THE IMMUNE RESPONSE IN HOMOLOGOUS TRANSPLANTATION

Thesis by

James Henry Berrian

In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1957

ABSTRACT

Part I discusses the immunogenetic basis of transplantation incompatibility and the nature of the immune response which adversely affects incompatible tissue transplants.

Part II describes attempts to detect specific reactions between tissue extracts and antisera by means of light-scattering measurements. The few positive reactions obtained tend to support, rather than refute, previous claims made for individually specific serum antibodies formed during the rejection of transplants.

Part III deals with an experimental demonstration of the destruction of mouse tissue by immune spleen cells, using in vivo cultured diffusion chambers. The results support the hypothesis that immune cells directly initiate the destruction of transplants.

In Part IV reactions between immune cells and isolated transplantation antigens are studied in vitro. It is possible to react immune cells as well as the transplantation antigens with fluorescent labels without abolishing their immunological effectiveness. With a biological test as the basis, evidence is described that transplantation antigens are removed from solution when suspended with immune cells in vitro.

In Part V a method is presented for describing the uptake, destribution and binding of fluorescent labeled molecules using polarization of fluorescence as an indication of the motion of molecules and their interaction in the intracellular environment.

Part VI describes a simplified test for mouse isohemagglutinating antibodies, which only requires purified hyaluronic acid and saline as the suspension medium.

Part VII describes some details of the structure of elastin and attempts to explain the immunological tolerance of elastin and its soluble derivatives in terms of its mobile molecular structure.

ACKNOWLEDGEMENTS

The author in deepest gratitude wishes to extend the following acknowledgements.

I owe my interest and most of my knowledge in immunology to Ray D. Owen. His sincere interest and stimulating companionship have been responsible for the completion of my graduate training. The collaboration of Leslie Brent made the studies in Part IV possible. Moreover, I am indebted to him for the encouragement and guidance during our association which have contributed immeasurably to the completion of this thesis and to my knowledge of transplantation. I am indebted to the United States Navy and the Command of the Naval Medical Research Institute for their generous support and for the opportunity of completing my graduate training. To Lt. R. L. Jacobs, USN, I owe a special debt of gratitude for his advice and untiring assistance. It is my wife, Bette, who, more than any other, deserves credit for the completion of this work. Her devotion, encouragement and assistance led to its accomplishment.

TABLE OF CONTENTS

TABLE OF CONTENTS
(Continued)

PART I

General Introduction and Review

Homologous transplantation involves the surgical exchange of tissues between different individuals within the same species. Such homotransplants or "homografts" are almost invariably destroyed after a short period of residence on the recipient animal. It is now established with reasonable certainty that a state of immunity acquired by the recipient against heritable cellular antigens of the graft brings about its destruction. The evidence for this immuno-genetic theory of transplantation imcompatibility is largely a development of the past decade. It strongly indicates the involvement of the same sort of host response called forth in other sorts of immunity, such as that against infectious agents. Analysis of transplantation immunity, at the present, scarcely extends beyond the formulation of this analogy. The current status of the subject will be briefly summarized in the first part of this thesis. A thorough review of the subject is that by Brent (1).

An implicit requirement of the theory is that antibodies are the causal agents of homograft destruction. These have thus far escaped identification. A search for these antibodies will be described in the sections following the introductory review.

The Genetic Origin of Homologous Incompatibility

Homotransplants between individuals within heterogeneous strains of animals, or between the members of different highly inbred strains, have a high probability of being rejected by the recipient. By contrast, "isografts" or transplants exchanged between monozygotic twins (2,3,4), or between individuals within certain highly inbred strains of laboratory animals (5,6), survive quite as well as "autografts". The latter are grafts excised from an individual, and subsequently returned to that individual.

In view of the above results, it is immediately apparent that the initial stimulus responsible for transplantation incompatibility has its origin in genetic disparity between the donor and the recipient. Transplants exchanged between individuals of different species, termed "heterografts", are universally rejected. For such exchanges the genetic disparity is greater than for exchanges within a single species, and this condition is manifested in a characteristic mode of response that differs from that evoked by a homologous transplant (7). The response in each case, nevertheless, leads to the same final result - destruction of the transplanted tissue.

The genetical basis of transplantation immunity has been most fully explored in mice, using homologous tumors as the transplants (8,9). This analysis has now been extended to include normal tissues (10). Certain genetic rules governing transplantation can be formulated, based on the results of these fundamental investigations.

The recipient of a graft must possess all of the same dominant alleles at certain "histocompatibility" loci as are present in the donor if the graft is to be accepted. The F1 hybrids of two strains having different, but homozygous, alleles at histocompatibility loci will accept grafts from either parental strain. Only a proportion of F2 hybrids will accept the grafts from either parental strain, and this proportion will be related to the number of histocompatibility alleles segregating in the cross. The most important conclusion, originally drawn by Little (8), is that the acceptance of a homologous transplant is dependent on the simultaneous presence of a number of Mendelizing genes in the recipient.

The fact that a homograft will be rejected in a recipient lacking any one of several histocompatibility alleles present in the donor suggests the basis for an immune response against the transplant. The only underlying assumption that need be made is that each different allele at histocompatibility loci directs the production of a unique molecular species which is capable of antigenicity in a recipient having different alleles.

The individual is rendered tolerant of unique molecular structures to which he becomes exposed in early development. In later development the situation is reversed, and newly introduced molecular configurations elicit immunity rather than tolerance. The phenomenon of immunological tolerance against antigens introduced in fetal life

is now well documented (11). It seems to account for the fact that an individual does not respond with immunity against the products of genes which he has inherited, but he may react vigorously against the products of certain homologous alleles when these are introduced in grafted tissues. Such genetic products are described as "cellular antigens".

Transplantation Immunity Against Cellular Antigens

The acquired immunity hypothesis of transplantation incompatibility first drew attention in the first decade of this century, and a review of the early investigations surrounding it is available (7). It reached general acceptance following observations reported by Medawar in 1944 (12) and 1945 (13). He found that initially, rabbit skin homografts heal in place, become vascularized, and show hyperplastic growth. In this initial, latent period, homografts are indistinguishable from autografts. Following this phase, a sequence of events ensues which has become known as the "homograft reaction". Circulation in the graft ceases, its bed becomes infiltrated with inflammatory cells, and the epithelium is finally destroyed. Medawar then observed that a second graft from the same donor, placed on the recipient at some later time, was more promptly rejected than the initial graft. The "second set" graft, in contrast to the initial graft, did not heal soundly into place or become vascularized. The epithelium was destroyed in little more than half the survival time noted on the average for first set grafts. Furthermore, the recipients

retained this specific immunity against the tissues of the initial donor for extended periods of time. The cross reactivity noted between various donors and recipients in the heterogeneous rabbit populations sampled by Medawar was consistent with the expectation for control of the various antigenic patterns by the distribution of a finite number of genes throughout the populations.

Following Medawar's observations on the second set phenomenon, this criterion of acquired immunity against homologous transplants has been confirmed in numerous species (1). It has found use as a potent method for disclosing the distribution of transplantation antigens among various tissues and within tissue preparations. Medawar (13) and his colleagues were able to show, for example, that certain of the antigens responsible for transplantation immunity against skin are also shared by leucocytes. In this case, prior immunization with leucocytes from a selected donor elicited a state of immunity in the recipient, against a subsequent skin graft from the same donor.

It can only be concluded from this sort of experiment that the leucocytes, in this example, shared certain of the antigens present in skin. The experiment does not necessarily demonstrate that every species of transplantation antigen active in the immunity against skin is equally represented in the leucocyte population. This question has been settled by Billingham, Brent, and Medawar (14), who have demonstrated the usefulness of immunological tolerance as a

further method for studying the distribution of transplantation antigens. They have devised practical methods of inducing specific tolerance in newly born mice, by the intravenous injection of cell suspensions. Mice of one inbred strain so injected will tolerate leter skin grafts only if the entire spectrum of transplantation antigens in the skin donor is represented in the tolerance-inducing suspension. Spleen cell suspensions were shown to be the most effective in inducing later tolerance of skin grafts, and it has been concluded that these cells and probably all other nucleated cells share the same pattern of transplantation antigens. These antigens, therefore, probably lack tissue specificity.

Genetic studies of tumor transplants in inbred mouse strains have shown that an allelic difference between the donor and recipient at one locus, H-2, will bring about an immune response upon grafting (15). In addition to the destruction of the transplant, the recipient may develop serum antibodies which will agglutinate the blood cells of the donor. All strains which have thus far been divided into groups having the same H-2 alleles with transplantation as the criterion, have also been found to fall into the same groups on the basis of their hemagglutination reactions (16, 17). It is therefore evident that the gene complex at H-2 directs not only the specificity of the transplantation antigens in each case, but simultaneously the specificity of the hemagglutinogen pattern. This

relationship has been clearly demonstrated by the analysis of "isogenic resistant", IR, mouse strains (16). These strains differ from the strains of origin by a single difference at the H-2 locus. Transplants exchanged between a member of the strain of origin and its IR partner do not survive, and antibodies may be identified in the serum of the recipient which will agglutinate the cells of the donor.

Despite the fact that both hemagglutinogens and the transplantation antigens seem to be specified by the same allele in the cases just cited, they appear to be distinct entities. Tissues subjected to mildly destructive treatments such as freezing, lyophilization or slight heating are quite effective in bringing about hemagglutinin production, but they are ineffective in eliciting a state of transplantation immunity (18). No transplantation immunity is evoked by the injection of pure erythrocytes, even when these are injected with an adjuvant containing Mycobacterium (19).

Transplantation Antigens

Billingham, Brent, and Medawar have succeeded in preparing cell-free tissue fractions which are capable of inducing transplantation immunity (18). The activity is associated with cell nuclei. It is easily lost upon slight heating, freezing, or lyophilization. Desoxyribonuclease digestion also destroys the activity. The active material is extractable from cells with water in the absence of calcium ions, which cause its precipitation. In the absence of calcium the aqueous extract shows no loss in activity, upon increasing

the sodium chloride concentration to 0.15 M, a treatment which precipitates desoxyribonucleoprotein. Cells disintegrated in .15M NaCl yield fibrous precipitates which are antigenic. These authors tentatively suggested that the transplantation antigens are desoxynucleoproteins, but the evidence for this is at present largely circumstantial.

Transplantation antigens prepared in the above manner have not thus far been shown to induce tolerance. They do not seem to be capable of inducing a prolonged state of immunity, and the possibility exists that the derived product is an altered form of the native material. The observations have provided a point of departure for further investigations.

Although cytoplasmic hemagglutinogens and nuclear transplantation antigens appear to be markedly different in their chemical properties, there is at present no evidence that these differences extend to the specific moiety of each. It is evident that they initiate responses in the recipient along different channels. On the other hand, some evidence is available that the hemagglutinogens, or antisera against these, interfere with the response against the transplantation antigens (20). The survival of grafts on animals which have been previously immunized with lyophilized tissues is actually prolonged or "enhanced". In the case of transplanted mouse

tumors, the state of immunity against the tumor is sufficiently inhibited to allow it to destroy the recipient. The enhancement effect for normal tissue grafts only delays the onset of immunity, it does not permanently suppress it (21).

The Response Centers

Lymph nodes and spleen appear to play the dominant role in the response to homografts. The regional lymph nodes show marked hypertrophy upon immunization. Mitchison has shown that regional lymph nodes of an animal immune to a tumor graft are capable of passively sensitizing another individual (22, 23). Billingham, Brent and Medawar have called this sort of immunity "adoptive", and they have confirmed Mitchison's findings using normal tissues as the grafts (24). Only the regional lymph nodes and the spleen appear to be capable of transferring the immunity. On the other hand, a general sensitization of all immunologically competent tissue must occur during the development of the immunity. This is borne out by the fact that excision of lymph nodes draining the site of an initial graft does not alter the ability of the recipient to meet a subsequent graft with a second set response. The most effective transfer of immunity through lymph nodes or spleen is brought about by their injection into the presumptive donor a few days before grafting. If injected at the time of grafting, a larger dosage of the cells is required to bring about the same results.

