THE QUALITY OF ANTIBODIES.

Based on a study of antisera against influenza virus.

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Statement.

The work presented in papers I, II and V, was directed by Professor S. Fazekas de St. Groth and carried out jointly. The mathematical formulation in these papers is the work of Professor S. Fazekas de St. Groth. The experimental work presented in paper III has been done together with Dr. W.C. Laver and Professor S. Fazekas de St. Groth; the work in paper IV was carried out by Professor Fazekas de St. Groth and myself, in conjunction with Dr. A. Datyner. The experimental data in the last three papers (VI, VII and VIII) are the results of my own work.

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INTRODUCTION.

The quality of antibodies has been recognised and discussed at the conceptual level since the times of Ehrlich, but the lack of simple techniques of measurement has had two consequences. In practical immunology, this parameter is more often than not ignored and the therapeutic potency of a serum is not fully described; in theoretical immunology, although recognised, this parameter has not been measured in natural antigen-antibody systems, and its relevance to the current theories of antibody production not fully realized.

This thesis sets out to study this problem on the influenza virus-rabbit antibody system. The law of mass action is applied to virus-antibody interaction, and techniques are developed for measuring all the parameters entering the reaction. These and several classical methods are used then side by side to follow the changes in quantity and quality of antibody when rabbits are vaccinated by different routes and schedules.
I. THE ANTIBODY RESPONSE

By the turn of this century, the foundations had been laid for the study of the immune response to infection or inoculation with antigens. The state of knowledge at that time may be summarised thus:

(1) Adequate antigenic stimulus of an animal leads to the appearance of antibodies in the blood and other body fluids.

(2) The antibodies so produced are specific for the stimulating antigen.

(3) The response is produced by the tissues of the animal, and runs a well defined course in time.

(4) Antibodies so produced are associated with a state of immunity.

(5) This immunity is not transmitted from parent to offspring according to the laws of inheritance.

These observations were made over the last ten years of the 19th century, and it is rather surprising how little has been added to them in the following sixty years. New techniques such as radioactive tracers, the use of ionizing radiations, and fluorescent antibody have given us greater understanding of where and at what time antibody is formed, but the actual mechanism of synthesis of antibody - and for that matter all proteins - is still one of the great problems facing biology today.

Differences in the quality of antibody were also recognized
at the end of the 19th century, but truly fundamental work on antigen-antibody combination could be attempted only after it had been demonstrated that biological systems yielded to analysis by thermodynamic methods.

Our knowledge of what we now call humoral antibodies starts with the discovery of antitoxin by Behring and Kitasato (1890). They showed that the immunity of mice and rabbits which had been treated with tetanus cultures depended on the capacity of their serum to render innocuous the toxic substances elaborated by the tetanus bacillus. When these sera were injected into other animals they specifically protected them against challenge with the toxin. Normal serum had no such effect.

Soon afterwards it was found that the ability of the animal body to produce antitoxins is a general phenomenon and holds for plant, as well as bacterial toxins. The researches of Ehrlich are of great importance to immunology, both in defining the principles of quantitative assay (1898), and in sorting out the kinds of immunity (1892). His studies of the antibody response (1891), and his Croonian lecture (1900), "On Immunity with Special Reference to Cell Life" formulating one of the early theories of antibody production, remain the bases of many current views.

The general features of the antibody response were clearly shown by von Dungern (1903), who observed the lag or inductive phase, the subsequent rise to a peak over the following days, and
then a slow fall. The inductive phase was shorter after a second injection of antigen, the rate of antibody formation and the peak titre higher, while the duration of antibody formation was longer. He also noted that the response of the animal was about the same regardless of whether the later stimuli were applied when the antibody was still detectable, or after it could no longer be demonstrated in the blood.

The Primary Response.

Glenny and Sűdmersen (1921) showed that there was a considerable difference between the response to an initial dose of antigen given to a normal animal, and that given after basal immunity had been established. They distinguished these two types by the terms "primary response" and "secondary response", a difference already evident in the work of von Düngern (1903). Thus injection of an antigen into a normal animal is followed not only by the production of antibody, but also by an increased ability to respond to subsequent injections. This condition of "potential immunity" was described by Glenny (1925). At the limit, injection of an antigen may not be followed by detectable antibody production, but the increased ability to respond to subsequent injections is still manifest.

Antigens which can induce a measurable antibody response vary greatly in origin and composition; they include almost every organic substance of high molecular weight and cover also
an extensive range of organic and inorganic substances of lower molecular weight if these are conjugated to proteins to act as antigens. The antibody response to such a variety of substances may be expected to vary, as well it does, but its essential features are still detectable.

The primary response may be divided into three phases: the inductive period, the period of antibody increase, and the period of decline. The nature of each phase varies greatly with the antigen, its state and dose, with the host animal, with the technique of immunization and the technique of assay, and with other less well defined factors.

Appearance of antibody does not follow immediately the injection of antigen, as already noted by Ehrlich (1891) when preparing antitoxin against ricin in mice. For the first 4 days there was no detectable immunity, and when it became evident on the 5th day, it coincided with the appearance of antitoxin in the blood.

The inductive phase was observed to vary from 10 days to 9 weeks for diphtheria toxin in the guinea-pig (Glenny and Südmersen, 1921) but was usually three weeks. For pneumococcal immunity in the rabbit it was 2 to 4 days (Armstrong, 1925). Burnet and Fermer (1949) have observed that the inductive phase for a number of antigens in the rabbit may vary from 2 to 5 days for rickettsial agglutinins, 7 days for herpes neutralizing antibody, 3 to 4 days for bacteriophage C16 neutralizing antibody, and
from 8 to 13 days for staphylococcal antitoxin. A very short inductive phase has been claimed by Nunes (1950) for guinea-pigs inoculated intravenously with type I pneumococcus; agglutinins were detected five hours after inoculation. This observation, made in a well-studied system, is contradicted both by earlier and subsequent work, and is perhaps best explained by assuming previous exposure of the animals to the same or a related antigen.

Exposure of animals to X irradiation 24 hours or more before antigen injection decreases both their ability to form antibody and the rate at which it is formed, but the most significant feature is the increase in the length of the induction period. The increase is proportional to the X-ray dose (Taliaferro and Taliaferro, 1954; Makinodan and Gengozian, 1958). The abnormal length of the induction period is claimed to rest on radiation damage and inhibition of cellular division. This is not the full explanation, as antigen injected immediately before or after irradiation will induce normal amounts of antibody production (Kohn, 1951).

Taliaferro (1957) divides this phase of the response into two periods:

1. the very short preinduction period, during which some necessary event takes place to initiate the rest of the process. This is the stage when the antigen, or antigenically active fragment (Campbell and Garvey, 1960) makes contact with the antibody
producing cell.
(2) the induction period proper, in which the antibody-synthesizing mechanism is developed.

This division is based upon the effect of X-rays on early antibody production in the rabbit. The pre-induction phase is highly radiosensitive, whereas total body irradiation at a stage just before antibody production, will markedly retard the appearance of antibody in the serum, but the amount of antibody is not reduced and may actually be above normal in amount.

The inductive phase ends with the appearance of detectable antibodies. These rise to a peak or plateau and then slowly decline. The peak may be reached in from 2 days to 2 months, depending on the antigen and the other factors mentioned.

The distinctive characteristics of the primary antibody will be dealt with in the experimental section, and the general features of the phases of production and decline will be considered with the secondary response.

The Secondary or Anamnestic Response.

Anamnesis, the ability to recall previous experience by way of a response that is enhanced both in rate and extent, is the distinguishing feature of the secondary response. This ability to recall persists for a very long time after the primary stimulus, even when antibody is no longer demonstrable. The course of the anamnestic response may be divided into the
negative phase, the inductive phase, the phase of rapid increase, and the phase of slow decline.

The negative phase was first noted by Brieger and Ehrlich (1893) when examining the antitoxin content of milk of a goat immunized with tetanus toxin. Shortly after the reinjection of antigen there was a sudden temporary decrease in the antitoxin titre in the blood. This phase is of variable magnitude and is not observed unless the existing antibody levels are still high. Even then the reduction may not occur, and seems to depend on repeated injection of large doses of antigen (Dean and Webb, 1928). There have been two theories put forward to explain this phenomenon. The first is that the combination between injected antigen and circulating antibody will cause a sudden decrease in the circulating antibody. The second is that the injection of antigen may cause massive oedema, leading to depletion of body fluids and consequent dilution of the plasma (Ramon et al., 1929). Since the combining ratios of antigen and antibody are such that several grams of a toxin would be required to bind an appreciable portion of circulating antibody, the first explanation is difficult to accept. The second possibility may play some part in the case of toxic antigens but little or no oedema is observed with non-toxic antigens. Until the phenomenon of the negative phase is studied in greater detail, and while its mere existence remains questionable, there is no great point in trying to take sides in this matter.
The duration of the inductive phase is very brief in animals still synthesising antibody as a result of the primary stimulus but can be studied in animals showing no detectable antibody.

Burnet (1941a) has emphasised a distinguishing feature of the secondary response that is especially obvious when the anamnestic dose of antigen is given intravenously. He observed that antibody increased exponentially with time in rabbits responding to staphylococcal toxoid, herpes virus, and bacteriophage. The secondary response is, however, not always logarithmic, as evidenced by Burnet and Freeman's (1938) observations on rickettsia. In this case, as with specific carbohydrates of pneumococcus (Heidelberger, et al., 1946), there was no detectable secondary response. Logarithmic curves may be fitted to some sets of observations, but, generally, the response curves are not accurate enough and vary greatly depending on animal, antigen, and many other variables.

Whether the sudden increase in antibody is due to release of antibody stored in the tissues or to newly synthesised antibody, has been debated for some time. Holt (1950) explained the increase by release of antibody stored from the primary response. His belief was based on the geometry of the primary and secondary response curves of guinea-pigs immunized with diphtheria toxoid. The hypothesis has been tested more appropriately by the injection of radioactively labelled amino acids,
during the secondary response. The early work of Green and Anker (1954) in this field suggested that up to 30% of antibody was stored and liberated later, whereas the more carefully controlled experiments of Taliaferro and Talmage (1955), Taliaferro and Taliaferro (1957), Richter and Haurowitz (1960) show that little, if any antibody is stored in the tissues for later release. The experimental results of Garvey and Campbell (1959) are not in full agreement with the above workers, they maintain that a small proportion of antibody is stored in the tissues and released on subsequent injection of antigen. The results are compatible with some antibody being stored in the tissues, but show that the bulk is synthesised de novo.

Burnet and Fenner (1949) explained the increased antibody production by multiplication of the entity concerned with antibody formation. Secondary antigenic stimulus is followed by the appearance of increasing numbers of plasma cells containing antibody, at the sites of production (Fagraeus, 1946; Coons et al., 1955). These results indicate that the numbers of cells producing antibody are increasing. The increase in antibody titre from such cells may be enhanced to some extent also by an improvement in the quality of secondary antibody (Jerne, 1951).

The rate of antibody decline is dependent on the rate of antibody synthesis on the one hand, and on the rate of antibody katabolism on the other (Koshland and Englberger, 1957). The
The time taken for antibody to fall to a non-detectable level depends on the physical and chemical nature of the antigen and on the animal. The biological half life of antibodies in small animals with high metabolic rates is in general, shorter than in larger animals.

The estimated half life of homologous antibody in the mouse is about 1.5 days, whereas homologous antibody in the rabbit has a half life of 5.5 days (Dixon and Weigle, 1957). Antibodies with different molecular weights can have different rates of metabolic decay even though they were formed simultaneously in the same rabbit. Forssman type antibodies in rabbits immunized with sheep cell stroma have a half life of 2.8 days (molecular weight c.a. 900,000) while the smaller antibodies (molecular weight c.a. 170,000) have a half life of 6.5 days (Taliaferro and Talmage, 1956).

Studies by Schoenheimer et al. (1942) on incorporation of $^{15}$N glycine during the period of antibody decline showed that the antibody was being synthesised constantly although at a declining rate and that the rate of simultaneous breakdown exceeded that of synthesis.
II. FACTORS AFFECTING THE ANTIBODY RESPONSE

It has been known since the time of Behring and Kitasato (1890) that many factors influence the antibody response, and until very recently the effects were interpreted as changes in quantity only. Today it is recognized that most of the factors bear on both quantity and quality of antibody formed. In this section the causes of variation will be surveyed, and in the next, the consequences.

The Host Animal.

Animals such as the horse, cow and pig produce antibodies that are either of high (c.a. 900,000) or lower (c.a. 170,000) molecular weight, while rabbits, monkeys and man produce antibodies that are predominantly of the smaller kind. Yet, there is no significant difference in the antibody response of all these animals to stimulation with non-replicating antigens. Greater variation however, occurs between mammals and birds. For example, the fowl gives higher titre antiserum to diphtheria and tetanus toxoid when injected intravenously (Watanabe, 1939) whereas the guinea-pig gives the highest titre antiserum to these antigens when injected subcutaneously (Neil et al., 1935).

All animals in the same genus do not respond in the same way to infection or vaccination (Carlinfanti, 1947); several workers have indicated that inbred strains of animals differ in their capacity to produce antibodies (Culbertson, 1935), and
strain differences have been shown to occur in mice and in rabbits (Webster, 1946; Marshall and Fenner, 1958). The recent observations of Schell (1960) show that the inbred strain C57 Black mice are more resistant than "stock" outbred mice to ectromelia virus infection because of heritable differences in the rate of antibody production. C57 Black mice produce antibody earlier than the "stock" mice. The ability of some animals to show a greater primary or secondary response than other animals of the same species has been shown to be heritable (Sobey, 1954; Sobey and Adams, 1955; Claringbold et al., 1957; Sobey and Adams, 1961).

The age of the host animal is also an important feature when studying the antibody response. Young animals do not respond as well as adult animals to antigenic stimuli (Freund, 1930) and the studies of Billingham et al. (1953) established the existence of a "tolerant state". In mice, rabbits and chickens the tolerant state exists for a short time after birth, when animals do not recognise many antigens as foreign and do not produce antibodies to these antigens following injection, as they would normally do later in life. The length of the tolerant phase during early life varies from animal to animal; some, such as the human infant are born immunologically mature and show virtually no truly tolerant state although the rate of antibody formation may be slower than in adults.

Following the early tolerant phase, all animals slowly
develop the capacity to respond to antigenic stimulation by antibody production, and in later life there is no significant relationship between age and antibody production. The early work of Baumgartner (1934) suggested that the quality of antibody from young rabbits (4 to 7 weeks) was poorer than that from adult rabbits (6 to 13 months) but the methods of measuring quality were inadequate and the studies of Jerne (1951) could show no difference between the quality of antibodies from young or old animals.

Dose of Antigen.

In general, there is a certain minimal dose of antigen below which detectable antibodies will not be formed. Above this value the titre obtained varies with the dose administered, but in such a way that the increase in titre is much smaller than the increase in dose required to produce it. Eventually a state is reached when further increase in dose yields a proportionally diminishing return, and under certain conditions may actually decrease the antibody response (Felton, 1949; Dixon and Maurer, 1955). There is general agreement that increase in antigenic dose results in increased antibody formation, but the relationship between the two is not well established. Edsall et al. (1951) has proposed a relationship between the square root of the antigen dose and the maximal antibody level obtained in either the primary or secondary response. This observation was confirmed by Stevens (1956), who after a
review of the dose-response figures from the literature, found that this relationship was the most applicable. He also applied the empirically derived dose-response relationship of Smith and St. John Brooks (1912) to the data and found that the equation held at intermediate concentrations of antigen but not at the extremes.

\[ k_1 C^n = Ab \]

where \( k_1 \) is a constant, \( C \) is the concentration of antigen, \( n \) a constant depending on the chemical nature of the antigen and \( Ab \) is the maximal concentration of antibody. However Stille (1960) has shown that this formula does not apply to the antibody response of humans to influenza virus vaccine administered by the subcutaneous or intracutaneous route. The route of inoculation influenced the relationship; intracutaneous inoculation gave a straight line relationship between log dose and log antibody response, while subcutaneous inoculation gave a sigmoid relationship. The recent studies of Koshland (1957) showed no direct relationship between the amount of antigen present in a lymph node and the amount of antibody synthesised. In short, there is still no established relationship between the dose of antigen and the amount of antibody produced.

Schedule of Inoculation.

The antibody response of an animal to multiple doses of antigen during the inductive phase of the primary response is
usually assumed to be greatly superior to that provoked by a single dose. Ramon and Zoeller first drew attention to this in 1927, and their observation has been confirmed by others for toxins and bacterial vaccines in animals and man (Schütze, 1941). The careful work of Carlinfanti (1951) on the antibody response of guinea-pigs to subcutaneous inoculation of diphtheria toxoid shows beyond doubt that multiple inoculations of antigen were superior to a single inoculation of antigen. However all of these studies have been carried out by comparing single or multiple inoculations of antigens into a particular tissue, and the effect of different routes, or the interaction of routes and dosage have not been studied comprehensively in the past.

The repeated inoculation of animals with antigen improves also the quality of antibody produced (Jerne, 1951; Jerne and Avegno, 1956; Talmage et al., 1956). In all of these cases, schedules of repeated inoculations induced the anamnestic response and hence it is not known whether or not multiple inoculation in the primary inductive phase is the factor improving the quality of antibody.

**Route of Inoculation.**

Probably the most important factor affecting the antibody response and bearing on the related fields of transplantation immunity and hypersensitivity is the route of inoculation of antigen. It is also difficult to separate the physical state
of the antigen from the effect of route of inoculation but where possible they will be considered separately.

In general, it is found that antigens in the form of large particles such as bacteria or erythrocytes are more effective when given intravenously, whereas soluble antigens are best given into the tissues. If a soluble antigen is attached to a particulate material it is usually more effective by the intravenous route (Freund and Bonanto, 1941). The route of inoculation can also favour one or another antigenic determinant of the injected particle. For example, in the rabbit the intravenous injection of heat killed pneumococci or streptococci results in the production mainly of anticapsular antibodies; on the other hand the intracutaneous injection of these organisms leads to the production of antinucleoprotein antibodies almost exclusively, with the subsequent development of delayed type hypersensitivity to the antigen (Jullianelle, 1930; Derick and Swift, 1929).

The early work of Motohashi (1922) on antibody production after small intravenous inoculation of the splenectomized rabbit indicated that the amount of antibody produced by the rabbit was significantly reduced. The importance of the spleen in antibody production after intravenous inoculation was more clearly shown by Rowley (1950) when using the rat as the experimental animal. Intravenous inoculation of antigen into the splenectomized rat resulted in significantly lower antibody titres than in non-splenectomized animals, whereas the amount
of antibody formed following subcutaneous or intraperitoneal inoculation was the same for splenectomized or non-splenectomized animals. Similar observations were made by Taliaferro and Taliaferro (1950, 1951) in the splenectomized rabbit; they showed that if the spleen was removed 6 days after inoculation there was little or no reduction in antibody production. The significance of the spleen in antibody formation after intravenous inoculation is also shown by the work of Billingham and Sparrow (1955). They observed that there was "an unexplained mystery" about the intravenous route of inoculation in the rabbit, as an intravenous injection of whole blood from a donor animal actually prolonged the life of a secondary homograft in the recipient instead of curtailing it. Other routes of inoculation of blood increased the rejection rate of the homograft. Similar observations were made by Prehn (1959) when he showed that large intravenous doses of blood would enhance the growth of sarcoma homografts in a second strain of mice. Other routes of inoculation caused increased rates of rejection of the homograft. Intravenous inoculation after splenectomy caused rejection of the homografts as rapidly as observed after subcutaneous injection of either splenectomized or non-splenectomized animals. It is tempting to postulate that the "unexplained mystery" of intravenous inoculation may simply be due to immunological paralysis of the antibody producing cells, as Felton et al. (1955) have shown that the massive injection of
antigen produces immunological paralysis, while smaller doses of antigen elicit the production of circulating antibodies.

The route of inoculation can also determine the type of response an animal will show. For example, toe-pad inoculation in the rabbit with antigen in water-in-oil adjuvant will produce a type of delayed hypersensitivity, while intramuscular inoculation in adjuvant results in circulating antibody production (Leskowitz and Waksman, 1960). The physical state of the antigen and its dose are also important factors; very small doses of antigen in water-in-oil adjuvant (0.0001 L.f. diphtheria toxoid) given into the toe-pad of the guinea-pig will produce delayed type hypersensitivity with no demonstrable antibody production, while larger doses (1 L.f. diphtheria toxoid) will produce delayed type hypersensitivity followed by circulating antibody formation (Salvin and Smith, 1960). An intravenous booster dose of antigen will suppress the delayed type hypersensitivity and will cause the production of circulating antibodies (Uhr and Pappenheimer, 1958; Leskowitz and Waksman, 1960). These observations support the suggestion of Dienes (1936) that delayed type hypersensitivity may represent an early phase in the immunological response preceding antibody production.

Other routes of inoculation, such as intranasal or oral, have been shown to elicit a secondary response to non-replicating antigens, but no detectable primary stimulation (Edsall, 1946; Dolman, 1948).
The significance of the route of inoculation in the immunological response can be summarized by quoting Campbell (1959):

In immunology "we find a continuum of phenomena from immunity to allergy... The status of an animal with respect to this spectrum is in part determined by the route of administration of antigen. From the immune end of the spectrum to the allergic end, the routes are ordered: intravenous, subcutaneous, intramuscular, intradermal. With regard to acute infectious disease, immunity extends along the whole spectrum. In allergic diseases the left end relates to desensitization, the right end to sensitization. At the immune end of the spectrum, transplants of tissue show acceptance, at the right end, rejection. The situation is the same for cancer transplants. At the base of all these phenomena lie the molecular characteristics of the different types of antibody." The role played by the quality of antibody in these phenomena, and the effect of route of inoculation on the quality of antibody have received little or no attention to date.

**State of the Antigen.**

That the chemical nature of the antigen affects the antibody response, is shown by pneumococcal polysaccharides which evoke a strong primary antibody response not increased by secondary stimulation (Heidelberger et al., 1946), whereas most protein
antigens elicit a marked secondary response on re-injection. The chemical nature of the antigen will be considered in greater detail in section V.

The physical state of the antigen also affects the nature of the antibody response. The phenomenon that particulate antigens are more effective when inoculated intravenously has already been mentioned. The use of adjuvants to enhance and prolong the antibody response has been known for a long time. Ramon (1925), having noticed that accidentally induced inflammation at the site of inoculation favoured antibody formation, incorporated tapioca into the antigen preparation. Many particulate adjuvants have been used since that time, including alum, aluminium hydroxide, phosphates and urates, but the most successful technique was developed by Freund and Bonanto (1944) based on the water-in-oil emulsion of antigen, with or without the addition of tubercule bacilli. In this way Freund and Bonanto (1946) were able to induce antibody levels that persisted for 3½ years, after only two injections. The addition of tubercule bacillus to the oil only improves the antibody response to small quantities of antigen or very "poor" antigens (Weigle et al., 1960).

There are conflicting reports on the mode of action of adjuvants, but there are at least three points that emerge from these studies:

(1) Adjuvants cause local tissue reaction and attract anti-
body-producing cells.

(2) Antigen is released slowly from the site of administration.

(3) Antibody is also produced in the draining lymph node.

There is no doubt that adjuvants produce a local reaction developing into a granuloma (Freund, 1956), but there is some controversy about the quantity of antibody produced by the granuloma. Askonas and Humphrey (1955) showed that up to 80 per cent of the antibody was produced by the granuloma, while McKinney and Davenport (1961) show that only a small quantity of antibody is produced by granulomas when transplanted to other animals of the same inbred strain. The former studies were carried out invitro and eliminated any possible host versus homograft reaction that might occur; although McKinney and Davenport (1961) claim that there was no gross evidence of rejection of the homografts.

Removal of the granuloma at various times after inoculation will reduce the amount of antibody produced by the animal (Holt, 1949; McKinney and Davenport, 1961). These workers claim that this is evidence for the slow liberation of antigen from the depot, but it might well be that they are removing the major site of antibody production. Most workers in this field however, are agreed that there is slow liberation from the depot, and McKinney and Davenport (1961) have shown that multiple injections of antigen in aqueous suspension will produce the same antibody titre as that produced by a single inoculation of
antigen in adjuvant. A single injection of antigen in aqueous suspension by the route in question will not produce such a good response.

Elimination studies on isotopically labelled diphtheria toxoid (Koshland, 1957) from alum induced granuloma in the guinea pig have shown that a portion of the antigen is broken down at the site of injection, but some intact antigen passes to the draining lymph node. These studies have also shown that significant levels of antibody are produced by the draining lymph node, as have the studies of White et al. (1955). These workers inoculated the antigen into one hind leg of the rabbit, in adjuvant and showed a secondary response in the cells of the draining lymph node when a secondary stimulus was given in the opposite leg; and that a primary response occurred in the lymph nodes draining the site of the second inoculum.

Inoculation of antigen-antibody complexes (Terres and Wolins, 1961; Uhr and Pappenheimer, 1956), bacterial endotoxins (Kind and Johnson, 1959), or intraocular inoculation of soluble antigens (Parks et al., 1961) all produce adjuvant effects on antibody production, and show some or all of the features discussed above.

The effect of adjuvants on the quality of the antibody produced has not been established in any detail, although it is known that the quality of antibody improves slowly with time.
after one inoculation in adjuvant (Jerne, 1951).

Conclusions.

It appears, then, that the nature of the antibody response is influenced by a great number of apparently unrelated factors, many of which cannot be separated from one another. The quantity and quality of antibody have not been separated when examining the factors influencing the antibody response.

There are further factors affecting the antibody response, such as ionizing radiations, hormones, and metabolic inhibitors. These will be considered in detail when dealing with particular questions.
III. THE QUALITY AND QUANTITY OF ANTIBODY

The comparison of serum samples from different animals, or even from the same animal at different times after immunization, has led to the conclusion that there are other differences besides the absolute concentration of antibody that have to be taken into account before the two sera can be fully equated. If two antiserum against some highly toxic substance are compared, say in a precipitin test, they may be found to give identical titres. We might at first sight think therefore that they were of equal potency in protecting an animal against the toxin. This, however, is seldom the case, for one serum might contain 10 protective units per unit volume, while the second might contain 5,000 protective units in the same volume. We will refer to the character of antibody underlying this difference as quality.

The early workers in immunology often came up against this difficulty when comparing the protective potency of an unknown serum against a standard preparation. Ehrlich (1897) thought that the complexity of diphtheria antitoxin standardization was due entirely to variation in toxoid content and that comparison of toxoids with one known antitoxin standard would overcome the undesirable variation. On these grounds he proposed what has become known as the "Ehrlich Unit" of antitoxin. The principle of identical behaviour of antitoxins from different origins has led to the greatest confusion in this field. The assumption
that the neutralizing potency of different antitoxic sera can always be made identical by simple dilution of the more potent serum does not always hold.

It was first shown by Roux (1900) that the curative power of antisera did not correspond to the Ehrlich titre, and Kraus (1903) suggested that in addition to the actual quantity of antibody in a serum there was another characteristic which determined the rate of reaction of that serum. To this characteristic he proposed giving the name "avidity". The meaning of the word avid is "to crave" and has a restricted meaning, especially in its original use where it is restricted to the rate of reaction. Quality, or the degree of excellence of a product, has a broader meaning by covering both rate and extent, and thus is more appropriate when referring to antibody; it will be used in preference to avidity.

As most of the early work on the quality of antibodies was done on the diphtheria toxin-antitoxin system, it will be worth while looking at the development of the concept of quality in this system.

Manifestations of Quality Noted by Early Workers.

Dreyer and Madsen (1901) observed that a mixture of toxin and antitoxin which was neutral when injected subcutaneously into the guinea-pig was lethal for the rabbit when injected intravenously. However, if this mixture was allowed to stand for 24
hours, it was found that in some cases the toxicity for rabbits
had been neutralized (Danysz, 1902). Toxin-antitoxin mixtures
that were non-toxic for both the guinea-pig and the rabbit were
usually found to have a short flocculation time (Madsen and
Schmidt, 1926), while those that were toxic for the rabbit had
a long flocculation time. These results were confirmed by
Glenny et al. (1925) when they showed that certain mixtures of
toxin and antitoxin would produce no reaction in the guinea-
pig when injected intracutaneously in an undiluted form, but
were toxic if diluted 100 fold before injection.

These observations appear simple enough and could be in-
terpreted on the notion of rate only; but the work of Madsen and
Schmidt (1930) showed that slowly flocculating sera were not al-
ways of poor quality thus disallowing any facile generalization.

Actual figures showing the difference in the quality of
antisera were quoted as early as 1901 by Tizzoni. He showed
in his experimental work on tetanus antitoxin that a serum
with 40,000 flocculating units could show twice the protective
power of another serum with 500,000 units. These types of
results have been quoted by most of the early workers in this
field, but it was not until 1937 that Goodner and Horsfall
(1937) established the inequality of antibodies quantita-
tively. These workers when comparing the mouse-
protective value of anti-pneumococcal type I horse sera
showed that the number of protective units per milligram of
precipitable antibody varied from serum to serum. For example,
one serum had 540 protective units per milligram of specifically precipitable nitrogen, while another serum had 805 protective units per milligram of specifically precipitable nitrogen. Similar observations by Heidelberger and Kendall (1935) showed that on prolonged immunization of the rabbit with ovalbumin there was a regular change in the ovalbumin to antibody nitrogen ratio.

Differences in the quality of antisera are manifest in flocculation time (Ramon, 1923), ease with which the antigen-antibody complex dissociates on dilution (Glenny et al., 1925), the slope of the neutralization curve (Heidelberger and Kendall, 1934; Jerne, 1951; Talmage and Maurer, 1953), and the increase of cross reactions (Landsteiner, 1947). The differences may be accompanied by heterogeneity with respect to molecular weight, electrophoretic mobility, and behaviour on chromatographic columns. These latter properties will be considered in more detail in the section on the physical and chemical structure of antibodies.

Although most of the early work on the quality of antibodies was carried out on the toxin-antitoxin systems, it would be wrong to think that this phenomenon is confined to these systems. Baumgartner reviewed this field in 1934 and showed that quality is a fundamental variable of all types of antibodies.

**Measurement of Quality.**

Measurement is the comparison of an unknown with some
established standard. It is nowadays conventional to compare substances in a basic system of physical units such as the CGS... or MKS systems. This type of measurement is, however, seldom readily feasible in biological systems, and we usually compare the titre of antiserum samples. This type of measurement is quite satisfactory providing we recognize its limitations and accurately define the conditions of the tests. For example, in complement fixation, we may use x units of complement standardized against y per cent of red blood cells sensitized with z units of haemolysin, at fixed volume and time of incubation.

If all the variables are not covered, the system cannot be sound, and no valid comparisons can be made. The full implications of this method of comparison must be realized; the titre of an antiserum is seldom the measure of the quantity or the quality of antibody, it is a measure of a compound of both of these.

Turning to the measurement of the quality of antibody and looking at the methods used by early workers, it is quite clear today where the source of uncertainty and systematic bias lay. The methods used have been reviewed fully by Jerne (1951) and will only be considered briefly.

(1) The flocculation time is a rough measure of the rate of association of antigen and antibody (Madsen and Schmidt, 1930). It is unsound, as slowly flocculating antisera can be of higher quality than faster flocculating sera.
(2) The ratio between the antitoxin titre, when measured by intravenous injection of rabbits immediately after mixing and 24 hours later is essentially a measure of dissociation. This method was used by Madsen and Schmidt (1930), but as no details of the test volumes are given, the dilution step into the rabbit is always a variable, and inter-rabbit variation in susceptibility to toxin is another. Even if these were controlled, qualities could not be compared directly.

(3) The ratio between the antitoxin titres obtained by measurement in animals and by flocculation in vitro (Glenny et al., 1925), rests on the assumption that one test measures quantity only and the other quality only. Neither assumption is sufficiently correct to allow real comparisons, even if the two component methods had been standardized and did not vary from worker to worker.

(4) The dilution ratio of Glenny and Barr (1932) was the ratio between the antitoxin titres measured by the intracutaneous method at two different levels of initial toxin concentration. The time taken for antitoxin and toxin to reach equilibrium will depend on the concentrations of toxin chosen to use in the test and on the dilution factor between the two levels of toxin. At the levels of toxin and antitoxin chosen in this system, aggregation can occur, and these factors will influence the ratio obtained.

Jerne observed these weaknesses in the above mentioned methods for measuring the quality of sera, and proposed what he
called the "Avidity Constant" to take their place. This is more commonly known as the association constant in physico-chemistry, i.e., the concentration of antigen-antibody complexes divided by the product of the concentration of free antigen and free antibody.

When toxin and antitoxin are mixed together, they combine to form complexes. In the simplest case this reaction can be written diagrammatically:

$$T + A \rightleftharpoons TA \rightleftharpoons (TA)_n$$ (aggregates).

Where T is toxin, A is antitoxin, and TA are the simplest complexes. The basic reaction shown above is much faster than the ensuing reactions, but these do occur and complicate the issue. The simplest case only will be considered.

According to the law of mass action the rate of formation of TA is proportional to the product of the concentrations of T and A. The square brackets denote concentration.

$$r_1 = k_1 [T] [A]$$

The rate at which the dissociation of TA into its components takes place is proportional to the concentration of TA.

$$r_2 = k_2 [TA]$$

An equilibrium is reached when $r_1$ equals $r_2$, then

$$\frac{k_1}{k_2} [T] [A] = [TA]$$

Writing $\frac{k_1}{k_2} = K$, we have the association constant. The
reciprocal value, \( \frac{1}{K} \), is the dissociation constant.

The first assumption here is that antibody is univalent, and even though this is known not to be the case, in dilute systems it may be considered to be so.

The second point that must be established is that the interaction between toxin and antitoxin is a reversible reaction, and that an equilibrium is formed. The concept of reversibility in antigen and antibody reactions was introduced by Arrhenius and Madsen (1902) and in recent years has come into general acceptance (Jerne, 1951). However, as it is one of the basic concepts of antigen-antibody interaction, it should be shown to be valid for each test system under investigation.

Heterogeneity of antibody within a single serum sample, and heterogeneity of antigen must also be taken into account when establishing the interactions between antigen and antibody. Jerne (1951) followed the results of Pauling et al. (1944b) and assumed that the free energy of combination of the various antibodies with toxin was normally distributed. A similar assumption was made for antigen.

The final point that must be remembered is that \( K \) is the first association constant valid for monovalent antigen and antibody only. As long as the system is far from saturation and there is no electrostatic or steric interaction between neighbouring antigenic sites, its use is unobjectionable.

Even when complicating factors are taken into account,
measurement of the association constant ($K$) is the only thermodynamically sound way of expressing the quality of antibodies. Definition of the equilibrium between antigen and antibody is the only way to describe the quality of a serum fully. It will account for differences in flocculation time, for the ease with which the antigen-antibody complex can dissociate, for the slope of the neutralization curve, and also for changes in specificity of antibody with time after inoculation.

The value of this constant can be expressed in basic units, allowing valid comparisons between sera. However, owing to the assumptions that have to be made in this system, the association constant is only meant to express the average quality of antibody molecules in a serum. Measurement of this value and of the quantity of antibody in basic units will be sufficient in practice to define the potency of a serum.

The practical methods for determining the quality of antibodies differ from system to system, but, in general, any method that will allow the measurement of the dissociation constant will give a measure of the quality of antibody. In practice this means that either free antigen, free antibody or the proportion of one or both in the antigen-antibody complex has to be determined. With small antigens and especially haptens, standard physicochemical methods can be used to this end, without any major modification. Indeed, these are the tools of immunochemistry,
and measurements of equilibria are one of its main occupations ever since Arrhenius coined the term. With particulate antigens such as bacteria and viruses there are no established techniques for similar measurements, and part of the present work will deal with the development of techniques that will permit the estimation of equilibrium parameters.
IV. THE PHYSICAL AND CHEMICAL PROPERTIES OF ANTIBODIES

Antibodies are proteins associated with the globulin fraction of serum. The work of Tiselius and Kabat (1939) showed that absorption of sera with the immunizing antigen caused a marked decrease in the $\gamma$-globulin fraction only. The term $\gamma$-globulin is derived from electrophoretic analysis and has been defined by the U.S. Commission on Plasma Fractionation and Related Processes as that fraction of plasma which does not contain proteins with electrophoretic mobility greater than $2.8 \times 10^{-5} \text{ cm}^2 \text{ v}^{-1} \text{ sec}^{-1}$ in veronal buffer pH 6.6, ionic strength 0.1.

All antibodies however are not $\gamma$-globulins, as Tiselius and Kabat (1939) conclude. At early stages after immunization, horses form antibodies that migrate between the $\beta$ and $\gamma$ globulins, and this electrophoretic peak has been termed the T component. At later stages after immunization, the antibody has an electrophoretic mobility in the slow moving $\gamma$ globulins. Using electrophoresis in starch blocks and ultracentrifugal methods (Stelos, 1956; Stelos and Taliaferro, 1959), it was possible to separate rabbit haemolysins against Forssmann type antigens, into two peaks of antibody activity: the $\gamma_1$ haemolysin sedimented with the 19 S globulin, while the $\gamma_2$ sedimented with the 7 S globulin. The $\gamma_1$ globulins appeared early after immunization, while the $\gamma_2$ globulins appeared later.
Recent studies by Bauer and Stavitsky (1961) on the different molecular forms of antibody synthesised by rabbits during the primary response to a single injection of soluble and particulate antigens show that the antibody response is heterogeneous. The initial antibody formed was a $\gamma_1$ macroglobulin of 19 S, while the later antibody formed was a $\gamma_2$ globulin of 7 S.

This change in the character of antibody that occurs during the time course of immunization of animals has been clearly demonstrated by Porter (1955), Humphrey and Porter (1956) and by Askonas et al. (1956). Antibodies in rabbit sera to pneumococcus type III polysaccharide, influenza virus (strain MEL), and ovalbumin, showed a change in elution pattern on partition chromatography. There was a change from slow elution rates to fast elution rates as the time after vaccination was increased. The only exception observed was after immunization of rabbits with antigen in Freund's water-in-oil adjuvant. In this case the elution pattern did not change even after a second injection of antigen in adjuvant.

Antibodies of one type or another have been detected throughout all the globulin fractions of serum. Skin sensitizing antibody, Rh antibodies, and syphilis reagins have been found in the $\alpha$- and $\beta$-globulins (Campbell et al., 1954; Laurell, 1955; Cann et al., 1952) as well as in the $\gamma$-globulins.
The Size and Shape of the Antibody Molecule.

Total \(\gamma\)-globulin of rabbit and man contains a principal component with a sedimentation constant of 7 S. Assuming an intrinsic viscosity of 0.06 and a partial specific volume of 0.74, Oncley et al. (1947) calculated a molecular weight of 150,000. However, recent studies on the three fragments of rabbit antibody separable by papain have given a slightly higher molecular weight of 185,400 ± 1,400 (Charlwood, 1959). Equine, bovine, and porcine antibody prepared by dissociation of pneumococcal specific precipitates, showed the presence of a component with much higher sedimentation constant (19 S) and Kabat (1939) calculated a molecular weight of about 1,000,000 for this type of antibody. The size of the antibody molecule also varies with the time after immunization (Stelos, 1956; Bauer and Stavitsky, 1961) but the predominant antibody form in the rabbit at a reasonable time after first inoculation (3 weeks) is the 7 S antibody.

