboratories, Toronto. Sent at a 1:1200 dilution of packed cells in propagating media (0.5% lactalbumin hydrolysate, 2.0% horse serum in Hank's balanced salt solution).
2. Complement-fixation antigens for Poliovirus Type 1 and Poliovirus Type 3 - purchased from Microbiological Associates, Baltimore, Maryland, U.S.A.
3. C-reactive protein antiserum (rabbit) and C-reactive protein (Positest) purchased from Schieffelin and Co., New York 3, N.Y.
4. Salk-type Vaccine - Diphtheria, Pertussis, Tetanus and inactivated Poliovirus Types 1, 2 and 3. Administered by Dept. of Public Health, City of Winnipeg and obtained from Connaught Medical Research Laboratories, Toronto.
5. Oral live attenuated Poliovirus Types 1 and 3 (Sabin strains) - supplied by Connaught Medical Research Laboratories, Toronto.
6. Electrophoresis Power Pack - Reco Model E-800-2. Research Equipment Corporation, Oakland, California.
7. Plastic for Electrophoresis migration chambers - purchased from Plaxilab Ltd., Winnipeg.
8. Centrifuge - International Portable Refrigerated Centrifuge - Model P.R.-2, International Equipment Co., Boston, Mass., U.S.A.
9. pH - Meter - Beckman Zeromatic.
10. Di-ethyl ether - Anaesthetic grade, stored at 4°C.
11. Ethyl Alcohol - 95%.
12. 0.01% calcium chloride solution in distilled water.

APPENDIX 1 - cont'd

13. 0.5 Molar Acetic Acid

14. 0.5 Molar Sodium Bicarbonate solution in distilled water - stored at 4°C.

15. Agar - Special Noble (Difco) 2% for slides and agar blocks in buffer.

16. Buffer - Made up as follows: -

Sodium Veronolate (Barbital sodium) B.D.H.	- 8.5 gms.
HCl (N)	- 11.5 cc.
Distilled (Deionized) water to	- 1000 cc.

This represents an ionic strength (μ) of 0.05 M. and a pH of 8.4.

17. Petroleum Ether (Mallinckrodt Chemical Works, Montreal)

18. Microscope Slides - 1" x 3" and lantern projection slides (glass) 3 1/4" x 4".

19. Fixative - Made up as follows: -

Ethanol (95%) - 70 cc. added to 100 cc. of deionized distilled water (70 vols. per cent ethanol). Then 5% Glacial Acetic acid made up in above 70 vols. per cent ethanol.

20. Stain - Made up as follows:

Mixed in order -	Amido-Black 10 B. (Harleco)	- 0.5 g
	Mercuric chloride	- 5.0 g
	Acetic Acid	- 5.0 cc.
	Distilled water to	- 100.0 cc.

Filtered and used.

21. Rinse - 5% acetic acid in distilled water.

APPENDIX 2 - METHODS OF PERFORMING TESTS

1. Zone Electrophoresis -

Electrophoretic Cell (See Fig. 3) - Made of plexiglass. Although not absolutely electrically inert, it is quite satisfactory. The box is constructed as follows: - Walls and centre partition are made of Plexiglass 1/4" thick, and the agar supports are 1/8" thick. Cementing of pieces is done with a paste made up of small pieces or shavings of Plexiglass dissolved in chloroform. This gives an excellent waterproof seal. The electrodes are made of platinum wire and covered with plastic tubing from an I.V. saline set. This allows products of "gassing" to travel up the tube and prevents bubbles forming in the buffer chamber. The electrode terminals must be made of a non-corrosive material (or alternately, the platinum wire can be passed directly through the wall of the chamber and the space around sealed with Perspex-chloroform paste).

Preparation of Agar - Used both for blocks and for slides. Heat buffer until moderately warm (approx. 65°C) and for every 100 ml. of buffer used add 1.0 gms. of Noble agar. Then heat further until solution is clear. This is 1% agar in buffer.

Some of this is used to make the agar blocks. Some is used to make the slides. The latter is done as follows: - Some agar is poured into a convenient flat container on a bench and allowed to set. The remaining agar is kept liquid by gentle heat. The set agar always finds its own level and therefore provides a perfectly flat surface on which to lay the glass slides. The still-liquid agar remaining is then poured over the slides in a quantity sufficient to make a 3 mm.

thickness when set. This amount depends on the size of the container. This procedure is always carried out with the container kept in the same spot on the bench. After the agar is set the slides are then covered with buffer and can be stored in the refrigerator until ready for use. They must be stored for at least 24 hrs, otherwise the separation of the proteins is not as good.

Applying the Specimen - A groove is made in the centre of each slide, 1 cm. in width and is made by laying a 0.5 cm. length of the filter paper wicks used in the Spinco paper electrophoresis method on edge for about 2 minutes, allowing it to soak up some of the salts and water. This leaves a shallow groove which will take approximately 0.005 ml. of serum. This can be accurately measured with a pipette, or can be applied with a tuberculin syringe with a #26 hypodermic needle. The slide is then left on the bench for about 3-4 minuntes until the serum is absorbed into the groove. It is then placed upside down, supported between the agar blocks. The centre cavity of the agar is then filled with petroleum ether until the surface of the slide is completely covered.

Operation - The cell is now ready to operate. The current should be adjusted to 25-30 milliamps per 1" width of agar (i.e. for one microscope slide - 25 ma., for two - 50 ma., or for one lantern slide - 100 milliamps.). With the Reco power pack or its equivalent, this usually means a potential difference between the blocks of approximately 120-140 volts and a running time of about 45 minutes. If the voltage or amperage is lower the run takes longer, electro-endosmosis is less, and separation is not as good. Run at constant voltage. The run may be interrupted to replace petroleum ether if this has evaporated too quickly. This does no harm provided the time interval is not too long.

Fixing, Drying and Staining - When the run is completed the slide is placed immediately into the fixative. At this point one can see the coagulated protein lines without staining, but the less dense lines may not show up too well. The time here is 30 minutes.

The next step is drying the agar strip. This is important and poor results are obtained when this is not adequately done. This is done by covering the agar on the slide completely with a thin filter paper, and placing in an incubator for a few hours at a temperature of 37°C.

Following drying, the strip is placed in the stain for 1-2 hours. The strip is then placed in the rinse solution until the background is perfectly clear. The strip can then be mounted as a permanent preparation and can be scanned by a densitometer and quantitative studies done.

2. Immuno-Electrophoresis -

This method is performed in exactly the same manner as for zone electrophoresis except that prior to electrophoresis, grooves and wells are made in the agar slides and following electrophoresis the agar is not fixed. The grooves and wells are made as follows: -

A groove is cut in the agar exactly in the centre, in the long axis of the slide anf for 3/4 of its total length (this leaves 1/8 of the length of uncut agar at each end). The groove should be

0.1 cm. in width. This can easily be done by binding two Bard-Parker scalpel blades together, adjusting the width to 0.1 cm. and using this to cut agar evently. Exactly at the centre point of the trough and 0.5 cm. lateral to it on either side a small hole is punched in the agar by using a #16 B.D. hypodermic needle with the point cut off.

The agar from the groove and from the punch holes can be easily lifted out by means of a fine probe.

The serum to be tested is placed in the punch holes and electrophoresed as for zone electrophoresis.

Following electrophoresis the antiserum is placed in the trough and the slide is placed in a petri dish in which a saline-soaked piece of cotton gauze is put to keep the atmosphere humid. The dish is kept at room temp. for 24 hrs. in a draught-free area. The precipitation arcs should be developed in 24 hrs. and are at a maximum in 3 days.