This dosage-time relationship seems to indicate that preformed antibodies within the transferred tissue are not directly responsible for the immunity, but that reactive materials are formed during the required period of residence within the host. The passive transfer of immunity by lymph node cells requires that they be in a viable condition.

The Immune Response

Once established, transplantation immunity seems to persist rather permanently. The channel of mediation appears to be the systemic circulation, and vascular approximation to the graft is required for its destruction. Corneal transplants survive, for example, so long as they do not become vascularized, even on an immune recipient (25, 26). The agents required for fulfilment of the homograft reaction must have access to the graft, and apparently these are confined within the vascular channels so long as the vessels remain patent. Algire has found that tissues enclosed within a filter capsule which is impermeable to cells survive indefinitely(27). However, if the capsule is made of a filter membrane having a pore size sufficient to allow cells from the recipient to enter, then the graft is promptly destroyed. Experiments with such chambers have further shown that when washed spleen cells from an immune recipient are enclosed in the capsules with tissues from the donor strain, both tissues will be destroyed (28). Spleen cells from non-immune animals are not capable of bringing about the same result. Furthermore,

in the former case, immune cells brought about the destruction of cells of the donor strain, when the chambers were placed in recipients of the same strain. In such an instance, there can be no question of the chamber recipient having contributed to the immune reaction within the chamber, unless it may have reacted against the immune cells. Since the results were independent of the strain used for the chamber recipient, it is probable that the immune spleen cells contributed the hypothetical antibodies involved. The experiments did not completely exclude the possibility that circulating antibodies might have played a role in the destruction of the grafts, if these had not been enclosed in the chambers.

Nature of the Antibodies

All of the available evidence indicates that transplantation antibodies are to be sought within the constituents of whole blood. The most effective proof of this point has been provided by Egdahl and Hume (29). They found that intact kidney homografts in dogs were destroyed in either a first or second set situation, when the only contact to the organ was made through polyethylene tubes providing vascular connections to the arterial and venous circulation of the recipient.

A number of attempts have been made to demonstrate cytotoxic effects of both cells and serum in vitro. Medawar cultured skin with specifically immune lymph node cells and immune serum (30). The results were entirely negative. So were the results of Allgower,

Blocker, and Engley, who failed to observe cytotoxic activity when skin was cultured with homologous immune serum or extracts of failing skin homografts and their beds (31). Weaver, Algire, and Prehn obtained similar negative results when the combinations of tissues giving positive results in diffusion chambers were cultured in vitro (28). Negative results in such experiments are, of course, inconclusive, for they may only indicate that the proper conditions for the reactions may not have been reached, even though the reactants themselves may have been present.

Billingham and Sparrow found that incubation of epithelial cell suspensions in immune serum curtailed the survival of the cells when they were returned to the donor (32). Certain tumor cells likewise seem to be influenced by incubation in immune sera (33, 34), which may have a direct cytoxic effect in vitro (35).

Numerous attempts have been made to transfer homograft immunity passively with immune serum of varying dosages and injection routines (1). These all failed to induce accelerated transplant destruction, but the opposite effect has been observed, that of enhancing the survival of the graft.

It was mentioned earlier that immunization of homograft recipients with erythrocytes or with lyophilized tissues did not result in transplantation immunity, although serum hemagglutinins were formed. This result is generally taken to indicate that the antibodies against

stable cytoplasmic antigens do not play a part in the destruction of the graft. However, there is some evidence that erythrocytes will immunize the recipients of tumor grafts in such a manner as to reduce the survival of the tumor (36, 37). The contrasting behavior of tumor grafts and normal tissue transplants when confronted with immune serum or with an existing immunity against stable cytoplasmic antigens does not fit well into the explanation of transplantation antigenicity advanced by Billingham, Brent and Medawar (18). The suggestion has been offered that tumor transplants are more sensitively balanced than normal tissue grafts with regard to the factors that influence their survival or destruction (1).

Recently, Bollag reported the nephelometric demonstration of serum antibodies in homografted rabbits that reacted in an individually specific manner with aqueous extracts from tissues of the donor (38, 39). Two features of Bollag's investigation have attracted attention. First, the tissue extracts were prepared in a manner strikingly similar to the aqueous extracts of mouse tissues which were found by Billingham, Brent and Medawar (18) to possess transplantation antigenicity. Bollag's antigens were highly unstable, suggesting a further similarity of the two preparations. The serum antibodies were likewise unstable, suggesting that Bollag might be dealing with a different system than that involved in hemagglutination. A further peculiar feature of Bollag's observations was the failure to observe cross-reactivity among the members of his rabbit

population. One hundred combinations between ten random antisera and ten donors did not result in a single instance of cross-reaction. This result is unparalleled elsewhere in immuno-genetics; it implies that not only did each donor differ from the corresponding recipient by at least one antigen not found in any of the other nine donors, but that even antigens serologically related to those differentiating each donor from the corresponding recipient were absent, in every case, from the other donors.

The general failure to find circulating antibodies which will passively transfer homograft immunity to another host has led to the suggestion that the immunity is analogous to that observed in delayed hypersensitivity against bacteria and chemical compounds.(1, 40, 41). Delayed hypersensitivity to tuberculin, diptheria toxoid, and picryl chloride are the prototypes most studied. The hypersensitivity in these cases is not associated with circulating antibody, although it is transferrable by cells. The delayed onset of the reaction upon challenging the sensitized animal with the antigen correlates with that observed for the second set skin graft, and like the latter a cellular reaction is characteristic of the inflammatory lesion. This sort of sensitivity is known to be associated with a unique channel of response. Protein antigens such as ovalbumin do not ordinarily give rise to delayed hypersensitivity when injected intra-dermally, but when the injection is accompanied by an adjuvant

containing tubercle wax a delayed type of response is obtained (42). This indicates that the materials associated with an antigen upon its introduction into the recipient may affect the course of the response. One effect of such adjuvants is the formation of granulomatous lesions at the antigen depot, and it may well be that the diversion of the response into the delayed type is associated with the uptake of antigen by cells at the site of introduction prior to its presentation to the lymphoid centers.

The phenomena associated with delayed hypersensitivity closely resemble those observed for homograft immunity. However, even though this analogy is supported by the scanty evidence now available, it brings little further understanding to either problem, for the immune components involved in delayed hypersensitivity are as much a mystery as those of homograft immunity.

It was the identification of antibodies which was set as the task in the search to be described, in the clear realization that these first attempts might result in little more than the development of techniques which might be useful in the further study of this problem. Confirmation of Algire's observations and those of Bollag, which at first sight seemed in conflict, was first undertaken. Following these experiments, attention was directed toward identifying reactions between antigenic extracts, which were known to contain the transplantation antigens, and immune lymph node cells. These studies will be described in the sections to follow.

PART II

Nephelometric Study of Reactions Between Tissue Extracts and Serum

Introduction

Some features of Bollag's nephelometric procedure were attractive (39). He added tissue extracts of increasing concentration from a doubling-dilution series to a constant amount of antiserum, measuring the scattered light perpendicular to the incident beam after each addition. In the absence of a reaction between the components of the mixture, a normal dilution curve should be obtained, when the measured light values were plotted against the concentration. This procedure seemed to provide a baseline and, depending on the stability and sensitivity of the measuring apparatus, any deviation from the expected curve would indicate the occurrence of a reaction.

Furthermore, it was evident that nephelometry under ideal conditions should disclose aggregation reactions in their earliest stages, when only two or more molecules combined. These early reactions would probably be more specific than the later stages when visible precipitation was incipient. Relatively short reaction times would allow a further advantage if unstable reactants, which might quickly lose their activity in vitro, were involved.

The foregoing considerations and those pointed out earlier in the introductory section led to the following attempt to confirm and

extend Bollag's findings.

The sensitivity of the method seemed to be sufficient to attach some significance to negative results if these were found. If no marked reactions were observed between the serum from immune animals and tissue extracts, this fact would further argue against the involvement of serum antibodies in the response of the recipient against the tissue grafts.

General Procedure

Pairs of rabbits were bled to provide normal serum samples. One animal of each pair was then selected as the donor of immunizing tissue and tissue extracts for testing. The other animal was designated as the recipient of the donor's tissues. The donor was subjected to one of several surgical procedures for the removal of tissues. An immunizing cell suspension was prepared from the tissues, and this was injected into the recipient. An extract was then prepared from the remainder of the tissue.

In testing, increments of an extract were added to a constant amount of serum, and the light scattered at 90° was measured for the mixture after each addition, by means of a light-scattering photometer.

Sera prepared from blood taken before, and at various intervals after, immunization were compared for their ability to react with various autologous and homologous tissue extracts, the criterion for the reaction being a deviation from the expected normal dilution curve.

Surgical Procedures

Adult albino rabbits were used in the investigations. The surgical procedures to which they were subjected included 1. skin homografting, 2. splenectomy, 3. nephrectomy, and 4. cecectomy. Nembutal was used for initial sedation, and the hair was clipped from the operative field. The skin was scrubbed with soap, rinsed, and tincture of Zephiran diluted 1:1000 was applied for disinfection. Aseptic conditions were maintained throughout surgery. Ether was used to supplement Nembutal as required for anesthesia.

Skin grafting The method used was that of Medawar (5). The skin was removed down to the level of the panniculus carnosis muscle over an area 6 cm. X 10 cm. on the lateral thorax, providing a recipient bed. Pinch grafts of 1 cm. diameter were removed from the skin covering the dorsum of the ear. Subcutaneous tissue was removed from the grafts as cleanly as possible, and the grafts were placed in Petri dishes on filter paper moistened with saline for cold storage. The grafts were applied in rows on the recipient bed without crowding, flattened on the bed, and sterile sulfadiazine powder was dusted over the entire site. This was followed by a dressing of Vaseline impregnated tulle, over which a gauze compression pad was placed. The dressing was held in place by a tight girth of plaster bandage.

Surgical removal of organs Conventional surgical procedures were followed. Emphasis was placed on the avoidance of sepsis, complete hemostasis, a minimum of injury to the animals, and repair of the

wounds. Careful attention was given to insuring the comfort of the animals and their recovery.

Splenectomy This was accomplished through a subcostal incision. Hemostasis was brought about by clamping off and ligating blood vessels. The spleen was then excised and placed in cold saline for storage. The incision was repaired in layers with interrupted 2-0 silk sutures. Sub-total splenectomy was performed by ligating the posterio-ventral half of the spleen with fine gut.

Nephrectomy Nephrectomy was approached through a diagonal lumbar incision at the outer border of the latissimus muscle. The kidney was dissected free of connective tissue down to its capsule, and the renal vessels were cleanly exposed. These were then clamped off, ligated, and the kidney excised.

Cecectomy Excision of the cecum was carried out through a midline, abdominal incision. The cecum was exposed and packed off to prevent contamination from intestinal contents in the event of leakage. Mesenteric vessels were ligated to a point about 1 cm. distal to the junction with the ileum. Two special intestinal clamps were used for sealing off the cecum, which was severed by cutting between the clamps. The organ was removed, placed in cold saline, spread open by cutting along its length, and washed free of intestinal contents in several changes of saline. It was then placed for storage in cold saline containing 100 mgs. of streptomycin and 25,000 units of penicillin

per 100 ml. of solution. The clamped end of the remaining cecal stump was cleaned well with tincture of Zephiran (1:1000). A single continuous 4-0 gut suture was used to close the stump by first sewing a series of loops around the clamp, which was then cautiously removed as the loops were tightened. The end of the stump was then inverted by a series of Lembert sutures. The lumen of the rabbit cecum is wide and its wall is too fragile to permit simple ligation. The method employed here, while more tedious than simpler procedures never led to complications or post-operative mortality.