The antibody molecule, taken as a cylindrical particle would have an elliptical cross section with axes of 19 and 57°A and with a length of 230 to 240°A (Kratky et al., 1955). These measurements were made by low angle X-ray scattering on preparations of human \(\gamma\)-globulin shown to be homogeneous in the ultracentrifuge. Recent studies by Hall et al. (1959) indicate that the rabbit antibody molecules have a length of 250°A and are
asymmetric rod-like particles, about 30 to 40\textsuperscript{0}A in diameter. These studies were carried out on purified rabbit antibody by electron micrography. Direct observation of the rabbit antibody attached to tobacco mosaic virus, using electron microscopy after negative staining, showed a perfectly round molecule with diameter 70\textsuperscript{0}A (Valentine, 1959). Valentine suggests that these are either artifacts caused by dehydration of the antibody during preparation, or that antibodies are dumb-bell-shaped molecules connected by protein structures of less than 10\textsuperscript{0}A diameter. The results of physicochemical measurements as well as the work of Porter (1955, 1958, 1959) who showed that after papain treatment, antibody is separable into three fragments, suggest that the former explanation of Valentine's is the most likely.

Valence of Antibody.

The multivalence of antibody has become a generally accepted fact, and the evidence points to two combining sites per antibody molecule. This holds for antibody of sedimentation constant of 7\textit{S} and molecular weight 180,000. The work of Pauling et al. (1944a) is perhaps the most elegant demonstration of bivalence. Antibodies were prepared in separate animals against two haptens, conjugated to protein molecules, phenylarsenic acid and benzoic acid, and when these sera were tested against a simple dihapten no precipitation occurred. Equilibrium
dialysis experiments in purified hapten-antihapten systems have
given a value of two for the valency of antibody (Eisman and
Karush, 1949; Lerman, 1949). Experiments carried out on
antigen-antibody complexes dissolved in antibody excess, have
also shown a valency of two for antibody when examined by
electrophoresis or ultracentrifugation (Pappenheimer et al., 1940;
Singer and Campbell, 1952; Weigle and Maurer, 1957; Kleczkowski,
1959). The most conclusive evidence is the work of Porter
(1955, 1959), who obtained 3 fragments after papain digestion of
antibody, two specific combining groups, and one larger group
carrying the antigenic determinants of the intact molecule.

There are isolated reports in the literature presenting
evidence for the monovalence of antibody (Boyd and Hooker, 1939;
Traub et al., 1953), but the weight of evidence behind the
bivalent theory indicates that these observations have probably
been misinterpreted. There is good evidence to show that
higher molecular weight antibodies contain more than two
combining sites. Enzymatic treatment of horse antipneumococcal
antibody will convert it into precipitating antibody with a
molecular weight below 100,000. Assuming that the native
antibody consists of 5 to 6 subunits polymerized end to end, the
undegraded antibody would have a valency of at least 10
(Pappenheimer, 1953).

The evidence presented above indicates that antibodies are
bivalent; the question remaining is whether the combining groups
on each end of the antibody molecule are of the same specificity. Lanni and Campbell (1948) coined the word "heteroligating" to describe hypothetical antibodies of two specificities. Their work and that of Nisonoff et al. (1959) on the adsorption of rabbit sera after injection with two haptens each coupled to bovine \(\gamma\)-globulin showed that each hapten removed only antibody against itself and that heteroligating antibody could not be demonstrated.

Non-precipitating antibody (Pappenheimer, 1940), and blocking antibody (Wiener and Katz, 1951) have been described as univalent owing to their failure to agglutinate the antigen. However, the blocking antibody in certain Rh antisera has been shown to agglutinate the cells if the cells have been pre-treated with enzymes.

The evidence all indicates that antibodies are bivalent and homoligating. The described "univalent antibodies" might be called more appropriately "non-ligating antibodies" and the cause of the "poor" reactions is most likely the poor quality of the antibody rather than the possession of only one combining site.

**Chemical Composition of Antibody.**

The amino acid composition of rabbit pneumococcal antibody determined by acid hydrolysis shows that it is high in serine, threonine, valine and proline, but low in methionine and
histidine (Smith et al., 1955). The N-terminal amino acid sequences of normal rabbit \(\gamma\)-globulin, and antibodies prepared in the rabbit against ovalbumin and pneumococcal polysaccharide are all the same (Porter, 1950). They all had one N-terminal alanine residue per molecule and a terminal tetra peptide alanyl-leucyl-valyl-aspartyl with glutamic acid probably in the fifth position.

The N-terminal analyses of antibodies from other species have shown several N-terminal amino acids in one preparation (McFadden and Smith, 1955).

The work of Gitlin and Merler (1961) on the peptide mapping of rabbit antibodies to different pneumococcal polysaccharides indicates that there may be differences in both primary and tertiary structures between the different antibodies. Fractions I and II of Porter's (1959) which contain the combining areas of the antibody molecule were shown to give almost identical peptide maps for antibodies against one antigen, but differences were detected between antibodies against different antigens.

Size and Configuration of the Antibody Combining Site.

Studies on the size of the antibody combining area have shown that a very small region of the total is involved in actual combination with antigen. It is of the order of 1 to 3 per cent of the area of the antibody. The actual measurement of the area of this active region is of importance in itself,
but of even greater importance is the structure of this area making it complementary to the antigen.

Direct measurement of the combining site by chemical or physical means is at the moment difficult or impossible, and all measurements have been done by equating the minimal size of the antigen with the antigenic area. The early work of Landsteiner and van der Scheer (1938) indicated an area of $700\,\text{A}^2$, whereas the studies of Kabat (1954, 1956, 1960) show that the area is smaller and probably of the order of $200\,\text{A}^2$. In a number of investigations of human antidextrans, he showed by hapten inhibition studies, that the power to inhibit precipitation increased with increasing numbers of sugar residues, from isomaltotriose to isomaltohexaose. The heptasaccharide caused a slight but definite increase over the hexasaccharide, but any further increase in size caused no increase in inhibiting power. Thus the largest unit to which an antibody combining site may be complementary is a heptasaccharide. These studies have also shown that the antidextran molecules are not all alike with respect to the complementariness of their combining sites; an antiserum is made up of a heterogeneous population of antibodies which vary in the size of the complementary area from one to another. They range in complementariness from a tri, tetra, penta, hexa to even a heptasaccharide. Thermodynamic studies of Karush (1956), Nisonoff and Pressman (1958) have given support to the values calculated by Kabat and have given further
information on the complementariness of antibodies for closely related antigens. These workers also found heterogenicity in the size and complementariness of antibodies.

Studies on the antibody combining site itself, by overiodination and destruction of combining power or protection of the combining region with antigen, indicate that some tyrosine and histidine residues must be present in this area (Pressman and Sternberger, 1951). The effect of mild acetylation on the antibody combining site showed that the ε-amino groups of lysine may also be involved.

From the above review it would appear that the region responsible for the serological activity of antibody is made up of not more than 10 amino acids. The nature of the forces holding antigen and antibody together remain a matter of conjecture at present. It was originally thought that the charge on the antigen played a major role, but since uncharged molecules such as dextrans are held as well as protein antigens, this cannot be the whole explanation. The predominant forces involved in holding antigen and antibody together must be hydrogen bonds and van der Waals forces. Molecular complementariness and the displacement of water molecules from the combining areas are probably the major factors, although the role of charged groups in some combinations may be equally important.
Specificity of Antibodies.

The specificity of the antibody for the immunizing antigen has been studied extensively by Landsteiner (1947), and the chemical groupings on the antigen have been shown to play the primary role in determining specificity. There is little evidence as to why certain chemical groups play a more prominent role than others in determining this specificity, especially as work on artificially conjugated protein antigens and haptens would suggest that there is no limit to the possible structures to which antibody can be formed.

The work of Landsteiner and van der Scheer (1936) has also shown that this specificity is not absolute. There are antibodies formed even against simple conjugated haptens that will combine with closely related substances as well as with the determinant group. The intensity of the heterologous reaction depends on the degree of similarity between the homologous and heterologous group. Landsteiner concludes:

"These facts can scarcely be interpreted otherwise than by assuming a multiplicity of antibodies, .... Thus to a multiplicity of serological reactions there need not correspond a coordinate mosaic of chemical structures in the antigen. One may conclude that the antibodies formed in response to one determinant group are, though related, not entirely identical, but, as evident from their cross reactions with heterologous antigens, vary to some
extent around a main pattern and what is ordinarily spoken of as antibody is generally a mixture of specifically different components."

When we come to compare complex protein antigens where the chemical structure is not known, it is difficult to decide how many of the heterologous reactions are due to a mosaic pattern of different determinant groups, and how many are contributed by variations of complementariness. Cross reactions have been shown to occur between protein antigens, such as the serum albumins of various species (Hooker and Boyd, 1936; Maurer, 1954; Porter, 1957), and that the degree of cross reaction depends on the route and the time course of immunization. The longer the time after inoculation of antigen, the lower the specificity, and the higher the degree of cross reactions.

The specificity of antibody is the basis of all immunochemical studies and serological typing, while the cross reactions produced by antibodies are used as a measure of antigenic similarity. The chemical basis for specificity and cross reactions in complex structures such as bacteria and viruses is not well established; however, the recent studies of Rappaport (1961) show that a start is being made in this field. Rappaport has compared the precipitin curves obtained from two strains of tobacco mosaic virus against homologous and heterologous rabbit antisera. The experimental results all favour the concept that the virus particle is made up of a single repeating antigenic
determinant, and that mosaics of different determinants probably do not occur. However, as only one third of the virus particles can be covered by antibody, owing to steric hindrance, it is impossible to completely exclude the mosaic concept. The single determinant hypothesis is in keeping with X-ray and physicochemical studies on this virus, as it can be broken down into its sub-units under certain conditions of pH and ionic strength and under other conditions the sub-units recombine to form virus-like rods.

The Heterogenicity of Antibodies.

Throughout the preceding sections on the quality and quantity of antibody, and the physical and chemical properties of antibodies, it has become evident that the antibody population in a particular serum is not uniform. Antigens such as purified haptens and single-component polymers such as dextrans, do not elicit a single species of antibody at any stage after immunization. It is not surprising, therefore, that complex antigens lead to an even greater degree of heterogenicity in the antibody response.

Now that we have observed this phenomenon of heterogenicity, how do we take account of it when studying the interaction between antigens and antisera that are a mixture of antibodies of different qualities? Pauling et al. (1944b) have proposed that the free energy of combination of antigen and antibody is normally distributed and that a population of antibodies can be
characterized by an average equilibrium constant and a "heterogeneity factor" corresponding to the standard deviation about the logarithm of the average equilibrium constant. Two alternative distributions which are very similar to the normal, but overcome the difficulty of integrating the area under the normal curve, were proposed by Sips (1948, 1950). Use of this distribution however, does not allow for the variable precision of measurement along these curves, and thus does not reduce the error of estimates based even on a large number of observations. Fazekas de St. Groth (1962) has proposed the use of Fisher's angle transformation (1922), which requires no special weighting procedure and yields information proportionate to the number of observations.

Conclusion.

The change in the quality of antibody with time after inoculation of an animal with an antigen has been described. These changes in quality can be accompanied by changes in the physical and chemical properties of the antibody. They may be observed as:

(1) Changes in electrophoretic mobility, and elution rates from chromatographic columns.

(2) Changes in the size of the antibody molecule: macro-globulins of 19 S appear early, followed later by the usual 7 S form of antibody.

(3) Changes in specificity: the degree of cross reactions
increases.

(4) Changes in the average size of the combining site of the antibody molecule have not been convincingly demonstrated, although Kabat (1956), Kabat and Bezer (1958) have observed that some antisera have a consistently higher proportion of antibodies with combining sites complementary to a hexasaccharide.

(5) The chemical studies on the primary and tertiary structure of antibodies are at too early a stage to show whether differences do occur.

It should be noted that the majority of these changes appear to be shifts in the average properties of the antibody population, and that at all stages there is demonstrable scatter about a mean, whatever criterion of quality is being applied.
V. ANTIGENICITY

It is customary to define antigens as substances or agents which will induce the formation of antibody when injected into an animal. The prerequisites for an antigen are:

(1) The molecular weight of the injected material should be relatively high, of the order of 10,000, or more. There is some correlation between the molecular weight of an antigen and the ability to initiate antibody formation (Merrill, 1936). It is usually assumed that small molecular weight antigens, such as penicillin, elicit antibody production by combining with host factors and thus producing high molecular weight material (Eisen, 1959). Not all high molecular weight proteins are antigenic, gelatin has been thought to be poorly antigenic, either because it is a denatured protein and is rapidly eliminated from the animal, or because it is low in tyrosyl and cystyl residues. The reason for gelatin's poor antigenicity has still not been worked out, for even after coupling gelatin to tyrosyl residues it is still not fully antigenic (Clutton et al., 1937).

(2) The antigen should be foreign to the host, but studies with blood group substances (isoantigens) and with sperm, lens, thyroid, and kidney proteins (autoantigens) in the same animal have shown exception to this rule (Landsteiner, 1947).

(3) Another prerequisite for determining antigenicity is that the determinant group should have a rigid structure.
(Haurowitz, 1952). The highly specific action of aromatic compounds is probably due to the rigidity of the benzene rings, while the easily distorted chains of the fatty acids are unable to elicit the formation of specific antibodies.

(4) The determinant groups must be present on the surface of the antigen molecule, as chemical groups on the interior of the antigen would not be accessible to antibody molecules.

Relatively little is known about the chemical structures of protein antigens that will elicit antibody formation. Landsteiner (1947) has shown that the specific action of the protein antigen is not due to the protein molecule as such, but is due to definite chemical groups on the protein. It may well be asked which chemical groups are important and what are the prerequisites of antigenicity.

Landsteiner (1947) demonstrated in his experiments with azoproteins that haptons with acidic groups were particularly efficient as determinants, while Haurowitz (1942) has demonstrated that strongly basic groups were equally important. To complete the cycle, Kabat (1954, 1956, 1960) has shown that non-polar dextrans and levans can act as antigens. Thus it would appear that polarity may not play such an important part in determining antigenicity.

The antigenicity of proteins is hardly altered when a few of their amino acid groups are acetylated, deaminated, or
iodinated (Haurowitz et al., 1941; Singer, 1957) but rigorous
treatment by any of these methods will abolish the original
specificity of the protein completely. Thus it appears that each
type of group may play some part in determining antigenicity, but
it is still open to question how much each amino acid group
contributes. Thermodynamic studies on closely related protein
antigens should give some answers to these problems in the next
few years.

The difficulty in deciding what are the antigenic determin-
ants on protein antigens has been mentioned in the section on
specificity. In tobacco mosaic virus, the evidence points to a
single repeating determinant, but the concept of a mosaic of
determinants has not been excluded. Under these conditions
and especially with mixtures of closely related antigens,
heterogenicity can be envisaged, but there are no sound methods
as yet, of determining this factor.
VI. THE TEST SYSTEM

If we wish to study the interactions between antibodies and antigens of natural pathogens, it is logical to opt for a relatively simple system. The simplest antigenic particles will be found among the viruses, for this group represents the smallest organised structures that carry their own genetic material. There are many viruses to choose from, but as the aim is essentially immunological it is best to choose a virus that can be obtained in reasonable quantity, can be purified readily and whose properties allow the interaction with antibody to be tested in many systems. The system should be suited to quantitative study, and it is an advantage if the antigen has a number of closely related antigenic variants to facilitate these studies.

Influenza viruses and their antibodies fulfil these requirements in every way. A short survey of the salient immunological features of this system will both establish the point and serve as framework and foundation for the experimental sections that follow.

Influenza Virus as Antigen

Influenza virus was first isolated by Smith et al. (1933) and in the following years many strains were recovered in different parts of the world (Francis, 1934; Burnet, 1935). These viruses behaved similarly, and are classified today as
influenza A viruses. In 1940 Francis isolated a new type of influenza virus which was antigenically distinct from the A type viruses and which became the prototype of influenza B viruses. Then in 1946 a modified type A virus was isolated in Australia (Anderson and Burnet, 1947) and displaced within the next year the earlier endemic A strains throughout the world; this subtype is referred to as A prime influenza. A further type of influenza virus, influenza C, that was antigenically distinct from either influenza A or B was described by Taylor (1949) and confirmed by Francis et al. (1950). To this list can be added the virus of swine influenza (Shope, 1931) which, antigenically at least, falls in the group influenza A viruses. A new type of influenza virus appears every few years, but a list of the major types gives no idea of the great plasticity of these viruses. A comprehensive list would fill many pages, and there are well documented reports of the isolation of more than one antigenic type of virus from a single local outbreak (Magill and Sugg, 1944; Burnet and Stone, 1946). The surface antigen of the virus is strain-specific while the "soluble" antigen present in the tissues during infection (Hoyle and Fairbrother, 1937) or released by disruption of the virus particle (Hoyle, 1952) is type-specific; there is no common antigen shared by the A and B groups: each possesses its own specific soluble antigen.
Influenza viruses are spherical particles of 80 to 100 mµ in diameter (Elford et al., 1936). A large number of measurements have been made on this virus and the present estimates are of the order of 80 mµ (Lauffer and Stanley, 1944). Newly isolated strains can occur as long filaments (Mosley and Wyckoff, 1946), but these forms are largely absent from egg-adapted laboratory strains. Electron microscope studies of the virus (Hoyle et al., 1961; Horne and Wildy, 1961) show the presence of an outer coat of radially orientated rods covering the whole surface. Influenza viruses can all be adapted to the allantoic cavity of the chick embryo and can be grown to high concentrations; they have been purified to a state suitable for chemical studies (Hoyle, 1952; Laver, 1962). Chemical analysis has shown that the virus is composed of ribonucleic acid 0.74%, protein 62%, lipid 34% and carbohydrate 3.5% (Frisch-Niggemeyer and Hoyle, 1956). Disruption of the virus particle with ether (Hoyle, 1952; Hoyle et al., 1961) or with mild detergent (Laver, 1961) yields fragments of approximately one million molecular weight. Laver (1962) has shown that all influenza strains tested have a single N-terminal amino acid residue, aspartic acid. Preliminary studies indicate that the C-terminal amino acids are the same for some A strains but differ from the B strains. The peptide maps obtained from closely related A strains may differ in as few as 2-3 peptides (Laver, 1962). The
virus particle may be pictured therefore as a sphere containing many identical protein subunits, surrounding a ribonucleic acid core. The part played by the lipid has not been elucidated, but it would appear from ether and detergent treatment that it must play some part in holding the subunits together. The biological role of the outer zone of radially orientated rods has not been clarified; it may contain the antigenic areas, the haemagglutinin and the viral enzyme.

Two properties make influenza virus particularly suited for the study of virus-antibody interaction: the possession of a haemagglutinin and the possession of an enzyme (Hirst, 1941, 1942, 1943). Both of these properties can be neutralized by specific antiserum. When virus is mixed with red blood cells, the virus attaches to the glycoprotein receptors on the red cell, and providing the concentration of virus is high enough, agglutination takes place. The union between virus and the red cell is not permanent however for the enzyme, neuraminidase (Gottschalk, 1954, 1956) present on the surface of the virus causes it to elute. The cells so treated are not reagglutinable by this virus, but are by viruses lower down the receptor gradient (Burnet et al., 1946). The virus on the other hand can agglutinate a fresh lot of cells. The combination between virus and red cell can be made permanent by treatment of the cells with dilute periodate (Fazekas de St. Groth, 1949) or by
inactivation of the enzyme (Burnet, 1952). The enzyme action of influenza virus on red blood cells, or on the receptors of cells of the lungs or allantois can be simulated by an exoenzyme of several bacterial species (Receptor Destroying Enzyme, R.D.E.) and the cells treated are no longer agglutinable (Burnet et al., 1946).

Serological Reactions of Influenza Virus.

Influenza virus and specific antibody show all the standard serological reactions, as well as some which are peculiar to this virus.

The virus will combine with antibody to give precipitates either in saline or in agar (Magill and Francis, 1938; Jensen and Francis, 1953). These techniques are insensitive as they require high concentrations of both virus and antibody, and are thus not as suitable as other techniques for studying virus-antibody interactions. These techniques are useful for determining impurities present in virus preparations, and for locating virus or antibody in immuno-electrophoretic techniques. The development of micro-precipitin tests (Smith et al., 1956) and micro gel diffusion tests (Grasset et al., 1956) have reduced the quantity of reagents required but like the macro techniques they are sensitive to non-specific inhibitors present in normal serum (Belyavin, 1957).

The use of inert carriers from which the virus does not
elute will increase the sensitivity of agglutination techniques up to 100 fold. Treatment of the red cells with periodate (Fazekas de St. Groth, 1949) will make the union between virus and red cell permanent, and thus makes the technique as sensitive as, and comparable to those of passive haemagglutination (Keogh et al., 1948; Boyden, 1951).

Complement is fixed when influenza virus and antibodies combine (Smith, 1936; Fairbrother and Hoyle, 1937; Lush and Burnet, 1937) and this immunological method has been used to compare antigenic structure (Henle et al., 1944, 1958; Fulton, 1958), and in serological surveys (Lief and Henle, 1960). Complement fixation is not sensitive to the non-specific inhibitors of influenza virus present in normal serum.

Besides the two-component systems, neutralization of interaction between influenza virus and some indicator component provides further techniques for studying virus-antibody interaction. There are several potential indicator systems that can be used, each based on a specific property of the virus, such as infectivity, haemagglutination, toxicity, interference and enzyme action.

The neutralization of virus infectivity can be tested in mice (Horsfall, 1939), in the embryonated egg (Burnet, 1940, 1941b), in surviving tissues (Fulton and Armitage, 1951; Fazekas de St. Groth et al., 1956c) or in primary cultures of mammalian cells (Lehmann-Grube, 1962). The estimation of neutralization in all
these systems is quantal and is determined by haemagglutination, haemadsorption, death of mice or pathological changes in tissue cultures or mouse lungs.

Active haemagglutination by influenza virus and the specific inhibition of this property by antibodies is one of the simplest and most rapid techniques in immunology. This technique suffers from many disadvantages however, and the outcome of the test can depend on the species of cells used (Clark and Nagler, 1943), the temperature at which the test is carried out (Fazekas de St. Groth, 1949; Tamm, 1954), the previous treatment of the virus (Briody, 1948) or the red blood cells (Stone, 1947) or even on the red blood cells from different fowls of the same breed. Normal and immune sera to influenza virus contain "non-specific" inhibitors of virus-haemagglutination (Hirst, 1942; Francis, 1947) of several kinds. Despite all these disadvantages, this technique remains of great value when studying virus-antibody interactions provided the variables are recognised and controlled as much as possible.

Large doses of influenza virus are toxic for mice or rabbits (Henle and Henle, 1948; Mims, 1960; Kato and Hara, 1961) and this effect can be neutralized by specific antiserum. There is variation in toxicity however from strain to strain and the effect depends also on the hosts, so that this method has not been used for detailed studies of virus neutralization.
Another test that falls in the same category is the neutralization of interfering capacity. When inactivated influenza virus is allowed to react with cells it is absorbed, and such treatment of susceptible cells will prevent or reduce the yield of infectious virus from such cells (Andrewes, 1942; Henle and Henle, 1943, 1945).

Neutralization of the virus enzyme by specific antibody offers another method for studying virus antibody interaction. The virus acts on a wide variety of substrates, ranging in size from the red cell, through macromolecules such as ovomucin (Gottschalk and Lind, 1949) to small molecules such as sialyllactose (Kuhn and Brossmer, 1956). The extent of neutralization can be measured by the reduction of the inhibitory action on viral haemagglutination, or by reduction of the neuraminic acid released by the enzyme (Warren, 1959).

The Interaction of Virus and Antibody.

When virus and antibody are mixed together they will combine to form complexes:

\[ V + Ab \rightleftharpoons VAb \]

and at equilibrium the basic model of virus antibody interaction (Fazekas de St. Groth et al., 1956b) may be expressed in the following way:

\[ K = \frac{(sV-x)(A-x)}{x} \]
where \( K \) is the equilibrium constant, \( x \) the concentration of formed antigen-antibody complexes, \( S \) the number of antigenic sites per virus particle and \( V \) is the concentration of virus.

The simplifications and complications mentioned in the diphtheria toxin-antitoxin system are also applicable here. However, the simplification of monovalence of antibody in this system is more legitimate, for virus and antibody will form aggregates only in very concentrated systems, and in the usual test systems these conditions do not obtain.

A complication that may arise is interaction between antibody molecules on the virus particle. This problem can arise only when above 20-25% of the antigenic sites are occupied, and at these concentrations electrostatic interactions occur between neighbouring antibody molecules.

Another factor which affects the measurement of the equilibrium constant is the occurrence of secondary reactions (Jerne and Avegno, 1956; Gard, 1957; Lafferty, 1960). Dilution or reactivation experiments reveal a small fraction of antibody molecules that are less dissociable than would be expected from the position of the equilibrium and the observed average rate of association. As the fraction of less dissociable unions is strongly temperature and concentration dependent, it cannot rest on the heterogeneity of either antigen or antibody. This type of decreasing dissociability of antibody is probably also due to antibody proximity on the virus particle but can also be
explained by combination of each end of the antibody molecule with sites on the same virus particle (Lafferty, 1960). As both of these complications occur only at high concentrations of antibody, or with prolonged incubation of reactants before addition to the indicator system they do not cause significant deviation from the simple model when working with dilute reagents and short periods of incubation.

Before the measurement of the parameters in the above reaction can be attempted, particularly the measurement of K, which was defined as the quality of antibody, the validity of the basic assumption, i.e. reversibility, must be tested. This will form part of the experimental section, and the methods developed to permit the measurement of the parameters will be presented in detail.

Neutralizing Potency.

When comparing antisera by neutralization tests it has been noted that the slope of dose response curves is different for sera compared in the same host system (Walker and Horsfall, 1950; Jerne, 1951). The volume in which the tests is carried out also affects the slope of the dose response curve (Fazekas de St. Groth et al., 1958c), and hence the results obtained in different host systems are rendered incommensurable (Tyrell and Horsfall, 1953).
To overcome these difficulties and to enable the practical immunologist to make valid comparisons between sera Fazekas de St. Groth (1961) has proposed the "neutralizing potency" as the natural measure by which antisera can be compared. The quantal neutralization curve becomes linear with unit slope if plotted according to the equation:

$$\log \log V = \log A + \left\{ \log C - \log K - 0.362 \right\}$$

where $V$ is the virus input, $A$ is the antibody input, $C$ the number of critical sites per virus particle, $K$ the dissociation constant, and the constant $-0.362$, which equals $\log \log e$, arising from the use of common, rather than natural logarithms.

The bracketed term of this equation is the one which alters the slope of the conventional ($\log A$, $\log V$) curves. Here it governs the intercept of the dose-response line with the abscissa; and as the intercept is that dose of antibody which leaves one out of ten infective particles free, it is a logical unit of neutralization. If an antiserum is found to contain one neutralizing dose when diluted $10^{-d}$, its potency may be expressed as the negative logarithm, $d$, of this dilution. The neutralizing potency, $pN$, is given by:

$$pN = \log \log V - \log A + \log \text{vol}$$

where $\text{vol}$ stands for volume in cm$^3$. This correction for volume simply enters as an additive term.
The log log plot is linear only as long as the number of antigenic sites remains below the value of the equilibrium constant. At high virus inputs the log log plot flattens. This however is outside the range of doses used in practice. The pN is a compound of the quality \((\log C - \log K)\) and the quantity \((\log A)\) of antibody. Its use overcomes the difficulties set out above and allows direct comparison of antisera, always provided the \((\log \log V, \log A)\) plot is linear in the region tested. This concept of neutralizing potency will be used in comparing antisera prepared in rabbits against a set of influenza viruses.
Paper I.

NEUTRALIZATION OF ANIMAL VIRUSES.

III. Equilibrium conditions in the influenza virus-antibody system.
The model of virus-antibody interaction, as developed in the first paper of this series (1), consists of two quite unequally established halves. The first, based on the law of mass action, translates generally accepted principles into terms of a biologic system, and stands or falls with the validity of these. The second concerns a peculiarly biological phenomenon, the neutralization of infectivity, and asserts that even this phase can be fully and quantitatively accounted for by the physical mechanisms that govern the association of discrete submicroscopic entities.

Although the theory has been checked against and found to fit a set of data on poliomyelitis and Western equine encephalitis viruses and their antibodies (2), the evidence must, by its very nature, remain lacunary as long as there are crucial points not covered by specifically designed tests. In a sense, even a complete argument of this kind is negative in character; it can do no more than show that a particular hypothesis is not contradicted by experimental facts, that is, has a right to exist. Formal proof is in the definition and measurement of all parameters entering the reaction, and in accurately predicting what will happen when similar but unknown reagents are brought together under given conditions.

In this paper the first steps are made towards this goal: we shall show that the system of influenza viruses and their antibodies is suited to quantitative studies of neutralization, and then examine whether it complies with the basic tenets of the mass action
model. In particular, we shall try to find out whether equilibrium is reached under conditions of the standard test; whether disturbances of assumed equilibria are reversible at reasonable rates; and whether the system shows signs of gross inhomogeneity in its components.

All experiments to be described were performed on three strains of influenza virus. The strain SW(A) was used because in our system of assay it closely approximates the ideal (i.e., an equivalence between physical particles and infective units); the strain LEE(B), because by the same criteria it is farthest from the ideal; and the strain MEL(A), because it is intermediate between the former two and cross-reacts serologically with SW but not at all with LEE. Since, apart from antigenic individuality, we found no basic differences in the behaviour of these strains, all Tables and Figures to follow will give the results obtained with the strain SW only; the rest of the findings will be used in the companion paper to evaluate some parameters of neutralization.

MATERIALS AND METHODS

Virus. The strains MEL(A) (3), LEE(B) (4) and SW (strain 15 of swine influenza) (5) were grown allantoically from dilute seed (about $10^2 D_{50}$) for 40 hr. at 35°C. The harvested allantoic fluids were pooled, diluted with two parts of Standard Medium (SM) (6),
ampouled in 0.5 ml lots, snap-frozen in a mixture of solid CO₂ and methanol, and stored at -70°C. The infectivity of such preparations was found unchanged over a period of at least one year.

**Antisera.** Healthy adult rabbits were given 50 µg of purified influenza virus (one cycle of adsorption-elution, followed by high-speed spinning) intravenously, a similar anamnestic dose 40, 60 and 80 days later, and bled on days 83, 85, 87. Sera were stored without any preservative at -15°C, and used in the experiments without inactivation. Their neutralizing titres remained stable over a period of at least a year.

**Neutralization test.** The method of Fazekas de St. Groth, Withell and Lafferty (7) was used without modification: 6 x 6 mm bits of allantois-on-shell were cut from 11-day White Leghorn eggs and suspended in 0.30 ml SM. The virus-antibody mixtures, made up in chilled SM and preincubated in an ice-bath for 30 min., were delivered with standardized dropping pipettes, 0.05 ml per cup of host tissue. After an incubation period of 64 hr at 35°C under continuous shaking, the host tissue was removed and a drop (0.025 ml) of 5% fowl red cells added to each cup. Complete agglutination was taken as the sign of infection, and the point of 50% infectivity was worked out by the simplified Reed and Muench procedure (8).

Dilution series were usually set up in twofold steps, with at least 8 replicates per dilution. The infectivity of the virus
preparation was checked, along with each experiment, in duplicate: one set put up before, the other after the neutralization test. Unless these "early" and "late" controls were found to coincide, the test was taken as invalid.

EXPERIMENTS and DISCUSSION

SCOPE OF THE TECHNIQUE

Limitations. Although the assay of neutralizing antibodies is quantal in nature, this need not detract from its usefulness. It has been shown (7) that the information derived by this method from a single egg is at least as much as could be gained by quantitative pock or plaque counting methods at the same cost in time and labour. The limitations are rather more fundamental, and stem from the very fact of equilibrium. Since influenza viruses spend a relatively long time outside the host cells before engaging in the first cycle of multiplication, any differences that may exist in the distribution of free and neutralized particles at the moment of adding the serum-virus mixture to the host tissue would level out by the time the average particle has been taken up by the cells. This lack of kinetic differences, demonstrated earlier (7), renders futile any kinetic experiment unless some means of quenching the reaction at a given point be found.

Even the study of equilibrium conditions is restricted by the
demands of the host tissue. While it is possible to let virus and antibody interact at any temperature between 0 and 40°C without drastic loss in infectivity, the number of infective units cannot be estimated over the whole of this range. Indeed, the multiplication of virus will proceed only in such a narrow sector of temperatures that the determination of thermodynamic constants is all but barred to experimental investigation in this system. What remains is the study of equilibria by varying the absolute amounts and concentrations of virus and antibody, or the comparison of quantitatively different reagents—heterologous antigens and sera of different origin.

Complications. The scope of such tests is wide enough to cover most questions likely to be of importance in immunology. It is necessary however to find out whether the answers obtained are direct or complicated by some peculiarity of the assay system.

I. One such complication would be the suggested dampening effect of antisera (2). If in the original mixture of virus and antibody a few particles stayed unneutralized, these could go through a cycle of multiplication. The yield, added to virus-antibody complexes still present in the medium, would be reduced by combining with antibody, and only a fraction of it could initiate a second cycle of multiplication. The third generation of particles, exposed to the same hazard, would likewise fail to grow at full rate, and it
### TABLE I

**ABSENCE OF DAMPENING EFFECT:**

**DEMONSTRATION BY REMOVAL OF ANTIBODY DURING VIRAL MULTIPLICATION**

<table>
<thead>
<tr>
<th>ID&lt;sub&gt;50&lt;/sub&gt; neutralized (log&lt;sub&gt;10&lt;/sub&gt; units)</th>
<th>Serum titre (log&lt;sub&gt;10&lt;/sub&gt; units)</th>
<th>Controls*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time of transfer into antibody-free medium</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>3.57</td>
<td>3.08</td>
<td>3.13</td>
</tr>
<tr>
<td>2.57</td>
<td>3.20</td>
<td>3.38</td>
</tr>
<tr>
<td>1.57</td>
<td>3.50</td>
<td>3.68</td>
</tr>
<tr>
<td>0.57</td>
<td>4.37</td>
<td>4.60</td>
</tr>
</tbody>
</table>

* based on 6 replicate titrations; the geometric means and their standard errors are given.
is easy to see that under certain conditions the test would register as negative not because the virus was fully neutralized, but because its rate of multiplication has been cut down. We examined this question when the technique was being developed; the answer strongly suggested that if any dampening existed, it was quite negligible in our system of assay. Since the point is fundamental to later interpretations, we sought formal proof in the following experiment.

Several trays of surviving bits of allantois-on-shell were inoculated with a set of virus-antibody mixtures made up in bulk. At various times after inoculation the bits of a few trays were picked up one by one with fine forceps, rinsed in 2.5 ml of Standard Medium, and transferred to the corresponding cup of another tray filled with SM. To ensure that the yield of even a single infective unit would register as positive, this latter set of trays also received a fresh, uninfected bit of tissue in each cup and was incubated for two days from the time of transfer. (Table I).

The neutralizing titres are the same whether antibody is present throughout the test or is removed before the end of the first intracellular cycle. Since the absence of free virus cannot be blamed on the continued presence of antibody, the conclusion seems inevitable that within an hour of coming into contact with host cells the infective particles are either neutralized by antibody or have entered on a course of unimpeded multiplication.
Figure 1 Absence of dampening effect: Demonstration by adding excess antibody during viral multiplication.

The broken lines represent the neutralizing titres in a standard assay with single, double and quadruple doses of antibody. The experimental points show the difference, in log_{10} units, from the single dose when doubling (circles) or quadrupling (dots) the amount of antibody, at the times given on the abscissa.
II. The same point can also be tested by an alternative method. Here excess antibody is given after the initial virus-cell contact has taken place.

Mixtures of virus and antiserum were made up in bulk, pre-incubated for half-an-hour at 0°C, and then inoculated into cups of SM containing bits of 11-day allantois-on-shell. After intervals ranging from 1 to 24 hrs appropriate dilutions of the antiserum were added in the form of 0.025 ml drops to each cup, raising the concentration of antibody to double and fourfold respectively in terms of the controls which received the same volume of SM instead. The trays were replaced on the shaking machine, and the test was read after 64 hrs of incubation. From previous knowledge of their potency, sera were used at such concentrations that a twofold increase would correspond to an approximately eightfold increase in the number of infective units neutralized, i.e. even the greatest effect would be somewhat less than the cyclic increment of influenza viruses. Figure 1 gives the results of one such test.

Within statistical variation the experimental points are indistinguishable from the controls, showing - now in a positive fashion - that even after one hour's contact with susceptible cells the fate of the initial virus-antibody complex is settled, and there is little that modest quantities of additional antiserum can do.

In later work it will be shown that the dampening effect, theoretically possible, may be demonstrated under very special circumstances and may even serve to decide certain issues. In the
**TABLE II**

**Time of Antibody Action**

<table>
<thead>
<tr>
<th></th>
<th>Antiserum added (hours after infection)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Controls*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-1</td>
<td>1/60</td>
<td>1/3</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td>8</td>
<td>10</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>3.37</td>
<td>3.41</td>
<td>3.35</td>
<td>3.33</td>
<td>3.34</td>
<td>3.15</td>
<td>3.23</td>
<td>3.23</td>
<td>2.98</td>
<td>2.27</td>
<td>3.47 3.30</td>
</tr>
<tr>
<td>titres</td>
<td>3.17</td>
<td>3.39</td>
<td>3.08</td>
<td>3.20</td>
<td>3.15</td>
<td>3.23</td>
<td>3.13</td>
<td>3.14</td>
<td>3.05</td>
<td>1.90</td>
<td>3.31 3.41</td>
</tr>
<tr>
<td>(log_{10})</td>
<td>3.23</td>
<td>3.29</td>
<td>3.34</td>
<td>3.17</td>
<td>3.10</td>
<td>3.53</td>
<td>3.20</td>
<td>3.17</td>
<td>3.09</td>
<td>2.40</td>
<td>3.23 3.42</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.18</td>
<td>3.35</td>
<td>3.25</td>
<td>3.18</td>
<td>3.36</td>
<td>3.44</td>
<td>3.20</td>
<td>3.18</td>
<td>2.93</td>
<td>2.54 3.53 3.50</td>
</tr>
<tr>
<td>Mean titre</td>
<td>3.24</td>
<td>3.36</td>
<td>3.26</td>
<td>3.22</td>
<td>3.24</td>
<td>3.34</td>
<td>3.19</td>
<td>3.18</td>
<td>3.01</td>
<td>2.28</td>
<td>3.40</td>
</tr>
<tr>
<td>± standard error</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td></td>
<td>0.046</td>
<td>0.026</td>
<td>0.063</td>
<td>0.037</td>
<td>0.066</td>
<td>0.089</td>
<td>0.021</td>
<td>0.019</td>
<td>0.036</td>
<td>0.137</td>
<td>0.037</td>
</tr>
<tr>
<td>Excess log ID$_{50}$ neutralized**</td>
<td>0.53 ± 0.102</td>
<td>0.97</td>
<td>1.02</td>
<td>2.26</td>
<td>5.76</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Infective dose: $10^{1.54}$ ID$_{50}$ of SW virus

*Standard neutralization tests, with 30 min preincubation of the virus-serum mixtures

**Calculated from the results of parallel standard neutralization tests with the same antiserum against a millionfold range of infective doses.
particular type of assay used in these studies the necessary conditions are never realized, and the phenomenon is not seen.