When the arcs are fully developed the slides are placed in normal (0.85%) saline and washed in seven changes of saline, as follows: -

1st day - change 3 times

2nd day - change 2 times

3rd day - change 2 times

This washing elutes out uncombined excess protein from the antiserum and the precipitation lines only are left. Following washing the slides are covered with filter paper and allowed to dry thoroughly in a hot air incubatør (37°C).

Once dried they are then stained as for zone electrophoresis.

APPENDIX 2 - cont'd

3. Complement-Fixation Test -

The Perspex tray method is used, as described by Fulton, F. and Dumbell, K.R.: *J. gen. Microb.*, 3: 97, 1949. 100% hemolysis of the indicator red blood cells is taken as the end-point, using 2 1/2 minimal hemolytic doses of complement.

4. Cytopathogenic-Inhibition Test -

In general this consists of testing 2-fold dilutions of serum against a dilution of virus known to produce cytopathogenic changes involving most of a monolayer of first generation Rhesus monkey-kidney cells grown on roller tubes, at a four-day reading. This virus dilution equaled 100 $T.C.D._{50}$. The specific method is described in "The Laboratory Manual," Virus Laboratory, Department of Bacteriology and Immunology, University of Manitoba.

5. Production of Rabbit Anti-Human Antisera -

10 ml. of human serum (in this case from one individual, but preferably pooled) is inoculated intramuscularly into a minimum of 3 rabbits in the following manner - no adjuvant is used: -

1st injection - 3 ml. I.M. in divided doses - 3 days later - 3 ml. I.M. in divided doses - then 1 ml. twice a week for 2 weeks. Rabbits are then exsanguinated or 10 ml. removed and further booster doses of 1 ml. given weekly for the remainder of the rabbit's life.

6. Preparation of Purified C-reactive Protein -

12 human sera were pooled. Each showed a 1+ or 2+ reaction with A.C.R.P. by the capillary tube method. Use was made of an ether-precipitation method of serum protein fractionation as described by Kekwick, R.A. and MacKay, M.E.: M.R.C. Spec. Report Series, #286, 1954.

Total volume of pooled sera = 12 cc. The serum was adjusted to pH 5.50 by adding dropwise 0.5 M. $NaHCO_3$ solution and checking with the Beckman pH meter. It was then divided equally into four 15 cc. centrifuge tubes.

The ionic strength was lowered by diluting each volume of serum with 3 volumes of distilled water at 0°C. in the international centrifuge. Diethyl ether was then added to 18.5 vols. per cent (approx. 2.0 cc. of ether to each 12 cc. of dilute serum) and the temperature was lowered to -3.5°C.

This precipitated the globulins. The tubes were then centrifuged at 1500 r.p.m. for 1/2 hr. at -3.5°C. The supernatant contained mainly albumin, α-globulins and some β-globulins as shown by zone electrophoresis.

The pH of the supernatant was then adjusted to 5.0 with 0.5 M. acetic acid. Then 1/2 vol. of 50% (v/v) ethanol was added and the temperature lowered to -5°C. This precipitated the albumin and C.R.P. This was spun at 1500 r.p.m. for 1/2 hr. and the supernatant discarded. The sediment was resuspended in 5 cc. of 0.01% calcium chloride solution and then dialyzed against tap water at room temp. for 4 hours. (Ca^{++} in tap water causes C.R.P. to flocculate). C.R.P. = fine flocculate. This was then spun down at 4°C in the international centrifuge, and resuspended in 2 cc. of normal saline.

Zone electrophoresis showed this solution to have a protein component with a mobility similar to albumin and the α-globulins and it reacted as a 2+ by the capillary tube method with A.C.R.P.

TABLE I

COMPARISON OF IMMUNE RESPONSE FOLLOWING NATURAL INFECTION AND VACCINATION WITH DEAD AND LIVE POLIOVIRUS VACCINES

	ANTIBODY RESPONSE	ANTIBODY SURVIVAL TIME	INTESTINAL IMMUNITY	PHARYNGEAL RESISTANCE	CAPACITY TO DISSEMINATE VIRUS	PERSISTENCE OF IMMUNITY
KILLED VIRUS VACCINE (SALK)	GOOD, POOR OR NIL (18)	DEPENDS ON ORIGINAL LEVEL ACHIEVED (18)	RARE- SOME WHEN ANTIBODY TITRE IS 1:1024 OR HIGHER NEVER COMPLETE (36)(37)(38)(39)	PRESENT USUALLY even if ONLY A MODERATE ANTIBODY TITRE IS PRESENT	CAN DISSEMINATE EASILY (3)	ONLY AS LONG AS ANTIBODY REMAINS
LIVE ORAL ATTENUATED	GOOD, POOR OR NIL (3)	NOT YET KNOWN WITH CERTAINTY YET APPEARS UNRELATED TO ORIGINAL ANTIBODY LEVEL ACHIEVED (13)	PRESENT SAME AS IN NATURAL INFECTION	PRESENT (13)	ONLY LIMITED DISSEMINATION (13)	NOT YET KNOWN PROBABLY LIFE-LONG (13)
NATURAL INFECTION	GOOD, POOR OR NIL (12)(3)(40)	PROBABLY LIFE-LONG. CERTAINLY UNRELATED TO ORIGINAL LEVEL ACHIEVED (42)	PRESENT- RELATED TO THE EXTENT OF THE ORIGINAL INFECTION	PRESENT (13)	ONLY LIMITED DISSEMINATION (22)	LIFE-LONG (22)

TABLE II

AGE OF CHILDREN IN MONTHS AT TIME OF VARIOUS PROCEDURES

Case No.	1st DPT and P	4th DPT and P	1st Blood Taken	Feeding oral Vaccine	2nd Blood Taken
1	5	12	13	14	16
2	3	11	12	13	15
3	4	18	19	20	22
4	8	18	18 1/2	20	23
5	3	11	12	13	15
6	4	11 1/2	13	15	17
7	5	15 1/2	16 1/2	18	20
8	4	11	12	-	-
9	5 1/2	13	14	-	-
10	4	12	13	-	-

TABLE III

ANTIBODY LEVELS AS SHOWN BY VARIOUS SEROLOGICAL TESTS

Case Number	Virus Type Fed	TYPE I				TYPE III				Rectal Swab	C.R.P.
		Screen	2 day	4 day	C.F.	Screen	2 day	4 day	C.F.		
1	1 and 3	< 10	A < 4 C 512 +	< 4 512 +	4 32	< 10	A 32 C 512+	16 512 +	16 32	Pos. for Type 3	A. Neg. C. Neg.
2	1 and 3	< 10	A 4 C 256	< 4 128	4 4	< 10	A < 4 C 512+	< 4 512 +	0 16	Pos. not Typed	A. Neg. C. Neg.
3	1 and 3	< 10	A < 4 C 512 +	< 4 256	4 8	< 10	A < 4 C 512+	< 4 128	0 16	Pos. not Typed	A. Neg. C. Neg.
4	1 and 3	< 10	A 4 C 512 +	< 4 512 +	8 16	< 10	A 4 C 512+	4 512+	4 16	Pos. not Typed	A. Neg. C. Neg.
5	1 and 3	< 10	A < 4 C 128	< 4 128	0 4	< 10	A 8 C 512 +	< 4 512 +	4 16	Pos. not Typed	A. Neg. C. Neg.
6	1	< 10	A < 4 C 512 +	< 4 128	0 -	80-320	A 128 C 32	32 32	0 -	Pos. for Type 1	A. Neg. C. Neg.
7	3	80-320	A 128 C 128	128 128	- -	< 10	A 32 C 512 +	8 512+	4 128	Pos. for Type 3	A. Pos. C. Neg.
8	-	320 +	-	-	4	320 +	-	-	8	-	Neg.
9	-	320 +	-	-	4	320 +	-	-	0	-	Neg.
10	-	320 +	-	-	0	320 +	-	-	4	-	Neg.