Preparation of Tissue Extracts and Sera

Tissue Extracts The excised organs were washed with saline, blotted dry on gauze, and weighed. A cell suspension for immunization, where this was required, was prepared by pressing a portion of the tissue through a 40 mesh stainless steel sieve into buffered physiological saline. This suspension was injected without delay as recorded in Table One. The remaining tissues were processed to provide extracts. Each gram of tissue was pressed through a sieve into 3 ml. of buffered physiological saline (0.85% NaCl buffered at $_{p}H$ 7.4 with 0.01 molar sodium phosphate). This suspension was centrifuged at 1100 RCF. for 5 minutes, and the supernatant solution was discarded. 7.5 ml. of distilled water was added to the sediment for each gram of tissue on the basis of the initial weight. The preparation was allowed to stand for one hour, and it was centrifuged at

2500 RCF. The supernatant solution was discarded. The sediment was washed in two changes of 5 ml. of distilled water per gram initial weight by resuspension and centrifugation. Following washing, the sediment was resuspended in the quantity of distilled water required to provide the final concentration desired, and it was then homogenized. In Series A, a Waring Blendor was used for this purpose, whereas a Virtis 45 micro-homogenizer was employed in Series B. In each series homogenization was continued until samples examined under the microscope were found to contain few remaining cells. After the homogenization less than 10% of the initial tissue volume could usually be sedimented at 2500 RCF. for 10 minutes. Microscopic examination disclosed some intact cells in the final residue, but the largest proportion was cellular debris and connective tissue stroma.

In Series A, the homogenate was clarified by centrifugation in a Servall angle centrifuge at 20,000 RCF. In Series B, the extracts were filtered through Seitz EK filter pads. Following clarification the extracts were stored in vials at -18°C. The temperature was never allowed to exceed 5°C. during the period of time required for the preparation of the extracts, except for very brief periods where room temperature was necessary, such as in weighing. The preparations were designated as full strength (FS) extracts although the dilution varied among the preparations. The strengths of the extracts are recorded in Table Two as the number of milliliters containing the extract from one gram of tissue. Thus, for example, if the concentration of full

strength (FS) extract is listed as 1:20, twenty milliliters of the solution would contain the extracted material from one gram of the tissue. Further dilutions of full strength extract are based on volume, thus 1:1 indicates the full strength antigen, 1:2 indicates the addition of an equal volume of diluent to the full strength extract, etc.

Sera Blood was withdrawn from the marginal ear vein, allowed to stand at room temperature (25°C.) for one hour, and then centrifuged at 1100 RCF. for 10 minutes at 5°C. The serum was poured from the clot, and recentrifuged to remove the last trace of cells. The serum was then either used fresh or stored in vials at -18°C. The times of bleeding for test serum preparation, along with other details of the individual tests, are recorded in Table Three.

Turbidity Measurements

Two different instruments were used for the measurement of the scattered light at 90°. In Series A experiments an Oster-Aminco light scattering photometer was used, but the Brice-Phoenix dual photomultiplier photometer was used for Series B. Each instrument follows the same basic arrangement (Fig. 24). Light from an 85-100 watt mercury lamp (LS) is collimated through a series of lenses (L1-2) and diaphragms (D1-2-5). Provision is made for the insertion of neutral density filters and wavelength isolation filters in the incident light beam at F1-F2. The light beam illuminates the cell (SC) containing the scattering solution under study. Scattered

light is picked up by a photomultiplier tube (P2) which is provided for screening by a polarizer (POL) and fluorescence filter (F3). The Oster-Aminco instrument is equipped with only a single photocell (P2) which can be rotated in an arc around the center of the solution cell, to measure the light scattered at any desired angle. The Brice-Phoenix dual photomultiplier photometer has two photocells (PL-2) at a fixed angle of 90° to each other, which together swing around an arc about the center of the solution cell.

The dual photomultiplier photometer has a marked advantage over the single photocell apparatus. Even though both instruments were provided with line voltage regulators, the stabilization these pro-vided was insufficient to maintain the brightness of the light source constant within 1% of the scale reading which was the instrumental error tolerated in these experiments. To accomplish stability in the single photocell instrument, the line voltage was held constant by manual adjustment of a Variac inserted at the source. At the time of reading, the line voltage was monitored on an expanded scale voltmeter, and corrected to a preselected value.

In the dual photomultiplier arrangement, fluctuations in the brightness of the light source are without significance. In the arrangement used here, light emitted from the solution cell at 0° and 90° falls on P1 and P2, respectively. These two photocells generate voltages proportional to the intensity of the light striking

their cathodes. The ratio-proportionator is a servo-device which provides a voltage output proportional to the ratio of two voltages fed into it. The output voltage of P2 was entered as the denominator, and the output voltage of P1 was entered as the numerator. The ratio-proportionator is provided for arbitrary selection of the proportionality constant k, which adjusts the output voltage to a convenient value for recording. The intensity of the light emitted at 0° from the solution cell includes the transmitted component I_s^o. Only scattered light $I_s 90^\circ$ is emitted at 90°. The quantity measured at the recorder is thus: $[(I_t + I_s^o)/I_s 90^\circ]$. k. In practice, the value of k is adjusted to give a maximal excursion of the recorder for changes in the turbidity of the solution expected in the particular experiment.

From considerations brought out later in the theoretical treatment, it can be shown that the ratio $(I_t + I_s^o)/I_s 90^\circ$ is characteristic of the solution in the scattering cell, and independent of the brightness of the illuminating beam. This conclusion was verified by deliberately introducing variations in the brightness of the light source; these did not affect the recorder readings.

Procedure for Testing Immediately before each run, the reagents were filtered into dust-free vessels for dilution and testing. In Series A Corning F sintered glass filters were used for this purpose, whereas Millipore HA membranes fitted into syringe adapters were used in Series B. The latter was more convenient. After filtration, tissue

extracts were diluted in appropriate series. The serum dilution was placed in the solution cell, and the instrument was adjusted for amplification, recorder excursion, and dark current compensation. Increments of tissue extract were then added to the serum, and after stirring and equilibration the light scattering value was read after each addition. Readings were continued after each addition until there was no appreciable change in one minute. For the reactions studied here, equilibration was usually reached in less than 5 minutes. As the end point, a drift of less than $\pm$ 0.5% of the scale length in one minute, was selected. This was the criterion routinely followed in the measurements. The reagents were maintained in an ice bath during the preparation and measurements. The reaction mixture in the solution cell was not thermostated in Series A where the temperature of the solution cell varied little from 25°C. In Series B, it was necessary to cool the cell compartment to maintain this temperature. If, during the period of measurement, dust particles found their way into the solution in the test cell random fluctuations were noted in the recorder reading. In this event the solution was discarded, and the test was repeated.

Results

The immunizing routine of each recipient is listed in Table 1. The origin of the tissue extracts used for testing and their full strength concentrations are given in Table 2. Table 3 is a compilation of the individual tests. The first two items designate the series (A or B) and the run number. Item 3 gives the origin of the tissue extract used in the run and the designation of the rabbit donor. Item 4 indicates whether the extract was absorbed, and the strength of the 1:1 dilution used in the test. This is the concentration of extract used after additions from the dilution series, if a dilution series was used in the run, and may or may not correspond to the full strength (FS) of the extract. Item 5 describes the dilution series. Normally, doubling dilutions were used and the abbreviation (DDS) indicates this dilution series by the number of tubes through which the dilution was carried. For example, the first tube added of an 11 tube series would be the 1:1024 dilution. The only departure from the use of a doubling dilution series was made in some Series B runs, where additions were made from a "multiple dilution series" (MDS). This series consisted of five tenfold dilutions from 1:1 to 1:10,000. Five 0.2 ml. additions were made from the 1:10,000 tube, followed by five additions from the 1:1000 tube, etc., through the 1:1 tube. Item 6 gives the concentration of the serum used in the test and the animal from which it was taken. Item 7 denotes the time after initial immunization when the blood

sample was taken. Item 8 gives the initial immunizing tissue and Item 9 any subsequent immunization. Item 10 describes the sort of combination represented in the test. AUTO refers to a titration of the serum with tissue extract from the same animal. IMM-DO indicates a combination of a presumably immune recipient's serum with the extract of tissue from the individual against which the recipient was immunized, the donor. CONT-DO indicates the same sort of combination as IMM-DO except that the serum used was taken before immunization, hence it cannot be regarded as an immune serum. IMM-XR, indicates the combination of an immune serum with tissues from an animal other than the donor or recipient. Cross reactions would be expected to appear in this combination, hence the XR designation. CONT-XR is the designation given to a combination in which serum taken before immunization was reacted against tissue extract from an animal other than its future donor. Finally, Item 11 is reserved to indicate the result of the run. A positive symbol in this column indicates that some deviation from the expected normal dilution curve was observed, hence this run may be further examined to evaluate the significance of the noted deviation. A negative symbol indicates that no significant reaction was evident for the run. Following Table 3 the runs are figured. The curve passes through all the points recorded.

Table 1

Immunization of Rabbits

Recipient	Immunization and Route of Injection	Donor	Time (Days)
			Series A
1	500 mg. spleen cells, i.p.	5	0
5	500 mg. spleen cells, i.p.	1	0
8	500 mg. spleen cells, i.p.	9	0
	multiple skin grafts,	9	13
9	500 mg. spleen cells, i.p.	8	0
	multiple skin grafts,	8	13
43	1 gm. cecal cells, i.p.	42	0
47	1 gm. cecal cells, i.p.	46	0
45	2 gm. cecal cells, i.p.	44	0
49	2 gm. cecal cells, i.p.	48	0
			Series B
B	500 mg. cecal cells, s.q.	A	0
	multiple skin grafts,	A	15
D	500 mg. cecal cells, s.q.	C	0
	multiple skin grafts,	C	15
F	500 mg. cecal cells, s.q.	E	0
	multiple skin grafts,	E	8
	500 mg. kidney cells, s.q.	E	22
J	multiple skin grafts,	I	0
	multiple skin grafts,	L	19
	500 mg. kidney cells, s.q.	I	19
L	multiple skin grafts,	K	0
	multiple skin grafts,	K	19
	500 mg. kidney cells, s.q.	K	19

Table 2

Tissue Extracts

Tissue	Donor	Concentration of Full Strength Extract*
		Series A
spleen	5	1:10
spleen	4	1:10
spleen	1	1:10
kidney	9	1:8
kidney	8	1:8
lung	8	1:10
lung	9	1:10
spleen	8	1:8
liver	8	1:4
liver	9	1:4
cecum	42	1:10
cecum	46	1:10
cecum	44	1:20
cecum	48	1:20
		Series B
cecum	A	1:10
cecum	C	1:10
spleen	A	1:20
spleen	C	1:20
cecum	E	1:10
cecum	G	1:10
kidney	E	1:8
kidney	G	1:8
spleen	I	1:20
spleen	K	1:20
kidney	I	1:30
kidney	K	1:30

* See explanation of this concentration, text p. 21.

Table 3

1. Series (See text p. 26–27 for explanation of items.)
2. Number of test run
3. Tissue extract and animal designation
4. Absorbed (abs) or non-absorbed (na) and concentration of 1:1 extract
5. Dilution series used: type and number of tubes
6. Animal from which serum was taken and its strength
7. Time serum was taken after initial immunization (days)
8. Tissue used for initial immunization
9. Subsequent immunization with tissue listed at time after initial imm.
10. Combination
11. Deviation from *normal* dilution curve

1. Series
2. Number of test run
3. Tissue extract and animal designation
4. Absorbed (abs) or non-absorbed (na) and concentration of 1:1 extract
5. Dilution series used: type and number of tubes
6. Animal from which serum was taken and its strength
7. Time serum was taken after initial immunization (days)
8. Tissue used for initial immunization
9. Subsequent immunization with tissue listed at time after initial imm.
10. Combination
11. Deviation from normal dilution curve

Table 3 (cont.)