III. There is another source of potential confusion bound up with the anatomy of the host system. Some cells of the allantoic endothelium do not reach the free surface (9), but are contiguous over a large area with directly infectible cells. If infection of the top layer could spread either laterally to other cells or by way of the underneath layer, an infective particle entering a susceptible cell would start a process which could end only in the eventual infection of all cells, irrespective of whether later antibody is added to the system or not. This proposition can be tested directly by inoculating trays with different doses of virus and then adding appropriate doses of antiserum at various periods after infection. Such experiments have been performed, and the results of one are given in detail (Table II).

The main conclusion is that antiserum added well after infection is capable of stopping the process, and bringing about complete neutralization not only of the virus inoculated but also of its numerous offspring. The mechanism of this effect is not revealed by the evidence in Table II, but some of the possibilities are more likely than others.

It could be that antibody entered cells and neutralization took place inside the cytoplasm. Apart from general biological
objections based on the distribution of labelled antibodies in vivo as well as the structure and functioning of the cytoplasm, the chance of this happening is denied by the left hand side of Table II. If antibody was added simultaneously with or very soon after the virus, its effect was rather less than when the two were mixed in vitro. It seems that once an infective particle has attached itself to its future host cell, considerably more antibody is needed to neutralize it. If neutralization could proceed or, indeed, start intracellularly, no such difference should be present.

If antibody permeated the whole of the endothelium, that is, entered the intercellular spaces both between neighbouring cells and between the top and bottom layers, the final result could be as found in the experiment. In this case however one should also assume that not only free antibody but also formed virus-antibody complexes might be present in this space, and these would dissociate under conditions of the test shown in Table I, with the release of once more infective virus. The results show that this is not the case.

The remaining possibility, the simplest of the three, is that virus yielded by one cell enters the medium before finding its way to another, yet uninfected cell. This is the orthodox view, and the one most naturally fitting the observations of Table II. Accordingly neutralization is an extracellular process and the host tissue is to be regarded merely as an indicator system by which the
presence of free virus particles may be detected. In these terms the complication of lateral spread would be absent from our method of assay.

The experiment provides also a direct argument in support of this view. The longer after infection antibody is added, the more of it is needed to bring about neutralization. This additional dose of antibody can be expressed in terms of an increase in the amount of virus to be neutralized. These values—the product of log extra serum and log slope of the dose-response curve—are shown in the last row of Table II. The close quantitative agreement between these figures and an allantoic growth curve of influenza viruses is evident. It means that once the virus in the allantoic fluid has been rendered non-infective, the process of multiplication in the tissue also comes to an end.

The dissociation of formed virus-antibody complexes

It has been shown repeatedly, both in mice (10, 11) and eggs (12, 13) that neutralized influenza virus can be reactivated either by diluting the virus-antibody mixture or by adding some killed homologous virus. The validity of this evidence has been queried on technical grounds (2), although never refuted experimentally. That these objections are without foundation follows from the above findings, since the continued presence of antibody does not affect the results of a quantal neutralization test, and the assay measures the total number of infective units in the system, and not only those
which are sheltered from antibody by physical barriers. The findings in our system of assay are in general agreement with published evidence; there are however certain aspects which do not seem to have been considered previously, or at least not investigated experimentally.

The classical design for discriminating between readily reversible and irreversible antigen-antibody union is due to Danysz (14). Because of the importance of information obtainable from tests of this kind, several large experiments were performed on the strains SW, MEL and LEE. One, using SW and hyperimmune rabbit antiserum is described in detail.

As controls, ten dilutions of antiserum (1/200 to 1/100000) were mixed with equal volumes of four virus dilutions (3 to 3000 \(\text{ID}_{50}\)), in all possible combinations. Sixteen replicate bits of host tissue were inoculated with each of these mixtures, either within five minutes of bringing virus and antibody together ("early controls") or after 120 minutes' incubation at 0°C ("late controls"). The first of the main experimental groups consisted of the full volume of serum dilutions to which 1/12 of the full virus dose was added every ten minutes. The tubes were thoroughly shaken after each addition, and returned to the ice-bath. The second group received the full volume of serum and 1/3 of the full virus dose every forty minutes. Groups 3 and 4 were the reverse: to the full volume of virus 1/12 or 1/3
Figure 2  Danysz experiment.

- : full quantity of virus and antibody mixed directly (controls);

⊙ : 1/3 quantity of virus added to full quantity of antibody, at 40 min. intervals;

Θ : 1/12 quantity of virus added to full quantity of antibody, at 10 min. intervals;

△ : 1/3 quantity of antibody added to full quantity of virus, at 40 min. intervals;

○ : 1/12 quantity of antibody added to full quantity of virus, at 10 min. intervals.
respectively of the full antibody dose was added every ten or forty minutes. At the end of the second hour 0.05 ml of each mixture was inoculated into 16 replicate cups containing a bit of membrane-on-shell, and the test read after three days' incubation at 35°C. The infectivity of the virus was tested in 32 bits of tissue per half-log dilution, both at the beginning and at the end of the test.

(Figure 2.)

If dissociation of formed virus-antibody complexes is slow or absent, the groups to which virus was added in small instalments should show much higher infectivity (i.e. lower neutralizing power of the serum) than either the two control groups or the test groups which received the antiserum fractionally. This is expected because the full dose of antibody would combine with the small initial doses of virus in multiple proportions and, unless it could dissociate, not enough would be left to neutralize the last doses of virus.

If on the other hand, dissociation can occur, with every addition of virus or antibody a new equilibrium will be established and the final conditions will be the same in all six groups since the final concentrations of the reagents are the same, although reached by different routes. The experimental results are compatible only with the latter mechanism. At each concentration of serum the neutralizing titres were the same, irrespective of whether the reagents were mixed at the beginning of the experiment and added to the medium of the host tissue immediately, or after two hours'
delay, or whether the concentration of one or the other was gradually changed over a period of two hours. The differences observed are all within the bounds of random variation and there is no trend detectable.

That the experiment actually tested dissociation of formed complexes with re-establishment of equilibria, and not the monotonic approach to the final equilibrium, should be clear on remembering that, for all practical purposes, the final neutralizing titre was reached within five minutes after bringing the reagents together in vitro (Table II), and that the situation did not change appreciably between the 1st and 24th hour after inoculation into the host system (Table I).

In general, then dissociation is clearly present under all circumstances where it can be reasonably expected. The process may not go to completion under special conditions, as has been amply noted in the literature; the mechanisms underlying this secondary trend will be treated in a separate paper. But to first approximation, both for practical purposes and for the investigation of the more basic aspects of virus-antibody interaction, the primary neutral complex is to be regarded as reversible.

**Practical consequences of dissociation**

**Volume effects.** As has been already observed when developing the technique of the neutralization assay (7), the volume into which
Figure 3  Relationship between the volume effect and the dose of virus neutralized.

Plot of Equation 2, in the form \((d, \log Y)\). The dilution factor, \(d\), is used as abscissal scale.
the virus-antibody mixture is injected has an influenza both on the endpoint of neutralization and on the shape of the dose-response curve. This phenomenon is a corollary of the mass action model of virus-antibody interaction, and is particularly striking in quantal assays. The surviving fraction (log residual ID$_{50}$ - log input ID$_{50}$) is directly proportional to the fraction of virus not neutralized, log V/V$_0$. Taking the simplest set of assumptions (cf. Eq. 2.12 of the theoretical paper (1)),

$$\log \frac{V}{V_0} = c \log (1 - \frac{V}{sV_0}),$$

where $c$ is the number of critical sites per particle, $s$ the total number of antigenic sites per particle, and $y$ the number of antigen-antibody complexes per unit volume.

If a quantal assay, where $V = 1$ ID$_{50}$ by definition, is performed with the same reagents in two different test volumes,

$$\log V_0 = \frac{c}{c-1} \left( \log \frac{V}{sK} + \log \frac{d}{d-1} \right).$$

Here $v$ is the smaller test volume, $d$ is the ratio of the two test volumes, and $d$ is the ratio of the two doses of antibody needed for neutralization in the two tests.

Figure 3 is the plot of Eq. 2, in the form of log V$_0$ as a function of $d$. Two important features of this relationship are immediately evident. First, that at low virus input the curve asymptotically approaches , i.e., the ratio of neutralizing titres between
the two systems will equal the ratio of their volumes. Second, that at very high virus input the ratio of the endpoints will approach unity, i.e., the same amount of antibody will be needed to neutralize a given infective dose irrespective of the test volume. The swing-over occurs at the level where \( \frac{c-1}{c} = v/sK \), and will obviously vary with the test volume, the nature of the virus, and the nature of the virus-antibody union. There might be systems where the whole of the range that can be investigated experimentally falls on one or the other asymptotic limb of the curve. These are the situations which could lead to alternate affirmation and denial of the mass action model, each on equally sound partial evidence. The reagents we have been using in these studies are of such kind that both limbs as well as the central region of the curve may be observed experimentally. One typical test will be given to demonstrate this behaviour.

Parallel series of antiserum-virus mixtures were made up in plastic trays containing 0.35, 1.05 or 2.10 ml of Standard Medium per cup, and incubated for 4 hours at room temperature. (This time, as established in preliminary experiments, was about twice that needed to obtain maximal neutralization in the largest test volumes.) Bits of allantois-on-shell were then added to each cup, and the trays placed on the shaking machine for three days' incubation at 35°C. The neutralizing titres for each serum dilution were determined, and the \( d \)-values calculated and plotted against the corresponding log
Standard neutralization tests were performed in final volumes of 0.35, 1.05 and 2.10 ml respectively, thus fixing the values of $\varphi$ at 2, 3 and 6. The dots show the mean $d$-values corresponding to the infective dose on the ordinate, and the shaded area represents the 95% confidence limits. The theoretical curve is that of Fig. 3.
virus input. Since each pair of volumes defines a complete \((d, \log V_0)\) plot, Figure 4 contains all three of these.

The experimental means carry unusually large errors. This is due, almost entirely, to the use of a ratio \(d\), as statistical estimator. It will be shown later that the basic relationship can be plotted in another, perhaps less dramatic form, which is not subject to such unsteadiness. Even so, the trend is unmistakeable, and the experimental and theoretical curves do not differ significantly, both arguing for the correctness of the dissociation hypothesis and stressing the importance of the volume effect in practical assays. From the knowledge of this relationship the results of one type of test may be transformed into another. Clearly, there is no difference in principle between tests in surviving bits and in the whole allantois; the only other published evidence of neutralization tests in different test volumes (15) would indicate that the same laws hold also for similar assays in tissue cultures.

The consideration which led to Eq. 2 and Figure 4 will be further developed and used for the estimation of some of the parameters in the companion paper.

**Effects of non-infective virus.** The second aspect of the mass action model of practical importance concerns the combination of antibody not only with infective particles but also those elementary bodies which were either "incomplete" from the start or have been rendered non-infective naturally by thermal inactivation or
artificially by some other means. It is known that all preparations of influenza viruses have more particles visible under the electron microscope than there are infective units. With fresh preparations the ratio of infective to countable particles is fairly constant, and it has been shown (8, 16) that most if not all of the discrepancy can be accounted for by the insusceptibility of host cells. As the latter property varies from host to host, it follows that the infective to non-infective ratio of even the same virus preparation may vary from test to test. If we further suppose, as is likely, that not all virus seeds are prepared and kept under optimal conditions, the variation in the ratio of infective to non-infective particles emerges as a factor capable of causing important differences in neutralization tests.

According to the mass action model, the presence of non-infective particles would alter the basic equations in the following manner. Equation 1 becomes

\[ \log \frac{V}{V_0} = c \log \left( 1 - \frac{V}{sV_0(1+n)} \right) , \]

where \( n \) is the number of non-infective particles for each infective unit. Although Eq. 3 completely defines the effect of non-infective virus within the framework of the model adopted for the neutralization process, it does so in terms of the fraction of antigenic sites occupied by antibody. This is a mathematically convenient form,
Figure 5  The effect of non-infective virus on neutralization. Equation 3 is solved for $y$, giving

$$\log A = \log \left[ K^{-1} + s(n + 1) V_0 \frac{c-1}{c} \right] + \log \left[ \frac{1}{c} - 1 \right]$$

3a

For the particular family of curves, plotted as ($\log A$, $\log V$), the equilibrium constant $K$ is set at $10^{-10}$, and the number of critical antigenic sites $c$ at 100 per particle. The value of the variable $\log \left[ s(n + 1) \right]$ appears on the curves.
but gives little direct insight into the behaviour of the more conventional parameters of the reaction, namely the quantity of infective doses to be neutralized and the concentration of antibody present. We have therefore programmed a digital computer to solve Eq. 3 for a wide range of all parameters entering the process, and one family of such theoretical neutralization curves is plotted in Figure 5.

The striking feature of these curves is that their lower limbs overlap, i.e., are independent of the number of non-infective particles introduced into the system. The reason for this is evident from the functional form of Eq. 3a: at low virus input, and as long as \( s(n+1)V \frac{c-1}{c} \) is negligible compared to \( K^{-1} \), the first term will reduce to a constant, \(-\log K\). The higher the proportion of non-infective particles in the test inoculum the sooner the neutralization curve flattens, that is, the sooner the first term of Eq. 3a becomes dominant. At very high virus inputs, when \( K^{-1} \ll s(n+1)V \frac{c-1}{c} \), all curves run parallel, with a slope very close to unity. The fraction of non-infective particles affects here only the extrapolated intercept with the ordinate.

Predictions on the effect of non-infective particles, as stated in Eq. 3a, can be readily tested in the influenza virus-antibody system, by comparing experiment with the curves of Figure 5. For such tests virus preparations were inactivated by ultraviolet
Figure 6 Neutralization curves in the presence of non-infective virus.

Standard neutralization tests with SW virus and rabbit anti-SW serum are represented by dots. Curves A, B, C, D show the behaviour of the same system in the presence of a $10^1$, $10^2$, $10^3$, $10^4$-fold excess, respectively, of UV-killed virus.
radiation (Philips 35W "Germicidal" strip source at 15 cm distance; dialysed allantoic fluid or concentrate exposed for 3 minutes in 2 mm layer thickness); if inactivation is exponential, this treatment reduced infectivity by a factor of more than $10^{-18}$. Appropriate volumes of infective and killed virus were brought together to give mixtures of $(1 + 0)$, $(1 + 9)$, $(1 + 99)$, $(1 + 999)$ of infective to non-infective particles. Parallel dilution series of these were set out and mixed with several dilutions of antiserum kept at 0°C for 30 minutes, and then inoculated into trays of 11-day bits of allantois-on-shell. The neutralization curves, as read after three days' incubation at 35°C, are shown in Figure 6.

The close correspondence between observed and expected dose-response relationship is evident. These results support the mass action hypothesis by an independent proof; they also furnish powerful means for definition of the variables in absolute terms. Since the latter falls within the scope of the next paper, further development of the argument is deferred till then.

Homogeneity of reagents

Although in the theoretical paper (1) the consequences of heterogeneity of the system of virus and antiserum were considered, the hypothesis tested in all experiments above did not incorporate this contingency, and treated the reagents as if all antigenic sites on the virus particle were identical and as if the serum contained
Figure 7. Heterogeneity of reagents: Neutralization tests with artificial mixtures of antigens.

Control tests, with single antigens indicated on the ordinate, are represented by dots. Curves A, B, C, D, E show the behaviour of SW virus in the presence of $10^{1.85}$, $10^{2.85}$, $10^{3.85}$, $10^{4.85}$, $10^{5.85}$ D$_{50}$, respectively, of the MEL strain.
only a single species of antibody. Instead of proving that such a
set of assumptions is justified as a first approximation, we shall
rather examine what degree of heterogeneity is needed in practice to
upset predictions of the simplest model.

A rabbit serum hyperimmune to SW virus was set up against
the homologous and a heterologous strain (MEL), and also against
various mixtures of these two in standard neutralization tests. By
this means the effect of antigenic heterogeneity could be assessed.
In similar experiments SW virus was tested against mixtures of anti-
sera, providing systems of variable heterogeneity in the antibody
components.

With the reagents used, the first experiment (Fig. 7) gave
an all-or-nothing answer. Adding as little as one-in-a-million of
heterologous virus completely overrides the effective neutralization
of the vast majority of homologous antigen. Indeed, as the lower half
of Fig. 7 demonstrates, the relationship is indistinguishable from
the heterologous controls where MEL virus alone was exposed to the
action of anti-SW serum. This situation, as will be shown in a
separate paper, can be deduced from first principles, and is a
characteristic of quantal assays. Quantal neutralization is only as
effective as the weakest antigen-antibody link in the system. In
practice even minute amounts of a contaminating virus will shift a
neutralization curve, although not alter its shape.
Figure 8. Heterogeneity of reagents: Neutralization tests with artificial mixtures of antisera. Control tests, performed with single antisera are represented by dots and marked with S for anti-SW or M for anti-MEL. Curve A shows the behaviour of anti-SW serum in the presence of $10^{-1.4}$ of anti-MEL. Curves a, b, c, d, e, f are the neutralization curves of anti-MEL serum in the presence of $10^{-3.0}$, $10^{-3.3}$, $10^{-3.6}$, $10^{-3.9}$, $10^{-4.2}$, $10^{-4.5}$, respectively, of anti-SW serum.
The second experiment (Fig. 8) shows that, unlike the case of antigenic heterogeneity, a mixture of antibodies behaves essentially as its most firmly binding component. Quality can be matched to some extent by quantity (cf. curve A of Fig. 8), but this effect becomes noticeable only at a great excess of the less complementary antibody. From the right hand side of the Figure we learn that mixtures made up of a minority of homologous and at least a tenfold majority of heterologous antibody do not give the kind of dose-response curve we are accustomed to find in standard neutralization tests.

From the combined evidence of these two series of tests we may draw the conclusion that antigenic heterogeneity, even of minimal degree, will significantly alter the position of neutralization curves. Since, however, their shape remains unchanged, such a shift can be detected only by reference to some norm, as yet undefinable. In practice therefore antigenic heterogeneity is of little consequence: it simply means that we may not be observing the reaction of the whole antigenic population, only of part of it. Gross heterogeneity of the antibody population, on the other hand, would lead to neutralization curves incompatible with the simplest model, as set out in the family of curves in Fig. 5. Since the neutralization curves obtained were indistinguishable from the theoretical, one is bound to conclude that, at least with the reagents in hand, there was no indication of major inhomogeneity in the antibody component.
Since, however, neutralization tests are performed as a rule at antigen concentrations well below the equilibrium constant, a population of antibodies with closely-spaced $K$-values would once more give response curves coinciding with the simplest model. Thus a further conclusion is in order, namely, that neutralization tests over the conventional range are not well suited for the detection of heterogeneity among antibodies. While these conclusions may answer two simple practical questions, the problem of heterogeneity clearly deserves further study, both for its bearing on the behaviour of sera from animals given polyvalent vaccines, and for its relevance to the mechanisms by which antibodies are produced.

**SUMMARY**

The quantal assay system of influenza virus multiplying in surviving bits of the allantois is shown to be suited to the quantitative study of neutralization. Complications, such as bias due to the continued presence of serum or to shielding of infectible cells from the action of antibody, are not evident in standard tests.

It is demonstrated that 1.) the final equilibrium is the same whether approached from the side of virus or antibody excess; 2.) changes in test volume lead to predictable shifts of equilibrium; and 3.) the effect of non-infective virus particles is compatible only with random reversible combination of antibody molecules with antigenic sites. On this evidence a model of neutralization based
on the law of mass action is accepted as working hypothesis.

The assay is not sensitive to minor inhomogeneity of the reagents, and thus not suited to the study of heterogeneity either of the virus or of the antibody population.
REFERENCES

METHODS IN IMMUNOCHEMISTRY OF VIRUSES

I. EQUILIBRIUM FILTRATION

by S. FAZEKAS de ST. GROTH AND R. G. WEBSTER
METHODS IN IMMUNOCHEMISTRY OF VIRUSES
I. EQUILIBRIUM FILTRATION

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(Accepted for publication 31st October, 1961.)

SUMMARY.

An apparatus for the separation of free antibody from the virus-antibody complex is designed and tested.

It is demonstrated on the influenza virus-antibody system that the technique is not biased by systematic errors and is equally suited to kinetic and thermodynamic measurements.

The sensitivity and reproducibility of equilibrium filtrations are evaluated statistically.

INTRODUCTION.

Although the interaction of antigen and antibody has been studied over the past half century by perhaps the most rigorous and ingenious quantitative methods to be applied to biological systems, immunochemistry has not yet ventured outside its circle of in vitro tests, and its preferred antigens, too, can hardly be regarded as representative of the host of antigens against which immune responses in nature are directed. This deliberate narrowing of scope is not altogether surprising: the problems to be faced are complex enough without having to cope with the extra variables and vagaries of in vivo tests. Yet, with its powerful techniques immunochemistry could contribute greatly to the exploration of infective antigens and it seems somewhat archaic to make do in practical immunology with methods of assay and evaluation long since superseded in the study of the more readily definable, simpler systems.

The formation of an antigen-antibody complex gives rise to a new species of solute and simultaneously reduces the concentration of the initial reactants. Thus, in principle, any method capable of either separating the components of a heterogeneous system, or of estimating any one of the species making up the system, is suitable for determining the parameters of neutralization. In practice, the handling of viruses and their antibodies presents some special difficulties. Preparations of these antigens are usually impure and of low concentration, with little if any information about the antigenic groups, and antisera are seldom available in quantity. Thus if such tests are to be of any use, they must not only be simple to perform but also economical and exceptionally sensitive. The

experimental approach here is essentially eclectic: from among an array of potential techniques such combinations are to be selected as will— with the reagents at hand — quantitatively answer particular questions. We start with some methods based on the size of the reactants.

The classical procedure of equilibrium dialysis employs semipermeable membranes that retain macromolecules and let solvent and crystalloids pass freely. In immunochemical terms this restricts the method to work on artificial antigens, that is, to haptenic groups coupled to large carriers. The equilibrium is established between the separate small hapten and antibody in one compartment, and the concentration of free hapten is then determined by sampling the other compartment where antibody cannot pass. The system, of course, is also suited to competitive tests: the complete antigen, added to the antibody compartment, will shift the distribution of hapten and thus, indirectly, the binding between antigen and antibody can be studied. If the haptenic groups are not available in isolated form or, as is the rule with natural antigens, not even known, the method breaks down as it cannot sift reactants of comparable size.

With large particulate antigens such as cells, microorganisms and viruses, however, the technique is once more feasible. All that is needed is a membrane of suitable pore size to allow the passage of antibody molecules and retain the large antigenic particles. Such membranes, owing largely to the work of Zsigmond and his school, are now readily available in standardized form and provide a simple technique for the separation of antigen and antibody.

Since the diffusion coefficient of antibody molecules would be about ten times smaller than that of the average hapten, establishment of osmotic equilibrium in a virus-antibody system would take much time, allowing denaturation or inactivation of the reagents. We opted therefore for ultrafiltration as a means of separating free antibody from the virus-antibody complex.

**Materials and Methods.**

**Diluent.** The Ca-Mg-saline of Fazekas de St.Groth, Graham and Jack (1958) was used throughout.

**Virus.** Strain 15 of swine influenza virus (Shope, 1931) was grown allantoically in 11-day chick embryos. After two days' incubation at 35° C. the allantoic fluids were harvested, absorbed with 2 p.c. human erythrocytes, and the virus eluted into one-tenth of the original volume. Such concentrates were preserved by 0.08 p.c. sodium azide, and stored at 4° C.

**Titration of virus.** Haemagglutinin tests were done in plastic trays, as described by Fazekas de St.Groth and Graham (1954), but using 5 p.c. fowl red cells instead of 10 p.c. Infectivity was assayed in surviving bits of allantois-on-shell, by the method of Fazekas de St.Groth and White (1958).

**Antiserum.** A rabbit was given 5000 agglutinating doses of virus intravenously on five consecutive days, and then again on day 40, 60 and 80. The serum obtained from the 83-day bleed was stored at -15° C., and used without inactivation.

**Titration of antibody.** Antihaemagglutinin tests were performed according to Fazekas de St.Groth, Withell and Lafferty (1958) against four agglutinating doses of SW virus.
Instrumentation.

Apparatus. The choice of apparatus was governed by the need of handling small volumes (of the order of 1 ml.) and a large number of samples at a time. Of the commercially available filtering devices the Swinny-apparatus comes closest to meeting these demands. It has, however, certain disadvantages: passage through the narrow opening of a syringe makes it almost impossible to avoid occasional air-locks between filter and sample; some of the filtrate is retained in the cavity and duct of the instrument after passing through the filter; and the use of a syringe makes individual handling necessary. To correct these shortcomings the apparatus shown in Fig. 1 was designed.

Fig. 1. The filter assembly.

A: Lower part of stainless steel filter housing.
B: Lower, flat teflon gasket.
C: Perforated backing plate.
D: Filter membrane.
E: Upper, ring gasket.
F: Upper part of filter housing.
G: Quickfit B14 fitting, connected to compressed air source.
H: Springs to hold F and G together.

All measurements are in millimetres.
The filter membrane (D), of 13 mm. diameter and 1 cm.\(^2\) effective area, rests on a stainless steel crib-plate (C). Teflon gaskets (B and E) seal it on top and bottom. The housing is of stainless steel tubing, with a capacity of about 6 ml. above the filter membrane, and a dead volume of about 0.05 ml. between filter and lower aperture. The top end of the upper tube (F) is conically turned, to take interchangeable glass fittings (Quickfit B14). These glass links (G) are connected through a manifold to a compressed air source.

Since the upper tube has no narrowing, air-locks cannot spoil the experiment; the volume of fluid retained after filtration is equivalent to a single drop, and usually less than 5 p.c. of the sample; and any number of tests can be run in parallel, the connection of each tube taking only a few seconds.

Filters. The criteria for appropriate filters are (1) high rate of flow; (2) controlled pore size, to allow passing of antibody while quantitatively retaining virus; (3) inertness towards all reagents; and (4) minimal retention of fluid.

(1) Of the two kinds of filter\(^1\) judged suitable on general grounds, the Millipore filters had, over the pore size range of 10 to 100 m\(\mu\), about ten times higher flow rates than the corresponding Membrane filters. Using either type, it was found that with protein solutions such as serum, and especially with impure virus preparations, the rate of flow dropped considerably after as little as 0·5 ml. of a 1 p.c. protein solution had passed. Clogging of membranes could be prevented readily by adding about 5 p.c. (w/v) Kieselguhr to the preparation to be filtered, and letting it settle for about a minute in the filtering apparatus before turning on the pressure. This amounts to inserting a coarse clarifying filter in front of the ultrafilter membrane.

(2) When testing for permeability, both kinds of membrane were found satisfactory. Although Membrane filters have a wider scatter of pore size than Millipore membranes, the appropriate grades (Membranfilter 10, Ultrafeinfilter “g”, and Millipore VM and VF) all have the rated characteristics required to separate influenza viruses (~ 80 m\(\mu\) diameter) from antibody (~ 24 m\(\mu\) longest axis). No virus could be detected either as haemagglutinin or as infective units when a preparation of virus was filtered under 1 atm. pressure through one of the above listed membranes. This sets an upper limit to the permeability at \(10^{-5}\). The next higher grade of membranes, of 100 m\(\mu\) average pore diameter, let about 10 p.c. of the virus pass under similar conditions and is thus not suited to work on the influenza group.

(3) When similar experiments were performed to find out whether the membranes that retained virus would pass antibody, no quantitative recovery could be obtained, irrespective of pore size. Obviously, both kinds of membrane interacted with \(\gamma\)-globulin. To overcome such a complication, we first tried increasing the ionic strength of the medium to be filtered, and shifted the

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\(^1\) Gelatine “Membrane filters” (Membranfiltergesellschaft, Göttingen) and cellulose acetate “Millipore filters” (Millipore Filter Corporation, Bedford, Mass.).
pH to either side of neutrality. Although some of these changes as well as their combination improved recovery, the final yields still remained below the input. We then attempted to saturate the binding sites of the membrane with a protein homologous, at least in this respect, to antibody. A solution of gelatine was found satisfactory for the purpose. The summary of experimental results is given in Table 1.

### TABLE 1.

The binding of antibody by filter membranes.

<table>
<thead>
<tr>
<th>Diluent molarity</th>
<th>pH</th>
<th>Membrane</th>
<th>Filtrate log titre</th>
<th>per cent. recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15</td>
<td>7.2</td>
<td>untreated</td>
<td>2.20</td>
<td>31</td>
</tr>
<tr>
<td>0.15</td>
<td>9.5</td>
<td>untreated</td>
<td>2.23</td>
<td>33</td>
</tr>
<tr>
<td>0.15</td>
<td>4.5</td>
<td>untreated</td>
<td>2.11</td>
<td>25</td>
</tr>
<tr>
<td>1.71</td>
<td>7.2</td>
<td>untreated</td>
<td>2.53</td>
<td>66</td>
</tr>
<tr>
<td>1.71</td>
<td>9.5</td>
<td>untreated</td>
<td>2.47</td>
<td>58</td>
</tr>
<tr>
<td>1.71</td>
<td>4.5</td>
<td>untreated</td>
<td>2.50</td>
<td>62</td>
</tr>
<tr>
<td>0.15</td>
<td>7.2</td>
<td>gelatine-treated</td>
<td>2.71</td>
<td>100</td>
</tr>
<tr>
<td>0.15</td>
<td>7.2</td>
<td>unfiltered control</td>
<td>2.71</td>
<td>100</td>
</tr>
</tbody>
</table>

Anti-SW rabbit serum diluted 1:40 was passed through Millipore VM membranes.

In practice, the saturation of protein-binding sites can be done by passing a 0.5 p.c. solution of gelatine through the membranes in the filter assembly directly before an experiment. This technique, however, leaves the filters with a residual volume of fluid since pressures applicable under ordinary conditions are insufficient to overcome the capillary pressure of fluid in the membranes, and thus cannot clear the lower passage by blowing air through it. An alternative technique proved preferable. The membranes were floated on a solution of gelatine — we used the Standard Medium of Fazekas de St.Groth and White (1958), which contains 0.2 p.c. gelatine in a balanced salt solution — and then dried under an infrared lamp. The dry, gelatine-coated membranes are ready for use, or can be stored at room temperature for any length of time. For all experiments detailed below, the filters were treated with gelatine in this fashion.

(4) The volume of fluid held within the membrane becomes a serious complication only if, to achieve greater accuracy, not only the filtrate but also the retained fraction is tested for the presence of a particular component, and the true ratio of the two is estimated from the observed ratio. This would usually be the case with radioactively labelled reagents, and could lead to large sys-

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2 It is essential that the membranes should come into contact with the fluid on one side only. Dipping will inevitably lock air in at least some of the capillaries, and thus not only defeat the purpose of the treatment but also reduce the effective filtering area.
tematic errors, especially in the regions far from equipartition. The volume of fluid retained was determined gravimetrically. As a check we also passed $^{131}$I-labeled proteins and KI$^{131}$ through the filters. The latter would essentially measure whether there is an excess of I$^-$ ion in the volume of fluid retained, while the former could also show whether protein was held back preferentially.

TABLE 2.

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Capillary volume ($\mu l$)</th>
<th>Percentage of solute retained*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>KI$^{131}$-activity</td>
</tr>
<tr>
<td>'Membranfilter 10'</td>
<td>6.89</td>
<td>0.45</td>
</tr>
<tr>
<td>'Millipore VM'</td>
<td>10.83</td>
<td>0.00</td>
</tr>
<tr>
<td>'Millipore VM' treated</td>
<td>9.55</td>
<td>1.15</td>
</tr>
<tr>
<td>with gelatine</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* calculated after correcting for capillary volume.

From the results in Table 2 it is clear that both kinds of filter retain measurable volumes of fluid, Millipore membranes about half as much again as Membrane filters (10 $\mu l$ and 7 $\mu l$, respectively). Judged by the KI$^{131}$ activity, the volume would be about 20 $\mu l$ and 5 $\mu l$, respectively. The protein activity gives values of 38 $\mu l$ and 102 $\mu l$, thus indicating that in appropriately treated membranes retention is appreciable though not excessive.

As long as concentrations are compared, the volume of fluid held by the membranes is probably of no importance since it seems to have a composition close to the filtrate. As soon, however, as quantities are measured and referred to the input volume, corrections must be taken even if retention is proportional. In particular, appropriate controls will be needed when the membrane itself is to be tested, say, as a source of radioactivity coming presumably from components above the filter membrane. It is easy to conceive of situations where the contribution of the membrane itself is not only comparable to the sample to be assayed but, indeed, overwhelming.

On the grounds of the combined evidence of this section, gelatine-treated Millipore VM membranes (13 mm. diameter, 50 m$\mu$ average pore size) were used in all experiments for the separation of influenza viruses and their antibodies.

**The standard test.**

*Temperature control.* During the developmental stage we did not observe special precautions, the tests being run at room temperature (18-20°C.). Virus and antiserum, stored at 4°C. and −15°C. respectively, were diluted about an hour before the tests in saline kept at room temperature. With dilutions of tenfold or higher, the temperature of reagents could not have been more than a
degree below that of the environment. Since the technique was being designed with thermodynamic measurements in view, experiments were later conducted in constant temperature rooms, set at 4, 22 and 35° C., with a variation of less than 0·5° C. Both the filtering assembly and all reagents were kept at the appropriate temperature overnight.

Preincubation. Equal volumes of appropriately diluted serum and virus were brought together in small test tubes, well shaken and incubated for half-an-hour. As will be seen below (Fig. 3), this time is not critical with reagents of the usual concentrations, since the stage reached after five minutes is indistinguishable from equilibrium conditions. When working with very dilute systems, however, it is essential to perform a preliminary test of the kind shown in Fig. 3. As the rate of collisions between virus and antibody falls away with the inverse product of their concentrations, the period of 30 minutes, greatly in excess under ordinary conditions, might not be sufficient for attaining equilibrium when reactants are present in low concentrations only.

Volumes. Depending on the type of assay used for testing the filtrate, volumes ranging from 0·5 to 5 ml. were pipetted into the upper compartment of the filtering device. Kieselguhr (5 p.c. w/v) was added only exceptionally, when the concentration of total solids approached 1 p.c. and the volume exceeded 2 ml.

Collection of ultrafiltrates. Once in contact with the filter membrane, the samples will start seeping through slowly, even under their own weight. When the glass caps are fitted and the compressed air turned on to give an atmosphere excess pressure, the flow rate rises to about 0·7 ml./min. over the cm.² exposed area of membranes with 50 mµ average pore diameter. Thus, using 1 ml. volumes, the filtration is complete within a minute and a half, and even with the largest volumes it takes less than ten.

Demonstration of unbiased sampling.

It may be suspected that a period of even a few minutes is sufficient to allow significant drift from the original equilibrium. Intuitively, one might envisage an interplay of two opposed factors. First, the removal of free antibody from the upper compartment would tend to be compensated by dissociation of formed virus-antibody complexes. Secondly, the progressive reduction of volume would favour association. That these two processes exactly balance each other can be shown formally by writing down the two equilibria, the first for the original volume \( v \), and the second for a smaller volume \( w \), after \( (v - w)/v \) fraction of free antibody has passed through the filter.

\[
\frac{(sV - x)(A - x)}{xv} = K = \frac{(sV - y)(A - [(A - x)(v - w)/v] - y)}{yw}
\]

where \( V \) is the number of virus particles in the system, each with \( s \) antigenic sites; \( A \) is the number of antibody molecules, \( x \) and \( y \) the number of antigen-
antibody complexes formed when the volumes are \( v \) and \( w \) respectively. \( K \) stands for the equilibrium constant. It is evident that the condition of equality, that is the constancy of \( K \), is satisfied if, and only if, \( x = y \).

It is implied in this treatment that the filter does not discriminate between solvent and the solute of importance, in this case antibody. If filter-solvent, filter-solute and solvent-solute interactions are not negligible, all measurements on the filtrate will be biased. Evaluation will then have to rely on the more complex model based on the thermodynamics of open systems (Kedem and Katchalsky, 1958).

To test for the presence and degree of such bias, we performed the following experiment. Two doses of SW virus (730 and 365 haemagglutinating units respectively) were taken up in 0.50 ml. saline and mixed with an equal volume of rabbit anti-SW serum diluted 1/10. To allow more extensive testing, the globulin fraction of the serum was labelled with I\(^{131}\). After preincubation at

![Graph](image)

**Fig. 2.** Variation of the effluent during filtration. A hyperimmune rabbit serum was mixed with 365 (circles) or 730 (dotted circles) haemagglutinating doses of the homologous SW virus, or with saline (dots) as control. After 30 min. incubation the mixtures were filtered through a Millipore VM membrane, and the effluent collected dropwise.
room temperature for 30 minutes, the mixture was passed through a 50 m\(\mu\) Millipore membrane pretreated with gelatine. The filtrate was collected dropwise, i.e. in 0.05 ml. volumes. Each of the fractions was analysed for protein by the method of Lowry et al. (1951), for antibody by the antihaemagglutinin test, and for specific activity in a \(\beta\)-counter.

The curves in Fig. 2 show that, apart from the first two drops of the filtrate, the concentrations vary only within the expected accuracy of the respective method of assay. The first 0.1 ml. has a concentration about 12 p.c. lower than the rest, and its inclusion in the average will lead to an error of about 2 p.c. With larger volumes the error would be proportionately less. For more accurate work, therefore, the first fractions should be considered only as a means of estimating correction coefficients to be applied to the subsequent stationary state, where the concentrations of each solute are stable, i.e. the “exchange flow” has become negligible. We have not used this refinement as the maximal error so introduced is well below the discriminatory power of the usual antibody assay.

**Demonstration of equilibrium.**

**Rate of reaction.** Dilutions of virus and antibody were mixed in bulk, and 1.0 ml. lots placed directly into the upper compartment of the filtering device. At intervals up to two hours a set of samples was passed through the filter, and

![Figure 3](image-url)

**Fig. 3.** Rate of virus-antibody union. Anti-SW serum diluted 1:10, was mixed with an equal volume of SW virus diluted 1:5 (circles), 1:10 (dotted circles) or 1:20 (dots), and aliquots filtered at times shown on the abscissa. The ordinate gives the antihaemagglutinin content of the filtrates.
all filtrates assayed for antihaemagglutinin at the end of the test. Fig. 3 gives the result of one such experiment with SW virus and rabbit anti-SW serum.

The initial rate of reaction is rapid and, at the concentrations used, the process practically complete within five minutes. This is reassuring as far as equilibrium measurements go, but precludes accurate measurement of rate constants. The kinetics of association could be studied only on much more dilute systems, indeed, in a range which may well lie beyond the limits attainable by the most sensitive assays for antibody.