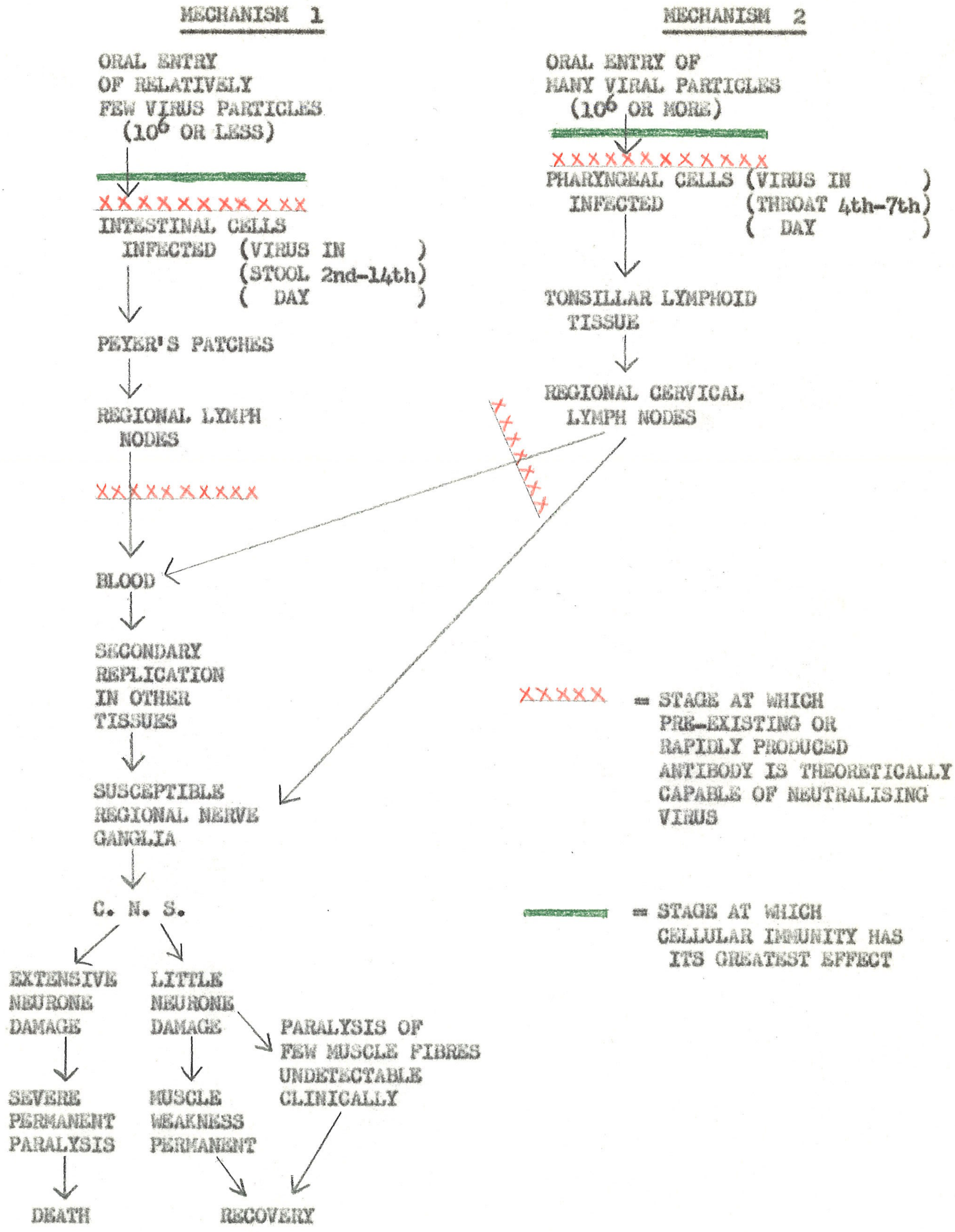


FIGURE 1

SUGGESTED MECHANISM of POLIOVIRUS INFECTION IN MAN

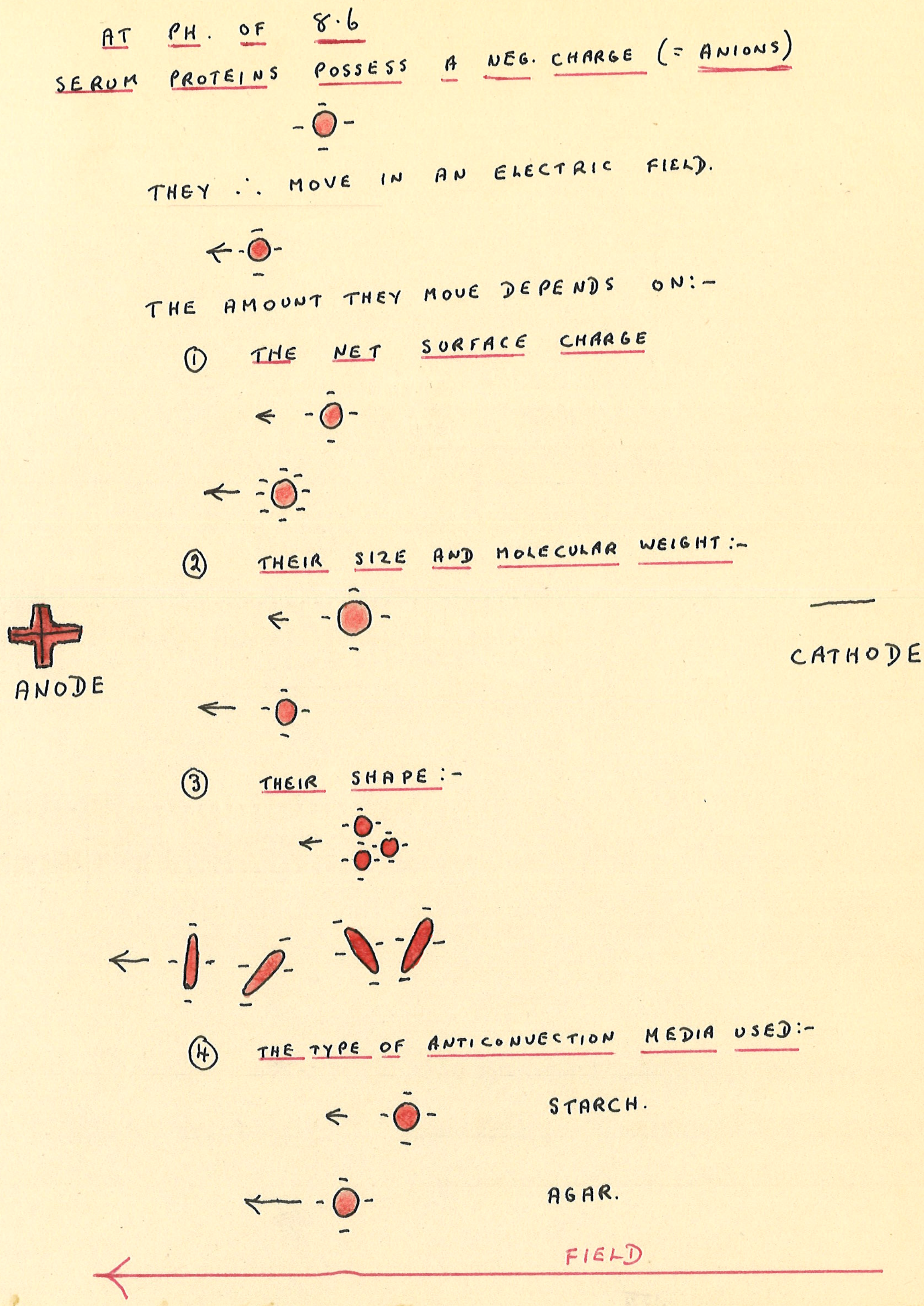


FIGURE 2

DIAGRAMATIC REPRESENTATION OF THE PRINCIPLES OF ELECTROPHORESIS