1.	A	A	A	A
2.	1	2	3	4
3.	5 spleen	5 spleen	5 spleen	4 spleen
4.	(na) 1:10	(na) 1:20	(na) 1:20	(na) 1:20
5.	1:1	1:1	1:1	1:1
6.	1 serum 1:2	1 serum 1:4	1 serum 1:4	1 serum 1:4
7.	13 days	13 days	13 days	13 days
8.	spleen	spleen	spleen	spleen
9.				
10.	IMM-DO	IMM-DO	IMM-DO	IMM-XR
11.	+	−	+	−

1.	A	A	A	A
2.	5	8	9	10
3.	1 spleen	9 kidney	8 kidney	8 kidney
4.	(na) 1:20	(na) 1:8	(na) 1:8	(na) 1:8
5.	1:1	DDS (10)	DDS (10)	DDS (10)
6.	5 serum 1:4	8 serum 1:1	8 serum 1:1	9 serum 1:1
7.	13 days	21 days	21 days	21 days
8.	spleen	spleen	spleen	spleen
9.		skin 13 days	skin 13 days	skin 13 days
10.	IMM-DO	IMM-DO	AUTO	IMM-DO
11.	+	−	−	−

Table 3 (cont.)

1.	A	A	A	A
2.	11	12	15	16
3.	8 lung	8 lung	9 lung	9 lung
4.	(na) 1:10	(na) 1:10	(na) 1:10	(na) 1:10
5.	DDS (20)	DDS (20)	DDS (20)	DDS (20)
6.	9 serum 1:1	8 serum 1:1	8 serum 1:2	8 serum 1:4
7.	21 days	21 days	21 days	21 days
8.	spleen	spleen	spleen	spleen
9.	skin 13 days	skin 13 days	skin 13 days	skin 13 days
10.	IMM-DO	AUTO	IMM-DO	IMM-DO
11.	+	−	+	+

1.	A	A	A	A
2.	17	13	18	19
3.	9 lung	8 spleen	8 liver	8 liver
4.	(na) 1:10	(na) 1:8	(na) 1:4	(na) 1:4
5.	DDS (20)	DDS (20)	DDS (20)	DDS (20)
6.	8 serum 1:4	9 serum 1:2	9 serum 1:2	9 serum 1:16
7.	21 days	21 days	21 days	21 days
8.	spleen	spleen	spleen	spleen
9.	skin 13 days	skin 13 days	skin 13 days	skin 13 days
10.	IMM-DO	IMM-DO	IMM-DO	IMM-DO
11.	+	+	+	+

Table 3 (cont.)

1.	A	A	A	A
2.	20	21	22	23
3.	8 liver	9 liver	9 liver	42 cecum
4.	(na) 1:4	(abs) 1:8	(abs) 1:8	(na) 1:10
5.	DDS (20)	DDS (20)	DDS (20)	DDS (10)
6.	8 serum 1:2	8 serum 1:1	8 serum 1:1	42 serum 1:1
7.	21 days	21 days	21 days	0
8.	spleen	spleen	spleen	
9.	skin 13 days	skin 13 days	skin 13 days	
10.	AUTO	IMM-DO	IMM-DO	AUTO
11.	+	+	+	−

1.	A	A	A	A
2.	24	27	25	31
3.	42 cecum	46 cecum	42 cecum	42 cecum
4.	(na) 1:10	(na) 1:10	(abs) 1:20	(abs) 1:20
5.	DDS (10)	DDS (10)	DDS (10)	DDS (10)
6.	42 serum 1:1	46 serum 1:1	43 serum 1:2	43 serum 1:2
7.	0	0	0	7 days
8.				cecum
9.				
10.	AUTO	AUTO	CONT-DO	IMM-DO
11.	−	−	−	−

Table 3 (cont.)

1.	A	A	A	A
2.	26	32	28	34
3.	42 cecum	42 cecum	46 cecum	46 cecum
4.	(abs) 1:20	(abs) 1:20	(abs) 1:20	(abs) 1:20
5.	DDS (10)	DDS (10)	DDS (10)	DDS (10)
6.	47 serum 1:2	47 serum 1:2	43 serum 1:2	43 serum 1:2
7.	0	7 days	0	7 days
8.		cecum		cecum
9.				
10.	CONT-XR	IMM-XR	CONT-XR	IMM-XR
11.	−	+	−	−

1.	A	A	A	
2.	29	33	37	39
3.	46 cecum	46 cecum	42 cecum	42 cecum
4.	(abs) 1:20	(abs) 1:20	(abs) 1:20	(abs) 1:20
5.	DDS (10)	DDS (10)	DDS (10)	DDS (10)
6.	47 serum 1:2	47 serum 1:2	43 serum 1:10	43 serum 1:10
7.	0	7 days	13 days	18 days
8.		cecum	cecum	cecum
9.				
10.	CONT-DO	IMM-DO	IMM-DO	IMM-DO
11.	+	+	+	+

Table 3 (cont.)

1.	A	A	A	A
2.	38	41	43	44
3.	46 cecum	46 cecum	46 cecum	44 cecum
4.	(na) 1:10	(abs) 1:20	(abs) 1:20	(abs) 1:20
5.	DDS (10)	DDS (10)	DDS (10)	1:1 .2 ml.
6.	47 serum 1:10	43 serum 1:10	47 serum 1:10	45 serum 1:10
7.	13 days	13 days	0	0
8.	cecum	cecum		
9.				
10.	IMM-DO	IMM-XR	CONT-DO	CONT-DO
11.	−	−	+	+

1.	A	A	A	A
2.	45	46	47	49
3.	48 cecum	48 cecum	48 cecum	44 cecum
4.	(abs) 1:40	(abs) 1:40	(abs) 1:20	(abs) 1:20
5.	DDS (10)	DDS (10)	DDS (10)	DDS (10)
6.	49 serum 1:10	45 serum 1:10	49 serum 1:10	45 serum 1:10
7.	0	0	7 days	7 days
8.			cecum	cecum
9.				
10.	CONT-DO	CONT-XR	IMM-DO	IMM-DO
11.	+	−	+	+

Table 3 (cont.)

1.	B	B	B	B
2.	3	4	5	6
3.	A cecum	A cecum	C cecum	C cecum
4.	(abs) 1:10	(abs) 1:10	(abs) 1:10	(abs) 1:10
5.	MDS	MDS	MDS	MDS
6.	B serum 1:10	D serum 1:10	B serum 1:10	D serum 1:10
7.	0	0	0	0
8.				
9.				
10.	CONT-DO	CONT-XR	CONT-XR	CONT-DO
11.	—	—	—	—

1.	B	B	B	B
2.	7	8	9	10
3.	C cecum	C cecum	A cecum	A cecum
4.	(abs) 1:10	(abs) 1:10	(abs) 1:10	(abs) 1:10
5.	MDS	MDS	MDS	MDS
6.	D serum 1:10	B serum 1:10	B serum 1:10	D serum 1;10
7.	6 days	6 days	6 days	6 days
8.	cecum	cecum	cecum	cecum
9.				
10.	IMM-DO	IMM-XR	IMM-DO	IMM-XR
11.	—	—	—	—

Table 3 (cont.)

1.	B	B	B	B
2.	11	12	13	14
3.	C cecum	C cecum	A cecum	A cecum
4.	(abs) 1:10	(abs) 1:10	(abs) 1:10	(abs) 1:10
5.	DDS (12)	DDS (12)	MDS	1:1 .1 ml.
6.	D serum 1:5	D serum 1:5	B serum 1:5	B serum 1:5
7.	9 days	9 days	9 days	9 days
8.	cecum	cecum	cecum	cecum
9.				
10.	IMM-DO	IMM-DO	IMM-DO	IMM-DO
11.	+	+	+	+

1.	B	B	B	B
2.	15	16	17	18
3.	A cecum	C cecum	A cecum	C cecum
4.	(abs) 1:10	(abs) 1:10	(abs) 1:10	(abs) 1:10
5.	1:1 .1 ml.	MDS	DDS (11)	DDS (11)
6.	D serum 1:5	D serum 1:10	B serum 1:1	D serum 1:1
7.	9 days	13 days	13 days	13 days
8.	cecum	cecum	cecum	cecum
9.				
10.	IMM-XR	IMM-DO	IMM-DO	IMM-DO
11.	-	+	-	+

Table 3 (cont.)

1.	B	B	B	B
2.	21	24	25	26
3.	A cecum	A cecum	A spleen	A spleen
4.	(abs) 1:10	(abs) 1:10	(na) 1:20	(na) 1:20
5.	DDS (11)	DDS (11)	DDS (11)	DDS (11)
6.	B serum 1:5	B serum 1:5	B serum 1:5	B serum 1:5
7.	17 days	21 days	21 days	24 days
8.	cecum	cecum	cecum	cecum
9.	skin 15 days	skin 15 days	skin 15 days	skin 15 days
10.	IMM-DO	IMM-DO	IMM-DO	IMM-DO
11.	—	—	—	+

1.	B	B	B	B
2.	27	28	19	20
3.	A spleen	A spleen	E cecum	G cecum
4.	(na) 1:20	(na) 1:20	(na) 1:10	(na) 1:10
5.	DDS (11)	1:1	DDS (11)	DDS (11)
6.	B serum 1:10	B serum 1:5	F serum 1:5	H serum 1:5
7.	24 days	24 days	5 days	5 days
8.	cecum	cecum	cecum	cecum
9.	skin 15 days	skin 15 days		
10.	IMM-DO	IMM-DO	IMM-DO	IMM-DO
11.	+	+	—	—

Table 3 (cont.)

1.	B	B	B	B
2.	22	23	29	30
3.	E cecum	G cecum	E cecum	E cecum
4.	(na) 1:10	(na) 1:10	(na) 1:10	(na) 1:10
5.	DDS (11)	DDS (11)	DDS (11)	DDS (11)
6.	F serum 1:5	H serum 1:5	F serum 1:5	F serum 1:5
7.	11 days	11 days	15 days	15 days
8.	cecum	cecum	cecum	cecum
9.	skin 8 days	skin 8 days	skin 8 days	skin 8 days
10.	IMM-DO	IMM-DO	IMM-DO	IMM-DO
11.	−	−	+	−

1.	B	B	B	B
2.	31	34	35	44
3.	E cecum	G cecum	G cecum	E kidney
4.	(na) 1:10	(na) 1:10	(na) 1:10	(na) 1:8
5.	DDS (11)	DDS (11)	DDS (11)	DDS (11)
6.	F serum 1:5	H serum 1:5	H serum 1:20	F serum 1:5
7.	15 days	18 days	18 days	28 days
8.	cecum	cecum	cecum	cecum
9.	skin 8 days	skin 8 days	skin 8 days	skin 8 days kidney 22 days
10.	IMM-DO	IMM-DO	IMM-DO	IMM-DO
11.	+	−	−	+

Table 3 (cont.)

1.	B	B	B	B
2.	45	46	47	48
3.	E kidney	E kidney	G kidney	G kidney
4.	(na) 1:8	(na) 1:8	(na) 1:8	(na) 1:8
5.	DDS (11)	DDS (11)	DDS (11)	DDS (11)
6.	F serum 1:5	F serum 1:5	F serum 1:5	H serum 1:5
7.	28 days	28 days	28 days	28 days
8.	cecum	cecum	cecum	cecum
9.	skin 8 days kidney 22 days	skin 8 days kidney 22 days	skin 8 days kidney 22 days	skin 8 days kidney 22 days
10.	IMM-DO	IMM-DO	IMM-XR	IMM-DO
11.	+	+	+	+

1.	B	B	B	B
2.	49	51	52	36
3.	G kidney	G kidney	E kidney	I spleen
4.	(na) 1:8	(na) 1:8	(na) 1:8	(na) 1:20
5.	DDS (11)	DDS (11)	DDS (11)	DDS (11)
6.	H serum 1:5	H serum 1:5	F serum 1:5	J serum 1:5
7.	28 days	28 days	28 days	9 days
8.	cecum	cecum	cecum	skin
9.	skin 8 days kidney 23 days	skin 8 days kidney 23 days	skin 8 days kidney 23 days	
10.	IMM-DO	IMM-DO	IMM-DO	IMM-DO
11.	+	−	−	+

Table 3 (cont.)