**Approach from two sides.** The most cogent demonstration of equilibrium is to show that the same final relationship of concentrations is reached whether approached from an excess of one or the other component of the system. In two experiments, therefore, the ratio of virus to antibody was gradually changed over a hundredfold range. In the first experiment small doses of antibody were added to the full dose of virus, and samples filtered ten minutes after each addition. The second experiment was set up the other way round: the concentration of virus was gradually increased in face of a fixed volume of antibody.

It should be noted that the final concentration of the reagents was about twice as high as in the kinetic experiment described in the previous section. Thus the ten-minute periods following successive additions of one or the other reagent should have been more than sufficient for attaining appropriate interim equilibria.

As seen from Fig. 4, the two curves tend to the same asymptote. Formally this amounts to the definition of an equilibrium, and experimentally to its proof.

**Irrelevance of heterologous antigens and antibodies in the system.**

To test whether components of the system other than the viral antigen and its homologous antibody contribute to the results, two sets of control experiments were
performed. In the first of these a dose of SW virus (sufficient to bind 80 p.c. of the dose of antibody used) was brought together with homologous antibody, both in the absence and in the presence of different animal sera, either normal or hyperimmune to some antigen other than SW virus.

**TABLE 3.**

<table>
<thead>
<tr>
<th>Additional reagent</th>
<th>Antibody titre of filtrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>nil</td>
<td>2.44</td>
</tr>
<tr>
<td>normal rabbit serum 1</td>
<td>2.63</td>
</tr>
<tr>
<td>normal rabbit serum 2</td>
<td>2.53</td>
</tr>
<tr>
<td>rabbit anti-LEE* serum 1</td>
<td>2.50</td>
</tr>
<tr>
<td>rabbit anti-LEE* serum 2</td>
<td>2.53</td>
</tr>
<tr>
<td>fowl anti-LEE* serum</td>
<td>2.44</td>
</tr>
</tbody>
</table>

0.25 ml. SW virus (diluted 1:10) was mixed with 0.25 ml. rabbit anti-SW serum (diluted 1:20) in the presence of 0.50 ml. of the additional reagent. After 30 min. incubation the mixtures were passed through a gelatine-treated 'Millipore VM' membrane. The antibody titres are given in log units.

* The prototype strain of influenza B virus.

As the results in Table 3 show, the filtrates did not differ significantly among one another. It may be concluded therefore that the non-antibody fraction of sera does not influence the equilibrium established between the homologous reactants.

In the second experiment the mixtures of SW virus and anti-SW serum were set up in the presence of various other antigens. After the usual preincubation of 30 minutes, the preparations were filtered, and the concentrations of free antibody determined in the filtrate (Table 4).

**TABLE 4.**

<table>
<thead>
<tr>
<th>Additional reagent</th>
<th>Antibody titre of filtrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>nil</td>
<td>2.44</td>
</tr>
<tr>
<td>LEE* virus 10⁵.5 ID₅₀</td>
<td>2.53</td>
</tr>
<tr>
<td>Vaccinia virus 10⁷ pfu</td>
<td>2.47</td>
</tr>
<tr>
<td>Rabbit pox virus 10⁵.5 pfu</td>
<td>2.47</td>
</tr>
</tbody>
</table>

0.25 ml. SW virus (diluted 1:10) was mixed with 0.25 ml. rabbit anti-SW serum (diluted 1:20) in the presence of 0.50 ml. of the additional reagent. After 30 min. incubation the mixtures were passed through a gelatine-treated 'Millipore VM' membrane. The antibody titres are given in log units.

* The prototype strain of influenza B virus.

Once again, the answer is unequivocal: the same fraction of antibody remains uncombined whether the system contains the homologous antigen only, or also sundry heterologous ones.
Reproducibility and sensitivity of the technique.

With the technical details standardized, it remained to establish the reliability of the method. To this end each of us made up virus-antibody mixtures in sextuplicate, using two concentrations of virus. After 30 minutes’ incubation at room temperature (20° C.) the mixtures were filtered, and antihaemagglutinin titres of the filtrates determined in duplicate. A test of identical design was performed with the same reagents two days later. The 96 titres so obtained were submitted to an analysis of variance. The main contrasts to be evaluated were the difference due to operators, to time of performance, to parallel tests with the two doses of virus, to replicate tests on the same virus-antibody mixture, and to antihaemagglutinin titrations.

The variance due to different doses of virus was highly significant (variance ratio $F(1, 88) = 3078$, where the $P = 0.001$ point lies at 11.5). This result is the same as can also be seen from the lower third of Fig. 2—in the mid-range of binding the method is exquisitely sensitive to variation in the concentration of antigen. As far as reproducibility is concerned, however, the response to dose is of no relevance. The contrasts of interest, or more precisely, the cumulative errors introduced by their presence, are listed in Table 5.

The basic error, due to antihaemagglutinin titrations, accounts for two-thirds of the maximal error obtaining when all sources of variation are simultaneously operative. Indeed, in practice about as much would be gained by performing a single equilibrium filtration and then assaying the filtrate in duplicate, as by a very large number of filtration tests followed by single antihaemagglutinin titrations. (Duplicate antihaemagglutinin assays would reduce the error from ± 24 p.c. to ± 19 p.c., triplicates to ± 17 p.c.; an infinite number of filtration tests would bring it only to ± 16 p.c.)

Clearly, the accuracy of the method is limited by the accuracy of the antibody assay. And since antihaemagglutinin tests are more reproducible and more economical than any test for antiviral antibody, it may be concluded that the

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Cumulative standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\log_{10}$ units</td>
</tr>
<tr>
<td>Antihaemagglutinin test (basic error)</td>
<td>$\pm 0.063$</td>
</tr>
<tr>
<td>+ replicate filtration assays</td>
<td>$\pm 0.078$</td>
</tr>
<tr>
<td>+ parallel filtration assays</td>
<td>$\pm 0.078$</td>
</tr>
<tr>
<td>+ time of performance</td>
<td>$\pm 0.090$</td>
</tr>
<tr>
<td>+ operators</td>
<td>$\pm 0.093$</td>
</tr>
</tbody>
</table>

The estimates are based on an analysis of variance computed from the results of 96 tests.
method of ultrafiltration compares more than favourably with immunological techniques at present available to the virologist.

REFERENCES.

Paper III.

METHODS IN IMMUNOCHEMISTRY OF VIRUSES.

III. Simple techniques for labelling antibodies

with $^{131}$I and $^{35}$S.
SUMMARY

Methods for labelling antibody with iodine ($^{131}$I) or sulphur ($^{35}$S) are described. Starting with the cheapest radioactive compounds, the proposed techniques are simple enough to allow preparation of labelled antibody in any biological laboratory. The final product is serologically unaltered and thus suited to the study of immunologic interactions in dilute systems or in micro quantities.

INTRODUCTION

The classical techniques of studying virus-antibody union have a number of limitations. For example, most of them are not sensitive enough for work in dilute systems, such as are preferred when estimating some parameters of immunological reactions. Moreover, all of these methods, whether based on binary interaction of antigen and antibody, or on competition for virus between antibody and an indicator component, estimate a compound of the quantity and quality of antibody; the two terms being inseparable in simple experiments. Neither is there a serologic procedure by which free and bound antibody could be assayed simultaneously. Special difficulties are encountered also on chromatographic or electrophoretic separation of members of an antibody population: the quantities are usually so small as to escape detection, even if methods are found that can be applied to antibody on a solid medium.

Many of these shortcomings can be eliminated and the scope of conventional techniques widened by radioactively labelling some of the re-
actants. There are several potential labels to choose from, and several methods of incorporation. When testing these, we aimed at a procedure which is economical, simple, can be performed reproducibly and without any special equipment in a biological laboratory, and will work even on micro-quantities of virus or antibody. Two techniques answering these demands will be described, and exemplified by the labelling of an antiserum.

MATERIALS AND METHODS

**Diluent.** Calcium-magnesium saline, prepared according to Fazekas de St.Groth, Graham and Jack (1958), was used as buffered saline throughout.

**Buffers.** The buffer used for electrophoresis of serum proteins in cellulose acetate strips, was prepared according to Aronsson and Grönnwall (1957). The acetate buffer used in the methanol fractionation of serum was prepared by mixing 7.2 ml of 1M acetic acid with 12.0 ml of 1M sodium hydroxide, and making the volume up to one litre with distilled water.

**Methanol Reagent.** This reagent was prepared by mixing 607 ml of analytical grade methanol with 393 ml of distilled water.

**Virus.** Strain 15 of swine influenza virus (Shope, 1931) was grown in the allantoic cavity of 11-day chick embryos. The virus was concentrated tenfold by one cycle of adsorption and elution from 2 p.c. human red cells, and stored at 4°C with 0.08 p.c. sodium azide as preservative.
**Titration of Virus.** Haemagglutinin tests were carried out in plastic trays, as described by Fazekas de St. Groth and Graham (1954), using 5 p.c. fowl cells. Viral infectivity was estimated in surviving bits of allantois-on-shell, according to the method of Fazekas de St. Groth and White (1958).

**Antiserum.** Hyperimmune serum against the virus was prepared in adult rabbits. Each rabbit received 5,000 haemagglutinating doses of virus intravenously on days 0, 40, 60, and 80; 45 ml of whole blood being taken from the ear vein 3, 5, 7, and 9 days after the last injection. Sera were separated after one hour and stored frozen at -15°C until required.

**Titration of antibody.** Antihaemagglutinin titrations were carried out in plastic trays using 4 agglutinating doses of virus and 5 p.c. fowl red cells, and neutralization tests in surviving bits of the allantois, according to Fazekas de St. Groth, Withell and Lafferty (1958). The neutralizing potency of the serum was estimated by the method described by Fazekas de St. Groth (1961).

**Electrophoresis.** Serum samples were separated into their constituent proteins on cellulose acetate strips, according to Fazekas de St. Groth, Webster and Datyner (1962). The strips were stained with Procion Brilliant Blue dye (Fazekas de St. Groth, Webster and Datyner, 1962), and the relative quantities of protein estimated in a photoelectric densitometer ("Analytrol", Beckman-Spinco).
Preparation of $^{35}$S-labelled Amino Acids. Carrier-free radioactive sulphate was incorporated by *E. coli* strain B (Hershey).

A synthetic medium that would support maximal growth of *E. coli* was prepared from analytical grade reagents. The concentration of sulphur as ammonium sulphate could be varied to determine the limiting concentration for growth of the bacteria. The basic solutions were:

**A.**
- $\text{NH}_4\text{Cl} -- 1.0 \text{ gm.}$
- $\text{KH}_2\text{PO}_4 -- 3.0 \text{ gm.}$
- $\text{Na}_2\text{HPO}_4 -- 6.0 \text{ gm.}$
- $\text{MgCl}_2 \cdot 6\text{H}_2\text{O} -- 10 \text{ mg}$

Made up to one litre with distilled water.

**B.** Glucose -- 12.5 gm.

Made up to 50 ml in 0.01N HCl.

**C.** $\text{(NH}_4\text{)}_2\text{SO}_4 -- 1.0 \text{ gm.}$

Made up to 10 ml with distilled water.

The above solutions were sterilized by autoclaving at 120°C for 10 minutes. A medium that will support maximal growth of *E. coli* was prepared by mixing 10 ml A, 0.5 ml B, and 0.1 ml C.

**Radioactive Isotopes.** Carrier-free Na$^{131}$I and Na$^{35}$SO$_4$ were obtained from the Radiochemical Centre, Amersham, England.

**Determination of Radioactivity.** Measurements of radioactivity were carried out with an end-window Geiger-Müller tube connected to a conventional scaler. Samples were diluted to a level that did not require correction for coincidence losses but gave sufficient counts to make the random sampling error negligible ($\leq 1$ p.c.).
### TABLE 1

Comparison of Fractionation Procedures

<table>
<thead>
<tr>
<th>Method of Fractionation</th>
<th>Percentage of Protein in Major Electrophoretic Peaks</th>
<th>Percentage of Antibody in Fraction*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Globulin</td>
<td>Albumin</td>
</tr>
<tr>
<td></td>
<td>$\gamma$</td>
<td>$\beta$</td>
</tr>
<tr>
<td>Original serum</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulphate</td>
<td>57</td>
<td>28</td>
</tr>
<tr>
<td>Sodium sulphate</td>
<td>66</td>
<td>41</td>
</tr>
<tr>
<td>Rivanol</td>
<td>64</td>
<td>41</td>
</tr>
<tr>
<td>Methanol</td>
<td>100</td>
<td>41</td>
</tr>
</tbody>
</table>

*Estimated by antihaemagglutinin titration.*
EXPERIMENTAL

Optimal Method for Fractionation of Antisera. Serum was fractionated to reduce its non-antibody components to a minimum, without any loss of antibody. A successful procedure of this kind would ensure that a smaller quantity of external label would be required for antibody, and that at the same time the background of labelled non-antibody protein would be greatly reduced.

Several simple methods of obtaining a globulin fraction were tested. Globulin fractions were prepared with sodium sulphate (Kekwick, 1940), ammonium sulphate (Kendall, 1937), Rivanol ((6-9 diamino 2 ethoxyacridine lactate), Saifer and Lipkin, 1959) and with methanol (Flemmer and Hutchinson, 1945). The various fractions were made up to their original volumes in saline and tested for antibody activity and protein content in the major electrophoretic peaks.

The comparison of methods in Table 1 shows that the methanol fractionation was the most satisfactory, and the only one not leading to appreciable loss of antibody activity.

Fractionation of Hyperimmune Serum with Methanol. Hyperimmune rabbit anti-influenza serum (2.0 ml) and 1.0 ml of acetate buffer were mixed and cooled to 0°C; 7.0 ml of methanol reagent was also cooled to 0°C and added slowly to the cold antiserum under constant stirring. The mixture was allowed to stand at 0°C for 30 minutes and centrifuged at 0°C. The precipitate was washed once with methanol reagent plus
buffer and again centrifuged. Then the precipitate was taken up in buffered saline and dialysed against running tap water for 10 hours. During dialysis a euglobulin precipitate formed, which redissolved readily when the sac was placed in buffered saline for 1 hour.

**Iodination Procedure.** Treatment of alkaline protein solutions with iodine results in the combination of iodine with the tyrosyl and, to a smaller extent, histidyl residues of the protein, and the concomitant liberation of hydrogen iodide. The method to be described differs from reported techniques in using \( \text{H}_2\text{O}_2 \) as oxidizing agent, and \( \text{CCl}_4 \) for extraction of iodine. Both of these reagents have been used in different procedures; McFarlane (1956) used \( \text{H}_2\text{O}_2 \) as oxidizing agent in the presence of the protein to be iodinated and found alterations in the chemical and biological properties of the iodinated proteins. Masouredis (1957) made use of carbon tetrachloride (\( \text{CCl}_4 \)) to extract free iodine from the oxidizing system, but extracted the iodine into NaOH before reacting with protein.

The present method makes use of the same principles but combines them in such a way that the iodinated protein remains biologically unaltered. Carrier \( 10^{-3} \text{ M KI (0.25 ml)} \) was mixed with carrier-free radio-iodide, the volume brought up to 3.5 ml with distilled water and acidified with 0.2 ml of \( 1\text{N H}_2\text{SO}_4 \). The iodine was liberated by the addition of 0.2 ml of \( \text{H}_2\text{O}_2 \) (100 volumes). The reaction mixture was shaken with 2.0 ml of \( \text{CCl}_4 \) at intervals for 30 minutes to extract the free iodine
TABLE 2

Biological Activity of $^{131}\text{I}$-Labelled Antibody

<table>
<thead>
<tr>
<th></th>
<th>Antihaemagglutinin Titre</th>
<th>Neutralizing Potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original serum</td>
<td>3.64</td>
<td>4.93</td>
</tr>
<tr>
<td>$^{131}\text{I}$ Globulin</td>
<td>3.65</td>
<td>5.01</td>
</tr>
</tbody>
</table>

Antihaemagglutinin titre is expressed in $\log_{10}$ units per ml.

A hyperimmune rabbit serum against the virus of swine influenza was labelled with 2.0 mc of $^{131}\text{I}$. 
into the carbon tetrachloride phase. During this time 98 p.c. of the labelled iodine was extracted into the CCl₄ which turned slightly pink. The aqueous phase was then removed and the CCl₄ washed twice with 10 ml of distilled water. The globulin fraction, buffered at pH 8.9 with 0.1 M bicarbonate buffer, was added to the washed CCl₄ and mixed immediately. This globulin-carbon tetrachloride mixture was gently agitated for 10 minutes, the two phases separated by centrifuging, and the aqueous phase dialysed against running tap water for 12 hours and finally against saline for one hour.

Properties of ¹³¹I Labelled Antibody. As a result of the procedure described above, 20 p.c. of the input radioactivity was bound to protein. Since 50 p.c. of the iodine is not available for labelling of protein but is lost as HI, 40 p.c. of the theoretical incorporation was obtained. This amounts to 5.10⁻² μmoles of iodine per 11.95 mg of protein in this experiment, and hence to one atom of iodine per 3.96.10⁻¹⁶ mg of protein. Taking 1.7.10⁵ grams as the molecular weight of rabbit γ globulin gives, on the average, 0.71 I-atoms per antibody molecule. Table 2 shows that the serological activity of the labelled antibody remained unaltered during treatment. This table gives the results of tests carried out on one serum, but it is representative of the results obtained with many different sera, carried out at different times.
The limiting concentration of inorganic sulphate for E. coli strain B

<table>
<thead>
<tr>
<th>Concentration of sulphate as mg/ml (NH₄)₂SO₄</th>
<th>Growth of organisms</th>
<th>Logarithmic phase of growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0.0125</td>
<td>++</td>
<td>= approximately 10⁶ organisms</td>
</tr>
<tr>
<td>0.025</td>
<td>+++</td>
<td>= approximately 10⁷ organisms</td>
</tr>
<tr>
<td>0.05</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>0.075</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>++</td>
<td></td>
</tr>
</tbody>
</table>

Cultures were inoculated with approximately 10⁶ washed organisms that were in the logarithmic phase of growth.
Incorporation of Sulphate (\(^{35}\)S) into Bacterial protein. Unlike in vitro labelling where the fraction of interest can be purified beforehand, in vivo methods are accompanied by great wastage of the injected label by indiscriminate incorporation into all substances formed at that time. Even though the animal may be producing antibody at maximal rate, the amount of radioactive amino acids in antibody will be negligible compared with that incorporated into all other proteins synthesised over the same period.

Experiments have shown that approximately 0.25 p.c. of the serum proteins was a particular antibody (Fazekas and Webster, 1962). As this would represent a minute fraction only of the daily protein turnover, most of the labelled amino acids are likely to be incorporated into products other than antibody. Under these conditions it is essential to have a cheap source of labelled amino acids. Such a source was obtained by making an autotrophic bacterium incorporate inorganic sulphate (\(^{35}\)S) into its amino acids. *E. coli* was grown on synthetic medium with limiting concentrations of inorganic sulphate. Table 3 shows that the incorporation of 0.075 mg of ammonium sulphate into the synthetic medium will cause minimal limitation of growth of the organism.

For the labelling experiments 1.25 mc of \(^{35}\)S sulphate was added to the synthetic medium containing 10 ml of solution A, 0.5 ml of B and 0.075 milligrams of ammonium sulphate. The medium was inoculated with approximately \(10^6\) washed organisms that were in the logarithmic growth phase and incubated at 37°C for 24 hours. The organisms were
centrifuged and washed through 20 ml of saline, six times. Under these conditions 71.0 p.c. of the labelled sulphate was found to be incorporated into the bacteria.

Hydrolysis of Bacterial Protein. $^{35}$S labelled methionine and cystine were prepared from the washed bacteria according to Tarver (1957). The bacteria were extracted with 5 p.c. trichloracetic acid at 90°C for 15 minutes, and then washed once with the cold reagent. The residue was freed of most of the trichloracetic acid by washing with alcohol and ether, and then hydrolysed under reflux for 5 hours with 25 ml of a mixture of 80 p.c. 6N hydrochloric acid and 20 p.c. formic acid, but no carrier cystine or methionine was added. The hydrolysate was evaporated to dryness in a desiccator at 37°C, and taken up in 2 ml of Standard Medium (Fazekas de St. Groth and White, 1958) for inoculation into rabbits.

Incorporation of $^{35}$S Amino Acid Hydrolysate into Antibody. As very little of the labelled amino acid hydrolysate is incorporated into antibody, it is essential that the animals should be synthesising antibody at maximal rate. This occurs 4-6 days after the intravenous injection of an adequate secondary or tertiary stimulus in the rabbit (Webster, 1962). The rate of incorporation of $^{35}$S labelled amino acids from the amino acid pool of the animal, into antibody is very rapid. Kinetic studies have shown (Taliaferro and Taliaferro, 1957) that detectable levels of $^{35}$S amino acids were incorporated into antibody within 20
TABLE 4

Incorporation of $^{35}$S into Rabbit Antibody

<table>
<thead>
<tr>
<th></th>
<th>Total Radioactivity (counts per minute)</th>
</tr>
</thead>
<tbody>
<tr>
<td>in washed bacteria</td>
<td>in amino acid hydrolysate</td>
</tr>
<tr>
<td>$8.43 \times 10^6$</td>
<td>$3.62 \times 10^6$</td>
</tr>
</tbody>
</table>

* Assuming that 0.25 p.c. of the labelled protein in serum is antibody.
minutes and that the process was complete within 240 minutes.

On the fifth day after boosting rabbits for the third time with 5,000 haemagglutinating doses of swine influenza virus, the $^{35}$S amino acid hydrolysate was injected intravenously. The rabbits were bled out 24 hours later, and the serum separated and stored at $-15^\circ$C without preservative.

Properties of $^{35}$S Labelled Rabbit Anti-Influenza Serum. Table 4 shows that 43 p.c. of the label present in the bacteria was recovered in the amino acid fraction after hydrolysis of the protein and, assuming a recovery of 50 ml of serum from the rabbit, 3.5 p.c. of the injected label was incorporated into serum proteins. The number of radioactive counts in the antibody was low but it would be possible to raise this number by increasing the input label, or by using more sensitive counting devices. The biological properties of the antibody are identical with those of unlabelled antibody.

DISCUSSION

The advantages of the method described for labelling antibody with $^{131}$I are that it is cheap, simple, reproducible, retains all the biological activity of the original serum and, provided that the level of radioactivity required is not inordinately high, can be carried out in any biological laboratory. The advantages of the method, compared with excellent published methods (McFarlane, 1956, 1958, Banerjee and
Ekins, 1961) of iodinating serum proteins, lie in its simplicity, no special glassware or chemicals being required.

The use of methanol for obtaining globulin fractions of serum, was found by McFarlane (1956) to increase the elimination rate of such proteins from rabbits, but Dixon and Weigle (1957) found that the elimination rate varies greatly from animal to animal. Our tests covered only in vitro behaviour, and by this criterion there was no difference between the original antiserum and its fraction produced by methanol. The other possible criticism of the method is the use of high pH during labelling: Masouredis (1957) found that high pH was as significant as over-iodination in altering proteins. However, in the proposed procedure the period of exposure to such harsh conditions is minimal, and our results show no loss as judged by the reactivity of antibody before and after treatment.

The major disadvantage of using $^{131}$I as a radioactive label, is its relatively short half life (8.1 days); but as the reagent is obtainable carrier-free and is inexpensive, it is possible to attain almost any desired level of radioactivity without increasing the degree of iodination beyond a single radioactive atom, on the average, per protein molecule. The other disadvantage inherent in this general method of labelling, is that the labelled molecule is not chemically identical with the original, and more than from 1-2 atoms of iodine per antibody molecule can alter the immunological reactivity (Johnson, Day and Pressman, 1960).
A recent report on the labelling of influenza antibody with $^{131}$I (Powell, 1961) probably suffers from this latter disadvantage. In that study the radioactive label per antihaemagglutinating unit of antibody was high, but the antibody content and its internal labelling of antibody with $^{35}$S offers a cheap source of tagged antibody, chemically indistinguishable from unlabelled antibody, and which has a reasonably long half life (87.1 days). However, this method is somewhat more complex and, as with all in vivo methods of labelling antibody, over 99.9% of the activity is incorporated into other proteins.
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Paper IV.

TWO NEW STAINING PROCEDURES FOR QUANTITATIVE ESTIMATION OF PROTEINS ON ELECTROPHORETIC STRIPS.
INTRODUCTION

The advantages of zone electrophoresis are often largely lost by the need to elute separated fractions for reliable assay, or by using methods of detection that are essentially qualitative or at best approximate. In the course of work on the antibody fraction of immune sera this need for a quantitative micromethod became only too evident. We decided therefore to develop a staining procedure which 1) was sensitive enough to allow estimation of proteins in microgramme quantity; 2) would follow Beer's law over a reasonable range of concentrations; 3) could be performed directly on paper and cellulose acetate strips, or on agar and starch gel; and 4) could be evaluated by simple, commercially available equipment. To this end a good number of standard dyeing techniques used in the textile industry was tested, and two families of dyes found which met all of our starting criteria. We report here on the methods deemed optimal, and give the evidence required to establish the scope and reliability of the new techniques.
MATERIALS AND METHODS

Electrophoresis equipment. The apparatus used was similar to that described by Kohn\(^1\), which is a modification of Grassmann's apparatus\(^2\). The tank, made of Perspex, had internal dimensions of 32 x 18 x 5 cm, and was capable of taking four bridges side by side, each carrying two strips 2.5 cm wide. Two partitions were inserted between the electrode compartment and the compartment in contact with the bridges, as we have found that without this arrangement the pH of the buffer in the outer compartments tended to change on prolonged electrophoresis. To avoid sagging of the strips which invariably led to collection of condense water on their under surface, we stretched three fine Nylon threads 3 cm apart across the bridges; these gave the needed horizontal support during electrophoresis. As a consequence, it was possible to reduce the air space around the strips to a minimum, and thus ensure quicker saturation of the vapour phase and obviate excessive evaporation and the resultant uneven movement in different regions of the zone. *

* Working drawings of the equipment are available on request.
Buffer system. The tris-hydroxymethyl aminomethane (80.0 g/l)- boracic acid (8.0 g/l)- disodium ethylene-diamine-tetraacetate (6.0 g/l)- sodium azide (0.8 g/l) buffer was essentially that described by Aronsson and Grönwall, and used throughout the main experiments. The choice was made after comparing several commonly used buffer systems and finding that separation of serum components was both the cleanest and most rapid in this buffer, thus confirming Aronsson and Gronwall's claims. The buffer has a pH of 8.95, and its conductivity is $3.4 \text{ m}\Omega^{-1}$.

Support. Cellulose acetate strips (Oxoid Div., London, E.C.4) of 36 x 5 cm size were quartered, giving four strips of 18 x 2.5 cm. (Cutting up is best done on wet strips, to avoid cracking of the brittle material.) The strips were floated on buffer, with their "0" sides up: by soaking from one side no air was trapped within the electrophoretic zone. This process is complete within a few minutes, after which the strips were submerged and stored in the buffer at room temperature.

Electrophoresis. Before being laid on the bridges, the wet strips were lightly passed between two sheets of absorbent tissue. At least an hour was allowed for equilibration between mounting the strips and switching on the current. The samples, usually 4 µl, were applied with a capillary pipette, along a line 2 cm long. We
have tried several commercially available applicators without satisfaction. These, designed for paper strips, rely on the sample being soaked up instantly on application. On cellulose acetate even volumes as small as $1 \mu l/cm$ take about a minute to soak in, and therefore gradual delivery and spreading by hand was found preferable. However, even with the greatest care it is difficult to perform this step reproducibly, and the unevenness of application will be shown to be the major contributor to the error component of the technique.

Electrophoretic runs were done at a potential gradient of 10V/cm. Due to the low conductivity of the buffer system, the current per strip amounted to 0.75 mA only, and thus the heating effect arising from $3 \text{mW/cm}^2$ was negligible. Consequently, tests could be conducted at room temperature ($18-20^\circ$).

**Scanning.** The stained, dried cellulose acetate strips were floated on a paraffin fraction of appropriate refractive index (Flozene 85) according to Kohn\(^1\), and thus rendered transparent. The central 6 cm of the strip, carrying the protein pattern, was cut out and slipped into a **scanning cell**: a $7.5 \times 2.5 \text{ cm}$ microscopic slide mounted on a rim 2 mm high, and sealed along one of the long and the two short edges to a $6 \times 9 \text{ cm}$ photographic plate. The
resulting chamber, of \(71 \times 23 \times 2 \text{ mm}^3\) volume, was filled with Flozene before receiving an electrophoretic strip. Thus, within the cell, there was no refractive interface in the path of the light beam. All patterns were evaluated by a recording strip-scanner (Beckman/Spinco 'Analytrol'), using the film densitometer attachment. The scanning cell was clamped directly to the carrying plate of the microanalyser. We employed the smallest slit supplied with the equipment (0.1 mm), and scanned at a speed of 1.86 cm/min. that is, about three minutes per strip. This amounts of a magnification factor of 4.65-fold. All measurements refer directly to the areas under the curves, as integrated by the machine. Checks were made only for areas less than 0.5 cm\(^2\), in which case the millimetre squares on the scanning sheet were counted.

**Test samples.** Six protein preparations were used in standardizing the staining techniques:

Bovine serum albumin, Armour Fraction V, twice recrystallized.

Human serum albumin, Nutritional Biochemicals Co., crystalline.

Ovalbumin, Nutritional Biochemicals Co., twice recrystallized, salt free.
Rabbit γ-globulin, Armour Fraction III containing 80% γ-globulin, the rest of the plasma proteins being reduced to different extent.

Lysozyme, L. Light & Co. Ltd.

Insulin (bovine), Commonwealth Serum Laboratories, "pool C", twice recrystallized.

All test samples were made up to about 20 mg/ml concentration in buffer; the true concentrations were derived from the Kjeldahl-N value multiplied by the factor appropriate for the respective substance.

In the case of rabbit γ-globulin, an inhomogeneous preparation, the conventional average factor 6.25 was used.

EXPERIMENTAL

Principles of Screening and Basic Tests

When choosing the dyes, our first criterion was set by the equipment we wished to use for evaluating electrophoretic patterns. This scanner is supplied with filters between 450 and 650 mµ, at 50 mµ steps. The 'matched' filters of our instrument were found to differ from each other by as much as 25 mµ in their transmittance maxima, and by as much as 15 mµ from their rated peak values. We
had to look therefore for dyes whose protein complex would show broad absorption maxima around the mean values set by the filters, and preferably in the range from 550 to 650 μ where the highest sensitivity of the photo-cells falls. The second criterion was set by the need for quantitative evaluation, and hence only such dyes and staining procedures were considered which resulted in optical density proportionate to protein concentration. The third criterion demanded that the dye should show no affinity for fully acetylated cellulose, and thus leave a clear background on which the stained protein would stand out. Simplicity was regarded as a further requirement, automatically ruling out a large class of dyes bound to their substrate after complex mordanting and fixing reactions.

The basic experiments were conducted on replicate strips: one microlitre of rabbit serum was electrophoresed for 150 minutes, giving a separation of about 4.5 cm between the albumin and γ-globulin peaks. Fifty to hundred such strips were prepared at a time and submitted to factorial experiments in which the concentration of the dye, the time of staining, the diluent, and the medium and time of differentiation were the variables. After eliminating several large classes of dyes, among them the commonly used Amido Black, Lissamine Green, Azocarmine and Bromphenol Blue,
we retained two groups of compounds as most promising. The reactions of these will be described in what follows.

**Procion Blue**

**Dyeing characteristics.** The Procion dyes, released in 1957, have the general structure

\[
\text{SO}_3^- \text{Chromophore} \text{NH} \text{-} \text{N} \text{-} \text{N} \text{-} \text{N} \text{-} \text{Cl}
\]

The Cl-substituted triazinyl group is highly reactive and will combine with alcoholic and amino groups through elimination of HCl. There is further evidence, obtained by independent methods in several laboratories \(^4, 5, 6\), that such substitution occurs also at the peptide bonds of nylon; it is most likely therefore that similar combinations are formed with proteins, too. At alkaline pH these dyes react with water: it is essential therefore to use freshly prepared, acidic baths for dyeing. Since covalent bonds are formed, with the equilibrium well towards the dye-protein complex, the process may be regarded as quantitative and, for practical purposes, irreversible under ordinary conditions.
Of the set of triazinyl dyes Procion Brilliant Blue RS (ICI) was chosen as it had the highest colour intensity of those tested, with a broad peak of absorption centred on 602 μ. The absorption was found to be 88 and 93% of the maximum at 575 and 625 μ respectively. It is also worth noting that the absorption spectra of the free dye and of its protein complexes do not differ appreciably, a fact that follows from the insulation of the chromophoric moiety of the dye molecule from its triazinyl ring, through which coupling takes place.

**Standard staining procedure.** After experimenting with dye-baths set at particular hydrogen-ion concentrations and containing various salts in order to shift the partition of the dye in favour of the protein, we found that rather better results were obtained by avoiding aqueous solutions and making up Procion Blue in methanol. As a result, all protein fractions stained as intensively, yet the background did not retain the dye and fixation of proteins as a preliminary to staining was rendered superfluous.

The procedure found optimal is as follows:–

1. After electrophoresis immerse the cellulose acetate strips directly into the dye-bath (5 g/l Procion Brilliant Blue RS and 20 ml/l conc. HCl in methanol)
for 5 minutes at room temperature. The time is not critical: twice as long steeping will give the same results.

(2) Without drying transfer the strips to a bath of pure methanol for 5 minutes. Most of the background will be removed, the stained bands of protein become clearly visible.

(3) Repeat step (2), to remove last traces of dye from the cellulose acetate.

(4) Dry strips, either stretched on wooden frame or, preferably, in a photographic glazer, at 60°C for about 5 minutes. By the second procedure the curling of the strip at the edges can be avoided, and thus subsequent insertion into the scanning cell is facilitated.

**Relationship of optical density and concentration.** Four proteins (ovalbumin, bovine serum albumin, human serum albumin and lysozyme) were made up to concentrations ranging from 0.2 to 2%, and 4 μl volumes of each dilution applied over 2 cm width of cellulose acetate strips. After 30 min. electrophoresis at 10V/cm potential gradient, the strips were stained with Procion Blue,
dried, made transparent, and scanned at the wavelength of 600 μm. Figure 1 gives the results of one such experiment, obtained by using cam B2 of the photometer. By this arrangement optical density is recorded, and the concentration-response relationship will be linear over the range where Beer’s law is obeyed.

(Figure 1)

Evidently, the curves are well fitted by straight lines over their whole length, and all extrapolate to zero concentration. This combination of findings indicates that Beer's law may be taken as a valid descriptive model of the optical behaviour of Procion Blue-protein complexes, at least over the range tested. This extends from optical density readings of 0.07 to 1.50, and corresponds to 3 to 40 μg protein applied across 1 cm width.

The same strips were also scanned with cam B1, where the recorded response is proportionate to transmission. At low levels of absorption the relationship should be indistinguishable from linear since ln(1-c) ≅ -c, provided c ≪ 1. However, as the initial rate of change here is much steeper than when optical density is being plotted against concentration, the sensitivity of the method should be appreciably increased over the appropriate range. The curves in Figure 2 are seen to be linear up to concen-
Figure 1. Optical density scans of proteins stained with Procion Blue.

(Bovine serum albumin ■, human serum albumin ●, lysozyme □ and ovalbumin ○ were electrophoresed on cellulose acetate strips, stained with Procion Blue and scanned at a wavelength of 600 μm in a Spinco 'Analytrol' integrating photometer.

The curves are fitted by the method of least squares. Deviations from Beer's law are indicated by broken lines. Each point represents the mean of four determinations.
(Figure 2)

Concentrations corresponding to an optical density of 0.6, i.e., to at least 20 µg of protein per cm of strip. The level of detection is lowered to about 1 µg protein/cm. The scatter about the curves is, on the whole, greater than in the concentration–optical density plots, largely due to the uncertainty of the readings at the lower end of the scale.

Coomassie Blue

Dyeing characteristics. A number of triphenylmethane dyes were found to give strongly coloured protein bands without irreversibly combining with cellulose acetate. Of these we found Coomassie Brilliant Blue R 250 (ICI) most suitable, on account of its exceptionally high colour intensity and of the position of its absorption peak. This dye, belonging to the Magenta family, is classified in the Colour Index under the name of Acid Blue 83 (CI 42660) and has the structure

![Chemical Structure of Acid Blue 83](image)
Figure 2. Transmittance scans of proteins stained with Procion Blue.

(Bovine serum albumin ■, human serum albumin ●, lysozyme □ and ovalbumin ○ were electrophoresed on cellulose acetate strips, stained with Procion Blue and scanned at a wavelength of 600 μm in a Spinco 'Analytrol' integrating photometer. The curves are fitted by the method of least squares. Deviations from Beer's law are indicated by broken lines).

Each point represents the mean of four determinations.
In slightly acid media, the dye-anion is electrostatically attracted to the \( \text{N}^+ \text{H}_3 \) -groups of the protein, and within this primary combination van der Waals forces hold the reactants together. The dye-protein complex is firm, although fully reversible by dilution under appropriate conditions of pH. It is known that on drying the dye dissociates and migrates towards the hotter surface - a point to be remembered when designing or evaluating practical procedures.

Coomassie Blue dissolved in 0.01 M citrate buffer at pH 3 has its peak of absorption of 555 mµ, with 74% and 83% of the maximum absorbed at 525 and 575 mµ respectively. The protein-dye complex is characterized by a peak slightly broader than that of the free dye, and a maximum at 549 mµ. Apart from its brilliance, it was this almost negligible spectral shift between the free and bound dye-anion which led us to choose it for our purposes.

**Standard staining procedure.** Unlike Procion Blue, Coomassie Blue dissolved in methanol does not differentiate between proteins and cellulose acetate, and thus has to be used in aqueous solutions. Consequently, the proteins have to be fixed to the supporting strip before staining. The procedure found optimal is as follows:
(1) Fix protein by immersing electrophoretic strip in 200 g/l sulfo salicylic acid in water, for 1 minute.

(2) Without rinsing, transfer strips to dye-bath (2.5 g/l Coomassie Brilliant Blue R 250 in glass-distilled water) for 5 min. (The use of water free from traces of heavy metals is essential, as these fundamentally alter the staining properties of the dye and prevent its subsequent removal from the background.)

(3) Remove background stain by four rinses of 5 min. each in distilled water.

(4) Dry strips either on wooden frame or, preferably in photographic glazer at 90°C for 15 mins.

**Relationship of optical density and concentration.** The same four proteins as used in the testing of Procion Blue were made up at concentrations ranging from 0.01 to 1.0%, and 4 μl of each was applied over 2 cm width of cellulose acetate strips. After electrophoresis at 10V/cm for 30 min. the strips were fixed in sulfosalicylic acid and stained according to the standard procedure described above. The dried strips were made transparent and scanned at 550 mμ, first by using cam B2 (optical density recorded) and then by using cam B1 (transmission recorded). The results are shown in
Figures 3 and 4.