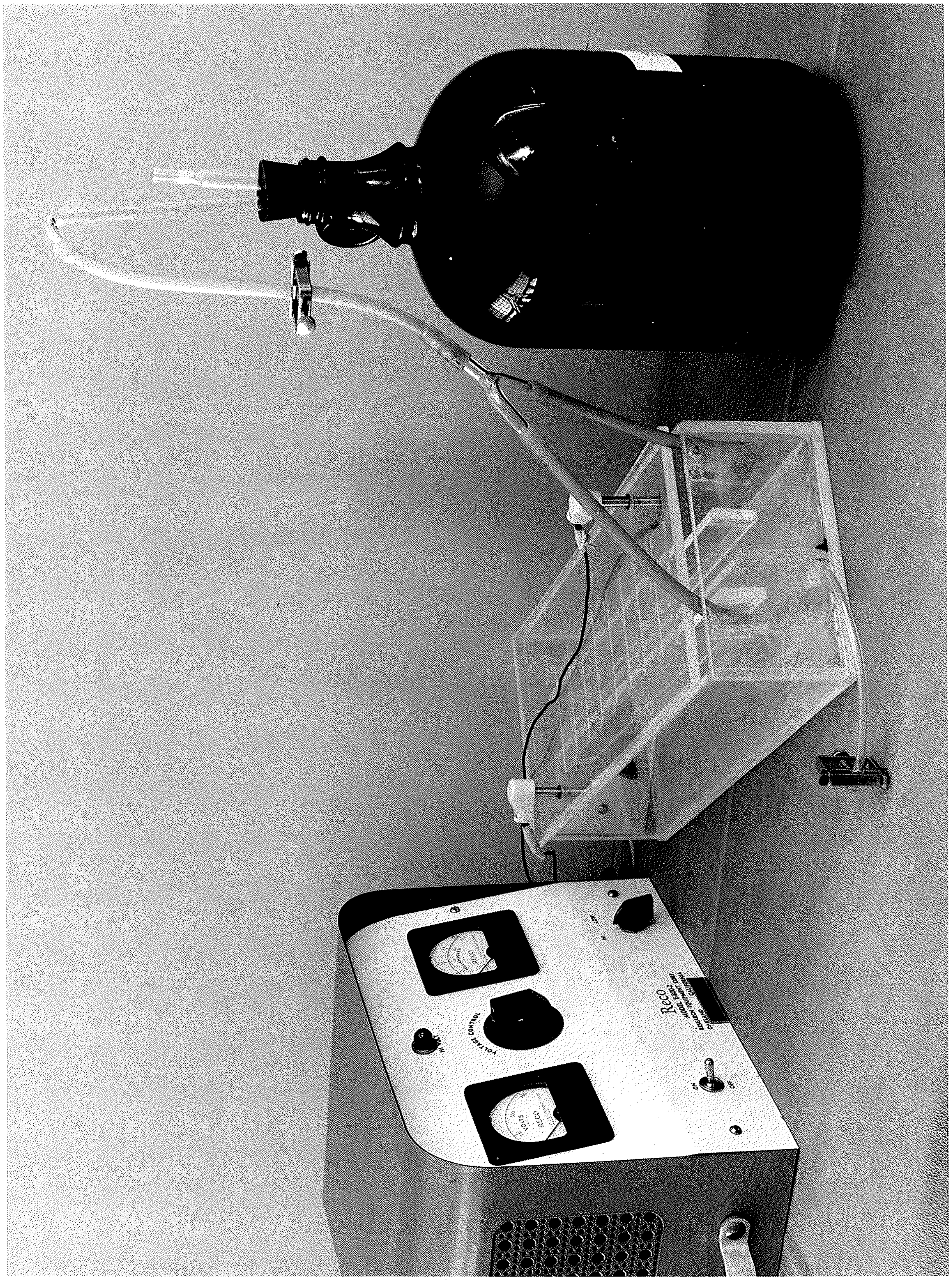
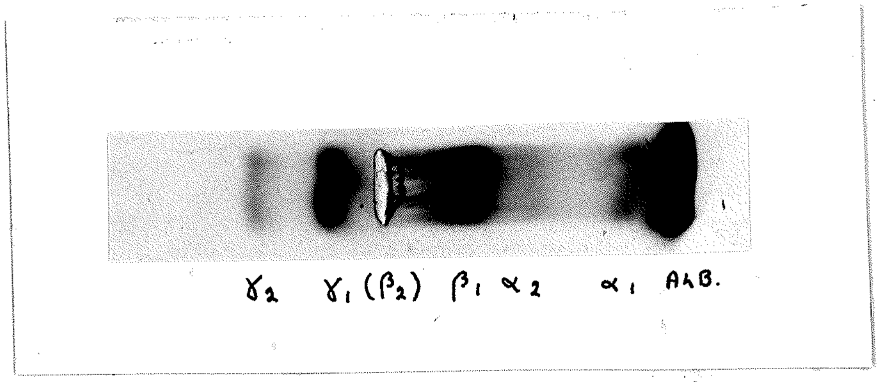


FIGURE 3

PHOTOGRAPH OF EQUIPMENT



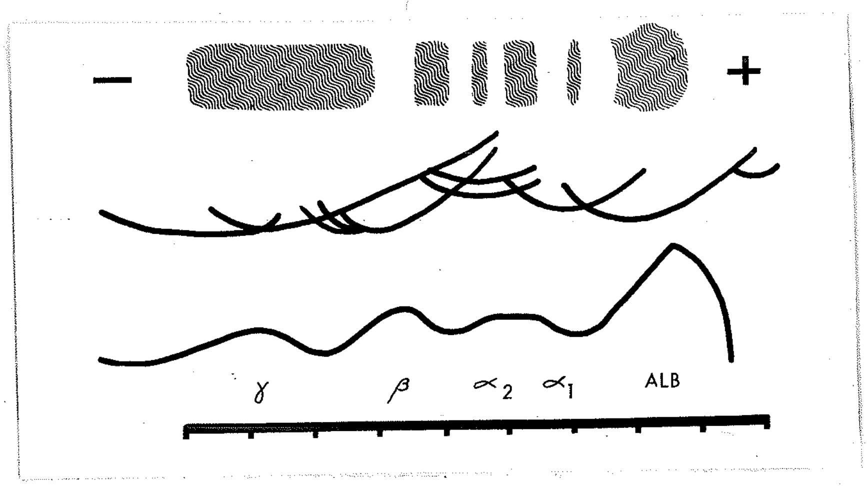
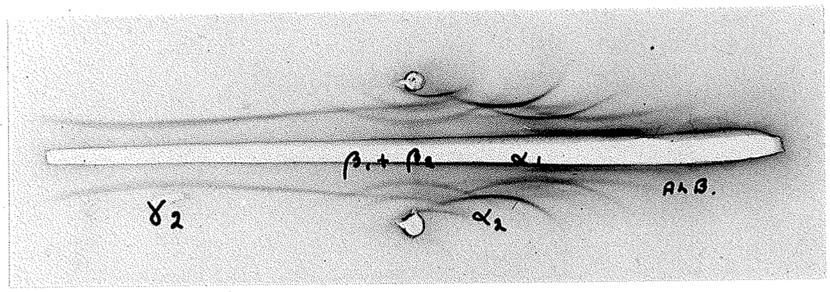
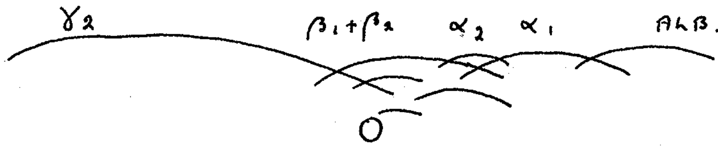