1.	B	B	B	B
2.	37	38	39	40
3.	I spleen	K spleen	I spleen	K spleen
4.	(na) 1:20	(na) 1:20	(na) 1:20	(na) 1:20
5.	DDS (11)	DDS (11)	DDS (11)	DDS (11)
6.	J serum 1:5	L serum 1:5	L serum 1:5	J serum 1:5
7.	9 days	9 days	9 days	9 days
8.	skin	skin	skin	skin
9.				
10.	IMM-DO	IMM-DO	IMM-XR	IMM-XR
11.	+	—	—	—

1.	B	B	B	B
2.	41	42	43	53
3.	I spleen	I spleen	I spleen	I spleen
4.	(na) 1:20	(na) 1:20	(na) 1:20	(na) 1:20
5.	DDS (11)	DDS (11)	DDS (11)	DDS (11)
6.	J serum 1:5	J serum 1:5	J serum 1:5	J serum 1:5
7.	9 days	9 days	9 days	9 days
8.	skin	skin	skin	skin
9.				
10.	IMM-DO	IMM-DO	IMM-DO	IMM-DO
11.	+	—	—	—

Table 3 (cont.)

1.	B	B	B	B
2.	54	55	56	57
3.	I kidney	I kidney	K kidney	K kidney
4.	(na) 1:30	(na) 1:30	(na) 1:30	(na) 1:30
5.	DDS (11)	DDS (11)	DDS (11)	DDS (11)
6.	J serum 1:5	J serum 1:5	L serum 1:5	L serum 1:5
7.	21 days	21 days	21 days	21 days
8.	skin	skin	skin	skin
9.	kidney 19 days	kidney 19 days	kidney 19 days	kidney 19 days
10.	IMM-DO	IMM-DO	IMM-DO	IMM-DO
11.	+	−	+	−

1.	B	B	B	B
2.	58	59	60	61
3.	I kidney	K kidney	I kidney	K kidney
4.	(na) 1:30	(na) 1:30	(na) 1:30	(na) 1:30
5.	DDS (11)	DDS (11)	DDS (11)	DDS (11)
6.	J serum 1:5	L serum 1:5	J serum 1:5	L serum 1:5
7.	23 days	23 days	23 days	23 days
8.	skin	skin	skin	skin
9.	kidney 19 days	kidney 19 days	kidney 19 days	kidney 19 days
10.	IMM-DO	IMM-DO	IMM-DO	IMM-DO
11.	−	−	−	−

Table 3 (cont.)

1.	B	B	B
2.	62	63	64
3.	I kidney	K kidney	K kidney
4.	(na) 1:30	(na) 1:30	(na) 1:30
5.	DDS (11)	DDS (11)	DDS (11)
6.	J serum 1:5	L serum 1:5	L serum 1:5
7.	23 days	23 days	23 days
8.	skin	skin	skin
9.	kidney 19 days	kidney 19 days	kidney 19 days
10.	IMM-DO	IMM-DO	IMM-DO
11.	—	—	—

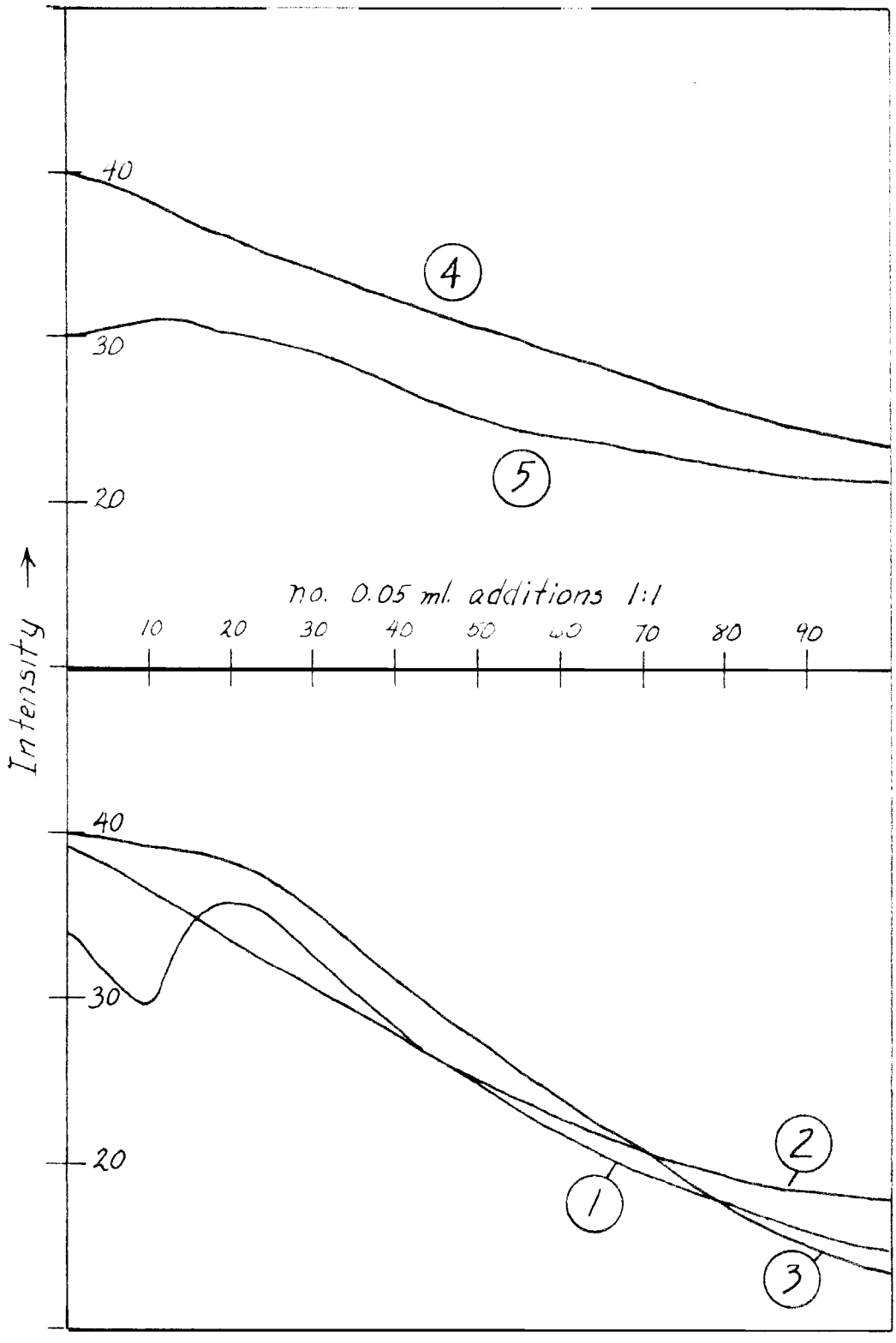

40
30
20
4
5
Intensity
no. 0.05 ml. additions 1:1
10
20
30
40
50
60
70
80
90
40
30
20
1
2
3

Figure 1

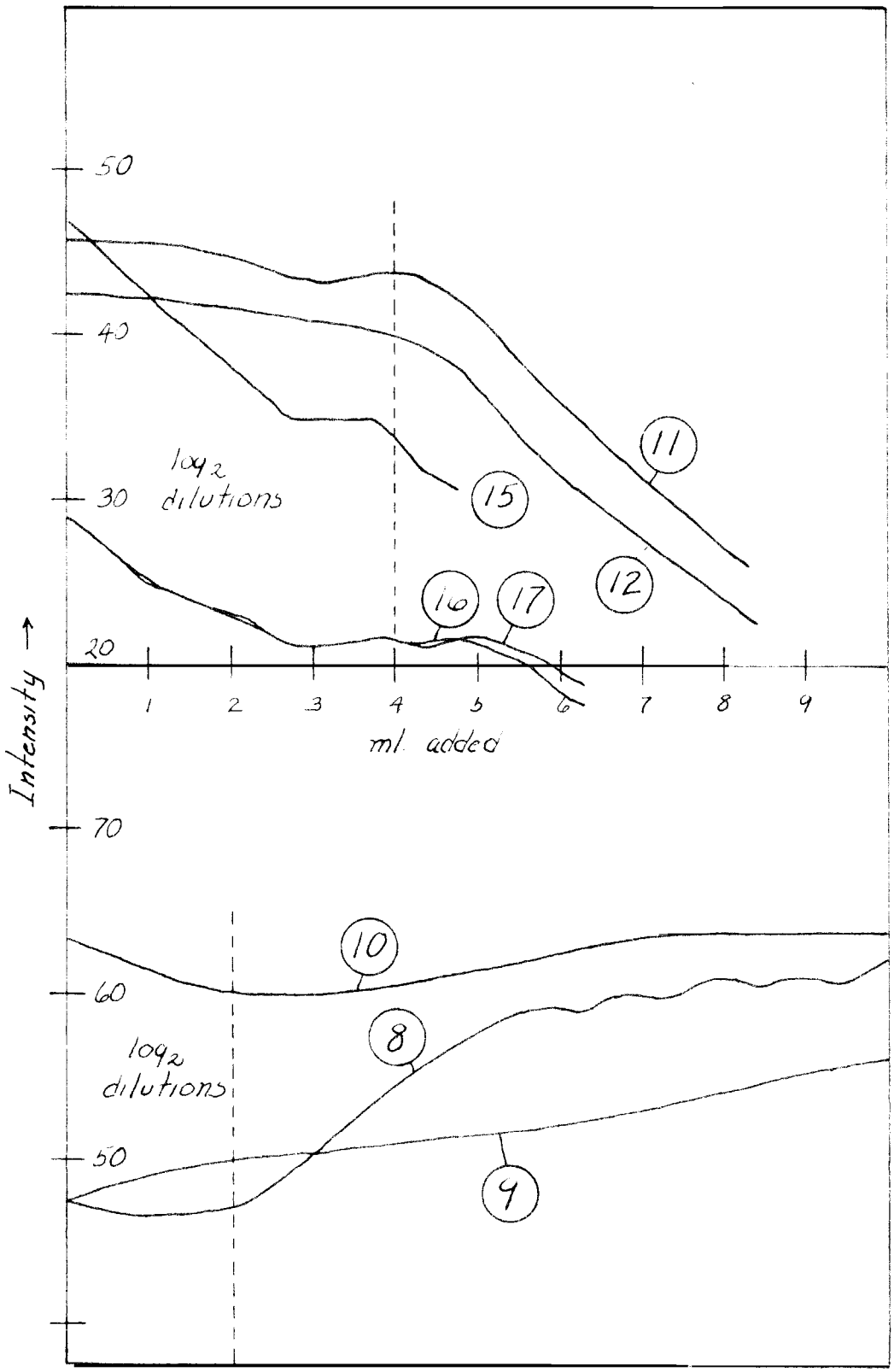

50
40
30
20
log2 dilutions
11
15
12
16
17
1
2
3
4
5
6
7
8
9
ml added
Intensity →
70
60
50
10
8
9
log2 dilutions