(Figure 3)

Although, due to its high colour intensity, Coomassie Blue was evaluated on a set of test proteins two to ten times more dilute than the series used for Procion Blue, deviations from Beer's law become obvious from the optical density reading of 0.8 upwards, corresponding to concentrations of 0.5% or higher (Figure 3). However, all the curves pass through the origin and hence may be used for quantitative estimation of protein-bound dye over their linear limb. Significant readings can be made in the concentration range of 0.05 - 0.5% of the original solutions, or between 1 and 10 μg protein applied across 1 cm width.

(Figure 4)

Recording transmission (Figure 4) leads to similar conclusions. Indeed, the scope of quantitative evaluation here is greater, extending over a 20-fold range, from 0.2 μg to 4 μg of protein/cm width. Even though the intercepts are estimated with rather lower accuracy than on the optical density versus concentration plots, the curves are once again seen to extrapolate the zero, thus validating direct estimation of protein-bound dye over the lower, linear parts of the plots.
Figure 3. Optical density scans of proteins stained with Coomassie Blue.

(Bovine serum albumin ■, human serum albumin ○, lysozyme □ and ovalbumin ○ were electrophoresed on cellulose acetate strips, stained with Coomassie Blue and scanned at a wavelength of 550 μm in a Spinco 'Analytrol' integrating photometer. The curves are fitted by the method of least squares. Deviations from Beer's law are indicated by broken lines. Each point represents the mean of four determinations.)
Figure 4. Transmittance scans of proteins stained with Coomassie Blue.

(Bovine serum albumin ■, human serum albumin ○, lysozyme □ and ovalbumin ○ were electrophoresed on cellulose acetate strips, stained with Coomassie Blue and scanned at a wavelength of 550 μm in a Spinco 'Analytrol' integrating photometer. The curves are fitted by the method of least squares. Deviations from Beer's law are indicated by broken lines. Each point represents the mean of four determinations).
Quantitative estimation of proteins

**Differences between substrates.** As a basis of comparison two commonly used chemical methods were chosen: the determination of nitrogen by the micro-Kjeldahl procedure, and the photometric method of Lowry et al.\(^7\), based on the titration of tyrosyl residues. A 1\% solution of crystalline bovine serum albumin served as standard, and all concentrations will be expressed in these terms. The molecular weights of the test substances ranged from \(6 \times 10^3\) (bovine insulin) to \(1.7 \times 10^5\) (rabbit \(\gamma\)-globulin). Each of these proteins was highly purified and, with the exception of rabbit \(\gamma\)-globulin, crystalline or at least electrophoretically homogeneous.

(Table 1)

As expected, the estimates of concentration obtained by the four techniques differ from each other according to the kind of protein tested. The Kjeldahl-N values differ appreciably only in the case of lysozyme, a protein known to be of considerably higher than average nitrogen content. The average difference from the bovine serum albumin standard is \(\pm 7\%\), while Lowry's method is seen to deviate by \(\pm 12\%\). Averaged over the first five proteins, the Procion Blue values have a scatter of \(\pm 6\%\) in terms of the standard, and the
TABLE 1

Comparison of methods for the estimation of proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Protein Content</th>
<th>Micro-Kjeldahl</th>
<th>Lowry</th>
<th>Optical Density</th>
<th>Transmittance</th>
<th>Procion Blue</th>
<th>Optical Density</th>
<th>Transmittance</th>
<th>Coomassie Blue</th>
<th>Optical Density</th>
<th>Transmittance</th>
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</thead>
<tbody>
<tr>
<td>Bovine serum albumin *</td>
<td></td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
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<tr>
<td>Human serum albumin</td>
<td></td>
<td>0.99</td>
<td>0.96</td>
<td>0.97</td>
<td>0.94</td>
<td>1.15</td>
<td>1.16</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Ovalbumin</td>
<td></td>
<td>0.98</td>
<td>0.99</td>
<td>0.90</td>
<td>0.89</td>
<td>1.33</td>
<td>1.39</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Rabbit γ-globulin</td>
<td></td>
<td>1.00</td>
<td>0.81</td>
<td>1.03</td>
<td>0.97</td>
<td>1.06</td>
<td>1.05</td>
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<tr>
<td>Lysozyme</td>
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<td>1.16</td>
<td>1.09</td>
<td>1.05</td>
<td>1.05</td>
<td>0.82</td>
<td>0.75</td>
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<tr>
<td>Insulin **</td>
<td></td>
<td>0.98</td>
<td>1.16</td>
<td>&gt;0.41</td>
<td>&gt;0.42</td>
<td>&gt;0.52</td>
<td>&gt;0.61</td>
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<td></td>
</tr>
</tbody>
</table>

The figures represent means of 10-12 determinations, and show the estimated protein content in terms of the bovine serum albumin standard (*).

** The Procion and Coomassie Blue values are underestimates, due to loss of insulin during the dyeing procedure.
Coomassie Blue estimates deviate by ± 21%. The readings for insulin are inordinately low after either of the staining procedures. However, control tests showed that both insulin and its Procion Blue-complex are soluble in methanol, and sulfosalicylic acid does not fix this protein to cellulose acetate. Thus the low readings are due to leaching out of the substrate into the dye-bath, and hence the inclusion of insulin into the average would be unfair. The same holds for some small peptides on which we performed similar experiments - an alternative procedure covering these will be published separately.

If the proteins tested may be regarded as a representative set, this would mean that a quantitative estimate of an unknown protein would fall between 0.86 and 1.16 with 95% probability, using the Kjeldahl procedure, between 0.76 and 1.24 when assayed by the Lowry test, between 0.87 and 1.11 when recording optical density with Procion Blue, and between 0.85 and 1.09 when assayed by transmittance. Coomassie Blue would cover the range of 0.70 to 1.45 and 0.60 to 1.54 for optical density and transmittance respectively. With known substrates, of course, definition of a factor would allow transformation of results obtained with any one technique into those of any other, or into terms of dry weight. The
figures obtained with lysozyme show up the particular limitations of Coomassie Blue: the chances of complementariness between the relatively rigid, plate-shaped dye molecule and the small proteins of partly random secondary structure are low, and the intensity of staining falls away disproportionately when compared to the dye-albumin or dye-globulin complexes. However, even after disregarding reactions with the smallest substrates, Coomassie Blue would still show a variation of 0.85 to 1.42 and 0.80 to 1.50 in terms of the bovine serum albumin standard. Procion Blue, on the other hand, compares well with the two chemical methods. Indeed, its range of variation is less than that of the Lowry technique, due no doubt to the more even distribution of sites within proteins at which the dye can form bonds than the distribution of their tyrosyl residues.

**Accuracy.** Tests under this heading were performed on a 'globulin fraction' of rabbit serum, originally prepared for the estimation of its antibody content. It was chosen because it contained comparable quantities of albumin, α-, β- and γ-globulin, and thus the size of the four protein bands could be estimated without the risk that any of them would fall on the non-linear limbs of the response curves. The preparation contained 1.28 mg N/ml, as
determined by the micro-Kjeldahl procedure, and thus 8.0 μg of protein/ml. Four dilutions (0.25:1, 0.50:1, 0.75:1, 1:1) were made up and each applied to 2 μl volumes/cm across 6 cellulose acetate strips. The total quantity of protein applied was thus 4, 8, 12 and 16 μg/cm width. After a run of two hours at a potential gradient of 10V/cm, a third of the strips was stained with Procion Blue, a third with Coomassie Blue and a third, for sake of comparison with one of the popular staining techniques, with Amido Black. Each of the strips was scanned – both for optical density and transmission – at two levels of the bands, to allow independent evaluation of the evenness of applying the samples. This provided 32 estimates at each of the 4 levels of concentration, with each of the three dyes. The experimental data are given in full (Figure 5), and the percentual distribution of the main classes of serum proteins in this preparation has also been worked out from each of the scans (Figure 6). A representative scan of the 'globulin fraction' is presented in Figure 7. The error of the percentual estimates was obtained by submitting the data to an analysis of variance, while the slopes of the regression lines, fitted by maximum likelihood methods, were compared by covariance (Figure 5)
FIGURE 5. Quantitative estimation of serum proteins.

(Dilutions of a 'globulin fraction' of rabbit serum were electrophoresed for 2 hr at a potential gradient of 10V/cm in the Aronsson-Gronwall buffer on cellulose acetate strips. The protein bands were stained with Procion Blue, Coomassie Blue or Amido Black and scanned at 600 mµ, 550 mµ or 550 mµ wavelength respectively in a Spinco 'Analytrol' integrating photometer. The steeper curves represent scans for transmittance, the flatter ones for optical density. The lower two dilutions stained with Amido Black did not permit evaluation by optical density readings. The curves are fitted by the method of least squares. Deviations from Beer's law are indicated by broken lines.

The symbols □ and □ refer to readings at two levels of the first strip, and the symbols ◇ and ◇ to readings on a replicate strip.)
analysis. The latter also served to test the significance of the regressions and to detect deviations from linearity.

The Procion Blue readings show good linearity throughout, even when transmittance was recorded: the highest concentration of protein in any one peak (5 µg/cm width, for the γ-globulin band of the 1:1 sample) still falls on the straight limb of the response curve. Moreover, all lines intercept the ordinate at points insignificantly different from zero. Thus, the slopes may serve as direct estimators of protein concentration in each of the bands. These slopes carry errors of ±9.3, ±15.3, ±8.3 and 7.6% for the albumin, α-, β- and γ-globulins respectively, when transmission is recorded. (Overall average ± 10.1%). The corresponding errors for optical density readings are ±8.2, ±21.9, ±13.9, ±10.7, averaging ±13.6%. The somewhat increased variance of the theoretically superior optical density scores stems largely from the uncertainty of evaluating the α-globulin peaks of the most dilute samples, where about 0.3 µg of protein/cm width had to be estimated.

Coomassie Blue gives consistently linear plots when optical density is recorded, and the errors of the slopes for albumin, α-, β- and γ-globulin come to ±4.9, ±11.1, ±8.4 and ±8.1%, respectively (average ±8.1%). Readings of transmission on the same
strips significantly deviate from linearity for the higher concentrations of albumin and β-globulin, the two fractions moving in very narrow bands and thus characterized by high colour intensity on staining. Also, the best fitting straight lines computed from these two sets of data intercept the ordinate at points significantly different from zero. If the curves are constrained to pass through the origin, the estimated slopes carry errors of ± 11.0, ± 13.6, ± 5.5% and ± 8.7%, averaging ± 9.8%.

Amido Black does not emerge as a satisfactory dye for the evaluation of protein concentration on electrophoretic strips: all plots are non-linear, and three of the four curves do not pass through the origin. If errors were computed, they would average over ±25%, even without constraining the curves to pass through the zero point. Furthermore, evaluation by optical density measurements is not feasible in this concentration range: the two lower quantities of protein tested (2 and 4 μg/cm) gave no significant readings for α- and γ-globulin, and a greatly distorted overall pattern was observed even at the higher concentrations. These effects are evident also in the transmittance readings, the only ones plotted in Figures 5 and 6.

On analysis of the components of error, the overwhelming
contribution of differences between scans on the same strip stands out. On the average, about 60% of the variance is accounted for by this source. Errors arising from deviations from linearity, from differences between replicate tests and from the interaction of these factors would add up to an overall error not exceeding ±4% for either Procion or Coomassie Blue, scanned either for transmittance or optical density. And none of the individual sources would cause more than ±2% error on their own. Clearly, the most promising avenue to future improvement of the technique leads through a method that would ensure uniform application of samples over the width of the cellulose acetate strips.

(Figure 6)

The main source of error is minimized if relative concentrations only have to be estimated. An evaluation of the data in this form (Figure 6) shows that the average standard deviation of the Coomassie Blue results is ±1.3% for transmission readings (range: 0.8% for β-globulin - 1.6% for albumin), and ±1.9% for optical density (range: 1.0% for β-globulin - 3.7% for γ-globulin). The percentage is in terms of the total quantity of protein applied. The corresponding data for Procion Blue are ±2.9% (range: 2.5 - 3.4%) for transmittance and ±2.0 (range: 1.5 - 2.3%). The Amido
Figure 6. Relative estimation of serum proteins.

(The average percentage of the protein fractions is shown as horizontal lines surrounded by shaded areas of 2 standard deviations' width. The short lines connected vertically stand for two readings on the same strip, and correspond to the symbols ■ , □ and ● , ○ of Figure 5, respectively.)
Black estimates carry partly overlapping, large errors and differences of less than 20% in terms of overall concentration cannot be asserted with any degree of confidence.

(Figure 7)

DISCUSSION

The two new techniques for estimating the quantity of protein directly on electropheretic strips were developed and tested on cellulose acetate as support. Once the basic information was available, however, a number of parallel tests were run also on filter paper, agar and starch gel. Both Procion and Coomassie Blue proved as satisfactory under these conditions as in the experiments presented above, and we may conclude therefore that the two staining procedures are equally well suited to work on any of the more popular supports used for zone electrophoresis at present.

Compared with other available techniques, staining with Coomassie Blue stands out by virtue of its great sensitivity. When recording transmittance, as little as 0.5 micrograms of protein per cm width (i.e., less than 1% of the protein in 1 µl of serum) can be estimated to better than ±10% accuracy. Procion Blue is about three times less sensitive, while Azocarmine, Amido Black and Bromphenol
Figure 7. Scan of the 'globulin fraction' of rabbit serum.
(Two microlitres of the 'globulin fraction' containing 0.8% protein were applied to 1 cm width of a cellulose acetate strip, electrophoresed for 2 hr. at 10V/cm, stained with Coomassie Blue and scanned at 550 mp wavelength in a Spinco 'Analytrol' integrating photometer.)
Blue are five to ten times less so. Transmittance readings in this range show satisfactory linear relation to concentration, and are hence to be preferred when minority components of mixtures or dilute solutions of proteins are to be evaluated.

Determination of optical density was found, by and large, four times less sensitive than transmission measurements; its value lies in the extended range of concentrations over which the area under the peaks is directly proportional to the quantity of proteins stained by either of the two techniques developed above. Whereas Coomassie Blue would be the dye of choice when the demand was for sensitivity, the Procion Blue technique is superior by the criterion of reproducibility and accuracy. The dye-protein bond of the latter being covalent, there is little risk of day-to-day variation between tests of variation due to operators. Neither overstaining nor overdiffereniating alters the amount of Procion Blue bound, in masked contrast with most other procedures where the delicate balance between bleaching the background and losing some of the protein-bound dye can be maintained only by the most rigorous standardization of techniques. Another feature of Procion Blue - and of triarzinyl dyes in general - is their uniform behaviour towards peptides and proteins of widely differing physical and chemical
characteristics. This property is well documented by the evidence of Table 1: the factor relating intensity of staining to dry weight varies less than half as much as it does with Coomassie Blue, and less than a tenth of what can be expected of Amido Black (cf. Figures 5 and 6), provided its erratic performance on plasma proteins may be taken as an indication of general behaviour.

The main shortcoming of the techniques is the unsolved problem of evenly applying microlitre volumes of fluid to poorly absorbent surfaces. The overall error of measurement is more than doubled by this factor: its elimination would allow an accuracy of rather better than ±5% in estimating protein in bands of 1 to 20 µg dry weight content. The magnitude of the error arising from uneven application of the samples can be best seen from the difference in accuracy when the same data are evaluated in absolute and relative terms. While the standard deviation of a single reading in Figure 5 is about ±10%, the relative protein content of the four serum fractions can be determined with a standard deviation of about 2.5% (Figure 6), using the same experimental observations. As a consequence, the best practical approach available at present is multiple scanning of the same strips, covering preferably their entire width. Until a satisfactory method of applying the test samples is found,
this is the only way of salvaging all the quantitative information these techniques can yield.

Both procedures are simple in their final form, and suitable for routine and large-scale work. The areas in which the two dyes can be employed to best advantage partly overlap. We have used Coomassie Blue and cellulose acetate as support where the greatest sensitivity was required and where quantitative considerations are only secondary. Thus in electrosyneresis and electrodieresis of immune complexes, and in testing the purity of antibody fractions. For similar reasons, Laver has followed by this method the separation of influenza virus into its subunits, and Elliott and Green, using starch electrophoresis, the purification of enzymes involved in protein synthesis. For quantitative assay of serum proteins Procion Blue is preferable, partly because it shows more uniform affinity towards the various fractions than do other staining procedures, and partly because it is free from day-to-day variation. Also, in this case sensitivity is no major concern since a microlitre of serum spread over one centimetre width happens to fall in the optimum range of this technique, with no significant deviations from Beer's law by any of the fractions. Procion Blue has been used for comparisons of serum and milk proteins by Lascelles and
Garlick (to be published), and by us in immunochemical experiments 13, 14, 15.
SUMMARY

Two new procedures are described for the estimation of protein by direct photometry on electrophoretic strips. The protein complexes of Procion Blue and Coomassie Blue are shown to follow Beer's law up to 50 and 20 µg/cm, respectively. The lower limits of detection are 2 and 0.5 µg/cm. Within these ranges the absolute amount of protein can be estimated with an accuracy of about ±10%. The major contribution to the error arises from uneven application of the samples. Relative concentrations within a mixture of proteins can be evaluated to an accuracy better than ±3%.

Technical details of the procedures and of the equipment required are given in full, and their areas of usefulness discussed.
REFERENCES


13. WEBSTER, R.G., LAVER, W.G. & FAZEKAS de ST. GROTH, S.
14. FAZEKAS de ST. GROTH, S., & WEBSTER, R.G.
Paper V.

NEUTRALIZATION OF ANIMAL VIRUSES. IV. PARAMETERS

OF THE INFLUENZA VIRUS-ANTIBODY SYSTEM.
**SUMMARY**

The equilibrium between three strains of influenza virus and their homologeous antibodies is determined by two independent methods, with an accuracy of better than ± 10%.

Counts of virus particles, of haemagglutinating and infective units are presented for the same preparations, and two techniques are developed for estimating the number of antibody molecules within sera (accuracy about ± 30%).

From these data the number of antigenic sites per virus particle is calculated, and shown to be of the order of 2000 for each of the viruses tested. The equilibrium constants of the antigen-antibody reaction fell within the range of $9 \times 10^{10}$ and $5 \times 10^{11}$ (in cgs units), and were significantly different for each pair of reactants. These estimates carry errors between ± 25 and ±50%.

The parameters of virus-antibody interaction are also estimated by two special *in vivo* techniques. The number of antigenic sites per virus particle is the same as obtained by *in vitro* measurements, but the equilibrium constants are consistently lower. This both indicates some heterogeneity within the antibody population and points to the preferential involvement of the more firmly binding antibody molecules in the process of neutralization.
INTRODUCTION

The accumulated evidence, both from earlier work on mice or developing eggs and from the companion paper on surviving bits of the allantois, is consistent with a dynamic equilibrium between virus and antibody. Unions of antigenic sites and antibody are formed and broken all the time, and the average virus particle has a definable chance of being free and thus infective, or combined with antibody and thus neutralized. The system is not a simple one and the conventional tests, however carefully done, will give an answer only in the crudest operational terms. It is possible to determine, with any required degree of accuracy, what dilution of a particular antiserum will neutralize a given dose of virus under given conditions. It is impossible to derive from the same test an answer to equally interesting and more fundamental questions, such as how many antibody molecules are needed to neutralize the average infective unit; how many of these are actually combined with the virus particle at any time; and, consequently, what would be the neutralizing power of the same serum against another dose of virus, or the same dose tested in another host system.

Since the mass action model of neutralization, formulated
in the first paper of this series (1), has not been contradicted by experimental findings, it is tempting to put its equations to a more positive use by stating them in a form that allows the isolation of some of the parameters. The corresponding experiment should then enable us to measure these, and to estimate particular constants and variables in absolute terms.

The design and performance of such tests is the aim of this study. Theory and practice are inseparable in this approach, and will appear side by side in the following sections. All materials and basic methods are the same as described earlier (2); details of special techniques will be given where required.
PARAMETERS OF THE ANTIGEN-ANTIBODY UNION

The basic equilibrium between antibody molecules and antigenic sites on the virus particle is

\[ K = \frac{[sV(1+n) - y][A-y]}{y} \]

where \( K \) stands for the equilibrium constant, \( y \) for the concentration of antigen-antibody complexes, \( s \) for the number of antigenic sites per virus particle, \( V(1+n) \) for the total concentration of virus particles of which \( V \) are infective and \( nV \) non-infective, and \( A \) for the concentration of antibody molecules.

If \( y \) is expressed as a fraction of all antibody molecules (i.e., \( y = \alpha A \)), Eq. 1 may be written

\[ K = \frac{[sV(1+n) - \alpha A][A-\alpha A]}{\alpha A} = \left[ sV(1+n) - \alpha A \right] \frac{1 - \alpha}{\alpha} \]

Consider now a second equilibrium established between \( h \)-times as much virus as above but the same amount of antibody, in a system characterized by the same constants. Here the concentration of antigen-antibody complexes will be \( \beta A \), and the equilibrium therefore is defined as
By combining equations 2 and 3 either through $K$, the equilibrium constant, or through $s$, the number of antigenic sites per particle, we obtain a single equation from which either $K$ or $s$ may be estimated:

$$s = \frac{A}{V(1 + n)} \left( (\alpha - \beta) \left[ b \frac{1 - \beta}{\beta} - \frac{1 - \alpha}{\alpha} \right] \right)^{-1}$$

$$K = A \left( b \alpha - \beta \right) \left[ \frac{\beta}{1 - \beta} - b \frac{\alpha}{1 - \alpha} \right]^{-1}$$

The problem can be solved also more elegantly, by simultaneously estimating the two constants (3). Accordingly, Eq. 2 is either divided through by $A$, giving

$$(1 - \alpha) = \frac{sV(1 + n)}{A} \cdot (1 - \alpha) - \frac{K}{A}$$

or by $(1 - \alpha) K$, giving

$$\frac{1}{1 - \alpha} = \frac{sV(1 + n)}{K} \cdot \frac{1}{\alpha} - \frac{A}{K}$$

When plotting the appropriate variables, both Eqs. 5 and 6 define a straight line and the value of $s$ can be worked out from the slopes, while the intercepts are estimators of $K$. 
Of the constants appearing in Eqs. 5 and 6, the virus input $V$ can be determined directly in an infectivity test, and the values of $\alpha$ from simple equilibrium measurements. There are also conventional means of estimating $n$ by electronmicroscopic counts, and $A$ by several immunochemical methods. Even $s$ can be set an upper limit by steric considerations, and it should be possible to estimate it directly from the knowledge of the other parameters. This we proceed to do now.

**EQUILIBRIUM MEASUREMENTS**

**Ultracentrifugal Separation**

When serum and virus are brought together, part of the two reactants will remain free, part will be combined. If sufficient time is given an equilibrium will be reached in which the number of antigen–antibody links formed and broken in unit time is the same. If such an equilibrium mixture is exposed to gravitational force sufficient to sediment all virus particles – and a fortiori all virus–antibody complexes – but not free antibody, the supernatant will give a direct measure of $(A - y)$. If $A$ is not measured in absolute terms, the ratio of residual and input antibody titres will still
equal \( (1 - \alpha) \), as defined above. By keeping the input concentration of antibody constant and varying the dose of virus over such equilibrium tests, the results will define a series of \( (1 - \alpha) \) values.

Experiments of this kind were performed on the same three representative strains of influenza virus, MEL, LEE and SW, which were used in the companion paper. First, the validity of the technique was tested in some preliminary experiments. Antisera were diluted 1:100, mixed with various doses of homologous virus and incubated for 30 minutes to allow equilibration. Control tubes received virus or antibody only, the missing reagent being replaced by saline. All mixtures were then spun in a Spinco preparative ultracentrifuge (angle head No. 40) for 45 minutes at an average \( g \) of 74,000. After the run the top third and the central third of the supernatants were carefully pipetted off and all samples containing antibody titrated for antihaemagglutinin by standard methods. The titre of virus controls was determined as haemagglutinin. While 1% or less of virus remained in the supernatant under conditions of the experiment, no loss of antibody was detected in the control. This partition was complete insofar as there was no difference between the top and central
thirds of the fluid columns. The rest of the tubes showed that, depending on the dose of virus, more or less antibody remained in the supernatant. This information served to set the range of concentrations for the main experiments.

All tests were performed with purified virus and bleeds of hyperimmune sera as used in the previous paper (2). For equilibrium measurements 0.60 ml of a serum dilution and 0.60 ml of a virus dilution were brought together, incubated for 30 minutes at 0°C and then dispensed into two small lusteroid tubes. Six of these tubes were placed in a perspex adaptor fitting one of the 38.5 ml cups of the Spinco preparative head No. 30. Thus 72 samples of 0.60 ml could be spun simultaneously. The gravitational force employed was 66,000 g for 60 minutes, at 2°C. At the end of the run 0.50 ml of the supernatant was removed and titrated for antihaemagglutinin. The results of a representative experiment on each of the three strains are given in Table I.

(Table I)

These experiments were so designed that the fraction of free antibody, i.e., the term (1-α), should not exceed 0.20, and thus fall within the most informative range of equilibrium
TABLE I

Equilibrium measurements on three virus-antibody systems

I. Ultracentrifugal separation

(Virus and antibody were brought together and kept at 0°C for 30 min before spinning at 74,000 g for 60 min. The antibody content of the supernatants was determined as antihaemagglutinin against 4 agglutinating doses of the homologous virus (4). The stock antisera were used at final dilutions of 1 : 80 for anti-MEL, 1 : 100 for anti-LEE and 1 : 200 for anti-SW serum. Purified virus preparations were standardized against our stock viruses in terms of haemagglutinin content, the respective doses of $d = 1.00$ corresponding to a dilution of 1 : 22.23 for MEL, 1 : 0.53 for LEE and 1 : 1.88 for SW.)

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<th>log antibody/ml of supernatant</th>
<th>Fraction of antibody free (1 - α)</th>
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| 0.00 (controls)      | 2.10                          | 2.10                             |
| original serum       | 2.10                          | 2.10                             |
measurements (3). This is the reason for the very limited span of virus concentrations corresponding to any given concentration of antiserum. Since the data fulfil this requirement, they may be used directly for the estimation of equilibrium parameters. Equation 5 served as linear transform, and the curves were fitted by the method of least squares. The appropriate plots are shown in Fig. 1.

(Figure 1)

An analysis of covariance confirmed the impression gained from the graphs: there are no significant deviations from linearity, and thus Eq. 5 is a valid representative model of the data.

Equilibrium Filtration

Components of an equilibrium system consisting of virus and antibody can be separated also by passing the equilibrium mixture through filters of controlled pore size which retain virus but not antibody. The filtrate then represents free antibody and thus defines the term \(1 - \alpha\). The design of the necessary equipment has been fully described in a separate publication (4), which also contains the experimental evidence to establish the validity of the technique and to assess its accuracy and sensitivity.
FIGURE 1. Estimation of equilibrium parameters for three virus-antibody systems. 1. Ultracentrifugal separation. (The data of Table 1 are plotted according to Eq. 5: d(1-α)/α on the abscissa and (1-α) on the ordinate. The straight lines were fitted by the method of least squares; estimated values of the slopes (b) and intercepts (a) are shown on the graphs.)
A series of equilibrium filtrations was performed with the same virus-antibody mixtures as used in the previous section; the results of one such set are given in Table II.

(Table II)

The data of Table II are very similar to those of Table I, indicating the equivalence of the two methods. This is even more striking when the results are evaluated according to Eq. 5 (Fig. 2).

(Figure 2)

Statistical analysis shows that none of the corresponding parameters in Fig. 1 and Fig. 2 are significantly different. The combined estimates obtained by the two equilibrium techniques are collected in Table 3, expressed now in terms of our stock virus and serum preparations. Evaluation of the equilibrium constants and of the number of antigenic sites will be based on these, once the variables have been defined in absolute terms.

(Table III)
TABLE II

Equilibrium measurements on three virus-antibody systems

II. Equilibrium filtration

(Virus and antibody were brought together and kept at 0°C for 30 min before passing through a gelatine-treated Millipore VM filter at 1 atm. pressure. The antibody content of the filtrates was determined as antihaemagglutinin against 4 agglutinating doses of the homologous virus (4).

The stock antisera were used at final dilutions of 1:80 for antiMEL, 1:100 for antiLEE and 1:200 for antiSW serum. Purified virus preparations were standardized against our stock viruses in terms of haemagglutinin content, the respective doses of \( d = 1.00 \) corresponding to a dilution of 1:22.23 for MEL, 1:0.53 for LEE and 1:1.88 for SW.)

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MEL virus - antiMEL serum
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<td>1.60</td>
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</tr>
<tr>
<td>0.00 (controls)</td>
<td>2.25</td>
<td>2.22</td>
</tr>
<tr>
<td>original serum</td>
<td>2.22</td>
<td>1.000</td>
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</table>

**MEL virus - antiMEL serum (Contd.)**

<table>
<thead>
<tr>
<th>Dose of virus/ml (d)</th>
<th>log Antibody/ml of supernatant</th>
<th>Fraction of antibody free (1 - α)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.30</td>
<td>1.55</td>
<td>0.141</td>
</tr>
<tr>
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<td>0.141</td>
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<td>0.40</td>
<td>1.47</td>
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<td>1.47</td>
<td>0.118</td>
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<tr>
<td>0.40</td>
<td>1.20</td>
<td>0.063</td>
</tr>
<tr>
<td>0.50</td>
<td>1.35</td>
<td>0.089</td>
</tr>
<tr>
<td>0.50</td>
<td>1.35</td>
<td>0.089</td>
</tr>
<tr>
<td>0.50</td>
<td>1.07</td>
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<td>0.50</td>
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<td>0.056</td>
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<tr>
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<td>0.056</td>
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<tr>
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<td>1.15</td>
<td>0.056</td>
</tr>
<tr>
<td>0.70</td>
<td>1.20</td>
<td>0.063</td>
</tr>
<tr>
<td>Dose of virus/ml (d)</td>
<td>log Antibody/ml of supernatant</td>
<td>Fraction of antibody free ((1 - \alpha))</td>
</tr>
<tr>
<td>----------------------</td>
<td>-------------------------------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td></td>
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<tr>
<td><strong>LEE virus - antiLEE serum (Contd.)</strong></td>
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</tr>
<tr>
<td>0.70</td>
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<tr>
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<tr>
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<td>0.016</td>
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<td>1.10</td>
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<tr>
<td>1.10</td>
<td>0.62</td>
<td>0.016</td>
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<tr>
<td>0.00</td>
<td>2.40</td>
<td>1.000</td>
</tr>
<tr>
<td>(controls)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>original serum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.40</td>
<td>2.40</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SW virus - antiSW serum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.35</td>
<td>1.50</td>
<td>0.186</td>
</tr>
<tr>
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<td>1.50</td>
<td>0.186</td>
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<td>1.40</td>
<td>0.148</td>
</tr>
<tr>
<td>0.40</td>
<td>1.40</td>
<td>0.148</td>
</tr>
<tr>
<td>0.40</td>
<td>1.40</td>
<td>0.148</td>
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<td>0.40</td>
<td>1.47</td>
<td>0.170</td>
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<td>0.45</td>
<td>1.25</td>
<td>0.105</td>
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<tr>
<td>0.45</td>
<td>1.25</td>
<td>0.105</td>
</tr>
<tr>
<td>Dose of virus/ml (d)</td>
<td>log Antibody/ml of supernatant</td>
<td>Fraction of antibody free (1 - α)</td>
</tr>
<tr>
<td>---------------------</td>
<td>-------------------------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>0.50</td>
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<td>0.093</td>
</tr>
<tr>
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<td>1.23</td>
<td>0.100</td>
</tr>
<tr>
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<td>0.093</td>
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<tr>
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<td>1.20</td>
<td>0.093</td>
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<tr>
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<td>0.050</td>
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<tr>
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<td>0.046</td>
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<td>0.90</td>
<td>0.046</td>
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<tr>
<td>0.65</td>
<td>0.87</td>
<td>0.044</td>
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<tr>
<td>0.65</td>
<td>0.80</td>
<td>0.037</td>
</tr>
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<td>0.77</td>
<td>0.035</td>
</tr>
<tr>
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<td>0.035</td>
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<tr>
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<td>0.77</td>
<td>0.035</td>
</tr>
<tr>
<td>0.80</td>
<td>0.47</td>
<td>0.017</td>
</tr>
<tr>
<td>0.80</td>
<td>0.47</td>
<td>0.017</td>
</tr>
<tr>
<td><strong>0.00</strong></td>
<td><strong>2.25</strong></td>
<td><strong>1.000</strong></td>
</tr>
</tbody>
</table>

*(controls)*

| original serum     | 2.25                          | 2.22                            |
FIGURE 2. Estimation of equilibrium parameters for three virus-antibody systems. II. Equilibrium filtration. (The data on Table 2 are plotted according to Eq. 5: d(1-α)/α on the abscissa and (1-α) on the ordinate. The straight lines were fitted by the method of least squares; estimated values of the slopes (b) and intercepts (a) are shown on the graphs).
TABLE III

Evaluation of equilibrium parameters

(The data of Tables I and II were evaluated according to Equations 5 & 6. The figures represent mean values and their standard errors, multiplied by $10^3$.)

<table>
<thead>
<tr>
<th>Strain of virus</th>
<th>Quantity estimated</th>
<th>Centrifugal separation</th>
<th>Equilibrium filtration</th>
<th>Best combined estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Eq. 5</td>
<td>Eq. 6</td>
<td>Eq. 5</td>
</tr>
<tr>
<td>MEL</td>
<td>sV(l+n)/A</td>
<td>253.21 ± 7.14</td>
<td>248.77 ± 17.61</td>
<td>251.38 ± 23.05</td>
</tr>
<tr>
<td></td>
<td>K/A</td>
<td>0.41 ± 0.05</td>
<td>0.39 ± 0.04</td>
<td>0.44 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>K/sV(l+n)</td>
<td>1.64 ± 2.99</td>
<td>1.56 ± 0.11</td>
<td>1.74 ± 5.34</td>
</tr>
<tr>
<td>LEE</td>
<td>sV(l+n)/A</td>
<td>11.70 ± 0.91</td>
<td>11.18 ± 0.74</td>
<td>12.92 ± 1.26</td>
</tr>
<tr>
<td></td>
<td>K/A</td>
<td>0.27 ± 0.07</td>
<td>0.24 ± 0.03</td>
<td>0.25 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>K/sV(l+n)</td>
<td>22.64 ± 71.13</td>
<td>21.13 ± 1.51</td>
<td>19.43 ± 70.75</td>
</tr>
<tr>
<td>SW</td>
<td>sV(l+n)/A</td>
<td>29.08 ± 1.02</td>
<td>27.32 ± 1.64</td>
<td>23.34 ± 0.58</td>
</tr>
<tr>
<td></td>
<td>K/A</td>
<td>0.17 ± 0.02</td>
<td>0.14 ± 0.01</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>K/sV(l+n)</td>
<td>5.80 ± 11.43</td>
<td>5.00 ± 0.32</td>
<td>5.58 ± 11.64</td>
</tr>
</tbody>
</table>
COUNTS OF VIRUS PARTICLES

Infective Units

Seed virus, prepared as a large pool of allantoic fluid, was ampouled, snap-frozen in a mixture of solid CO\textsubscript{2} and methanol, and stored at -70\textdegree\textsuperscript{C}. Experience extending over years had shown that the infectivity of influenza viruses is perfectly stable under these conditions. The results of infectivity tests collected in Table IV cover a span of two years. Each experiment refers to a single ampoule, titrated in quadruplicate, using 3.16-fold dilution steps and 8 replicates per dilution. All tests were done in bits of the surviving allantois (5).

(Table IV)

The error of mean infectivities is about ±0.04 log units, or ±10\%, and the error of the mean of any one quadruplicate titration would come to about twice as much. Knowledge of the number of infective units is not needed for the evaluation of equilibrium data, since infective and non-infective virus participate equally in antigen-antibody combinations. However, the value of V, the number of infective particles, is one of the variables in all neutralization tests, and this can be worked out.
TABLE IV

Infectivity of virus stocks

(The allantoic fluids of 13-day chick embryos, infected 40 hours earlier with about 100 ID_{50} of the respective influenza virus strains, were pooled, dispensed in ampoules, snap-frozen and stored at -70°C. All titrations were done in bits of the surviving allantois (5), with eight replicates at each 3.16-fold dilution.

Each 'Experiment' was performed on the contents of one ampoule, the time span between I and V being two years.)

<table>
<thead>
<tr>
<th>Strain of virus</th>
<th>log ID_{50}/ml</th>
<th>Experiment</th>
<th>Mean ± standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
<td>III</td>
</tr>
<tr>
<td>MEL</td>
<td>9.21</td>
<td>9.27</td>
<td>8.66</td>
</tr>
<tr>
<td></td>
<td>8.80</td>
<td>8.80</td>
<td>8.93</td>
</tr>
<tr>
<td></td>
<td>9.15</td>
<td>9.14</td>
<td>8.97</td>
</tr>
<tr>
<td></td>
<td>8.80</td>
<td>9.05</td>
<td>9.09</td>
</tr>
<tr>
<td>LEE</td>
<td>8.38</td>
<td>8.05</td>
<td>7.85</td>
</tr>
<tr>
<td></td>
<td>8.15</td>
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<td>8.00</td>
<td>8.44</td>
<td>7.72</td>
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<td>7.88</td>
<td>7.89</td>
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<td>SW</td>
<td>8.22</td>
<td>8.39</td>
<td>8.19</td>
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</tr>
<tr>
<td></td>
<td>8.03</td>
<td>8.55</td>
<td>8.12</td>
</tr>
</tbody>
</table>
from the data of Table IV. The best estimate of \( V \) is obtained by multiplying the number of 50%-infective doses by 0.693. The factor is based on the fact that the dose-response curve is Poissonian in this system (6). Accordingly, the mean concentration of infective particles in our preparations was \( 10^{8.79} \) for MEL, \( 10^{7.91} \) for LEE, and \( 10^{8.08} \) for SW virus.

**Total Number of Particles**

Particles identifiable as influenza virus under the electron microscope were counted by the method of Sharp (7). Each preparation of virus was diluted to contain approximately \( 10^8 \) particles/ml. One millilitre volumes were transferred into each of three sector-cells of the Sorvall SU head, and spun at 26,000 g for 60 min. The virus deposited onto the collodion membranes was shadowed, and five electron micrographs were taken from each grid, at an instrumental magnification of 4250-fold. The negatives were printed at a linear enlargement of 5.85-fold, and parallel prints counted by each of us. The estimates obtained are \( 5.49 \times 10^{10} \) particles of MEL virus per millilitre, \( 1.23 \times 10^{10} \) LEE/ml, and \( 1.29 \times 10^{10} \) SW/ml, each carrying an error of about \( \pm 15\% \).
Control tests on second pseudo-replicas showed that 98% or more of the virus had been removed from the agar block by the first collodion membrane, and thus the underestimate arising from this source is not significant in terms of the inherent error of the method. This error is somewhat larger than could be expected on random sampling variation only, and one of the contributing factors seems to be the uneven distribution of virus from field to field. The preparations, of course, were crude allantoic fluids and contained, apart from a majority of discrete particles, some clumps of virus which cannot be counted with the same accuracy.