FIGURE 4

PHOTOGRAPH OF ZONE MICRO-ELECTROPHORESIS OF NORMAL HUMAN SERUM AND DIAGRAM ILLUSTRATING THE IDENTITY OF THE ARCS OBTAINED





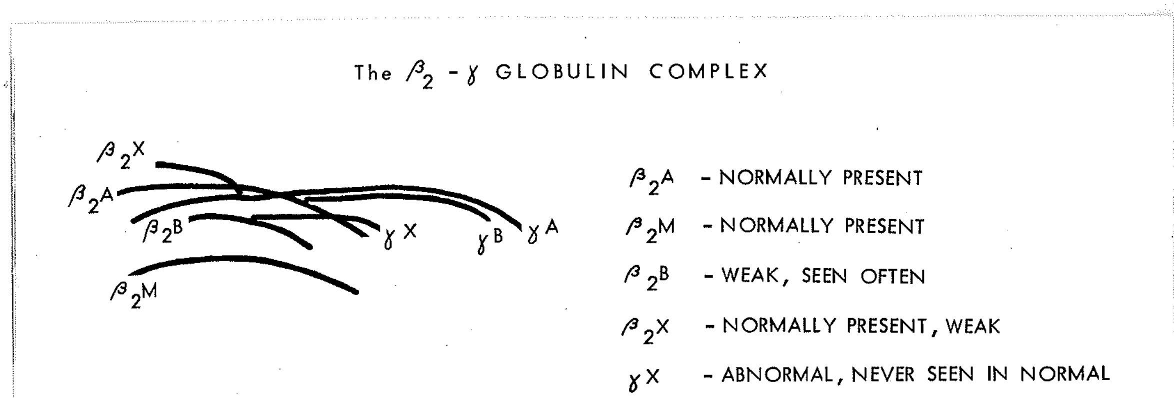


FIGURE 5

PHOTOGRAPH OF IMMUNO-ELECTROPHORETIC PATTERN OF NORMAL HUMAN SERUM AND DIAGRAM ILLUSTRATING THE INDENTITY OF THE ARCS OBTAINED

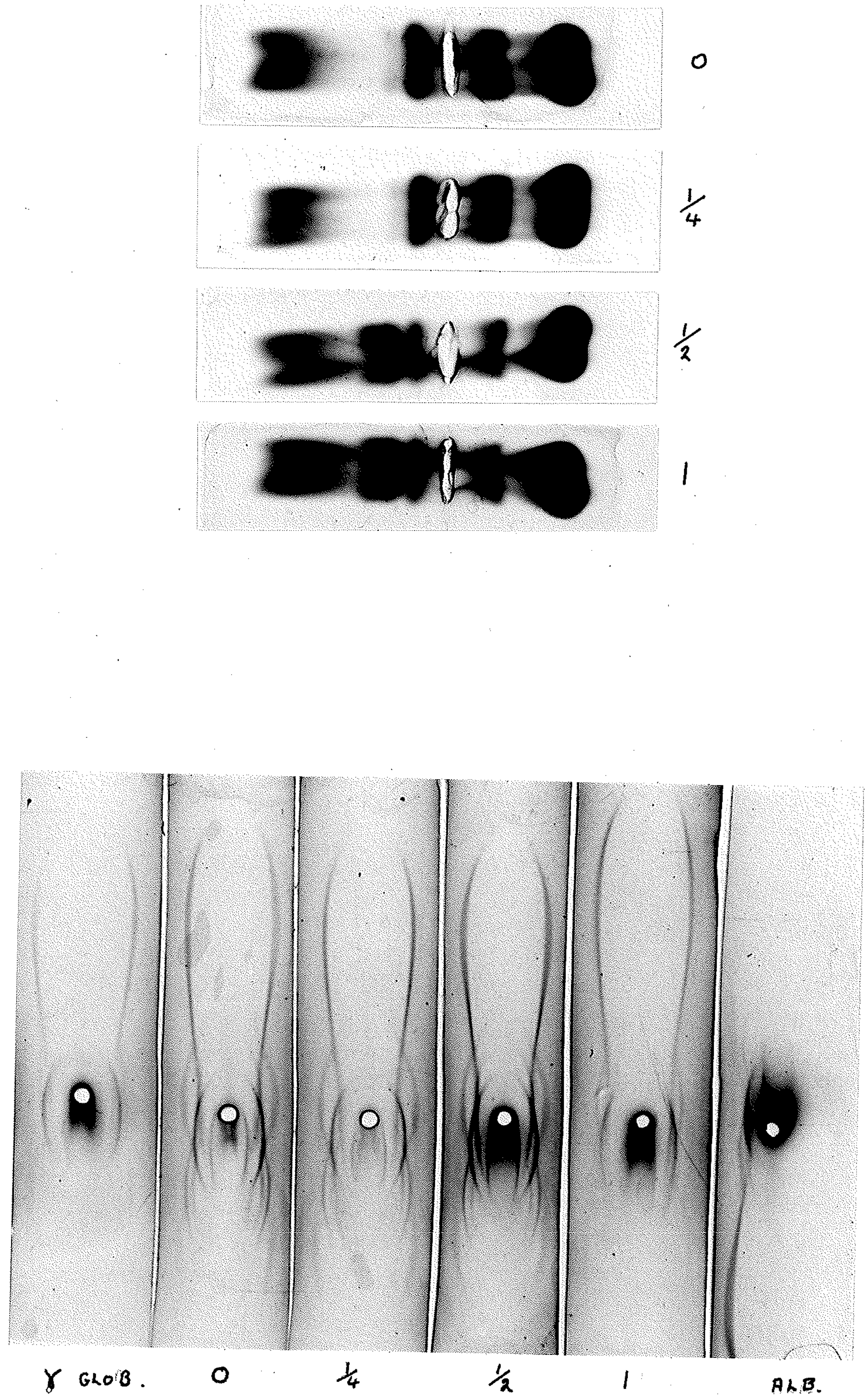
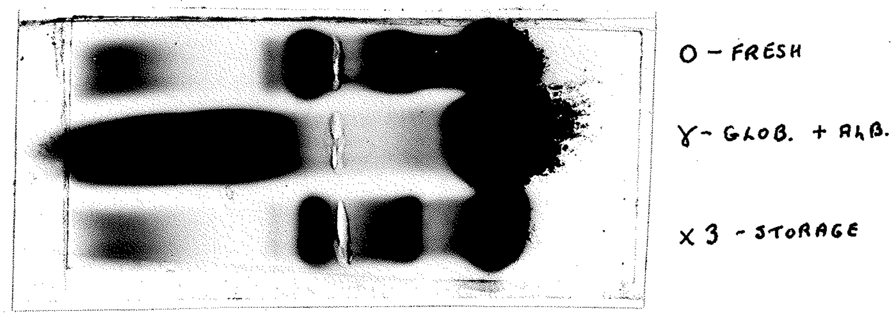