Figure 2

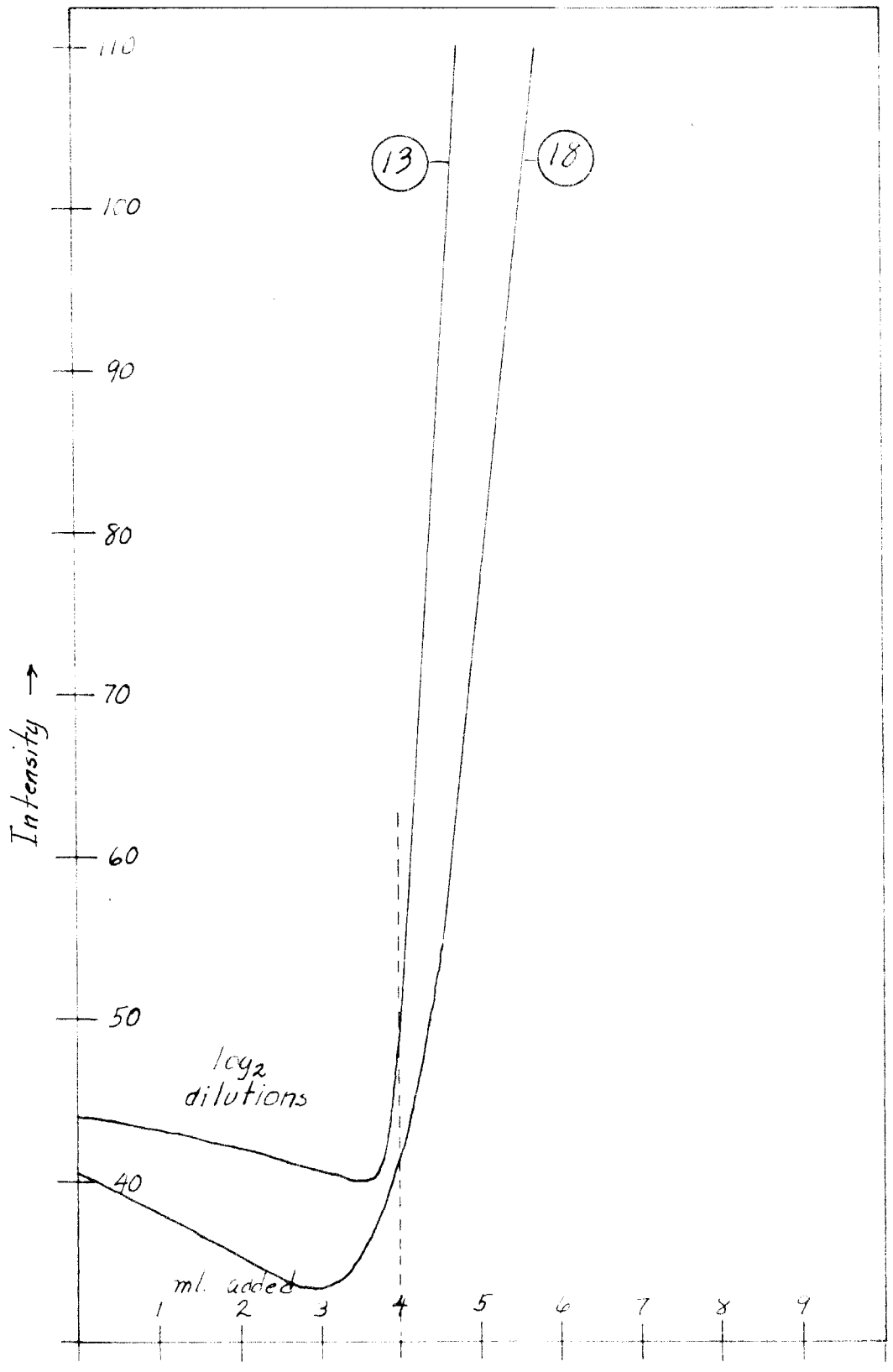

110
100
90
80
70
60
50
40
Intensity
13
18
log2 dilutions
ml. added
1
2
3
4
5
6
7
8
9

Figure 3

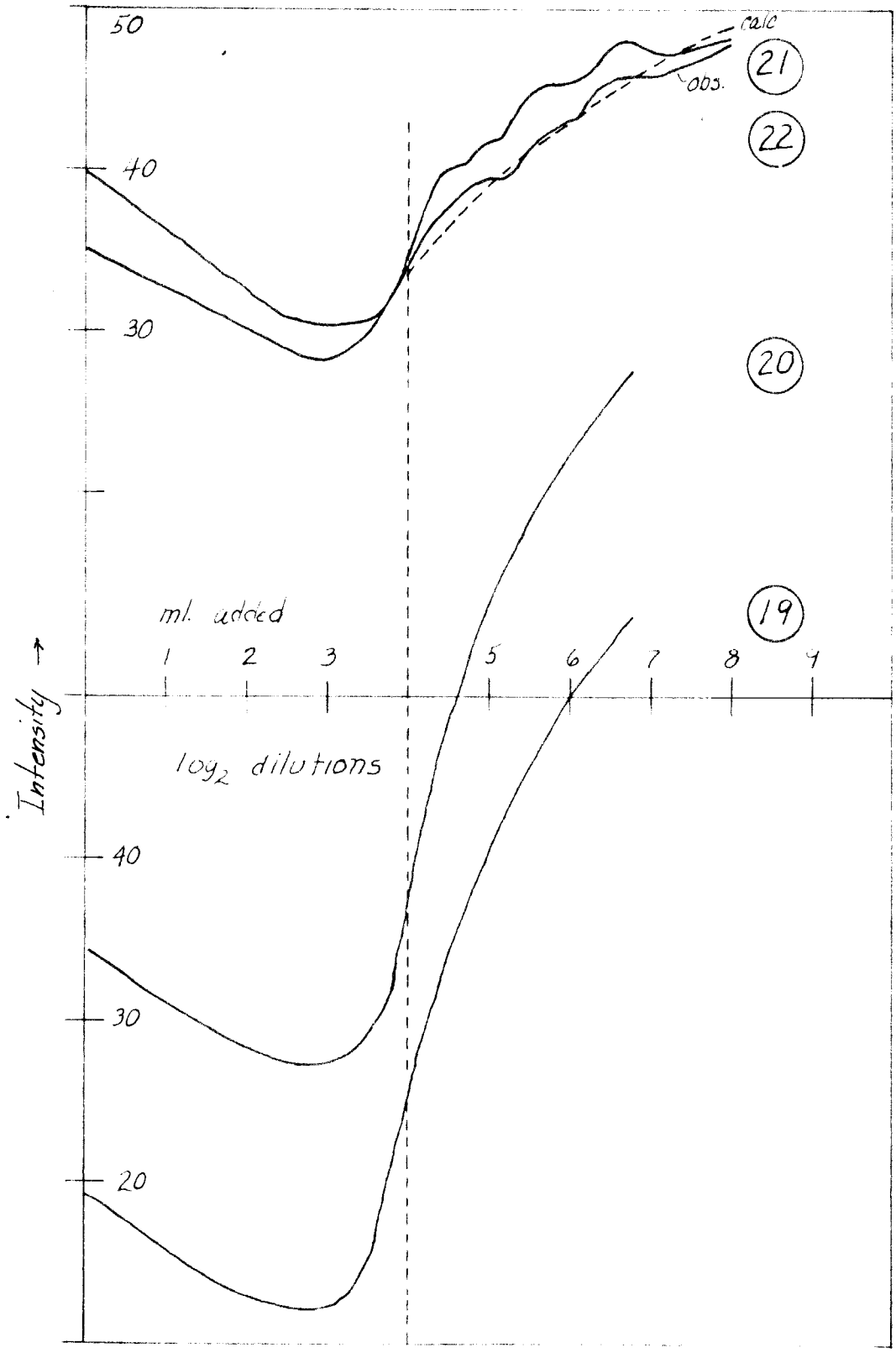

50
40
30
40
30
20
calc
obs.
21
22
20
19
ml. added
1
2
3
5
6
7
8
4
Intensity →
log2 dilutions

Figure 4

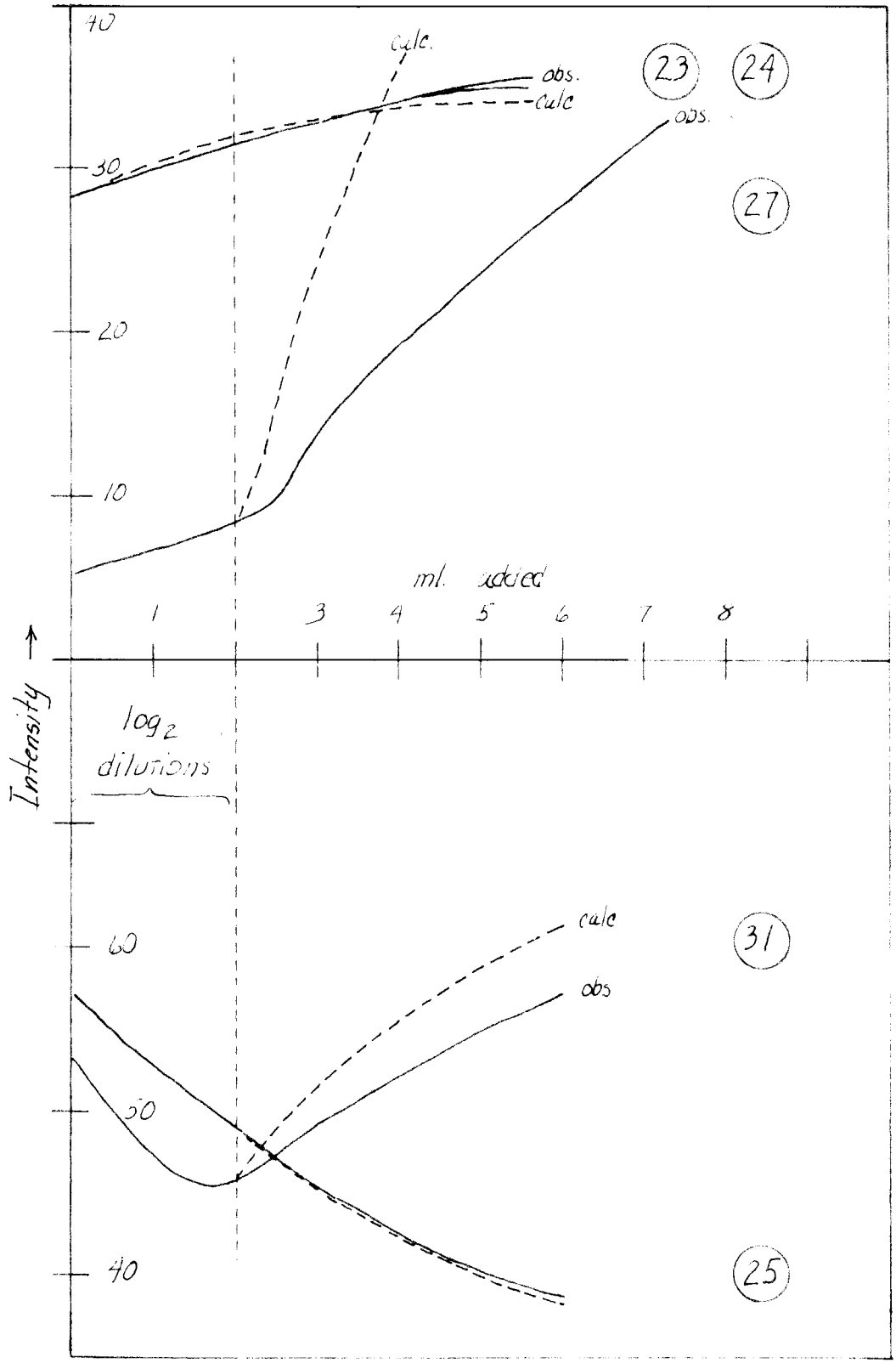

40
calc.
obs.
calc
23
24
obs.
30
27
20
10
ml. added
1
3
4
5
6
7
8
Intensity
log2 dilutions
60
calc
31
obs
50
40
25