**Haemagglutinating Units**

Although the haemagglutinin test cannot be regarded as a procedure giving absolute counts of virus, once the ratio of physical particles to haemagglutinating units has been established, it should provide reliable relative estimates. Even so, inter-strain comparisons are not permissible and the technique of haemagglutinin tests must remain invariate, including the use of erythrocytes from a single donor. By observing these rules we found that our preparations had $10^{3.69}$ haemagglutinating units/ml for MEL, $10^{3.04}$ for LEE, and $10^{3.06}$ for SW virus. All tests were done with red cells of the same fowl, a haemagglutinating unit
being that quantity of virus which causes partial agglutination (the conventional endpoint) of 0.25 ml of a 1% suspension of erythrocytes.

The number of virus particles has been estimated by haemagglutination only on preparations which have not been counted under the electron microscope, such as the concentrates used in equilibrium measurements and the UV-killed virus employed in some of the neutralization tests to be described below.

**THE NUMBER OF ANTIBODY MOLECULES**

The γ-globulin of rabbits has a molecular weight of about $1.7 \times 10^5$ (8). It is known that neutralizing antibodies are found in this fraction of plasma, as could be confirmed in the case of our hyperimmune sera where only the γ-globulin peak was diminished when sera absorbed with specific antigen were submitted to free electrophoresis in a Perkin-Elmer apparatus. This method, however, is unsuited to our purposes: it requires large quantities of antisera and prohibitive amounts of virus; evaluation is difficult and reproducibility poor; and tests cannot be conducted at levels of concentration comparable to those obtaining in equilibrium or neutralization assays. We have developed therefore
techniques capable of estimating the concentration of antibodies in micro-quantities and with an accuracy acceptable by virologic standards.

**Absorption of Specific Activity.**

If the globulin fraction of an anti-influenza serum is uniformly labelled with radio-iodine, an average of about 1 atom of $^{131}$I per molecule will provide about a thousand times as many disintegrations per minute as is the antihaemagglutinin or neutralizing titre of that serum. Thus if specific antibody can be separated from the rest of the serum fraction, even minute amounts of it can be estimated reliably.

The experimental procedure is, in principle, the same as used in the ultracentrifugal equilibrium assays above. The globulin of our three standard antisera was precipitated $0^\circ$C, by gradually adding 60% (v/v) cold methanol. These fractions were then labelled with $^{131}$I, according to the method of Webster et al. (9). As has been demonstrated in the original paper, this procedure does not diminish either the quantity or the combining capacity of antibodies, and attaches about one I-atom/1.5 x $10^5$ avograms, i.e., substitutes on the average in one tyrosyl residue of each
γ-globulin molecule. If such preparations are now mixed with virus and then spun in the ultracentrifuge, antibody combined with virus will be carried down and its ratio to the non-antibody proteins can be estimated by comparing the specific activities of supernatant and deposit. As controls - to assess what fraction of the globulins combines non-specifically with our antigens - we labelled sera taken before vaccination from the same rabbits. These will be referred to as 'zero-day bleeds' in Table V which gives the results of these experiments.

After the centrifugal run the supernatants were removed, and the deposits resuspended in the original volume of saline containing a second dose of virus, and the mixture re-spun. By this means the small but highly radio-active fraction of the supernatant trapped in the pellet could be removed. It was found that a second resuspension did not further reduce the sedimentable specific activity. We have also observed that some labelled protein is firmly bound to lusteroid tubes and gives an undesirably high blank; this can be largely prevented by coating the tubes with protein before use. As part of our standard procedure all tubes were filled before an experiment with 10% normal rabbit serum in saline for 10 min., and then dried in air.
Control antihaemagglutinin tests on the supernatants showed that, for practical purposes, all (99.93%) antibody could be deposited with the virus, and that non-immune serum contributed little to the radioactivity of the deposits. On the assumption of uniform labelling and of molecular weights of $1.7 \times 10^5$, the antibody content of our three standard sera comes to $10^{14.29}$ molecules/ml for anti-MEL, $10^{15.25}$ molecules/ml for anti-LEE and $10^{15.00}$ molecules/ml for anti-SW.

**Electrodieresis**

When influenza viruses and their homologous antibodies are brought together in concentrated form, they give a precipitate (10). If this reaction is allowed to proceed within a molecular sieve, the precipitate will be trapped, and the free reagents can be removed electrophoretically. This is the principle of the method designed for estimating antibody in 1 to 5 µl of serum, and goes under the name of electrodieresis (11).

In practice, 4 µl of a purified virus suspension containing about $10^{11}$ particles/ml was streaked across 2.5 cm of a cellulose acetate strip ('Oxoid' Div., Oxo Co., Great Britain) and allowed to soak in. Two minutes later 4 µl of antiserum was streaked over
**TABLE V**

**Antibody content of stock sera**

I. Estimation by specific absorption.

(The $^{131}$I-labelled globulin fraction of sera was absorbed with excess homologous virus, and the radioactivity of the deposits (d) and supernatants (s) measured after depositing the virus-bound components in the ultracentrifuge. The means and standard errors are based on 14 determinations.)

<table>
<thead>
<tr>
<th>Sample absorbed with</th>
<th>Radioactivity (counts/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>antiMEL</td>
</tr>
<tr>
<td>zero</td>
<td></td>
</tr>
<tr>
<td>nil (d)</td>
<td>652 ± 106</td>
</tr>
<tr>
<td>virus (d)</td>
<td>638 ± 114</td>
</tr>
<tr>
<td>day bleed</td>
<td></td>
</tr>
<tr>
<td>virus (s)</td>
<td>137100</td>
</tr>
<tr>
<td>% bound by virus</td>
<td>0.0102%</td>
</tr>
<tr>
<td>hyper-immune bleed</td>
<td></td>
</tr>
<tr>
<td>nil (d)</td>
<td>1046 ± 213</td>
</tr>
<tr>
<td>virus (d)</td>
<td>680 ± 151</td>
</tr>
<tr>
<td>virus (s)</td>
<td>173200</td>
</tr>
<tr>
<td>% bound by virus</td>
<td>0.2113%</td>
</tr>
<tr>
<td>Fraction specifically bound</td>
<td>0.201%</td>
</tr>
<tr>
<td>Protein content mg/ml)</td>
<td>27.2</td>
</tr>
<tr>
<td>Antibody molecules/ml)</td>
<td>$(1.94±0.40)\times10^{14}$</td>
</tr>
</tbody>
</table>
the antigen. After a period of 2 hr, allowed for equilibration, the electrophoretic run was begun at a potential gradient of 10V/cm and a current of 0.7 mA per strip, in the Aronsson-Grönwall buffer (12), at pH 8.9. Under these conditions the albumin peak moves about 2.5 cm/hr and γ-globulin about 1.0 cm/hr. The current was switched off after two hours, the strips stained with Procion or Coomassie Blue, dried, made transparent and scanned in an integrating photometer. (The electrophoretic equipment and the new quantitative staining procedures developed for this purpose have been published separately (13).) The peak remaining at the origin is the virus-antibody complex. After correction for virus, this area is to the area of the γ-globulin peak as the number of antibody molecules is to the number of non-antibody γ-globulin molecules. If labelled antiserum is used in the same test, corrections for antigen at the origin become unnecessary. This arrangement both enhances the sensitivity of the method and renders it less open to systematic error. The results of such electrophoretic runs are given in Table VI.

(Table VI)

The quantity of specific antibody is seen to be within the range of the values worked out in Table V. This provides
TABLE VI

Antibody content of stock sera

II. Estimation by electrodieresis

(Precipitates formed by 4 μl of hyperimmune serum and about $10^{8.6}$ particles of the homologous virus were submitted to electrophoresis on cellulose acetate strips. The protein content of the various bands was evaluated by the quantitative staining procedure of Fazekas, Webster and Datyner, (1962) (13), with an average error of ±1.9 μg).

<table>
<thead>
<tr>
<th>Protein content μg</th>
<th>Serum</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>antiMEL</td>
<td>antiLEE</td>
<td>antiSW</td>
<td></td>
</tr>
<tr>
<td>γ-globulin peak</td>
<td>47.5</td>
<td>56.6</td>
<td>63.4</td>
<td></td>
</tr>
<tr>
<td>antibody peak</td>
<td>1.7</td>
<td>2.1</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>antibody as % of γ-globulin</td>
<td>3.59</td>
<td>3.71</td>
<td>4.25</td>
<td></td>
</tr>
<tr>
<td>antibody molecules/ml</td>
<td>$(1.51±1.68)x10^{15}$</td>
<td>$(1.86±1.68)x10^{15}$</td>
<td>$(2.39±1.68)x10^{15}$</td>
<td></td>
</tr>
</tbody>
</table>
a second, independent estimate which, although considerably less accurate, does not depend on the assumptions made when evaluating the results of ultracentrifugal separation.

**EQUILIBRIUM CONSTANTS AND NUMBER OF ANTIGENIC SITES**

Combining the best estimates of slopes and intercepts obtained in equilibrium measurements (Table III) with estimates of the concentration of virus and antibody molecules in our stock preparations (Table IV, V, and VI) the parameters of virus–antibody interaction can be defined and allocated appropriate experimental errors (Table VII).

(Table VII)

One feature of Table VII deserving emphasis is the similarity among influenza strains of $g$, the number of antigenic sites per particle. The mean values do not differ significantly among each other and a pooled estimate would come to $1733 \pm 520$ sites per particle. This value is supported by further measurements, some already published (3), some to appear later in this series. The simplest hypothesis could thus postulate a repeating pattern covering the surface of influenza viruses with the individual
### TABLE VII

**Estimates of equilibrium constants and antigenic sites**
(Computed from the data of Tables III, IV, V, & VI).  

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Weighted mean and standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MEL - antiMEL</td>
</tr>
<tr>
<td>Concentration of virus (particles/ml)</td>
<td>$(5.49 \pm 0.94) \times 10^{10}$</td>
</tr>
<tr>
<td>Concentration of antibody (molecules/ml)</td>
<td>$(2.24 \pm 0.48) \times 10^{14}$</td>
</tr>
<tr>
<td>Equilibrium constant, $K$</td>
<td>$(9.18 \pm 2.39) \times 10^{10}$</td>
</tr>
<tr>
<td>Number of antigenic sites per virus particle</td>
<td>$(1.33 \pm 0.37) \times 10^{3}$</td>
</tr>
</tbody>
</table>
antigenic determinants, different for each strain, appearing about 2,000 times. Whether immunochmical methods can be expected to yield numerical estimates of less uncertainty depends entirely on the accuracy with which the number of virus particles and, especially, of antibody molecules can be determined. The equilibrium measurements themselves are reproducible within better than 10%, accounting for a small fraction only of the overall error.

The values of $K$, the equilibrium constant, refer to a heterogeneous population and should thus be considered as mean values centred on the species of antibody molecule whose equilibrium constant is of the order of concentration obtaining in the tests. These concentrations were 1:80, 1:100 and 1:200 of our stock anti-MEL, anti-LEE and anti-SW sera respectively, corresponding to $10^{12.45}$, $10^{13.26}$ and $10^{12.73}$ antibody molecules per ml. The observed equilibrium constants tell nothing about the distribution of antibody species characterized by different $K$-values, and a more penetrating study of this problem must await effective fractionation of antisera according to this criterion. In the meantime, it is mandatory to conduct equilibrium measurements within the same concentration range as used in estimating the quantity of
antibody molecules, as has been done above; extrapolation, as regards either the value of $K$ or the concentration of antibody, is unwarranted and bound to lead to systematic errors of interpretation.

**ESTIMATION OF PARAMETERS IN VIVO**

Finally, to find out whether the basic mechanism proposed to account for the behaviour of virus and antibody in vitro held also in neutralization tests, means were also sought for estimating the same parameters in vivo, in ternary systems where the fraction of surviving infective particles was being measured and not the concentration of free antibody.

The volume effect

One such procedure may be based on the study of variation in the test volume, as already used in the companion paper. If the basic equilibrium between virus and antibody, Eq. 2, is rewritten to contain a term for volume, we have

$$K = \frac{\left[ sV_o (1+n) - \alpha A_o \right] v^{-1} \left[ A_o - \alpha A_o \right] v^{-1}}{\alpha A_o v^{-1}}$$

$$= \frac{\left[ sV_o (1+n) - \alpha A_o \right] \left[ A_o - \alpha A_o \right]}{v \alpha A_o}$$

7
The symbols $V_0$ and $A_0$ stand here for the absolute numbers of infective particles and antibody molecules respectively, and not for their concentrations; $v$ is the test volume in cm$^3$.

The outcome of neutralization assays is evaluated in terms of the surviving fraction of virus, and this fraction may be kept constant by keeping the number of unoccupied antigenic sites, $\left[ sV_0 (1 + n) - \alpha A_0 \right]$, constant. This will be achieved by adding $d$ times as much antibody to the system when the volume is changed from 1 to $v$. Thus

$$K = \frac{\left[ sV (1 + n) - \alpha A \right]}{v \alpha A_0} \left[ dA_0 - \alpha A \right]$$

$$= \left[ sV_0 (1 + n) - \alpha A \right] \frac{d - \alpha}{v \alpha}$$

If such tests are performed in three different volumes $(V_1, V_2, V_3)$ requiring antibody increments of $d_1, d_2, d_3$, we have three equations of the type of Equation 8. Any of these can be used for eliminating the unknown parameter $\alpha$, and the other two in the estimation of the parameters of interest, $s$ and $K$. Generally three equations of the form

$$\frac{v_h (d_1 - d_j)}{v_j (v_i - v_h)} - \frac{v_i (d_j - d_i)}{v_h (v_j - v_i)} = \frac{sV_0 (1 + n)}{A_0} \left( \frac{v_h (d_i - d_j)}{v_j (d_h v_j - d_i v_i)} - \frac{K}{A} \right)$$

$$9$$
will be obtained, where the subscripts $h$, $i$, $j$ take the values of $(1, 2, 3)$, $(2, 3, 1)$ and $(3, 1, 2)$ in turn. Another transform of equation 8

$$\frac{v_j(v_i - v_h)}{v_h(d_i - d_j) - v_i(d_i - d_j)} = \frac{sV_o(1 + n)}{K} \left( \frac{v_i - v_h}{d_i v_i - d_i v_h} \right) - \frac{A}{K}$$

will give an alternative means of handling the experimental data. Thus, at each level of virus input, the number of antigenic sites and the equilibrium constant can be estimated by fitting straight lines to the points defined in Eq. 9 or 10. Evidently, in this way we are able to evaluate the behaviour of antigen and antibody in neutralization tests without knowledge of the mechanism of neutralization, that is without any additional assumptions.

The appropriate experiments have been performed with all three strains of influenza, at 8 or more concentrations of virus, the three test volumes standing in the ratio of $1:3:6$. By the use of one of the three equations of type 9 or 10 in turn, the fraction of antibody molecules bound to virus, $\alpha$, was eliminated and the parameters $s$ and $K$ evaluated simultaneously from the remaining two equations. The best combined estimates appear in Table VIII.
The displacement effect

An alternative procedure for evaluating equilibrium parameters *in vivo* may be based on the distribution of antibody between infective and non-infective particles. If at the end point of neutralization, the equilibrium between antigenic sites and antibody is described by Eq. 1

\[ K = \frac{sV(1 + n) - y}{y} (A - y) \]  

by adding \( m \)-times as much killed virus, the equilibrium will shift to

\[ K = \frac{msV(1 + n) - x}{x} (A - x) \]  

The original neutralization end point will be re-established by ensuring the same average occupancy of antigenic sites, i.e., setting \( \frac{sV(1 + n) - y}{y} = \frac{msV(1 + n) - x}{x} \) and hence \( my = x \). Accordingly,

\[ K = \frac{msV(1 + n) - my}{my} (dA - my) \]

that is, by adding \( d \)-times as much antibody, the end point of neutralization will be maintained in face of an \( m \)-fold increase in non-infective virus. From Eq. 11 and 12 \( (dA - my) = (A - y) \), and hence \( y = A (d - 1)/(m - 1) \). By this means the unknown quantity
\( y \) is eliminated and thus, once again, we have an equation which allows estimation of \( s \) and \( K \) from neutralization tests without any assumptions on the mechanism of neutralization.

Suitable linear transforms are

\[
\begin{align*}
\left[ \frac{m - d}{m - 1} \right] &= \frac{sV(1 + n)}{A} \left[ \frac{m - d}{d - 1} \right] - \frac{K}{A} \\
\text{and} \\
\left[ \frac{m - 1}{m - d} \right] &= \frac{sV(1 + n)}{K} \left[ \frac{m - 1}{d - 1} \right] - \frac{A}{K}
\end{align*}
\]

Equations 13 and 14 are useful in the range where \( d \neq 1 \) and \( d \neq m/2 \); this is the region of the inflection point in neutralization tests, where \( sV(1 + n)K \). (See Fig. 5 of (2)).

Experiments of the type represented in Fig. 6 of the companion paper were performed on the three stock strains; UV-killed purified viruses were used for shifting the inflection point. The results, evaluated according to Eqs. 13 and 14, appear in Table VIII.

(Table VIII)

The best estimates of \( s \) and \( K \), based on \textit{in vivo} measurements are \( 10^{2.98} \) and \( 10^{10.20} \) for MEL virus and its antibody, \( 10^{3.13} \) and \( 10^{10.86} \) for LEE, and \( 10^{3.49} \) and \( 10^{10.73} \) for SW. On the whole, the results obtained by these methods carry large experimental errors. In a sense, this is the price to be
TABLE VIII
Evaluation of equilibrium parameters in vivo
(The data of volume- and displacement-experiments were evaluated according to Equations 9 and 10, and 13 and 14, respectively. The figures represent combined weighted means and their standard errors, multiplied by $10^3$).

<table>
<thead>
<tr>
<th>Strain of virus</th>
<th>Quantity estimated</th>
<th>Method of estimation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Volume-experiment</td>
<td>Displacement-experiment</td>
</tr>
<tr>
<td>MEL</td>
<td>$sV(l+n)/A$</td>
<td>$192.35 \pm 17.00$</td>
</tr>
<tr>
<td></td>
<td>$K/A$</td>
<td>$0.07 \pm 0.01$</td>
</tr>
<tr>
<td></td>
<td>$K/sV(l+n)$</td>
<td>$0.37 \pm 0.19$</td>
</tr>
<tr>
<td>LEE</td>
<td>$sV(l+n)/A$</td>
<td>$10.68 \pm 4.32$</td>
</tr>
<tr>
<td></td>
<td>$K/A$</td>
<td>$0.04 \pm 0.01$</td>
</tr>
<tr>
<td></td>
<td>$K/sV(l+n)$</td>
<td>$5.21 \pm 5.40$</td>
</tr>
<tr>
<td>SW</td>
<td>$sV(l+n)/A$</td>
<td>$37.02 \pm 4.10$</td>
</tr>
<tr>
<td></td>
<td>$K/A$</td>
<td>$0.05 \pm 0.01$</td>
</tr>
<tr>
<td></td>
<td>$K/sV(l+n)$</td>
<td>$1.38 \pm 1.29$</td>
</tr>
</tbody>
</table>
paid for the self-imposed restriction of doing without gratuitous assumptions regarding the mechanism of neutralization. As will be shown in later papers of this series, considerably better estimates can be derived from in vivo measurements when specified - and experimentally verifiable - models of neutralization are used as basis for the appropriate calculations. As they stand, the results are inaccurate, that is, define rather wide intervals within which the true means must lie. Yet, the quantitative estimates obtained by a variety of in vitro and in vivo techniques may be legitimately compared; they are seen to be consistent within each set of observations. This finding is sufficient to establish the mass action model as the proper working hypothesis for the interaction of viruses and antibodies, both in vitro and in vivo.

The number of antigenic sites, as estimated by the two in vivo methods, falls within 50% of the estimates derived from equilibrium measurements (-23 and -32% for MEL, -16 and -39% for LEE, +33 and +37% for SW). Unless regarded as change coincidence, this would suggest that the same areas are both involved in the neutralization of influenza viruses and underlie their serological reactions in vitro. It is interesting to note that
the surface of a spherical particle of 0.080 μ diameter (i.e., 0.0201 μ²) could accommodate at close packing about 2500 circular areas of the dimensions of an antibody molecule (minor axis 14°A). Thus, it seems, that the experimentally determined number of sites comes very close to the maximum possible, and that the antibody molecules can be expected to attach by their tips and be arranged normal to the surface of the virus.

The estimates of $K$ derived from *in vivo* measurements are uniformly and significantly lower than the corresponding values obtained by equilibrium techniques. The difference is 5.85-fold for MEL, 7.25-fold for LEE, 2.60-fold for SW. These differences are independent of the estimates of the number of antibody molecules, as the same results are obtained when the ratios $K/s$ are considered, and these contain only the number of virus particles as variable. Since the concentration of antibody used in neutralization tests was about 20-50 times lower than in the equilibrium measurements, the difference may well rest on the heterogeneity of the antibody population. Such an interpretation implies than only the more firmly binding varieties of antibody are effective in abolishing infectivity.
Generally, the main source of uncertainty of equilibrium measurements in immunological systems, be it in vivo or in vitro, springs from errors bound up with estimating the number of antibody molecules. Satisfactory solution of this problem can be expected in two ways. If the antibody fraction can be separated from an antiserum, even if this fraction does not contain all of the antibody, the overwhelming background of non-specifically combining substances would be eliminated. Or, if the uniformity of antigenic sites and their constant number per virus particle is accepted, the value of $s$ can be treated as a known fundamental constant. This not only does away with the need of experimentally estimating the number of antibody molecules but, indeed, would provide a powerful method for their measurement. Such constancy of antigenic sites per particle is not only the simplest interpretation of our findings, but would be a necessary consequence of the subunit-structure of small viruses, increasingly evident from recent X-ray and electronmicroscopic studies. A compelling case for the identity of capsomeres and antigenic units in tobacco mosaic virus has been made by Rapaport (14).
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THE IMMUNE RESPONSE TO INFLUENZA VIRUS. 1. EFFECT OF
ROUTE AND TIME SCHEDULE OF VACCINATION ON NEUTRALIZING
ANTIBODIES
SUMMARY

The immune response of rabbits to viral antigens is investigated in a series of studies. The first of these contains the general design of the experiments, the methods of evaluation, and the data of neutralization tests covering primary and anamnestic responses.

Three strains of influenza virus (MEL, LEE and SW) served as antigens, and were administered with or without adjuvant, either as single or as serial injections, by the intravenous, intraperitoneal or subcutaneous route.

Neutralizing antibody could be detected in the circulation by 2-3 days after primary vaccination, and its rate of increase is compatible with an exponential mechanism. The anamnestic response starts after a similar delay, but its rate of increase is initially faster than exponential.

The quantity of neutralizing antibody produced depends on the route and schedule of vaccination, as well as on the antigen. For the same dose of virus the intravenous route is somewhat better than the intraperitoneal, and much better than the subcutaneous.
Higher antibody levels are reached after multiple injections irrespective of route, during the primary response; these differences disappear after anamnestic stimulation.

Aqueous vaccines elicit a more rapid and higher primary response than water-in-oil emulsions; this is particularly true for the viruses SW and LEE which actually fail to respond well to multiple injections by any route if first given a single intramuscular dose of the same virus in adjuvant.

More neutralizing antibody is produced by all routes and schedules against MEL than against SW, while LEE is the least immunogenic of the three antigens tested.

With the large dose of antigen used, secondary stimulation elicits maximal responses which tertiary injections can only maintain, not improve.
INTRODUCTION

While most theories in immunology have arisen from new sets of observations, the more recent ideas in this field are largely deductive and seek to show how the production of antibodies would fit into the modern conceptual framework of heredity and individuality. A single body of data has thus been interpreted from fundamentally different points-of-view, and as a result we are presented with separate syntheses that apparently account for most of the facts but happen to be mutually exclusive. Since a state of affairs like this can rest only on a lack of basic information, it seemed to us that the immediate task of immunologic enquiry was not so much the design and performance of crucial tests aimed to rule out one or the other of the contending hypotheses, but rather a systematic collection of data, comprehensive enough to cover the implications of present hypotheses and coherent enough to guard against ambiguities in the future.

In setting out to establish such a reference system, we looked for a natural antigen that is readily available, simple,
occurred in a large number of variant forms, was easy to assay, and could be tested by many serological and immunochemical techniques. Our choice fell on the viruses of influenza. There are a great number of serologically related strains within this group, all of which can be grown and are being maintained by standard techniques in many laboratories. The elementary particle is characterized by a single surface antigen (Fazekas de St.Groth, 1962), enters most reactions known in serology and can thus be tested by a variety of in vivo and in vitro methods. We made a point of using several of these methods side by side, since some of them measure predominantly the quality of antibodies while others their quantity; some are sensitive to inhomogeneity in the reactants, others less so; some are influenced by the rate of antigen–antibody combination, whereas others depend on the equilibrium between free and combined antigenic sites.

The first papers of this series employ three basic techniques in describing the time course of the immune response, and are concerned with the dependence of this response on the route and schedule of vaccination, as well as on the nature of the antigen.
MATERIALS AND METHODS

Diluents. Calcium magnesium saline, prepared according to Fazekas de St.Groth, Graham and Jack (1958), was used as buffered saline throughout. The standard medium (SM) used in infectivity and neutralization tests was prepared according to Fazekas de St.Groth and White (1958).

Virus Strains. The three strains of influenza virus used were MEL (Burnet, 1935), a typical A-strain; LEE (Francis, 1940), the prototype of B-strains; and SW (strain 15 of Shope, 1931), the classical strain of swine influenza.

Growth and concentration of virus. About $10^3$ ID$_{50}$ of virus was inoculated into the allantoic cavity of 11-day old chick embryos. After two days' incubation at 35°C in the case of MEL and SW, and three days in the case of LEE, the allantoic fluid was harvested and concentrated tenfold by one cycle of adsorption and elution from 2 p.c. human red cells. The concentrated virus suspensions were preserved with 0.08% sodium azide and stored at 4°C. Such preparations must have contained, as contaminative antigens, about one-tenth of the non-viral material present in allantoic fluids, as well as some constituents of human erythrocytes leached out during the process of adsorption-elution.
Titration of virus. Haemagglutinin titrations were carried out in plastic trays using 5 p.c. fowl cells as described by Fazekas de St. Groth and Graham (1954). Viral infectivity was assayed in surviving pieces of allantois-on-shell, according to the method of Fazekas de St. Groth and White (1958).

Preparation of non-infective vaccines. Three methods were used to abolish the infectivity of virus preparations.

Heat treatment consisted in incubation of the virus concentrates in a water bath at 50°C for one hour.

Ultraviolet treatment was preceded by dialysis of the virus preparations against 1000 times their volume of buffered saline for 24 hours, to remove urates and other UV-absorbing material. Then 10 ml volumes were exposed in 9 cm diameter Petri dishes for 5 minutes to the radiation from a 15 watt Phillips ultraviolet tube, at a distance of 20 cm. To ensure uniform exposure, the preparations were mixed constantly with a magnetic stirrer during irradiation.

For ultraviolet and formaldehyde treatment, the UV-treated preparations were made up to a final concentration of 0.03 g/100 ml.
formaldehyde and kept at 20°C for 48 hours. Residual formaldehyde was removed by dialysis against several changes of buffered saline.

The vaccines inactivated by the three methods described above were tested for residual infective virus in pieces of surviving allantois-on-shell. As the titrations started with undiluted virus whose haemagglutinin titre was high, it was necessary to transfer each egg bit after an adsorption period of three hours, thus avoiding haemagglutination caused by unabsorbed virus. The egg pieces were removed one by one with fine forceps, washed in 2.0 ml volumes of standard medium and transferred to a second tray containing standard medium and a freshly cut egg piece. In other respects, the period of incubation and the scoring of infectivity was the same as in orthodox virus assays. No infectivity could be detected by this method in any of the vaccines.

**Preparation of adjuvant-vaccines.** Concentrated virus was made up in SM to contain 10,000 HA doses per 0.25 ml, i.e., at double strength compared to the standard concentration of 5,000 HA units/0.25 ml in the non-adjuvant vaccines. An equal
volume of Freund's incomplete adjuvant (Difco) was added to these preparations, and the mixture emulsified in a VirTis homogenizer running at maximum speed for 5 min at 0°C. The resultant emulsion was stable: it did not spread or separate when an aliquot was dropped onto the surface of water. The water-in-oil emulsions were injected within a few hours of preparation, in 0.25 ml volumes. The dose of antigen administered was thus the same as given to other groups of animals in the form of aqueous suspensions.

**Experimental animals.** Adult outbreed rabbits of mixed breeds (Australian wild, Belgian giant, and New Zealand white) and of both sexes were housed in individual cages with unrestricted access to a pelleted complete diet and water. Additional green fodder was fed 3 times a week.

Three rabbits made up each treatment group. Each of the animals was bled two days before vaccination, and at regular intervals thereafter. About 15 ml of whole blood was collected from the ear vein, allowed to clot at room temperature and then incubated for 1 hr at 35°C. The separated sera were spun free of red blood cells, distributed in small screw-cap
containers and stored at \(-15^\circ C\), without preservative.

**Titration of antibody.**

**Neutralization tests** were carried out according to Fazekas de St. Groth, Withell and Lafferty (1958). Non-inactivated sera were diluted in 3.16-fold steps in 1.08 ml volumes of SM, and cooled to 0°C before adding 0.05 ml of SM containing the appropriate dose of virus (between \(10^3\) and \(10^4\) ID\(_{50}\)). The actual concentration of virus varied from one strain to the other, but was always constant for any particular strain. The virus-serum mixtures were preincubated for 30 minutes at 0°C and then 0.05 ml of each mixture was added to 8 replicate cups containing a square of allantois-on-shell in 0.35 ml of SM. Controls on viral infectivity were carried out at the beginning and end of each experiment. The trays were incubated for 60-64 hours at 35°C, under constant shaking. The endpoint of neutralization was judged by agglutination of one drop of 5% fowl red cells at the end of the incubation period. Neutralizing potencies (pN) were calculated according to Fazekas de St. Groth (1961).

**Antihaemagglutinin tests** were performed in plastic trays, using 0.25 ml saline as dilution volume, 4 haemagglu-
tinating doses of the homologous virus as antigen and 0.025 ml 5% fowl red cells as indicator (Fazekas de St. Groth, Withell and Lafferty, 1958). The red cells used in the antihaemagglutinin tests were taken from a single donor; this fowl was selected from approximately 100 birds, as its red cells were found insensitive to nonspecific inhibition when tested with RDE-citrate-65°C treated normal rabbit serum. All sera were treated by the method of Fazekas de St. Groth (1949) prior to these tests to destroy nonspecific inhibitors of haemagglutination.

Complement fixation tests followed the overnight procedure of Fazekas de St. Groth, Graham and Jack (1958). Sera inactivated as for antihaemagglutinin tests, were diluted in 0.25 ml volumes of Ca-Mg-saline. Then a standard drop (0.025 ml) containing 8 units of antigen and 3 HD$_{50}$ of complement was added to each cup, and the trays incubated at 4°C for 16-20 hr. The following morning a standard drop containing 4% washed sheep erythrocytes sensitized with 4 HD$_{50}$ of haemolysin was added, and the trays placed for 2 hr on a shaking machine at 35°C. The pattern of settled cells was read to judge the degree of haemolysis, 50% lysis marking the endpoint.
**GENERAL EXPERIMENTAL DESIGN**

The first series of experiments sought to establish the rate and extent of antibody production after primary and anamnestic stimuli of various kinds. The antigenic dose chosen was such that, given intravenously, it would elicit maximal production of antibody, i.e., doubling or quadrupling the dose did not alter the immune response. The following factors were varied in this set of experiments:

**Antigens.** The three strains of influenza virus differ in their surface antigens, MEL and SW showing some crossed reactions by all serological methods, while LEE is antigenically distinct from both. The dose of virus administered under each schedule was the same in terms of haemagglutinin; and since the ratio of haemagglutinating units to electronmicroscopically visible particles is practically the same for these three strains, the absolute dose of antigen received will also be very close to identical.

**Routes of vaccination.** The immunizing dose, always
contained in 0.25 ml of standard medium, was inoculated either intravenously into the marginal vein of the ear, or intraperitoneally through the lateral side of the rectus abdominis, or subcutaneously over the lumbar region. Water-in-oil-emulsions of the antigens were injected intramuscularly, into the glutaeus muscles.

Schedules of inoculation. Under this heading, two separate sets of factors were compared: the effect of single or multiple injections, and the effect of administration of the antigens in an aqueous suspension or as an emulsion of water-in-oil. The four Groups referred to in the Tables and Figures are the orthogonal arrangement of these factors. Thus, Group I = single dose of antigen, without adjuvant; Group II = multiple doses of antigen, without adjuvant; Group III = single dose of antigen, in adjuvant; Group IV = multiple doses of antigen, of which the first was in adjuvant.

Timing. The experiments start with the administration of the first dose of antigen, and the primary response was followed over the subsequent 40 days. The secondary dose (or doses) of antigen were given on day 40 (or starting with day 40, in the case of multiple injections), and the six samples of serum obtained over the
40 to 60 day period constitute the **secondary response**. The third inoculation (or set of inoculations) started on day 60, and thus the sera collected after this time make up the **tertiary response**.

The general scheme of the experiment is set out in Table 1.

(Table 1)

**Evaluation.** All serum samples were stored until the conclusion of an experiment, and then tested by three serological methods. Such multiplication of labour was deemed necessary not only to increase accuracy but, mainly, to find out whether a combination of assay techniques would yield information by which the quality and quantity of antibodies could be assessed separately. Although each of the three tests employed depends on the interaction of the same antigen–antibody system, they differ in sensitivity and fall into two groups with fundamentally different mechanisms. Complement fixation tests are binary in character and work at relatively high concentrations of antigen and antibody, thus the outcome will rest essentially on the quantity of antibody present, whether the binding between the two reactants is firm.
<table>
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<th>Inocula</th>
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<th></th>
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<td>Intra-</td>
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<td>Secondary</td>
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<td>Secondary</td>
<td>Tertiary</td>
<td>Primary</td>
<td>Secondary</td>
<td>Tertiary</td>
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<tr>
<td>Group I (single-aqueous)</td>
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<td>40</td>
<td>60</td>
<td>0</td>
<td>40</td>
<td>60</td>
<td>0</td>
<td>40</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group II (multiple-aqueous)</td>
<td>0,2,4,7,9,11</td>
<td>40,42,44</td>
<td>60,62,64</td>
<td>0,2,4,7,9,11</td>
<td>40,42,44</td>
<td>60,62,64</td>
<td>0,2,4,7,9,11</td>
<td>40,42,44</td>
<td>60,62,64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group III (single-adjuvant)</td>
<td>0*</td>
<td>40</td>
<td>60</td>
<td>0*</td>
<td>40</td>
<td>60</td>
<td>0*</td>
<td>40</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group IV (multiple-adjuvant)</td>
<td>0,2,4,7,9,11</td>
<td>40,42,44</td>
<td>60,62,64</td>
<td>0,2,4,7,9,11</td>
<td>40,42,44</td>
<td>60,62,64</td>
<td>0,2,4,7,9,11</td>
<td>40,42,44</td>
<td>60,62,64</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The figures show the days on which the three rabbits of a group received 5,000 haemagglutinating doses of either MEL, LEE or SW influenza virus.

* Received antigen in Freund\'s incomplete adjuvant (Difco) by the intramuscular route.
or not (Fazekas de St. Groth, 1962). Both the antihaemagglutinin test and neutralization assays are competitive: the virus is distributed between antibody and an indicator component (the red cell in the first case and the infectible host cell in the second). Since antihaemagglutinin tests operate at concentrations of antigen close to the value of the equilibrium constant of the virus–antibody interaction (Fazekas de St. Groth and Webster, 1962), the quality of antibody will contribute less significantly to the results than in the case of neutralization where the concentration of the test-antigen is several orders of magnitude below the equilibrium constant of even the most firmly binding fraction of the antibody population. It should be expected therefore that the ratio of antibody titres obtained by these three techniques would change whenever the quality of antibody changes, while the absolute magnitude of the titres would be more closely related to the quantity of antibody in the test samples, although not linearly.

The first paper deals with the neutralizing power of to antisera produced in response to the various antigenic stimuli. The observed titres are transformed into 'neutralizing potencies'
or pN (Fazekas de St. Groth, 1961), both to render the results independent of the dose of virus used in their determination, and to allow absolute comparisons between strains, i.e., evaluation of antigenic potencies. After presenting a set of preliminary experiments, carried out to test some technical aspects of design and method, the results will be given in detail, followed by discussion of points relevant to neutralization. Comparison of the information obtained by use of the three different techniques of assay will follow later.

**Stability of reagents.**

Before the results obtained in a study extending over several years can be legitimately compared, it is necessary to demonstrate that the reagents remained unchanged over the period of observations.

Antisera are usually assumed to be stable when stored in the frozen state; to furnish formal evidence on this point, a set of sera, one against each of the three strains of influenza virus used in these studies, was tested for neutralizing potency over a span of two years (Table 2).
TABLE 2

The Stability of Antibody

The neutralizing potencies of three hyperimmune antisera to 3 strains of influenza virus collected from experiments carried out on single batches of antisera that had been frozen and thawed at irregular intervals over two years. The Table gives the mean neutralizing potencies and the standard errors for two periods of 3 months during the 2 year period.

<table>
<thead>
<tr>
<th>Antisera</th>
<th>Neutralizing Potency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1960</td>
</tr>
<tr>
<td>anti-LEE</td>
<td>4.51 ± 0.04</td>
</tr>
<tr>
<td>anti-SW</td>
<td>4.79 ± 0.04</td>
</tr>
<tr>
<td>anti-MEL</td>
<td>4.31 ± 0.06</td>
</tr>
</tbody>
</table>
Evidently, storage at -15°C, even with frequent thawing and re-freezing, does not alter the neutralizing potencies of antisera. Similar control tests, using antihaemagglutinin or complement fixation tests as methods of assay, gave the same answer.

The infectivity of influenza virus preparations stored at -70°C does not change over a period of two years as has been demonstrated elsewhere, using the same preparations of virus as in the present study (Fazekas de St. Groth and Webster, 1962). The haemagglutinin of influenza virus eluates stored at 4°C with 0.08% sodium azide as preservative has been tested over a similar period and, provided the virus is stored in detergent-free glassware, the haemagglutinin titres remain unchanged over at least 2 years.

The response to live and killed influenza vaccines.

It is generally assumed that influenza viruses do not multiply in the rabbit, i.e., that these viruses can be looked upon as inert antigens in this animal. However, as some of the experiments in this series are based upon the influence of antigenic dose on the immune response, and the results are interpreted in terms of influenza viruses not multiplying in the
rabbit, it is essential to establish at the outset whether or not this is so.

Four groups of two rabbits each were vaccinated by the intravenous route with 5,000 haemagglutinating doses of SW influenza virus. The first group received infective virus eluate, the second heat inactivated virus, the third ultraviolet-treated virus and the fourth formaldehyde and ultraviolet-treated virus. All rabbits were bled prior to vaccination and at intervals thereafter, the sera collected and tested for antihaemagglutinin after the conclusion of the experiment. The results are presented in Figure 1. The antibody responses are seen to be identical within statistical (Figure 1) variation in all four groups. This experiment clearly shows that it makes no difference whether infective or non-infective virus is used as antigen in the rabbit. Accordingly, the virus eluates were administered in all following experiments as immunizing antigens, without any further treatment.