FIGURE 6

THE EFFECTS OF HEATING NORMAL HUMAN SERUM AT 56° C FOR $\frac{1}{4}$ HOUR, $\frac{1}{2}$ HOUR AND 1 HOUR AS SHOWN BY ZONE ELECTROPHORESIS AND IMMUNO-ELECTROPHORESIS



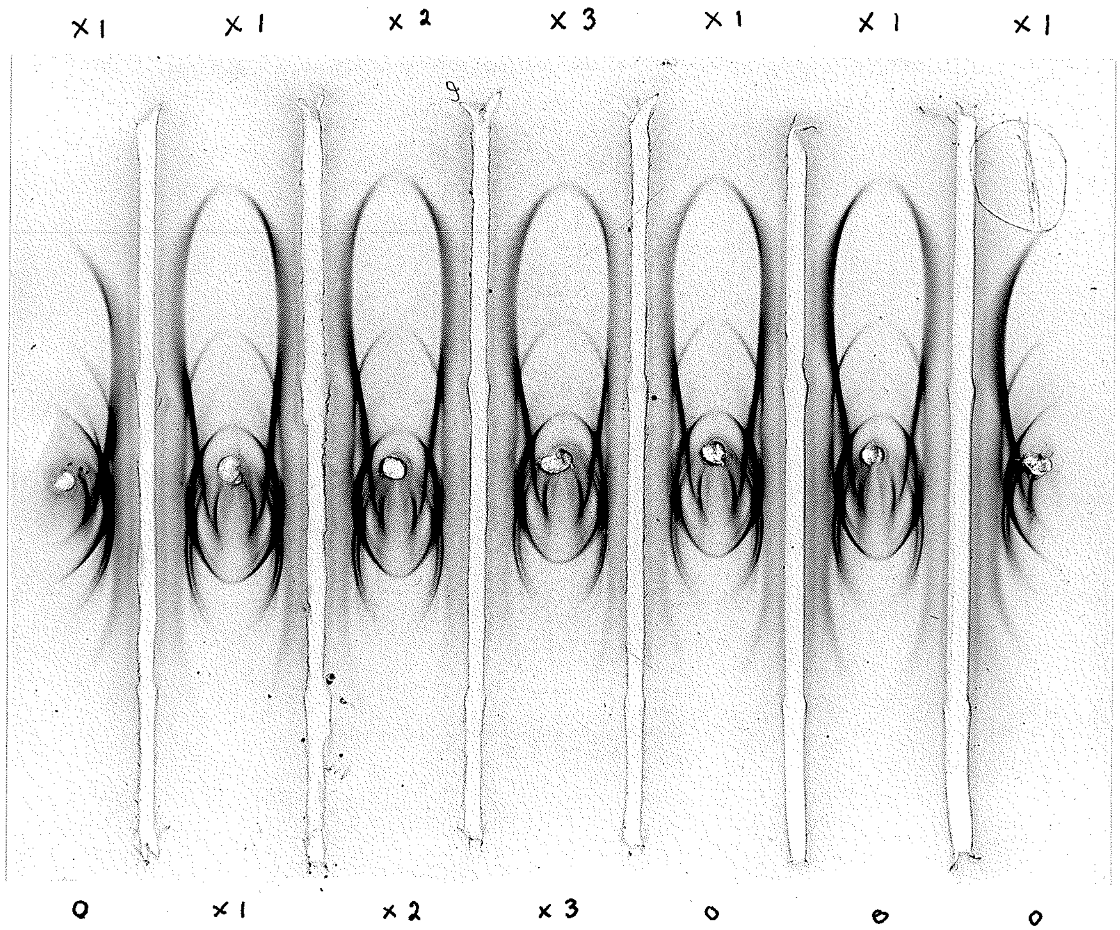
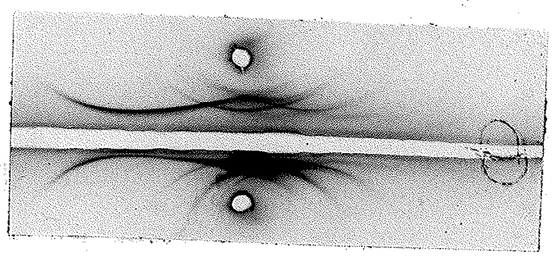


FIGURE 7

THE EFFECTS OF ROUTINE STORAGE OF NORMAL HUMAN SERA AS SHOWN BY ZONE AND IMMUNO-ELECTROPHORESIS

NO CHANGE.



INCREASED DENSITY OF β_1 α_2 BANDS. (? HAPTOGLOBINS)

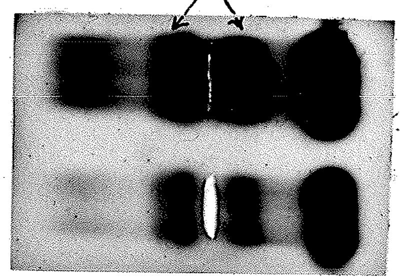
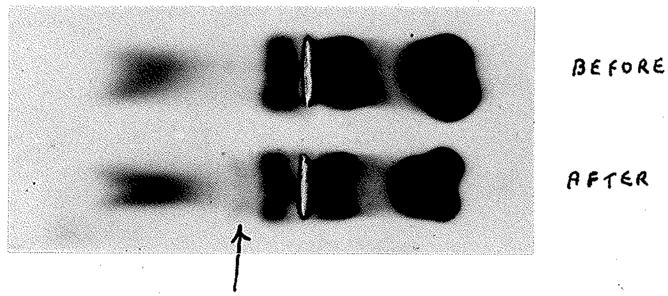


FIGURE 8

THE EFFECTS OF HEMOLYSIS ON NORMAL HUMAN SERUM AS SHOWN BY ZONE-ELECTRO-PHORESIS



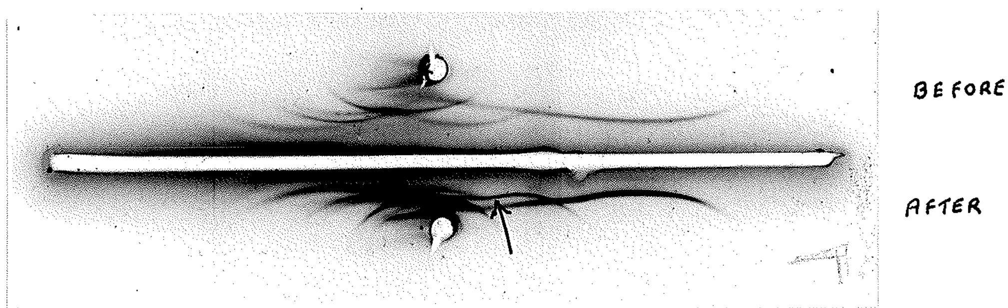
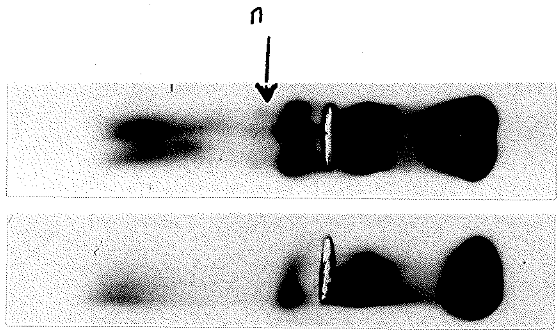