Figure 5

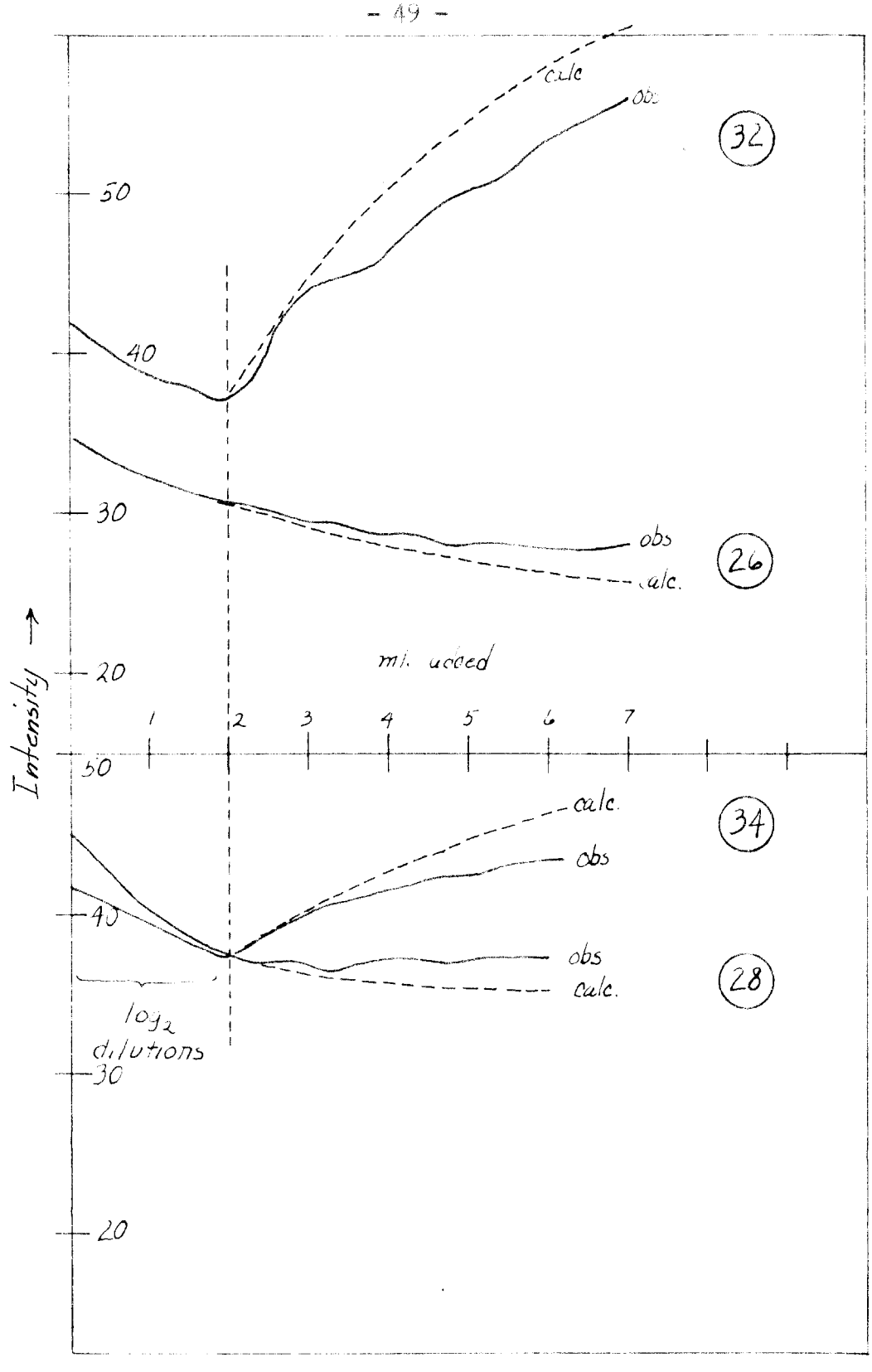

calc
obs
32
50
40
30
obs
calc.
26
Intensity
20
ml. added
1
2
3
4
5
6
7
50
calc.
34
obs
40
obs
calc.
28
log2 dilutions
30
20

Figure 6

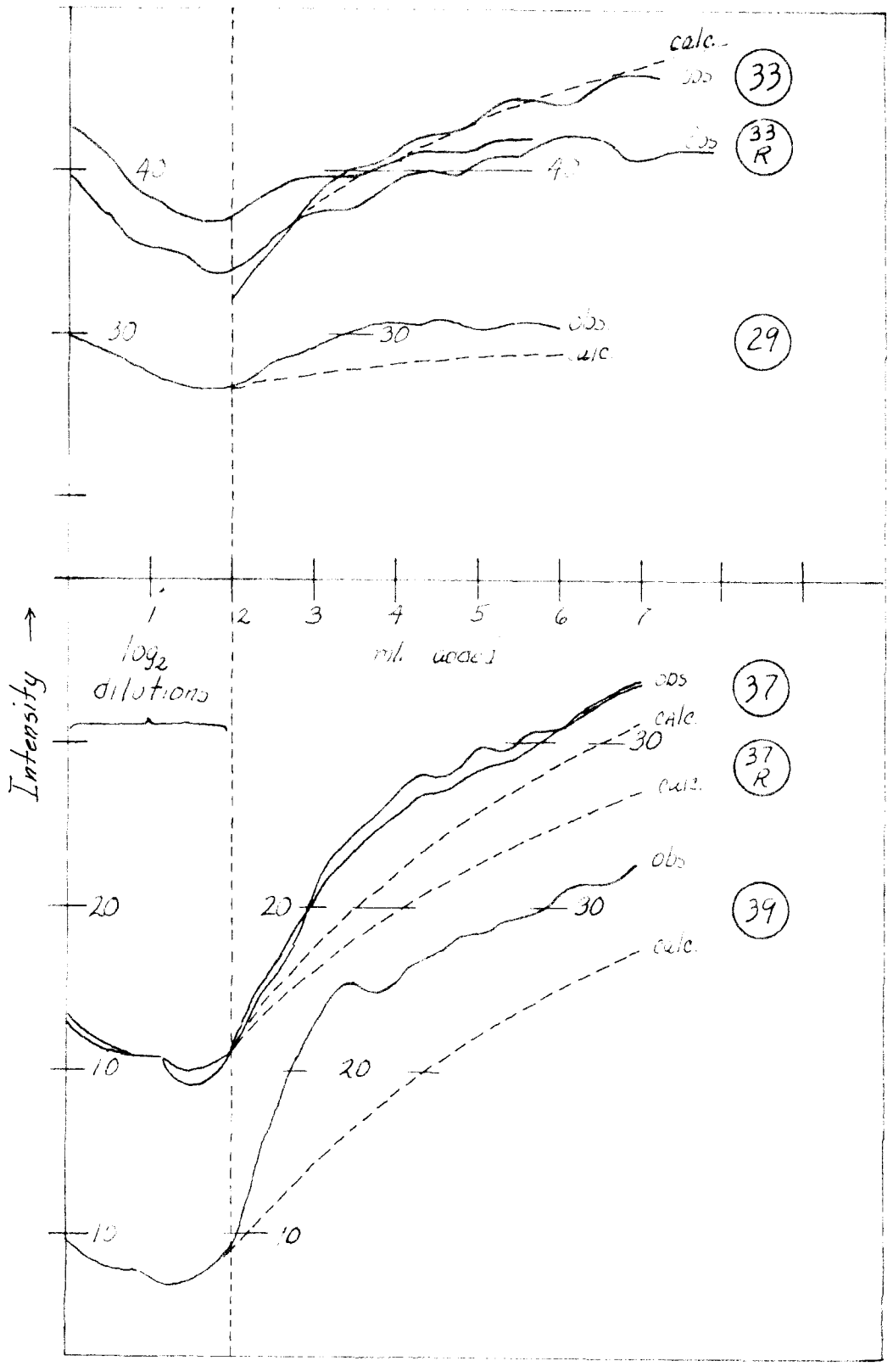

calc.
obs
33
33 R
40
40
obs
29
30
30
obs
calc
1
2
3
4
5
6
7
log2 dilutions
ml. used
Intensity
37
obs
calc.
30
37 R
calc.
obs
39
20
20
30
calc.
10
20
10
10

Figure 7

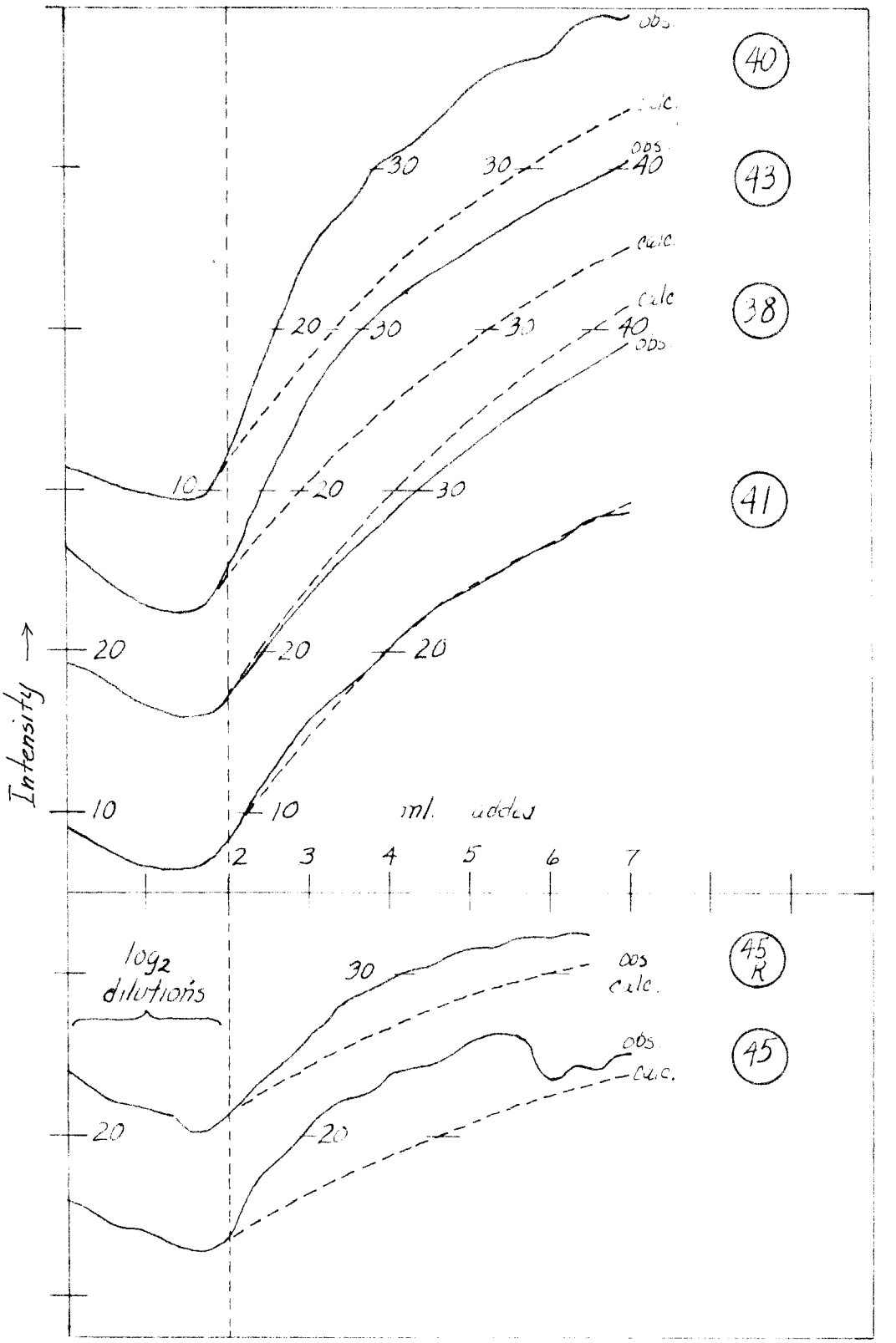

Intensity
ml. added
2
3
4
5
6
7
10
20
30
40
obs.
calc.
40
43
38
41
45 R
45
log2 dilutions