The production of neutralizing antibody.

Each of the experiments to be shown in the Figures of this and the subsequent two papers was performed on 36 rabbits,
FIGURE 1. The antibody response of the rabbit to live and killed influenza vaccines.

Each point represents the geometric mean titre of a group of two rabbits.
3 animals to each of the 12 treatments. After completion of a course of injections and bleedings the sera were assayed in orthogonal groups, always in multiples of 12, neutralization tests were usually done in sets of 72 per day. The inherent error of a single titration is ± 0.118 (Fazekas de St. Groth, Withell and Lafferty, 1958). For determination of the maximum levels of antibody production three or four successive bleedings were used and the average of these was bound to carry standard deviations of ± 0.246 for MEL, ± 0.267 for LEE and ± 0.335 for SW virus and their respective antibodies. The rates of antibody production were estimated by fitting straight lines to 3 or 4 points on the rising sections of each response curve, with an accuracy of ±0.010 for MEL, ± 0.007 for LEE and ± 0.011 for SW, in log_{10} units per day, i.e., an uncertainty of ± 3.3%, ± 2.3% and ±3.7% in the estimated doubling times.

Both these estimations and the comparison of starting times, rates of production and maximum levels attained were carried out by programming a digital computer to perform the required analyses of variance and covariance, and to compute all the means and standard errors to appear in this and subsequent
papers. The analyses were nonorthogonal due to serial
correlation of the primary, secondary and tertiary antibody
responses (which were measured on the same animals), and
due to some missing entries (6 out of 108 animals died during
the course of the experiments by misadventure). The non-
orthogonality has been allowed for when computing variances
and covariances.

The immune response to the 12 test-treatments is
given separately for the three strains of virus used as antigens.
Each point represents the geometric mean titre of a group of 3
rabbits; the curves were fitted by eye. To enable comparisons
between the various treatments, a number of Tables have been
prepared, giving in summary form the results of the statistical
calculations.

(Figure 2)

(Figure 3)

(Figure 4)

(Table 3)

(Table 4)

(Table 5)
FIGURE 2. The antibody response of rabbits to a type A influenza virus (MEL), as measured by neutralizing potency.

Antibody titres are expressed in $\log_{10}$ units per 1.0 ml volumes of serum. Each point represents the geometric mean titre of a group of three rabbits.

Schedules of vaccination:

Group I: Aqueous vaccine on days 0, 40 and 60, by the route indicated on the figures.

Group II: Aqueous vaccine on days 0, 2, 4, 7, 9, 11 as primary stimulus, on days 40, 42, 44 as secondary and on days 60, 62, 64 as tertiary stimulus.

Group III: Vaccine in incomplete Freund's adjuvant intramuscularly on day 0, aqueous vaccine on days 40 and 60, by the routes indicated on the figures.

Group IV: Vaccine in incomplete Freund's adjuvant intramuscularly on day 0, followed by aqueous vaccine on days 2, 4, 7, 9, 11 as primary stimulus, by the routes indicated on the figures. The secondary and tertiary courses of vaccination were the same as for the corresponding sub-sets of Group II.

Each inoculum of vaccine contained 5,000 haemagglutinating units of virus (approximately $10^{11}$ particles, or 0.06 mg).
FIGURE 3. The antibody response of rabbits to a type B influenza virus (LEE), as measured by neutralizing potency
FIGURE 4. The antibody response of rabbits to swine influenza virus (SW), as measured by neutralizing potency.
### Lag period preceding the appearance of neutralizing antibodies

<table>
<thead>
<tr>
<th>Vaccinating strain of virus</th>
<th>Schedule of administration</th>
<th>Route of inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>intravenous</td>
</tr>
<tr>
<td></td>
<td></td>
<td>intraperitoneal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>subcutaneous</td>
</tr>
<tr>
<td>MEL</td>
<td>Group I single-aqueous</td>
<td>2.34</td>
</tr>
<tr>
<td></td>
<td>Group II multiple-aqueous</td>
<td>2.38</td>
</tr>
<tr>
<td></td>
<td>* Group III single-adjuvant</td>
<td>2.42</td>
</tr>
<tr>
<td></td>
<td>* Group IV multiple-adjuvant</td>
<td>1.28</td>
</tr>
<tr>
<td>LEE</td>
<td>Group I single-aqueous</td>
<td>2.76</td>
</tr>
<tr>
<td></td>
<td>Group II multiple-aqueous</td>
<td>2.60</td>
</tr>
<tr>
<td></td>
<td>* Group III single-adjuvant</td>
<td>4.60</td>
</tr>
<tr>
<td></td>
<td>* Group IV multiple-adjuvant</td>
<td>16.70</td>
</tr>
<tr>
<td>SW</td>
<td>Group I single-aqueous</td>
<td>2.31</td>
</tr>
<tr>
<td></td>
<td>Group II multiple-aqueous</td>
<td>2.44</td>
</tr>
<tr>
<td></td>
<td>* Group III single-adjuvant</td>
<td>17.30</td>
</tr>
<tr>
<td></td>
<td>* Group IV multiple-adjuvant</td>
<td>19.50</td>
</tr>
</tbody>
</table>

The figures show the average time in days elapsing between primary inoculation and appearance of antibody in groups of 3 rabbits. The schedules of vaccination are set out in Table 1.

* The primary dose of vaccine in adjuvant was administered by the intramuscular route.
### TABLE IV

The rate of neutralizing antibody production

<table>
<thead>
<tr>
<th>Vaccinating strain of virus</th>
<th>Schedule of Administration</th>
<th>Primary Response</th>
<th>Secondary Response</th>
<th>Tertiary Response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>intra-venous</td>
<td>intra-peritoneal</td>
<td>sub-cutaneous</td>
<td>intra-venous</td>
</tr>
<tr>
<td>MEL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I</td>
<td>0.66</td>
<td>0.12</td>
<td>0.07</td>
<td>0.39</td>
</tr>
<tr>
<td>Group II</td>
<td>0.73</td>
<td>0.25</td>
<td>0.19</td>
<td>0.27</td>
</tr>
<tr>
<td>Group III</td>
<td>0.17</td>
<td>0.14</td>
<td>0.18</td>
<td>0.18</td>
</tr>
<tr>
<td>Group IV</td>
<td>0.20</td>
<td>0.16</td>
<td>0.04</td>
<td>0.19</td>
</tr>
<tr>
<td>LEE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I</td>
<td>0.07</td>
<td>0.05</td>
<td>0.05</td>
<td>0.27</td>
</tr>
<tr>
<td>Group II</td>
<td>0.20</td>
<td>0.07</td>
<td>0.05</td>
<td>0.26</td>
</tr>
<tr>
<td>Group III</td>
<td>0.05</td>
<td>0.06</td>
<td>0.09</td>
<td>0.22</td>
</tr>
<tr>
<td>Group IV</td>
<td>0.02</td>
<td>0.03</td>
<td>0.09</td>
<td>0.26</td>
</tr>
<tr>
<td>SW</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I</td>
<td>0.76</td>
<td>0.18</td>
<td>0.15</td>
<td>0.02</td>
</tr>
<tr>
<td>Group II</td>
<td>0.70</td>
<td>0.26</td>
<td>0.22</td>
<td>0.08</td>
</tr>
<tr>
<td>Group III</td>
<td>0.08</td>
<td>0.07</td>
<td>0.07</td>
<td>0.36</td>
</tr>
<tr>
<td>Group IV</td>
<td>0.09</td>
<td>0.07</td>
<td>0.06</td>
<td>0.39</td>
</tr>
</tbody>
</table>

The figures show the log<sub>10</sub> slopes fitted to the steepest portions of the respective time-log response curves, based on the geometric mean neutralizing antibody titres from groups of 3 rabbits. The schedules of vaccination are set out in Table I.
<table>
<thead>
<tr>
<th>Vaccinating strain of virus</th>
<th>Schedule of Administration</th>
<th>Primary Response</th>
<th>Secondary Response</th>
<th>Tertiary Response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>intra-venous</td>
<td>intra-peritoneal</td>
<td>sub-cutaneous</td>
<td>intra-venous</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>intra-peritoneal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>sub-cutaneous</td>
</tr>
<tr>
<td>MEL</td>
<td>Group I</td>
<td>4.22</td>
<td>3.07</td>
<td>3.13</td>
</tr>
<tr>
<td></td>
<td>Group II</td>
<td>4.63</td>
<td>4.79</td>
<td>3.91</td>
</tr>
<tr>
<td></td>
<td>Group III</td>
<td>4.30</td>
<td>4.90</td>
<td>4.38</td>
</tr>
<tr>
<td></td>
<td>Group IV</td>
<td>4.63</td>
<td>4.25</td>
<td>4.05</td>
</tr>
<tr>
<td>LEE</td>
<td>Group I</td>
<td>3.59</td>
<td>3.27</td>
<td>3.07</td>
</tr>
<tr>
<td></td>
<td>Group II</td>
<td>4.42</td>
<td>3.63</td>
<td>3.15</td>
</tr>
<tr>
<td></td>
<td>Group III</td>
<td>3.75</td>
<td>3.34</td>
<td>3.74</td>
</tr>
<tr>
<td></td>
<td>Group IV</td>
<td>3.52</td>
<td>3.30</td>
<td>3.63</td>
</tr>
<tr>
<td>SW</td>
<td>Group I</td>
<td>4.78</td>
<td>3.60</td>
<td>3.88</td>
</tr>
<tr>
<td></td>
<td>Group II</td>
<td>5.06</td>
<td>4.51</td>
<td>4.07</td>
</tr>
<tr>
<td></td>
<td>Group III</td>
<td>3.20</td>
<td>3.49</td>
<td>2.87</td>
</tr>
<tr>
<td></td>
<td>Group IV</td>
<td>2.79</td>
<td>3.31</td>
<td>2.95</td>
</tr>
</tbody>
</table>

The figures show the mean neutralizing potency (pN) of groups of 3 rabbits, averaged for at least three determinations over 10 days during the peak of antibody production. The schedules of vaccination are set out in Table I.
DISCUSSION

All analyses of variance and covariance showed significant differences between the immune response of rabbits within each treatment group. Since genetic variation of this kind could not have been studied effectively on groups of the size used in the experiments and on animals bred without any selection, this factor was eliminated from all comparisons by pooling the error sum of squares with all interaction sums of squares containing differences between rabbits as factors. As a result, the error variances were increased by a factor of 2.33 in the evaluation of the levels of antibody production, by 1.24 in the comparison of starting times, and by 1.42 in comparing the rates of increase in neutralizing antibody. These factors show also that the rabbits differed considerably in the amount of antibody produced, less so in the rate of production, and hardly at all in the lag period preceding the appearance of newly formed antibody. It should be added that all of the significant variance ratios due to the main contrasts remained significant at the 1% level even after increasing the error variance.
The Primary Response

All titrations were started at serum dilutions of 1:3.16. At this level all normal sera gave negative results with MEL and SW antigens, but were found to neutralize LEE virus; in fact, the pre-vaccination bleeds against this strain varied in titre between 1:5 to 1:20. Thus, the primary response against LEE could only be followed over a somewhat narrower range than against the other two strains. This background will not influence estimates of the maximum antibody levels attained, but may mask the true starting time and apparently flatten the rate of initial production.

Intravenous administration.

Single injections. With the large dose of antigen used in this study, the first rise of antibody titre (i.e., initial appearance of antibody against MEL and SW, and passing of the nonspecific baseline for LEE) was detected between days 2 and 3 after vaccination. This holds both after aqueous and water-in-oil vaccines for MEL, but LEE and especially SW adjuvant-vaccines elicit an antibody response that is significantly delayed in comparison to the response to watery suspensions of antigen.
The rate of antibody production is consistently higher following simple vaccines than after adjuvant vaccines. In the case of MEL and SW, where the estimates are not biased by a non-specific baseline, the initial rates are $0.66$ and $0.76\log_{10}$ units per day or doubling times of $0.45$ and $0.40$ days respectively. The corresponding figures after adjuvant-vaccines are $0.18$ and $0.08\log_{10}$ units per day, or doubling times of $1.78$ and $3.66$ days, respectively.

The maximum levels attained after simple and adjuvant-vaccines are identical within statistical variation, in the case of both MEL and LEE, in spite of the different starting times and rates of production. However, the response to an aqueous suspension of SW virus is significantly higher than to the same dose given in adjuvant.

**Multiple injections.** The responses are measurable from the second day onwards after multiple injections of the simple vaccines, the exceptions being the same as after single injections, viz., LEE and, especially, SW in adjuvant. The delay here is even more surprising, since the intramuscular dose of adjuvant-vaccine was followed by five identical doses
of antigen given intravenously, and thus the adjuvant must have exerted a depressive effect on the production of antibody or may have preempted the system to a particular type of response.

The rates of production are, if anything, somewhat higher than after a single dose, giving doubling times of 0.41 days with MEL, 1.47 days with LEE, and 0.43 days with SW in response to the simple vaccines. Adjuvant vaccines, once again, elicit significantly lower rates of response: 1.50, 6.00 and 3.00 days doubling time respectively for the three strains.

The maxima after aqueous vaccines are uniformly higher than after a single dose, the difference being 2.6-fold for MEL, 6.8-fold for LEE and 2.2-fold for SW. After adjuvant vaccines the levels are, on the average, the same as those found after single injections. Compared to aqueous vaccines, there are significant differences in the behaviour of the three strains: MEL stimulates antibody production to the same extent with or without adjuvant; LEE is eight times less immunogenic in adjuvant, and SW about a hundred times less so.
Intraperitoneal administration.

On the whole, a dose of vaccine given intraperitoneally produces about half as much antibody as the same dose given intravenously (56% with MEL, 42% with LEE and 41% with SW). These differences are even more striking when the different schedules of administration are compared.

**Single injections.** Whereas the response to MEL becomes detectable between days 2 and 3, LEE and SW produce measurable titres only between days 3 and 6 when administered as watery suspensions. The effect of the adjuvant shows up once more in the delayed appearance of antiSW antibodies (about 10 days).

The rates of production are all lower than after intravenous vaccination, being of the order of 2.5 days doubling time after simple vaccines. This is the rate of increase for MEL in adjuvant, too, but the other two strains stimulate the production of antibody at half this rate only.

The maximum levels reached are much the same after adjuvant and non-adjuvant vaccines, in contrast to the findings after intravenous administration of the same doses.
Multiple injections. The starting time is not
distinguishably different after multiple or single injections,
and shows the same characteristic strain differences.

The rates of production are definitely higher in the
two strains where unbiased estimates can be made (doubling
times of 1.22 and 1.15 days for MEL and SW, respectively),
after simple vaccines. These differences disappear when
the response after adjuvant-vaccines is compared: the rates
are the same whether single or multiple doses of antigen were
injected.

In the case of MEL the maximum levels reached approach
those observed after intravenous inoculations. LEE remains
slightly below the maximum after intravenous vaccination, and
the response to adjuvant-vaccine is about half as much only.
SW behaves like LEE when given as aqueous suspension, but
reaches only one-tenth of that level after adjuvant-vaccination.

Subcutaneous administration.

Compared to the average response taken over all treat-
ments by the intravenous route, the response to subcutaneous
vaccines is about five times lower (19% for MEL, 24% for LEE
and 16% for SW).

**Single injections.** After the simple vaccines antibody is measurable within 2-4 days, in all groups. This also holds for MEL given in adjuvant. The other two strains induce a more delayed response, antibody appearing on the average on the 10th and 14th day, respectively.

The rate of production is in every respect comparable to the rates observed after intraperitoneal injection of the same vaccines.

On the whole, the maximum levels reached are the same or only slightly below those after intraperitoneal vaccination. Thus the overall difference between the two routes must rest on the poorer response to multiple injections.

**Multiple injections.** The onset of antibody production is the same as in the groups receiving one dose of vaccine only, and the delaying effect of adjuvant is clearly noticeable in the case of LEE and SW.

The initial rates of antibody production are significantly higher after the two virus strains unaffected by non-specific inhibitors (2.9-fold increase for MEL, 1.6-fold for SW), provided
they are administered as aqueous suspensions. The same dose given as water-in-oil emulsion results in rates of production lower by a factor of 4 and 5 for MEL and SW, respectively.

The differential behaviour of the three test strains in respect to the efficiency of the adjuvant is once again evident: SW administered as a water-in-oil emulsion leads to the production of about ten times less antibody than the same dose of virus in water. The responses to LEE and MEL are better, but none of them attains the levels reached after intravenous inoculations.

The Anamnestic Response

The dose of virus used in eliciting a secondary and tertiary response was the same as given primarily - a relatively large quantity of virus. This fact, as well as the short interval of only 20 days between secondary and tertiary boosting accounts for the lack of significant changes in antibody titre between days 60 and 75. For this reason, the two sets of observations will be discussed together.

In several treatment groups the anamnestic response
starts with a negative phase: the bleedings taken 2 days after the boosting inoculum contain less antibody than the bleed taken immediately before boosting. This drop was not observed regularly, and varied even within any single group, i.e., among rabbits receiving identical treatment. The mechanism underlying this finding is unknown, but the dose of virus administered can be excluded as causative factor: the maximum amount of virus administered would have been insufficient to lower serum titres by more than a few per cent, and thus would have escaped detection.

When fitting straight lines to the initial slopes of the log antibody response to anamnestic challenge, all lines were found to extrapolate to points in time preceding the injection of the booster antigen. This finding has two immediate consequences: first, that starting times cannot be defined in the same way as has been done for the primary response; and, second, that the assumption of an exponential anamnestic increase in antibody is untenable. It follows that all the slopes considered in this section may be underestimates, either because the rapid initial rise of antibody was masked by antibody formed in response to primary stimulation and still present in the circulation, or because
at the stage where measurements became possible, the rate of elimination was not negligible as compared to the rate of production. A third possibility, namely, changes in the quality of antibody, will be considered both when comparing the results obtained by the use of the three methods of assay, and in later experimental work on the thermodynamic properties of primary and anamnestic antisera.

**Intravenous administration.**

**Single injections.** Judged by the first detectable increase, the anamnestic response becomes measurable from the second day after boostings. Irrespective of the previous history of the animals, the measured rates are characterized by doubling times of the order of a day. The tertiary response is hardly noticeable in these groups: the estimated slopes differ insignificantly from zero in all cases, and in several groups there is a genuine drop in antibody titres.

The maximum titres attained are comparable for the group that received their primary dose of antigen in water or in adjuvant, and are consistently higher than the primary maxima. The difference is, on the average, about 8-fold, with higher than average rise in the adjuvant-SW group whose poor primary
response has already been pointed out above. The tertiary maxima are very close to the secondary peaks, and with the possible exception of MEL (where an actual drop is observed) can be taken as identical.

**Multiple injections.** As far as the onset and rate of anamnestic production is concerned the groups receiving multiple doses of antigen do not differ from those receiving single doses. The animals which received SW in adjuvant and produced little antibody after primary stimulation are seen to catch up with the other groups by producing antibody at a particularly fast rate. By the time the tertiary stimulus was applied there was no difference between any of the treatments.

The maximum levels of antibody reached are 8-fold above the corresponding primary levels, due mainly to the great increase of the adjuvated groups. There is an approximately two-fold difference between single and multiple injections which, however, does not show up consistently for all pairs of treatments. In general, it may be said that by the end of the secondary, and especially of the tertiary, response the marked differences which characterized the immune state after primary stimulation have been levelled out.
Intraperitoneal administration.

Single injections. The rate of antibody production is strikingly higher than after primary stimulation, in all groups. Those having relatively low primary levels of antibody (LEE and SW) exceed even the rates of production after intravenous boosting. As a result such levels of neutralizing antibody are attained that the tertiary response proceeds at negligible or even negative rates.

The maxima are in every way comparable to those reached after intravenous boosting, and hence about 30-fold higher than the titres reached by the same animals during the primary response. The increase is most striking in the adjuvated groups of poor primary response (LEE and SW). The tertiary response is essentially restricted to maintaining the secondary levels of antibody, with the possible exception of the non-adjuvated SW group which shows a 2.5-fold rise and thus reaches the same level as the corresponding adjuvated group.

Multiple injections. The anamnestic response is underway in all groups by the second day after boosting and proceeds at a rate which is not different within these groups, indistinguishable from the rate of increase after single doses of vaccine, and
about twice that of the corresponding primary rates of production. All tertiary responses are of low or practically zero rates.

The maxima of these treatment groups compare favourably with any reached after secondary stimulation, and there is little further rise during tertiary production. The only exception is the adjuvated LEE-group, although the contribution of inter-rabbit variation cannot be excluded in this case.

**Subcutaneous administration.**

**Single injections.** Although the starting levels of antibody are the lowest in these groups, the rates of anamnestic production remain well below those observed after the other two routes of administration. This is particularly striking in the animals that had primarily received LEE or SW in adjuvant, and followed a poor initial response by a poor secondary one. Indeed, even tertiary stimulation does not lead to any marked rise of neutralizing antibodies.

The maximum levels are the lowest after these treatments (9% and 28% of the peak attained in the corresponding groups after intravenous or intraperitoneal vaccination with MEL, 21% and 19% with LEE, 4% and 27% with SW), with no significant improvement
on tertiary stimulation. The lack of anamnestic response is most evident in the adjuvated groups which contribute largely to the low overall averages.

Multiple injections. The effect of repeated booster injections by the subcutaneous route gives only slightly better rates of antibody production than single boosters. However, the resting levels of these treatment groups were somewhat higher on the starting day, and this might have masked any initial burst. Tertiary stimulation seems to result in detectable rates of increase only in the SW-adjuvant group; this rise, however, is based on the exceptional response of one rabbit whose final serum titres exceeded the average of the other two in the group by a factor of ten.

The level of neutralizing antibody in these groups is consistently and significantly higher than in those receiving a single booster injection only, although not quite as high as after the corresponding intravenous and intraperitoneal schedules. Tertiary rises are negligible, except in the SW-adjuvant group, where they rest in the exceptional behaviour of one out of three animals.
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THE IMMUNE RESPONSE TO INFLUENZA VIRUS. II. EFFECT OF ROUTE AND TIME SCHEDULE OF VACCINATION ON HAEMAGGLUTININ-INHIBITING ANTIBODIES.
SUMMARY

The influence of the route and schedule of vaccination on the response of rabbits to three strains of influenza virus (MEL, LEE and SW) is investigated. This paper contains the result of antihaemagglutinin tests covering the primary and anamnestic responses.

Circulating haemagglutinin was detected 2–3 days after primary vaccination with aqueous suspensions of all strains. After adjuvant vaccination with LEE and SW antigens there is little or no antibody produced in the primary response. The rise in antihaemagglutinin levels is somewhat slower than exponential both in the primary and anamnestic responses.

The quantity of antibody produced depends both on the route and schedule of administration, as well as on the antigen. The intravenous route is consistently better than the intraperitoneal, and both are superior to the subcutaneous route.

The primary responses are better following multiple injections in aqueous suspension, irrespective of route. These differences level out after anamnestic stimulation. Simple vaccination gives higher rates of production and higher maxima than adjuvant vaccination over the primary response, but once again
these differences disappear after secondary stimulation.

In order of descending immunogenic power, as detected by haemagglutination-inhibition, the strains are MEL, SW, and LEE. With the large dose of antigen used in these experiments, maximal production of antibody was elicited in the secondary response upon which tertiary stimulation could not improve.

**INTRODUCTION**

The same set of 2,000 sera on which the neutralization tests of the first paper of this series were performed served as experimental material for the present study. The antihaemagglutinin assay, used here to estimate antibody levels, differs in several fundamental points from the standard assay for neutralizing antibodies (Fazekas de St.Groth, 1962). First the antihaemagglutinin test is carried out in a closed system: the total number of reacting entities (virus particles, antibody molecules and indicator cells) remains unchanged throughout. In contrast, the number of infective units in a neutralization test diminishes with time, partly because a certain fraction enters infective cells and is thus withdrawn from the system, and partly because another fraction loses its infectivity by
thermal inactivation and thus cannot indicate whether it has been neutralized or not. Secondly, the prevention of haemagglutination can proceed by a single mechanism only: the potential contacting surface of the virus particle must be shaded by antibody, and a predictable number of antibody molecules attached to a fixed area of the virus will bring this about. Neutralization of infectivity is conceivable by several means, such as prevention of adsorption, hindrance of viroplasia, interference with intracellular multiplication and blocking or slowing down of cell-to-cell spread. Each of these mechanisms would require different numbers and arrangement of antibody molecules, and would also depend on the nature and physiological state of the host cells employed. Thirdly, haemagglutinin tests work only over a limited range of antigenic concentrations and even the lowest of these is of the order of the equilibrium constant characterizing the interaction of influenza viruses and antibody (Fazekas de St. Groth, 1961; Fazekas de St. Groth and Webster, 1962). As a consequence, a much larger fraction of the heterogeneous antibody population within any serum will combine with its antigen and thus score in antihaemagglutinin tests, while only the most firmly binding minority is capable of blocking infectivity.
To the basic differences can be added others, more of a technical nature. The error of neutralization assays is relatively large, due in part to the random distribution of the small numbers of infective doses (in the case of quantal tests, of a single one) used in scoring, and in part to the restriction of size on considerations of cost and labour. Antihaemagglutinin tests are inherently more accurate, being based on the behaviour of large numbers, and perhaps the cheapest of all serological procedures. On the other hand, properly designed neutralization tests are practically free from non-specific interference by components of serum, whereas there are at least three kinds of non-specific antihaemagglutinin inhibitors known to be present in all normal sera. There are methods of minimizing interference from these substances, and the three most common precautions were taken throughout these tests. Thus, all sera were pretreated to reduce their non-specific inhibitory titre; inhibitor-insensitive fowl cells (Anderson, 1946) were used; and the reaction was set up in small volumes, without pre-incubation of the virus-serum mixtures. Even so, the variable firmness of binding to cells ("receptor gradient": Burnet, McCrea, and Stone, 1946) and inhibitors ("inhibitor gradient": Stone, 1947) introduces additional complications which vary from strain to strain. In all,
antihaemagglutinin tests are more accurate than neutralization tests, reflect the quantity of antibody rather than its quality, and are likely to be less informative early in the immune response where their appearance might still be marked by residual non-specific inhibitors.

All materials and methods used in this study have been described in the first paper of the series. For a synopsis of the experimental design, Table 1 of that paper should be consulted.

**EXPERIMENTAL**

All of the antisera taken from the rabbits immunized with one of the strains of virus were assayed on the same day, thus eliminating possible variation due to reagents and operators. Antibody titres are expressed in terms of 1.0 ml volumes permitting direct comparison with the results obtained in neutralization and complement fixation tests (Papers I and III). The inherent error of a single titration is \( \pm 0.063 \log_{10} \) units (Fazekas de St.Groth and Webster, 1961). The statistical comparison of maximal antibody titres, rates of production and starting times of the immune response were carried out as in the first paper. The titrations of
maximum levels had a standard deviation of ±0.080 for MEL, ±0.088 for LEE and ±0.116 for SW virus and their respective antibodies. The accuracy of the rates of antibody production was ±0.002 for MEL, ±0.005 for LEE and ±0.007 for SW, in log_{10} units per day.

The antibody titres of the 12 treatment-groups are presented in Figures 1, 2, 3. Each point is the geometric mean titre of the 3 rabbits in each group, and the curves were fitted by eye. To facilitate comparisons between the groups, Tables 1 and 2 are presented giving in summary form, the main statistical results.

Figure 1
Figure 2
Figure 3
Table 1
Table 2

**DISCUSSION**

The analyses of variance showed significant differences between the immune response of rabbits in each treatment-group. Genetic variation was once again eliminated from all comparisons.
FIGURE 1. The antibody response of rabbits to a type A influenza virus (MEL), as measured by antihaemagglutinin.

Antibody titres are expressed in $\log_{10}$ units per 1.0 ml volumes of serum. Each point represents the geometric mean titre of a group of three rabbits.

Schedules of vaccination:

Group I: Aqueous vaccine on days 0, 40 and 60, by the route indicated on the figures.

Group II: Aqueous vaccine on days 0, 2, 4, 7, 9, 11 as primary stimulus, on days 40, 42, 44 as secondary and on days 60, 62, 64 as tertiary stimulus.

Group III: Vaccine in incomplete Freund's adjuvant intramuscularly on day 0, aqueous vaccine on days 40 and 60, by the routes indicated on the figures.

Group IV: Vaccine in incomplete Freund's adjuvant intramuscularly on day 0, followed by aqueous vaccine on days 2, 4, 7, 9, 11 as primary stimulus, by the routes indicated on the figures. The secondary and tertiary courses of vaccination were the same as for the corresponding sub-sets of Group II.

Each inoculum of vaccine contained 5,000 haem-agglutinating units of virus (approximately $10^{11}$ particles, or 0.06 mg).
FIGURE 2. The antibody response of rabbits to a type B influenza virus (LEE), as measured by antihaemagglutinin.
Days After Vaccination

Groups:
- Group I: Intramuscular
- Group II: Intranasal
- Group III: Subcutaneous
- Group IV: Subcutaneous
FIGURE 3. The antibody response of rabbits to swine influenza virus (SW), as measured by antihaemagglutinin.
<table>
<thead>
<tr>
<th>Vaccinating strain of virus</th>
<th>Schedule of Administration</th>
<th>Primary Response</th>
<th>Secondary Response</th>
<th>Tertiary Response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>intra-venous</td>
<td>intra-venous</td>
<td>intra-venous</td>
</tr>
<tr>
<td></td>
<td></td>
<td>intra-peritoneal</td>
<td>intra-peritoneal</td>
<td>intra-peritoneal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sub-cutaneous</td>
<td>sub-cutaneous</td>
<td>sub-cutaneous</td>
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<td></td>
<td></td>
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<tr>
<td>MEL</td>
<td>Group I</td>
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<td>0.06</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Group II</td>
<td>0.22</td>
<td>0.19</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>Group III</td>
<td>0.12</td>
<td>0.12</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>Group IV</td>
<td>0.11</td>
<td>0.13</td>
<td>0.11</td>
</tr>
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<td>LEE</td>
<td>Group I</td>
<td>0.03</td>
<td>0.02</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Group II</td>
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<td>0.02</td>
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<td></td>
<td>Group III</td>
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<td>0.02</td>
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<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
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<td>Group I</td>
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<td>0.01</td>
<td>0.02</td>
</tr>
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<td></td>
<td>Group II</td>
<td>0.20</td>
<td>0.11</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>Group III</td>
<td>0.01</td>
<td>0.02</td>
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<tr>
<td></td>
<td>Group IV</td>
<td>0.00</td>
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<td>0.00</td>
</tr>
</tbody>
</table>

The figures show the \( \log_{10} \) slopes fitted to the steepest portions of the respective time-log response curves, based on the geometric mean antihaemagglutinin titres from groups of 3 rabbits.
### TABLE II

**Maximum levels of antihaemagglutinin**

<table>
<thead>
<tr>
<th>Vaccinating strain of virus</th>
<th>Schedule of Administration</th>
<th>Primary Response</th>
<th>Secondary Response</th>
<th>Tertiary Response</th>
</tr>
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<tr>
<td></td>
<td>intra-venous</td>
<td>intra-peritoneal</td>
<td>sub-cutaneous</td>
<td>intra-venous</td>
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<tr>
<td>MEL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I</td>
<td>3.02</td>
<td>2.10</td>
<td>1.91</td>
<td>3.93</td>
</tr>
<tr>
<td>Group II</td>
<td>3.50</td>
<td>3.45</td>
<td>2.58</td>
<td>3.74</td>
</tr>
<tr>
<td>Group III</td>
<td>3.32</td>
<td>3.32</td>
<td>2.86</td>
<td>3.78</td>
</tr>
<tr>
<td>Group IV</td>
<td>3.30</td>
<td>3.35</td>
<td>3.02</td>
<td>4.01</td>
</tr>
<tr>
<td>LEE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I</td>
<td>1.93</td>
<td>1.83</td>
<td>-</td>
<td>2.65</td>
</tr>
<tr>
<td>Group II</td>
<td>2.74</td>
<td>2.04</td>
<td>-</td>
<td>3.06</td>
</tr>
<tr>
<td>Group III</td>
<td>-</td>
<td>-</td>
<td>1.75</td>
<td>2.58</td>
</tr>
<tr>
<td>Group IV</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.08</td>
</tr>
<tr>
<td>SW</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I</td>
<td>3.04</td>
<td>2.18</td>
<td>1.91</td>
<td>3.82</td>
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<tr>
<td>Group II</td>
<td>3.34</td>
<td>2.92</td>
<td>2.73</td>
<td>3.60</td>
</tr>
<tr>
<td>Group III</td>
<td>1.90</td>
<td>1.82</td>
<td>1.75</td>
<td>2.85</td>
</tr>
<tr>
<td>Group IV</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.13</td>
</tr>
</tbody>
</table>

The figures show the mean antihaemagglutinin titres of groups of 3 rabbits, expressed in terms of 1.0 ml volumes. The average figure was determined from at least 3 points during maximum antibody production over a period of 10 days.

\[ - = \langle 1.60 \]
by pooling the error sums of squares with all interaction sums of squares containing differences between rabbits as factors. These contributions are considerably larger when evaluated by anti-haemagglutinin than by neutralization tests, pointing to a marked inter-rabbit variation in the quantity of antibody, although a compound of quantity and quality varies less. This difference is shown in a more striking way by the contribution of inter-rabbit variation to the error variance, which is comparable for the rates of antibody production when assessed by the two techniques. The increments of the error variance are 13.59- and 1.40-fold for levels and rates respectively. All the significant variance ratios due to the main contrasts remained significant at the 1% level, even after increasing the error variance.

The Primary Response

All titrations were started at a serum dilution of 1:20. At this dilution there was no non-specific inhibition found in the pre-vaccination sera for any of the strains of antigen. For accurate studies on the starting time and rates of antibody formation it would be desirable to start the antihaemagglutinin titrations at even lower serum dilutions. However, the masking of small quantities of antibody by non-specific inhibitors on the one hand, and the
desirability of conserving serum for further serological and thermodynamic studies rules out this possibility. As a consequence, the true starting times of antibody formation may be masked and the initial observed rates may be underestimated, since in some cases the rate of production may have become comparable to the rate of elimination by the time the baseline was passed.

**Intravenous Administration.**

**Single injections.** With the large dose of antigen used in this series of experiments antibody against MEL and SW was first detected around the second day after vaccination when antigen was administered in aqueous suspension; against LEE it was about the fifth day. There may be a marked delay in starting time when the same dose of antigen is administered in adjuvant. With MEL such a delay is not observed and the response starts as after stimulation with antigen in aqueous suspension, but SW has a 15 day's lag phase and LEE does not stimulate measurable anti-haemagglutinin production during the primary response.

The rates of antibody production are higher when antigen is administered in aqueous suspension than in adjuvant. The rates in aqueous suspension are $0.17, 0.18$ and $0.03 \log_{10}$ units per day for MEL, SW and LEE, or a doubling time of $1.76, 1.66,$
or 10 days respectively. The corresponding figures after adjuvant-vaccine are $0.12, 0.02$ and $0.00 \log_{10}$ units per day, or doubling times of 2.5 and 15 days for MEL and SW, and LEE does not respond at all.

The maximal antibody levels attained after simple vaccination are identical for MEL and SW, while LEE is ten times lower. After adjuvant-vaccine the levels reached by MEL are similar to those reached after simple vaccination, but LEE and SW stimulate the production of little or no antibody during the primary response.

**Multiple injections.** Antibody was first detected 2-5 days after primary stimulation as after single injections in aqueous suspension; but once again adjuvant vaccination failed to initiate antibody formation for either LEE or SW. The lack of detectable antihaemagglutinin responses in these groups is quite surprising for the adjuvant dose was followed by 5 intravenous doses of antigen. The absence of detectable antibody suggests a suppression of the antibody forming mechanism and perhaps the initiation of some other type of response.

The rate of antibody production is significantly higher than with single doses of antigen, giving doubling times of 1.38
1.50 and 3.76 days respectively for MEL, SW and LEE antigens in aqueous suspension. Adjuvant vaccination gives a doubling time of 2.83 days for MEL, and is similar to the rate of antibody formation after a single dose of antigen in adjuvant.

The maximal antibody produced with simple vaccines was in all cases higher than with single doses of antigen, the difference being seven-fold for MEL, nine-fold for LEE and two-fold for SW. Adjuvant vaccination with MEL induces levels of antibody comparable to those obtained with a single adjuvated dose, while LEE and SW initiate no detectable antibody.

**Intraperitoneal Administration.**

On the whole an intraperitoneal inoculation of antigen produces about half as much antibody as the intravenous route (60% with MEL, 59% with LEE and 47% with SW). These differences are more striking when simple and adjuvant vaccines are compared.

**Single injections.** The delay in the appearance of antibody is 2–3 days for MEL and 5–6 days for LEE or SW following simple vaccination. In all cases the use of adjuvants increases this delay being 5–6 days for MEL, 20–22 days for SW, while no detectable antibody is formed with LEE antigen.

The rates of production are all significantly lower than
after intravenous vaccination, giving doubling times of 5, 15, and 43 days for MEL, LEE and SW respectively, after simple vaccination. Adjuvant vaccination doubles this rate for MEL and SW, LEE giving no antibody response at all.

Adjuvant-vaccination induces ten times higher levels of antibody than does simple vaccination when MEL is used as antigen, while the reverse is true with LEE and SW. These antigens induce little or no antibody when given in adjuvant, but give detectable levels in aqueous suspension.

Multiple injections. In the case of MEL, the starting times of antibody formation are similar after aqueous or water-in-oil suspensions, while the other antigens are characterized by having long inductive phases, especially after adjuvant vaccination.

The rates of antibody formation are significantly higher for MEL and SW (1.61 and 2.69 days doubling time), while the rate with LEE is comparable to single injections in aqueous suspension. Adjuvant vaccines give rates that are in every way comparable to those with single doses of antigen in adjuvant.

The maximum antibody levels are significantly higher than with single doses, and in the case of MEL the level is comparable with intravenous inoculation for either simple or adjuvant vaccination. SW virus gives one half and LEE virus only one eighth of
the maximal levels induced by these viruses administered by the intravenous route. Detectable antibody is not formed when LEE and SW antigens are inoculated in adjuvant.

Subcutaneous Administration.

Compared with the overall mean of the intravenous schedules, this route of vaccination stimulates about one third as much antihaemagglutinin only, (20% for MEL, 47% for LEE and 33% for SW). With the exception of MEL, the antibody response is poor.

Single injections. The starting time for antibody to MEL antigen is between 5 and 6 days, but for LEE and SW it varies from 5 to 40 days and then is only just detectable above the baseline of non-specific inhibition.

The rate of antibody formation is low, with an average doubling time of 20 days for all strains of antigen; ajuvated MEL, however, gives a doubling time of 3 days, while simple inoculation with LEE gives no detectable antibody.

The maximal levels of antibody formed are low or not detectable, perhaps slightly and irregularly improved after adjuvant vaccines.

Multiple injections. The onset of antibody formation is
similar to the groups receiving single injections of vaccine; the delayed response to LEE by both schedules, and to SW in adjuvant is again apparent.