FIGURE 9

SERUM OF CASE NUMBER 1 - IMMUNO-ELECTROPHORESIS



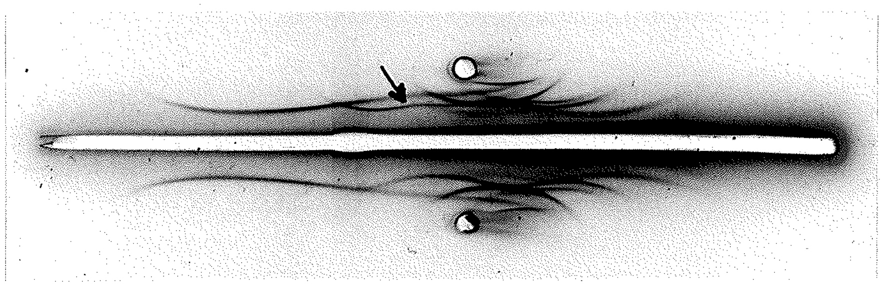


FIGURE 10

SERUM OF CASE NUMBER 2 - IMMUNO-ELECTROPHORESIS

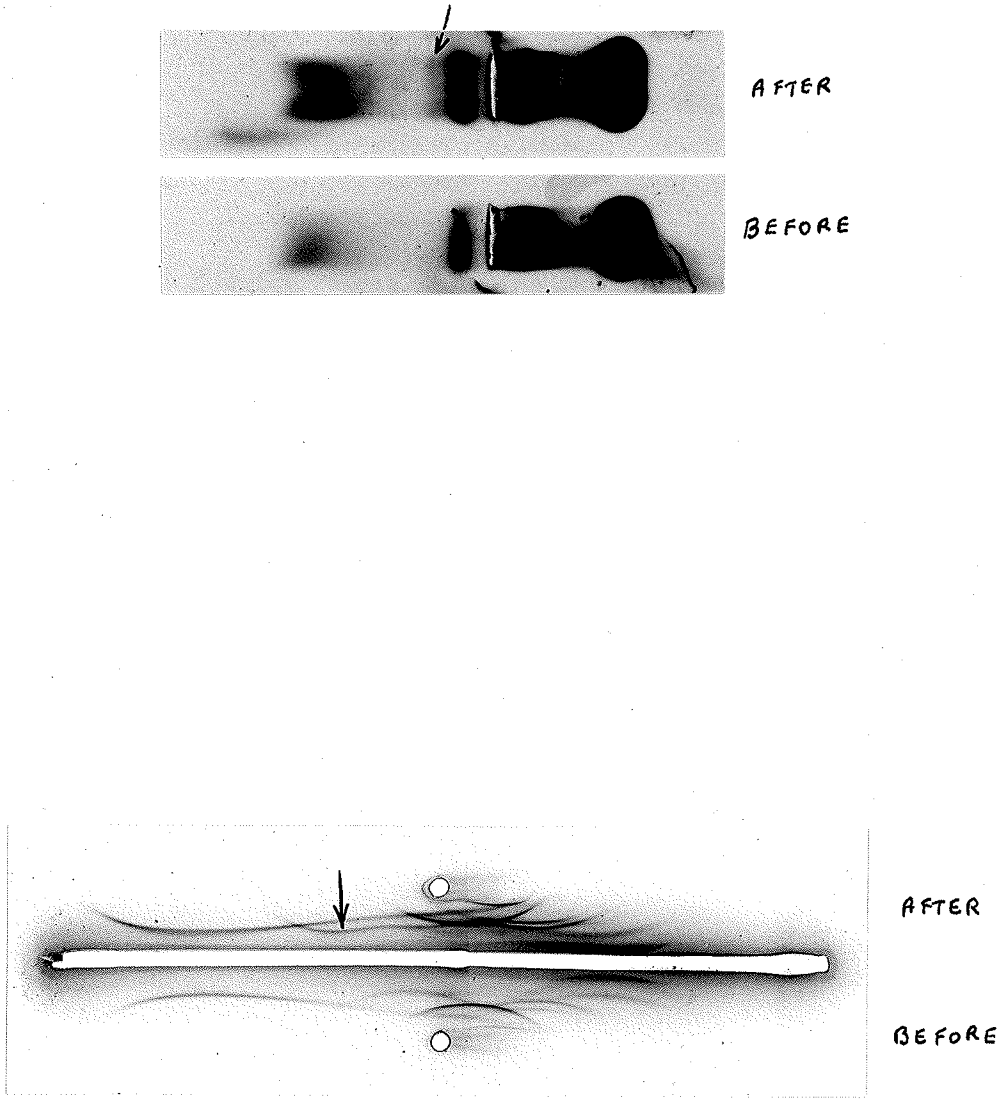
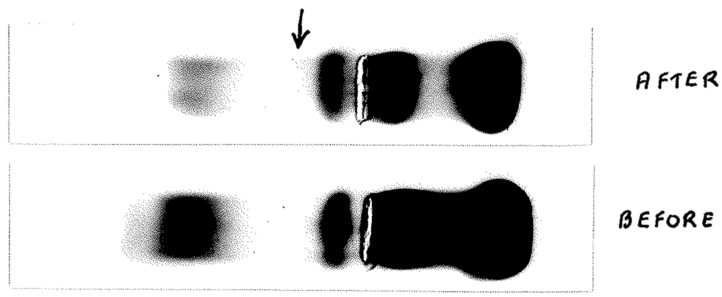


FIGURE 11

SERUM OF CASE NUMBER 3 – IMMUNO-ELECTROPHORESIS



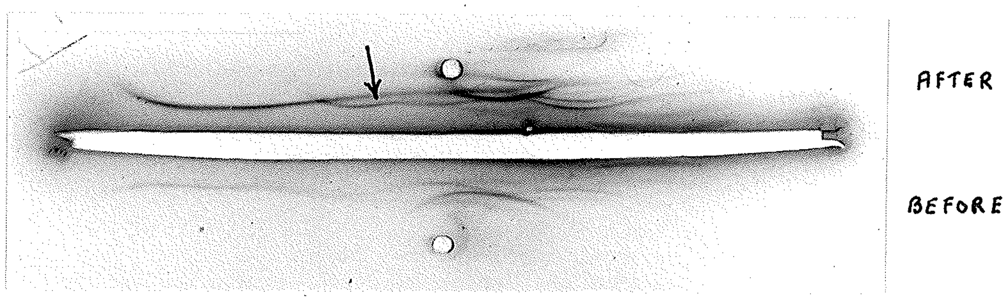
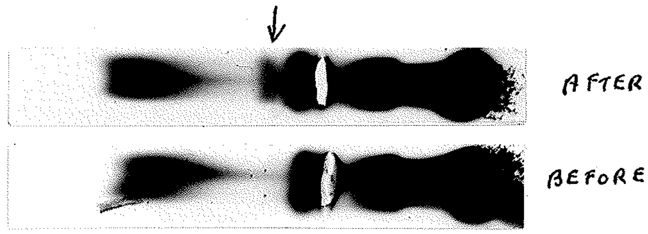


FIGURE 12

SERUM OF CASE NUMBER 4 - IMMUNO-ELECTROPHORESIS



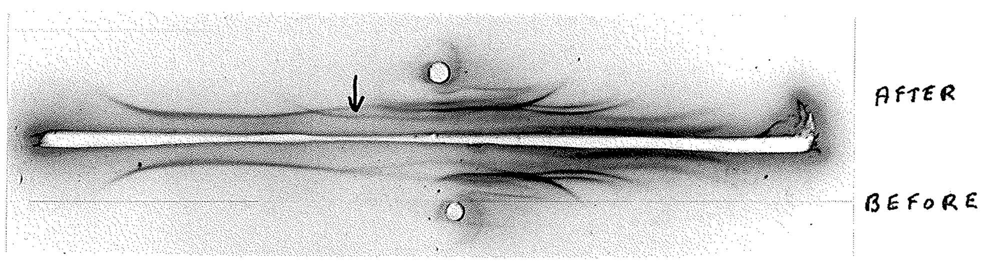
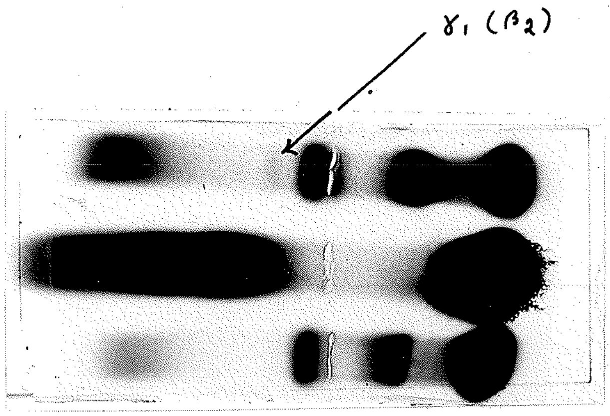


FIGURE 13

SERUM OF CASE NUMBER 5 - IMMUNO-ELECTROPHORESIS



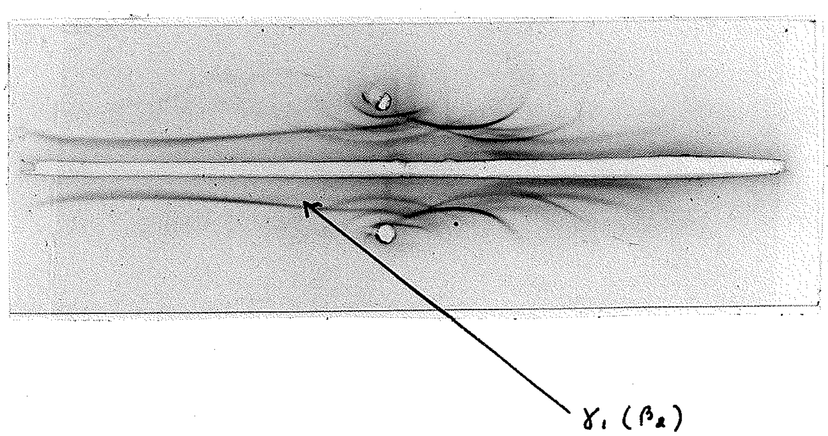


FIGURE 14

DIFFERENCES BETWEEN SERA FOLLOWING POOR AND ADEQUATE RESPONSE TO SALK VACCINATION

EXAMPLE OF ZONE AND IMMUNO-ELECTROPHORESIS

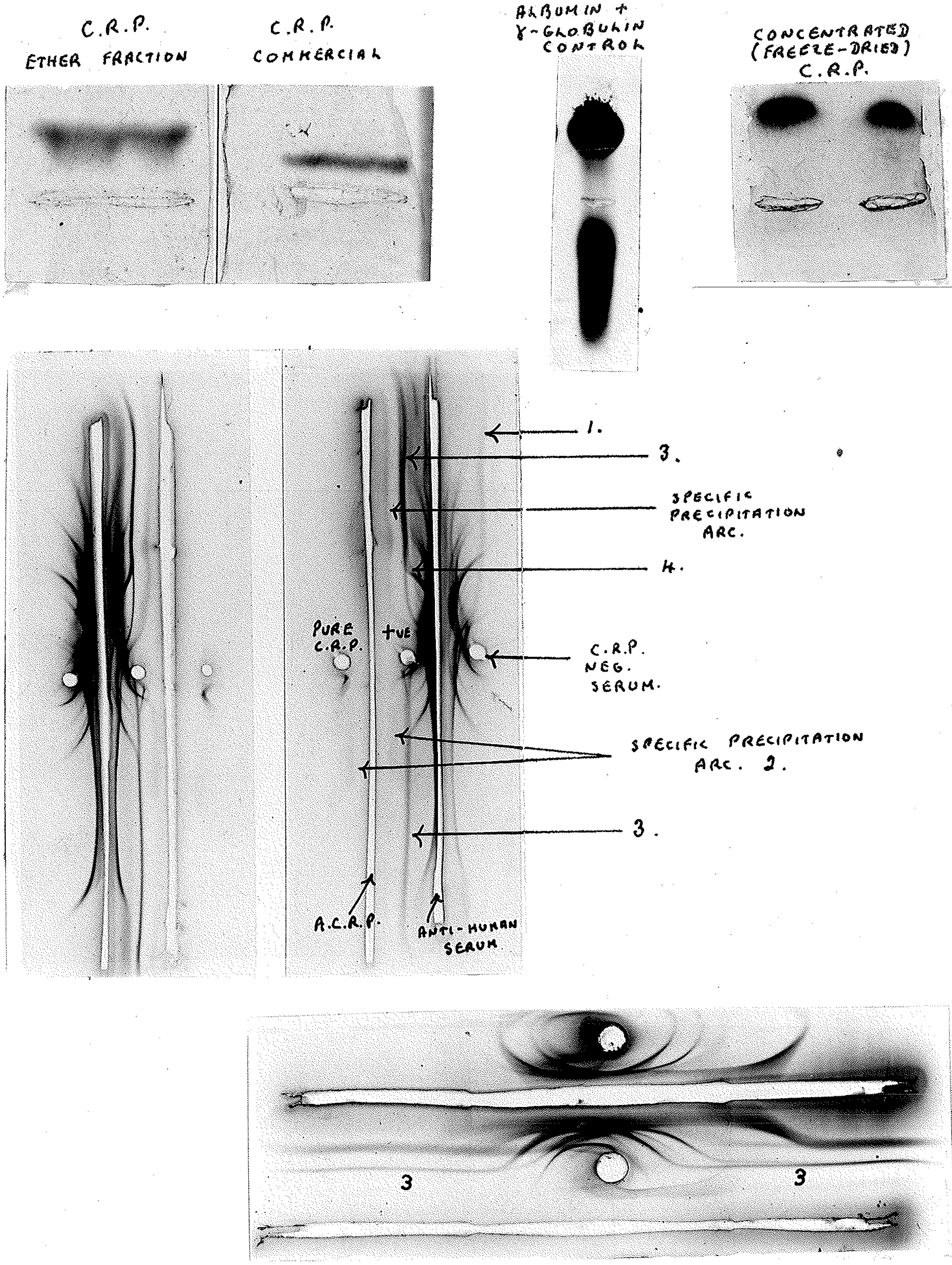


FIGURE 15

STUDIES OF C-REACTIVE PROTEIN
ZONE AND IMMUNO-ELECTROPHORESIS