The rate of antibody formation is much higher, giving a doubling time of about 2–8 days for MEL stimulated by either schedule and for SW following simple vaccination. Ajuvated SW and LEE by either schedule elicit no antibody formation in the primary response.

The antibody levels are much higher in those groups that do respond and are comparable to the levels obtained by the intraperitoneal route. Simple vaccination with MEL being the exception where the maximal levels are eight times lower.

**The Anamnestic Response**

For the reasons already mentioned in the first paper, viz., the relatively large dose of antigen and the short interval of time between the secondary and tertiary stimulus, the anamnestic responses will be considered together.

The number of treatment-groups showing a negative phase on secondary stimulation is minimal, and shows no correlation with treatments. In fact, the drop in titre in most groups falls within the expected statistical variation.
The difficulties already mentioned in fitting straight lines to the initially detectable rising sections of the antibody responses are more acute in the secondary and tertiary responses. All lines extrapolate to points in time preceding the booster injection so that the starting times cannot be determined. It also follows that the slopes will probably be underestimated, and the assumption of an exponential anamnestic response rendered untenable.

**Intravenous Administration.**

**Single injections.** From Figures 1–3 it can be seen that the anamnestic response begins between the second and third day after stimulation.

The average rates of antibody production are significantly higher than in the primary response, chiefly because all treatment-groups are now showing the production of antibody. The doubling times for groups receiving simple vaccines were 1.0, 1.4 and 2.1 days for MEL, LEE and SW, respectively, while those for the corresponding groups receiving adjuvant vaccines were 2.5, 1.2, and 1.2 days. The rates in those groups not masked by high levels of primary antibody show shorter doubling times. In the tertiary response the rates of antibody production are low, and in many instances do not differ from zero.
The maximum levels of antibody attained are the same by either schedule; SW in adjuvant being the only exception, and is 10 times lower than the group receiving simple vaccine. The maximum levels are comparable for MEL and SW, while LEE is 10 times lower. With simple vaccines the maximum levels are 8 times above those in the primary, while those groups showing no antibody in the primary response show, of course, much larger increases. The tertiary maxima show no increase over the secondary, and in most cases, the levels are either identical or lower.

**Multiple injections.** The starting times and rates of antibody production are not significantly different from those receiving single doses. The groups that have received adjuvant vaccines and responded with no detectable antibody primarily, once again show higher rates. The rates after tertiary stimulation are low and serve only to return the levels of antibody to those observed in the secondary response.

The maximum levels of antibody attained are on the average not different from those groups receiving single doses. The lower titres obtained with SW in adjuvant are once more evident, and supports the view that antibody response to this antigen is suppressed by the initial sensitizing stimulus. The tertiary maxima
are uniformly below the levels obtained in the secondary, do not differ according to schedules, and appear to have reached the upper limits of production.

**Intraperitoneal Administration.**

**Single injections.** The delay in appearance of antibody after secondary stimulation is uniformly 2 days, but the rates vary with strains and schedules and depend on the levels of primary antibody. Those treatment-groups with low levels of primary antibody have high rates and vice versa. Simple vaccination gave doubling times of 1.1, 2.1, and 1.2 days, while adjuvant vaccination gave doubling times of 3.6, 2.1, and 1.1 days respectively for MEL, LEE and SW. The rates in the tertiary response are quite variable but are lower than the secondary rates, and serve only to return the levels of antibody to those of the secondary maxima.

The maximum antibody titres are on the average 10 times higher than the corresponding primary levels, but are below the anamnestic levels reached after intravenous administration. Adjuvant vaccination gives higher levels than simple vaccination for MEL and SW antigens, while the case of LEE is equivocal. The treatment-groups showing the greatest increase in antibody levels are those with low primary levels. Tertiary stimulation in aqueous suspension caused a two-fold increase over the secondary antibody
level in the case of SW; other treatment-groups show no increase at all.

**Multiple injections.** The delay after secondary stimulation, before new levels of antibody are detected, is uniformly 2-3 days and there is no difference between simple and water-in-oil vaccines. The rates of antibody production on tertiary stimulation are negligible for MEL and SW antigens, while LEE antigen causes a doubling of antibody levels in 3 and 1.9 days respectively after simple and adjuvant vaccination.

The maximal levels of antibody compare favourably with the groups receiving vaccines by the intravenous route in the case of MEL while SW and LEE antigens elicit maximal titres that are on the average four times lower. Tertiary stimulation leads to minimal increase in antibody levels in the treatment-groups of LEE and SW that received the initial dose of antigen in adjuvant. This increase is about 1.5-fold and rests on the exceptional response of one rabbit.

**Subcutaneous Administration.**

**Single injections.** Although the starting times of antibody formation are not different from other groups, the rates and the maximal levels attained are very low. This is particularly striking in those groups of animals receiving LEE or SW antigens in either
aqueous or water-in-oil suspensions, where a poor primary response is followed by an equally poor secondary and tertiary response.

The maximal levels of anamnestically produced antibody are not different after simple or adjuvant vaccination, except in the case of MEL, where the adjuvated group attains a higher final level. The average levels are the lowest after these treatments (8 and 21% of the peak attained in the corresponding groups after intravenous or intraperitoneal vaccination with MEL, 23 and 40% with LEE, 7 and 12% with SW), with no significant improvement on tertiary stimulation.

**Multiple injections.** The rate of antibody production is higher in these groups, compared to the response after single injections, especially where no primary response was detectable. However, on the average they are still below the rates obtained after intravenous or intraperitoneal administration. Tertiary stimulation results in detectably higher rates only in the SW-adjuvant group, and this unusually high rate is caused by a single animal.

The levels of antibody attained in these groups are at
least twice as high as in the corresponding groups after single
injections, but still approximately eight times poorer than after
the other routes of inoculation, in all cases. The maxima
obtained with adjuvated SW and LEE vaccines are lower than
in the corresponding groups receiving simple vaccines. Tertiary
increases do not occur except in the SW-adjuvant group and this
is caused by the exceptional response of one rabbit in the group.
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THE IMMUNE RESPONSE TO INFLUENZA VIRUS. III. EFFECT OF ROUTE AND TIME SCHEDULE OF VACCINATION ON COMPLEMENT-FIXING ANTIBODIES.
SUMMARY

The complement fixation test against the virus antigen was employed as the third serological procedure for following the antibody response of the rabbit to three strains of influenza virus (MEL, LEE and SW). After the large antigenic dose used, antibody is first detectable between the second and third days following primary or secondary stimulation. Administration of the same doses as a water-in-oil emulsion increased the lag phase to between 5 and 20 days for LEE and SW antigens.

In treatment-groups giving high levels of primary antibody the observed rates of antibody production are not significantly different after primary or secondary stimulation. The other treatment-groups show a 3-4 fold increase on secondary stimulation, which is partly due to underestimation of primary rates.

The overall increase from primary to anamnestic levels of antibody was relatively small, being on the average 2.5 fold, and was dependent on the schedule of vaccination. The maximal levels of antibody vary with route, intravenous being slightly superior to intraperitoneal and much superior to the subcutaneous route.
Multiple injections of antigen in aqueous suspension stimulate the highest levels of primary antibody, but the differences between schedules is much smaller after secondary stimulation. Once again, the viruses can be ordered as MEL, SW, LEE in descending order of immunogenicity. With the high doses of antigen used in these experiments, tertiary stimulation does not improve on the secondary levels of antibody.

**INTRODUCTION**

The assay of antibodies by either neutralization or by haemagglutination-inhibition evaluates their efficiency in terms of a third, the indicator component of the system. The efficiency of an antiserum in preventing either the infection or the agglutination of susceptible cells rests as much on the quantity as on the quality of its antibody population. Unlike these competitive tests, complement fixation occurs in a system where the equilibrium between antigenic sites and antibodies is uninfluenced by any other component of the system. The depletion of complement (or, more precisely, of a fraction of the complement-complex) serves only as a measure of the number of antigen-antibody unions.
From the law of Mass Action, which governs this interaction, it can be predicted that at concentrations of the reactants below the value of the equilibrium constant the position of the equilibrium will depend both on the concentrations and on the equilibrium constant. This means that complement fixation titres are a function of both the quantity and quality of antibodies. However, practical assays are conducted at such levels of virus that the concentration of antigenic sites will exceed the value of the equilibrium constant (Fazekas de St.Groth, 1962). In this region the value of the equilibrium constant becomes negligible compared to the other parameters of the reaction, and the outcome of tests will depend essentially on the quantity of antibodies only.

Such a simple relationship can be expected to hold over practically the whole range of tests reported in this study. Quantitative equilibrium measurements on this system (Fazekas de St.Groth and Webster, 1961; Fazekas de St.Groth, 1961; Fazekas de St.Groth and Webster, 1962b) indicated that the concentrations of antigenic sites, as employed in complement fixation tests, are about an order of magnitude above the respective equilibrium constants of hyperimmune sera against all three strains
of virus employed. Similar conclusions can be drawn also from qualitative tests (Fazekas de St. Groth and Webster, 1962a), where the transition from quality-dependence to quantity-dependence has been demonstrated by several independent methods.

Complications are to be expected only when working with antibodies of poor quality (i.e., of high equilibrium constants), or on poorly cross reacting heterologous systems. Situations of this kind have been observed by comparing quantitative precipitation and complement fixation data (Osler and Heidelberger, 1948a, 1948b; Wallace, Osler and Mayer, 1950; Osler and Hill, 1955; Hill and Osler, 1955).

The materials and methods used in this study have been fully described in the first paper of the series, and a synopsis of the experimental design can be found in Table 1 of that paper.

**EXPERIMENTAL**

To eliminate variations due to operators and reagents, all the sera from the one rabbit immunized with one antigen were assayed on the same day. The titres are once again expressed in terms of 1.0 ml volumes.
Statistical comparisons of maximal antibody levels and rates of production were carried out by analyses of variance and covariance, as in the first two papers. The estimate of maximal levels of antibody had a standard deviation of ±0.068 for MEL, ±0.095 for LEE, and ±0.088 for SW and their respective antibodies. The accuracy of the rates of antibody production was ±0.005 for MEL, ±0.004 for LEE and ±0.007 for SW, in log_{10} units per day.

The mean antibody titres of the 12 treatment-groups are presented in Figures 1, 2, 3, the curves being fitted by eye. Tables 1 and 2 give a summary of the main statistical results.

Figure 1
Figure 2
Figure 3
Table 1
Table 2
FIGURE 1.

The antibody response of rabbits to a Type A influenza virus (MEL), as measured by complement fixation.

Antibody titres are expressed in $\log_{10}$ units per 1.0 ml volumes of serum. Each point represents the geometric mean titre of a group of three rabbits.

Schedules of vaccination:

Group I: Aqueous vaccine on days 0, 40 and 60, by the route indicated on the figures.

Group II: Aqueous vaccine on days 0, 2, 4, 7, 9, 11 as primary stimulus, on days 40, 42, 44 as secondary and on days 60, 62, 64 as tertiary stimulus.

Group III: Vaccine in incomplete Freund's adjuvant intramuscularly on day 0, aqueous vaccine on days 40 and 60, by the routes indicated on the figures.

Group IV: Vaccine in incomplete Freund's adjuvant intramuscularly on day 0, followed by aqueous vaccine on days 2, 4, 7, 9, 11 as primary stimulus, by the routes indicated on the figures. The secondary and tertiary courses of vaccination were the same as for the corresponding sub-sets of Group II.

Each inoculum of vaccine contained 5,000 haemagglutinating units of virus (approximately $10^{11}$ particles, or 0.06 mg).
FIGURE 2. The antibody response of rabbits to a Type B influenza virus (LEE), as measured by complement fixation.
FIGURE 3. The antibody response of rabbits to swine influenza virus (SW), as measured by complement fixation.
Intro pton*a!

Group II

DAYS AFTER VACCINATION

Group IV

DAYS AFTER VACCINATION
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<tr>
<th>Vaccinating strain of virus</th>
<th>Schedule of administration</th>
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<th>Secondary Response</th>
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The rates, in $\log_{10}$ increment/day, are the mean values of a group of three rabbits and are based on at least three bleeds each over a span of at least 6 days.
<table>
<thead>
<tr>
<th>Vaccinating strain of virus</th>
<th>Schedule of Administration</th>
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The titres, in log_{10} units/ml, are the geometric mean of a group of three rabbits tested over a span of at least 10 days.
DISCUSSION

In the analyses of variance and covariance the genetic variation between rabbits was eliminated from all comparisons by pooling the error sum of squares with all the interaction sums of squares containing differences between rabbits as factors. As a result the error variance was increased by a factor of 10.53 in evaluation of maximal levels of antibody produced and by 1.86 in comparing the rates of complement fixing antibody production. The increases in error variance, in particular the high increment for inter-rabbit variation in levels of antibody produced, is in keeping with the results obtained by antihaemagglutinin tests. Both of these techniques give a measure of the quantity of antibody present in a serum, and the smaller variances characterizing the behaviour of neutralizing antibodies show that, on the average, quality must make up for lack of quantity when sera was assayed for neutralizing potency. The great similarity of inter-rabbit variation when rates of antibody production are compared by the three serological techniques, makes the above figures even more meaningful.

The significance of variance ratios due to the main
contrasts was maintained even after evaluating them against the increased error term containing the higher interactions as well as the replication variance.

**The Primary Response**

All titrations were started at a serum dilution of 1:20, but even at this level many pre-vaccination sera fixed complement in the presence of influenza virus antigens, some giving 50% fixation even when diluted 1:100. The primary response can therefore only be followed over a narrower range than by the other two methods of assay. This background will not influence estimates of the maximal antibody levels but will mask the true starting times and may flatten the slope of the response. As a result the starting times of antibody formation have been read off the figures as calculations from the slopes and intercepts would have introduced systematic bias under the guise of objective assessment.

**Intravenous Administration.**

**Single injections.** With the relatively large dose of antigen used in these experiments antibody was first detectable above the baseline for all strains between the second and third day after primary stimulation. This holds generally for aqueous
vaccines; for water-in-oil vaccines the lag phase is significantly longer, lasting from 5 to 10 days.

The rates of antibody formation are low, 0.06, 0.01, and 0.11 log_{10} units per day or doubling times of 5, 30, and 2.7 days respectively, for MEL, LEE and SW in aqueous suspensions. The corresponding figures for adjuvant vaccines are 0.15, 0.04, and 0.06 log_{10} units or doubling times of 2.0, 7.5 and 5 days respectively. All these values are minimum estimates, due to the high baselines.

The maximal levels of antibody attained are identical after simple or adjuvant vaccination for LEE and MEL, in spite of the different rates and starting times. However, the maximum with SW antigen, attained after simple vaccination is significantly higher than after adjuvant vaccination, the difference being four-fold.

**Multiple injections.** The delays between primary stimulation and initial detection of antibody above the baseline levels are the same as in the treatment-groups receiving single doses of antigen. Adjuvant vaccination once again causes a marked delay in appearance of detectable antibody with LEE and SW antigens, even when the primary stimulating dose is followed at two-daily
intervals with intravenous injections of antigens. This must mean that the initial intramuscular dose of antigen in adjuvant in some way depresses the production of circulating antibody.

The rates of antibody production are somewhat higher after multiple doses of antigen in aqueous suspension, the doubling times being 1.8, 3.0, and 2.1 days for MEL, LEE and SW respectively. After adjuvant vaccination the rates are not significantly different from the companion groups receiving single doses of antigen.

The maximum antibody levels are uniformly higher after multiple doses of antigen, the difference being 2.6-fold for MEL, 2.5-fold for LEE and 1.9-fold for SW. On the other hand, adjuvant vaccines do not stimulate significantly higher levels of antibody after multiple booster doses, even though the latter were administered without adjuvant.

**Intraperitoneal Administration.**

On the whole, a dose of vaccine given intraperitoneally produces about two thirds as much antibody as the same dose given intravenously (43% with MEL, 95% with LEE, and 42% with SW). The differences are most striking between schedules that employ aqueous or water-in-oil suspensions of antigens; the former stimulate much
lower levels of antibody by this route while the latter give levels comparable to those achieved after intravenous vaccination.

**Single injections.** The starting times of antibody formation cannot be estimated accurately from the figures, but they do not seem to differ greatly from those after intravenous stimulation. Adjuvated LEE and SW antigens once again show the delayed initiation of detectable antibody.

The rate of antibody production is low, giving a doubling time of the order of 10 days for both simple and adjuvant vaccination.

The maximum levels of antibody attained are higher after adjuvant vaccination, especially with MEL antigen, while after simple vaccination all strains of antigen elicit identical titres, being about eight times below the corresponding intravenous treatment-groups.

**Multiple injections.** Starting times are not different from the previous treatment-groups, although the delay with LEE and SW antigens after primary adjuvant stimulation is, if anything, larger than after a single dose of antigen.

The rates of antibody production are slightly higher than after single doses, giving doubling times of about 3 days, when antigens are given in aqueous suspension. This is also true for
MEL antigen given as water-in-oil suspension; LEE and SW antigens in adjuvant give rates that are not significantly different from the groups receiving single doses of antigen.

The maximum levels of antibody attained after simple vaccination are higher than in the groups receiving single doses but are still twofold below the levels reached after intravenous inoculation. The adjuvated vaccines stimulate antibody production to levels that are not different from the response to single doses or, in fact, from the levels reached by the adjuvated intravenous groups.

Subcutaneous Administration.

Compared with the average response taken over all treatment-groups by the intravenous route, the response to subcutaneous vaccines is slightly less than half as much (47% for MEL, 49% for LEE and 37% for SW). Once again, there are major differences between aqueous and water-in-oil vaccines.

Single injections. Both the starting times of antibody formation and the rates of production do not differ from the corresponding groups after intraperitoneal vaccination. The doubling time for antibody is, on the average, 10 days and the differences between simple or adjuvant vaccination are insignificant.

The maximal levels of antibody are lower after simple than after adjuvant vaccination, the exception being SW antigen
where the same maximal levels are reached after either schedule. The scatter within these groups is so great and the levels attained so low that the observations have little value in themselves, and are presented mainly for comparison with the results obtained by the other two serological techniques on the same sera.

**Multiple injections.** The rates of antibody production are only slightly faster than after single doses of vaccine and are comparable with the intraperitoneal rates. The higher rates with simple vaccines are once again evident.

Simple vaccination induces levels of antibody in those treatment-groups that are significantly higher than in the groups receiving single doses of vaccine (10.2-fold for MEL, 1.9-fold for LEE and 4.5-fold for SW). On the average, these levels are comparable to those obtained by the intraperitoneal route, as are the levels after adjuvant vaccination.

**The Anamnestic Response**

The secondary responses are characterized by attainment of maximal levels of antibody that are not improved upon by tertiary stimulation. The absolute levels of antibody are mainly dependent on route and less so on the schedule of administration, and
therefore do not all reach the same level after tertiary stimulation. The relatively large dose of antigen used for anamnestic stimulation, and the short interval (20 days) between secondary and tertiary stimulation probably accounts for the lack of significant changes in antibody titre between days 60 and 75. For these reasons, the two sets of observations will be discussed together.

There is no detectable negative phase on secondary stimulation, suggesting that, when present, this phenomenon is not caused by a significant drop in the quantity of antibody. As the complement fixing titre of a serum is largely a measure of the number of antibody molecules present, the negative phase as observed by other serological techniques must rest on changes in quality.

The true starting times of antibody formation and the rates of formation are once again obscured by the high levels of primary antibody, but estimates of the maximal levels attained are not affected.

Intravenous Administration.

Single injections. Judged by the first detectable increase above the primary levels, the anamnestic response becomes measurable between the second and third day after stimulation.
Irrespective of the previous history of the animals, the measured rates are characterized by a doubling time of the order of 2 days. The tertiary response in these groups is hardly noticeable and the rates do not differ significantly from zero.

The maximal levels of antibody attained are comparable for simple and adjuvant vaccines and are consistently higher than the primary maxima. The difference is on the average four-fold for the three strains of antigen, and remains the same after tertiary stimulation.

**Multiple injections.** The starting times are not different from the groups receiving single doses of vaccine, being of the order of two to three days.

The doubling times for antibody are again approximately two days for simple or adjuvant vaccines, and are significantly higher than after single doses. Tertiary stimulation results in rates of antibody production that are detectably different from zero.

The levels of antibody produced are as high as can be attained by any of the methods tried and are on the average 2.8 times as high as the primary levels. The differences between simple and adjuvant vaccines are minimal and in fact, it can be
generally stated that the differences characterizing the primary responses have disappeared and the secondary and tertiary responses of the treatment-groups are quite comparable.

**Intraperitoneal Administration.**

*Single injections.* The detectable rates of antibody formation are in general related to the levels of antibody attained in the primary response: the lower the primary level, the higher the secondary rate. There are some exceptions to this rule, arising largely from inter-rabbit variation. One such is the case of LEE antigen in adjuvant, where a poor primary response is followed by a poor secondary. These groups in fact give a measurable rate of antibody production on tertiary stimulation.

Simple vaccines elicit the production of lower levels of antibody than do adjuvated vaccines. The levels attained with water-in-oil antigens are comparable with those after intravenous vaccination. Tertiary stimulation causes an increase in maximal levels only in those groups that show a poor primary and secondary response (SW in aqueous suspension and to a lesser extent, LEE in adjuvant); in the other groups the secondary levels are maintained only over the whole period from day 40 to 80.

*Multiple injections.* The delay in antibody increase above the primary levels is once again two to three days and does
not differ with antigen or treatment.

The rates of antibody production are not significantly different from the groups receiving single doses of antigen, or from the companion groups receiving intravenous doses of antigen. The doubling time is on the average 2 days, being about 2.5 times faster than the primary rates in these groups.

The antibody levels attained are not different for simple or adjuvant vaccines and are uniformly higher than after single doses of vaccine in aqueous suspension. The levels are comparable with those obtained after single doses of antigen in adjuvant or for that matter after intravenous administration. Tertiary stimulation does not increase the levels of antibody, but serves only to return it to what is apparently the maximum the animals can produce.

**Subcutaneous Administration.**

**Single injections.** The rates of antibody production are the lowest after subcutaneous injections, for all treatments. Even though primary levels of antibody are low, the rates of anamnestic production remain below those observed after the other two routes of inoculation.

This is less striking after simple vaccination with MEL and LEE where the doubling time compares favourably with other
routes, but is evident in all other treatment-groups. Tertiary stimulation serves only to return the levels of antibody to those attained after secondary stimulation, at rates not very far from zero.

The maximum levels are the lowest obtained, and are on the average, four-fold below those after intravenous or intra-peritoneal vaccination. Adjuvated antigens give twice as high titres with MEL and LEE, the case of SW virus is equivocal. Tertiary stimulation does not improve the levels attained.

**Multiple injections.** The rates of antibody production are not significantly altered by multiple injections, and like many of the anamnestic responses, are probably masked by the high primary antibody levels. Tertiary stimulation results in a detectable rate of increase in the adjuvant-SW group but this is due to the exceptional reaction of a single rabbit in the group.

The levels of antibody are in general two-fold above the groups receiving a single dose of antigen by this route, and there is no difference between the levels attained with simple or adjuvant vaccines. However, the absolute levels are still well below those reached after vaccination by the intravenous or intra-peritoneal routes. The only treatment-group showing an increased
level on tertiary stimulation is the adjuvated SW group, and this is due to a single animal in the group.
REFERENCES


CONCLUSIONS AND GENERAL DISCUSSION.
CONCLUSIONS AND GENERAL DISCUSSION.

Having defined the quality of antibody as the equilibrium constant characterizing its combination with antigen, and thus having opted for interpreting immunological phenomena in terms of the law of mass action, the first part of these studies was concerned entirely with showing that the working hypothesis rested on valid premises. Since the law of mass action is essentially a formal statement of the thermodynamics of reversible reactions in a closed system, these tests sought to demonstrate an equilibrium between influenza viruses and their antibodies, and then examine whether changes in the concentrations of the reactants (or in "their active masses", in the words of Guldberg and Waage) would bring about such changes as are predictable from the mass action law.

The first paper, on the qualitative aspects of virus-antibody equilibria, demonstrates by several independent methods that the interaction which leads to the neutralization of the infective particle can be fully interpreted as a reversible union of antibody with multiple antigenic sites on the virus. This paper also shows that at initial concentrations of reactants well above the equilibrium constant, the reaction will appear as if it were irreversible (irreversible reactions being the limiting case of the mass action law), while at concentrations below the equilibrium constant, the reaction is obviously reversible. The influence of non-infectious virus is examined and found to be in accordance with what can be
predicted from the basic model. Artificially created heterogeneity in systems of antigen and antibody were also examined: antigenic heterogeneity greatly affected the reaction, while heterogeneity of antibody simply shifted the position of the dose-response curve, without significantly altering its shape.

As the basic tenets of the mass action model were found to be satisfied by the experimental evidence, it was decided to put the model to quantitative use by measurement of all parameters entering the reaction. However, before this could be attempted, it was necessary both to devise methods for separation of components of the reaction, and also a method or methods for measuring the absolute quantity of reactants.

Equilibrium filtration was found to be a very satisfactory method for separating free antibody from the reaction mixture. The results obtained are the same after equilibrium filtration or equilibrium centrifugation, and the former has the advantage that the whole process of separation can be completed within two minutes. The possible disadvantages of using an open system have been examined experimentally and shown not to influence the equilibrium attained.

The second prerequisite for measuring parameters, i.e. the measurement of absolute quantities of reactants at very low concentrations was approached in the case of antibody, in two papers. The first of these, on the labelling of antibody with radioactive tracers shows that these methods can be applied to the system without loss of serological activity. The second paper, on the staining of serum
proteins with two new dyes, shows that this also is a potential method for estimating the concentration of antibody in absolute terms. Virus particles, of course, can be estimated by one of the conventional methods, already available at the beginning of this study.

The parameters of interest in the influenza virus-antibody system are the number of antigenic sites on the virus, and the equilibrium constant. These were measured both in binary and ternary systems. The results show that the number of antigenic sites on a particular virus particle is the same whether the determinations were made in a binary (equilibrium filtration or equilibrium centrifuging) or ternary system (neutralization tests). However, estimates of the quality of antibody varied with the type of system used.

The constancy of the number of antigenic sites shows that the model of influenza virus-antibody interaction is not contradicted and that the methods employed in this system should be applicable to the broader problems of immunology. The values obtained for the equilibrium constants depend on the system used, provided the system is heterogeneous with respect to either the antigenic or antibody population. In this case neutralization tests will measure predominantly the best quality antibody present, while binary systems and the antihaemagglutinin test will give a broader measure of quality. Since such discrepancies between the tests were consistently noted, the results furnish further proof for the heterogeneity of antibodies in all of the sera tested.
The heterogeneity of antibody does not invalidate the application of these techniques to the wider problems of immunology, such as the production of antibodies. Indeed, it demands that this aspect of the problem should never be ignored and, on the lowest plane, at least two techniques of assay should be applied even to the simplest questions, in order to obtain information both on quantity and quality of the antibody response.

A start has been made in this study to build up a large body of information in this area. The influence of the route and schedule of vaccination was investigated and a binary (complement fixation) and two ternary procedures (neutralization and antihaemagglutinin) were used to distinguish between quantity and quality of antibody.

The unitarian theory of antibodies (Zinsser, 1921) is assumed to hold for the influenza virus–rabbit antibody system. The identity of the antigenic areas for neutralizing and antihaemagglutinin antibodies has been established by measurements of parameters in this system, and the number of sites is so close to the theoretical saturation value of the viral surface that this fact itself excludes the possibility of two separate or even partly overlapping sets of antibodies underlying these two immunological phenomena. The identity of the antibodies detected by the three serological procedures can be further proven by absorption studies on homogeneous antibody preparations.

Each of the serological methods employed gives a different measure of quality and quantity of antibody. These differences have
been dealt with in detail in the text and it is only necessary to mention them briefly here. The complement fixation test and the antihaemagglutinin test give measures largely of the quantity of antibody present, while the neutralizing potency measures a compound of quality and quantity. Thus the maximum titres each contain a measure of quantity and the differences between the maxima may serve as a measure of quality.

The sensitivity of the techniques varies and depends on the concentration of reagents used in the tests. Neutralization is the most sensitive, followed by complement fixation and antihaemagglutinin tests. The findings based on the maximal rates and antibody titres in the primary and anamnestic responses are similar. They all show that the intravenous route of vaccination is slightly better than the intraperitoneal, and much better than the subcutaneous route, at eliciting production of antibody. This holds without qualification when the vaccine is administered in aqueous suspension. For adjuvant vaccines it is generally true, but in the primary response - especially in the case of SW and LEE antigens - is suppressed by some mechanism. The schedules of vaccination have the greatest influence on the primary response and, in general, multiple injections are superior to single injections, but neither schedule has any influence on the depressive effect initiated by the intramuscular injection of antigen in adjuvant. In descending order of immunogenic power, as demonstrated by each of the serological procedures, the strains are MEL, SW and LEE.
With the large dose of antigen used, the maximal levels of antibody are reached in the secondary response. This maximum is not the same for the various groups, and depends on route of inoculation and to a lesser extent on schedule.

The ratios of the antibody titres as measured by neutralization, complement fixation and haemagglutination inhibition are not fixed. The figure varies with the route and schedule of vaccination and, in general, it can be said that the higher the titre as measured by neutralization, the greater is the difference. Since neutralization tests are much more sensitive to the quality of antibodies than are the other two techniques of assay used in this study, this observation is best interpreted as a fairly uniform output of antibody as regards quantity (slight variation of, say, complement fixation antibody levels), while the quality depends greatly on route, schedule and timing of vaccination (wide variation in neutralizing maxima, as well as variable neutralizing potency to complement fixation ratios). The differences in antibody levels are most clearly seen on secondary stimulation, as here they cannot be attributed to, or confused by the sensitivity of the tests.

After secondary stimulation, the overall increase in titre as measured by neutralization was 6.3-fold, by antihaemagglutinin 4.2-fold, and by complement fixation 2.5-fold, for the three strains of antigen. The extent of this increase varied with route and schedule of vaccination, but not with antigen. As measured by neutralization, the mean increase for the three strains of virus (MEL, LEE and SW) was 5.5, 4.3, 5.6 and 11.0 fold respectively for
schedules I to IV, while the increase according to route was 9.1-fold intravenously, 7.9-fold intraperitoneally and 2.5-fold subcutaneously. As measured by antihemagglutinin, the increase according to schedule was 4.5, 2.6, 3.7, and 7.8 fold respectively for schedules I to IV, the routes of inoculation caused increases of 6.0-fold intravenously, 5.6-fold intraperitoneally and 2.1-fold subcutaneously. Complement fixation, which gives a measure of the absolute quantity of antibodies shows increases of 2.8, 2.0, 2.4, and 3.1-fold respectively for schedules I to IV, from the primary to the secondary maximal levels of antibody; according to routes, the increases were 3.0-fold intravenously, 3.1-fold intraperitoneally, and 1.7-fold subcutaneously.

As each technique is measuring the same antibodies, the disproportionate increases must be due to a sudden increase in the quality of antibodies on secondary stimulation. The greatest increase in the quality of antibody on secondary stimulation is observed in the treatment-groups that received an initial injection of antigen in adjuvant, followed by multiple injections in aqueous suspension, and responded poorly by all criteria in the primary response. This holds particularly for SW and LEE antigens, and less for MEL. These changes are also dependent on route, and in general, they can be ordered intravenous, intraperitoneal and subcutaneous, in descending order of efficiency.

The increase in the quality of antibodies on secondary stimulation is less noticeable in those groups that show high levels of primary antibody, where the neutralizing potency to complement
fixation ratio is $0.97 \log_{10}$ units by the twentieth day, and $1.38 \log_{10}$ units after boosting. This is especially so for aqueous vaccines given by the intravenous route when multiple doses of antigen are administered, and to a lesser extent after multiple doses by the intraperitoneal route.

Any comprehensive theory of antibody production therefore, has to account for these features:

(1) The very short inductive phase after primary stimulation with aqueous suspensions of virus;
(2) The high rates of primary antibody production;
(3) The depressive action of incomplete water-in-oil adjuvants, on antibody production;
(4) The increase in quality of antibody on secondary stimulation;
(5) The attainment of different maximal levels of antibody, depending on the route and schedule of vaccination, levels are not passed after tertiary stimulation.

The current theories of antibody production fall into two categories, the instructive theory, (Breinl and Haurowitz, 1930; Alexander, 1931; Mudd, 1932; Pauling, 1940) and the selective theory (Ehrlich, 1900; Jerne, 1955; Talmage, 1957; Burnet, 1957; Lederberg, 1959). In simple terms the question is whether the information for specific antibody is brought into the antibody producing cell by the antigen (instructive), or is inherent in the cell waiting to be evoked (selective). The first theory implies that the cells required for antibody formation are already present in sufficient numbers,
the second that a clone of cells develops as a result of antigenic stimulation.

How do the above observations fit into these two theories?

The early appearance of antibody after initial stimulation can be accommodated by either theory, but the very high rate of production is difficult to explain by clonal replication of selected cells. The doubling time for antibody as detected by neutralization is about 5 hours, and this is faster than the most rapid rate of replication observed or, rather, inferred by Nossal and Mäkelä (1962). The discrepancy is sufficient to cast serious doubt on the validity of the selective hypothesis in its simplest form. A possible improvement in the quality of antibody during the earliest phases of production could be invoked to account for the observations, but since directional variation of the kind of antibody produced is contrary to expectations from both hypotheses, such an interpretation would equally weaken both. Indeed, essentially the same conclusions can be drawn from the rise in complement fixing antibody titres, and since this technique is uninfluenced by the quality of antibody, the overall evidence from the primary response is compatible with the instructive model and contrary to the selective one.

The depressive action of adjuvant on the starting times, rates and maximal levels of primary antibody production, especially with SW and LEE antigens, cannot be fitted into either theory. The slow release of antigen from the adjuvant depot could be used as an explanation in the treatment-groups receiving single doses of antigen in water-in-oil suspensions. However, this could not be said
for the companion group, where the intramuscular dose of adjuvant-vaccine was followed by 5 doses of antigen in aqueous suspension at two-daily intervals. One can involve delayed-type hypersensitivity, or immunological blocking in one form or another to explain these observations but until a larger body of data is collected, and, indeed, the phenomenon investigated in greater depth, it is premature to elaborate as much as a working hypothesis on the evidence presented, which amounts to no more than a statement of the problem.

The increase in antibody levels on secondary stimulation is generally assumed to be an increase in the amount of antibody, but our observations indicate that the increase is essentially in quality. The instructive hypothesis in its original form has no explanation for increased titres in the secondary response. Modifications and extensions of this theory (Monod, 1959; Pappenheimer, 1957) postulate a permease-like induced mechanism capable of trapping antigen and thus rendering the same number of cells more effective as antibody producers on anamnestic stimulation. The selective hypothesis accounts readily for increased titres, by further proliferation of the competent clones originally stimulated by the primary antigenic dose. Heterogeneity is explained by the variable period of time spent on the template during secondary and tertiary folding of the peptide chain (Pauling et al., 1944b; Karush, 1957, 1958), according to the instructive model, and by the range of cells destined to produce \( \gamma \)-globulins of variable complementariness to the stimulating antigen. Neither theory envisages shifts in the distribution of the antibody population, in fact, neither is elastic enough to accommodate
the observed rapid increase in quality following secondary stimulation.

Differences in the maximal levels of antibody attained by the routes and schedules tested, which show no sign of increased levels of antibody on further stimulation by the same route, suggests that either the total number of cells has been stimulated under these conditions or that the size of the clone is governed by the route and schedule of vaccination. The first suggestion could fit in with the instructive theory provided some genetic qualifications are incorporated into it, but there seems to be no reason for one clone of cells being larger than another if the same homeostatic mechanism is supposed to limit their proliferation.

Thus only some of the observations can be fitted into one or other theory. The depressive action of adjuvant merits further study before its significance can be evaluated in terms of any theory of antibody production. The increase in the quality of antibody with time is not a new observation, but the sudden, and to all appearances, discontinuous increase in quality on secondary stimulation has not been observed before and cannot be fitted into either of the above theories without fundamental modifications. There has been one hypothesis put forward especially to account for changes in quality after repeated vaccination that should be taken into account. Najjar and Fisher (1955, 1956) proposed that when antigen and antibody combine, the physicochemical stresses expose new antigenic determinants on the antigen to which further antibody could be formed. After each encounter with antigen a new antigenic determinant is
exposed until eventually all groupings are exposed and finally anti-
body is formed against structurally altered antibody. This anti-
 antibody has been used in a later paper (Najjar and Robinson, 1959) to explain immunological paralysis.

However, this hypothesis gives no explanation at all for increases in quality of antibodies to single determinant groups, nor does it account for the fact that increase in quality can occur without increase in quantity, as already demonstrated by Heidelberger and Kendall (1935). Thus it can be discounted as offering any explanation for the changes in the quality of antibody.

Porter (1955), Humphrey and Porter (1956), and Askonas, Humphrey and Porter (1956), on the other hand, have suggested that the multiple forms of rabbit antibody are produced in different cells in the same organ, and each organ produces a greater or lesser quantity of chromatographically distinct antibody. Changes in quality are envisaged as changes in the organs producing antibody. This latter suggestion seems somewhat far-fetched, for their results are compatible with lymph nodes and bone marrow producing antibody in the earliest stages of immunization, while the spleen becomes an important producer as immunization continues. The above observations on the high rates and maximal levels of antibody produced after intravenious vaccination, and the possible early increase in quality of antibody by this route, combined with observations on splenectomy, suggest that the spleen, too, can produce antibody early after stimu-
lation, and that this antibody is of high quality.
Antigenic heterogeneity can also be used to explain the different qualities of antibodies being produced, but there is no obvious mechanism by which the quality could change with time or upon reinjection of antigen. One could indulge in further speculation and place the onus for different types of antibody on cells dealing with the antigen before it came in contact with the antibody-producing cells proper. If these cells are required to pre-treat antigen before antibody can be produced in the plasma cell series, as first suggested by Sabin (1939) and recently re-emphasised by Fishman (1961), the size of the antigenic determinant will depend on the enzymatic degradation inside the macrophage. This speculation only shifts the responsibility for differences in quality to the macrophage, it does not explain how the macrophage learns to send an improved or more complete copy of the determinant group to the antibody producing cell on secondary stimulation.

The inability to fit the collected data into the selective, the instructive, or any of the current minor theories of antibody production, and the speculative views on the mechanism for increase in quality, simply underscores the need for more data on which a consistent and comprehensive theory can be based. The application of the quantitative techniques discussed in the first papers, for the thermodynamic measurements on antigen-antibody systems, suited to work with natural antigens, should supply such data as are sadly lacking today.

Advances in our knowledge of basic immune mechanisms have certainly been made since the time of Ehrlich, but the mystery of
antibody formation still eludes us. The recent great strides in the understanding of the regulatory system in the synthesis of proteins (Jacob and Monod, 1961) show that impressive progress is being made in the case of single cells, and in the study of the simpler systems. It is hoped that the control of directed protein synthesis in the multicellular organism will also progress apace and throw more light on the mechanism of antibody formation.
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