Figure 8

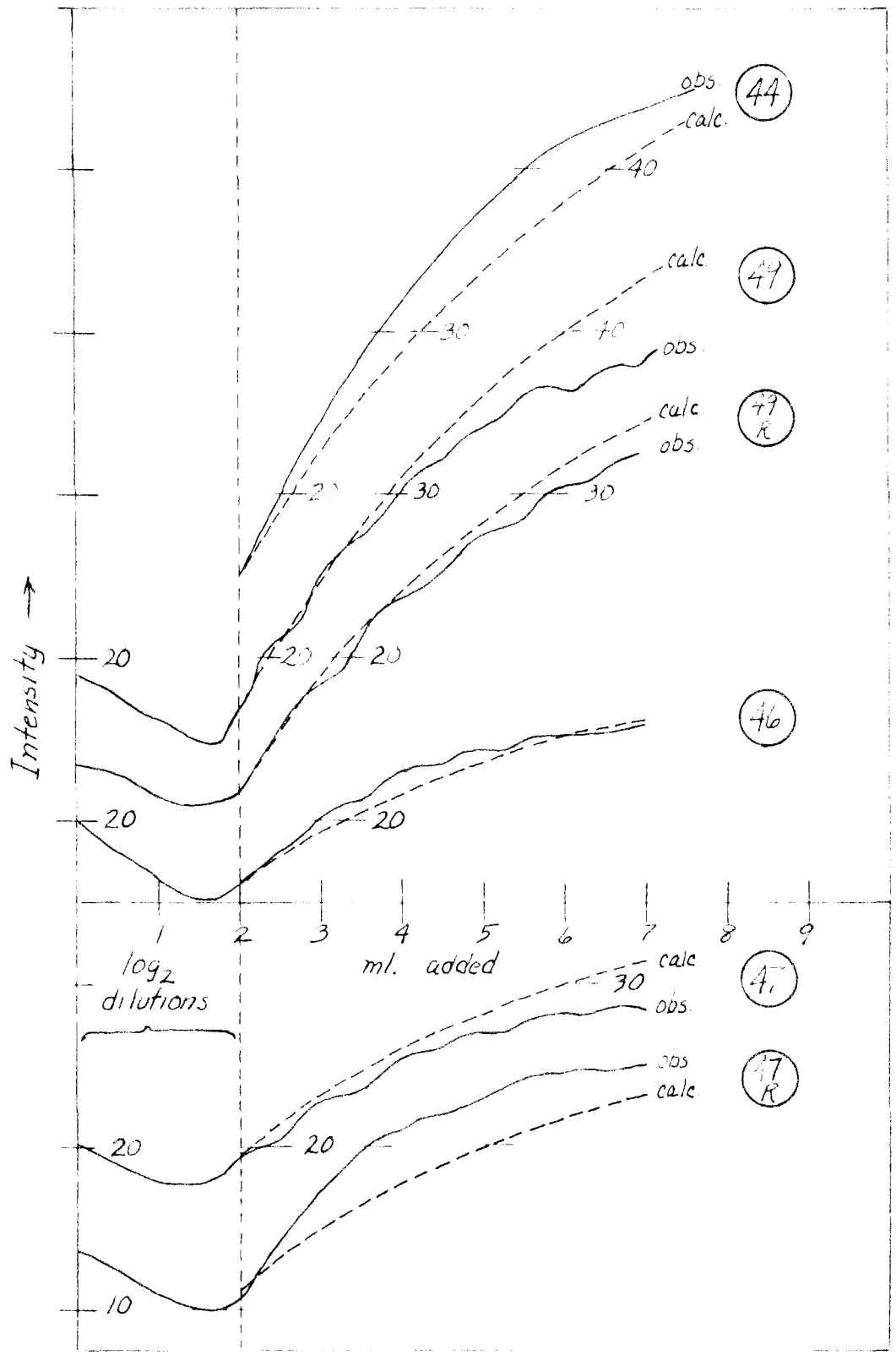

obs.
calc.
44
40
calc.
47
30
40
obs.
47 R
calc.
obs.
30
30
Intensity
20
20
20
46
20
1
2
3
4
5
6
7
8
9
log2 dilutions
ml. added
calc.
30
obs.
47
obs.
calc.
47 R
20
20
10

Figure 9

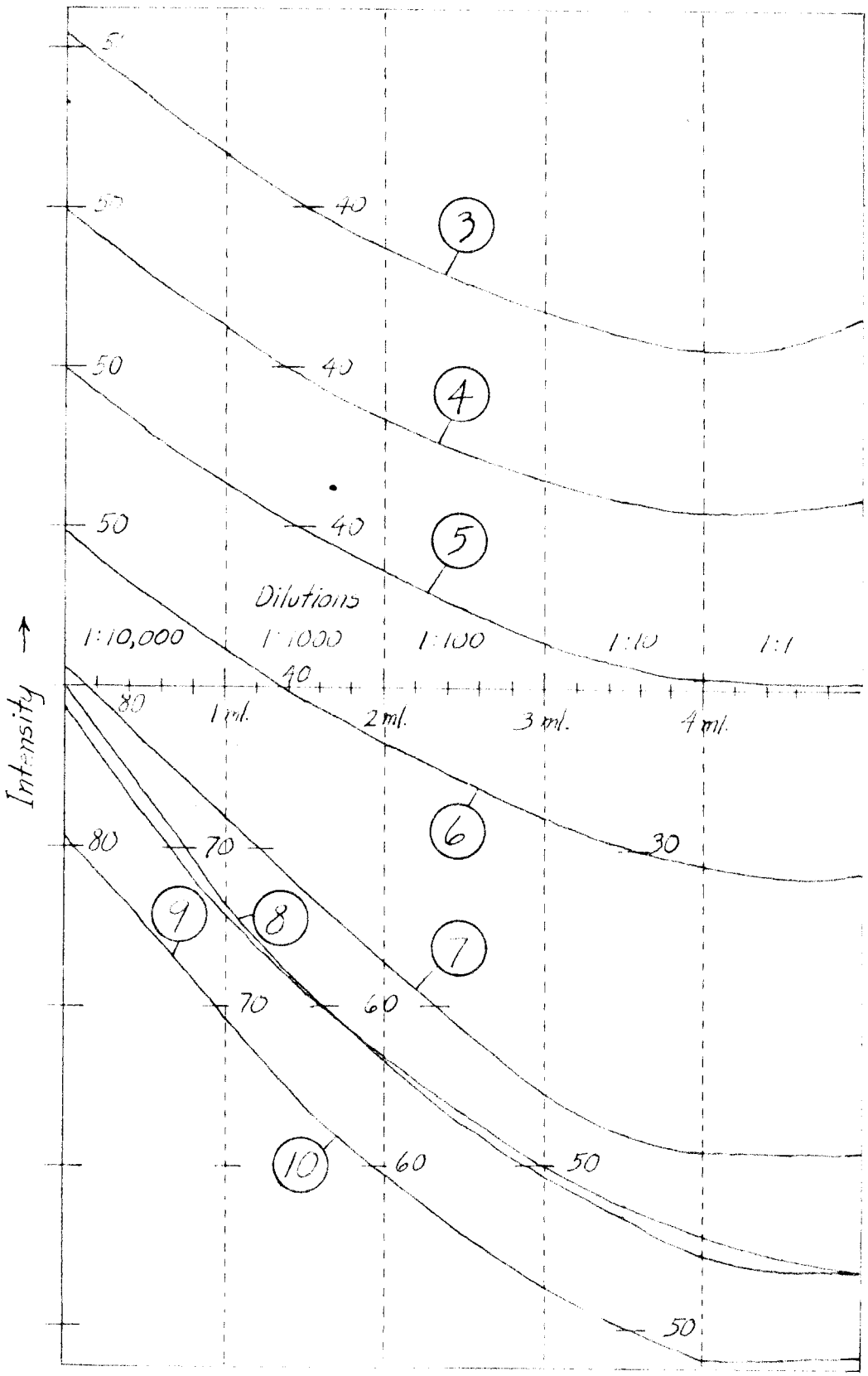

Intensity
Dilutions
1:10,000
1:1000
1:100
1:10
1:1
1 ml.
2 ml.
3 ml.
4 ml.
3
4
5
6
7
8
9
10
50
40
80
70
60
30

Figure 10

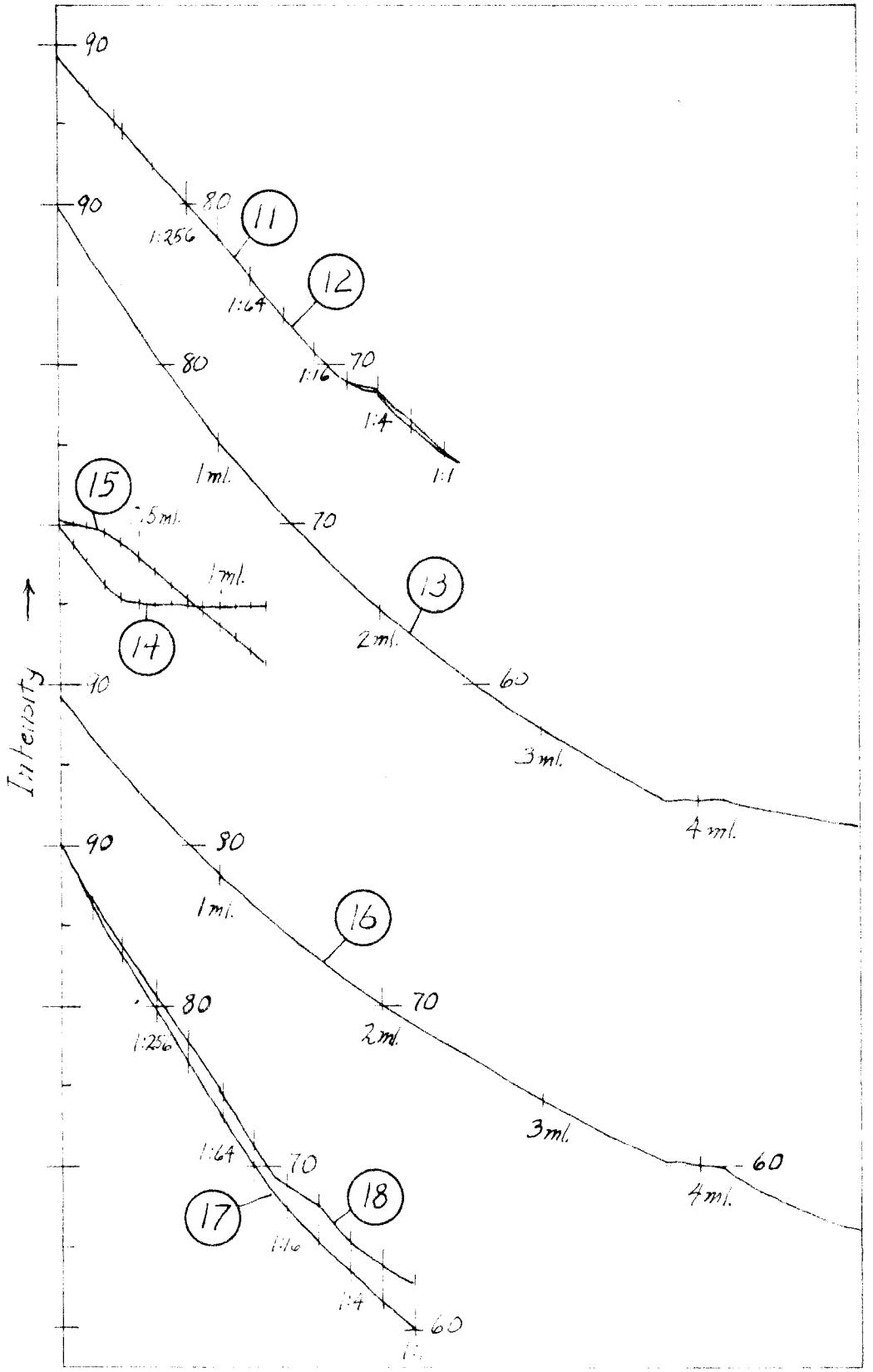

90
80
1:256
11
1:64
12
1:16
70
1:4
1:1
90
80
1 ml.
70
15
.5 ml.
1 ml.
14
13
2 ml.
60
3 ml.
4 ml.
Intensity
10
90
80
1 ml.
16
70
2 ml.
3 ml.
60
4 ml.
90
80
1:256
1:64
70
17
18
1:16
1:4
60

Figure 11

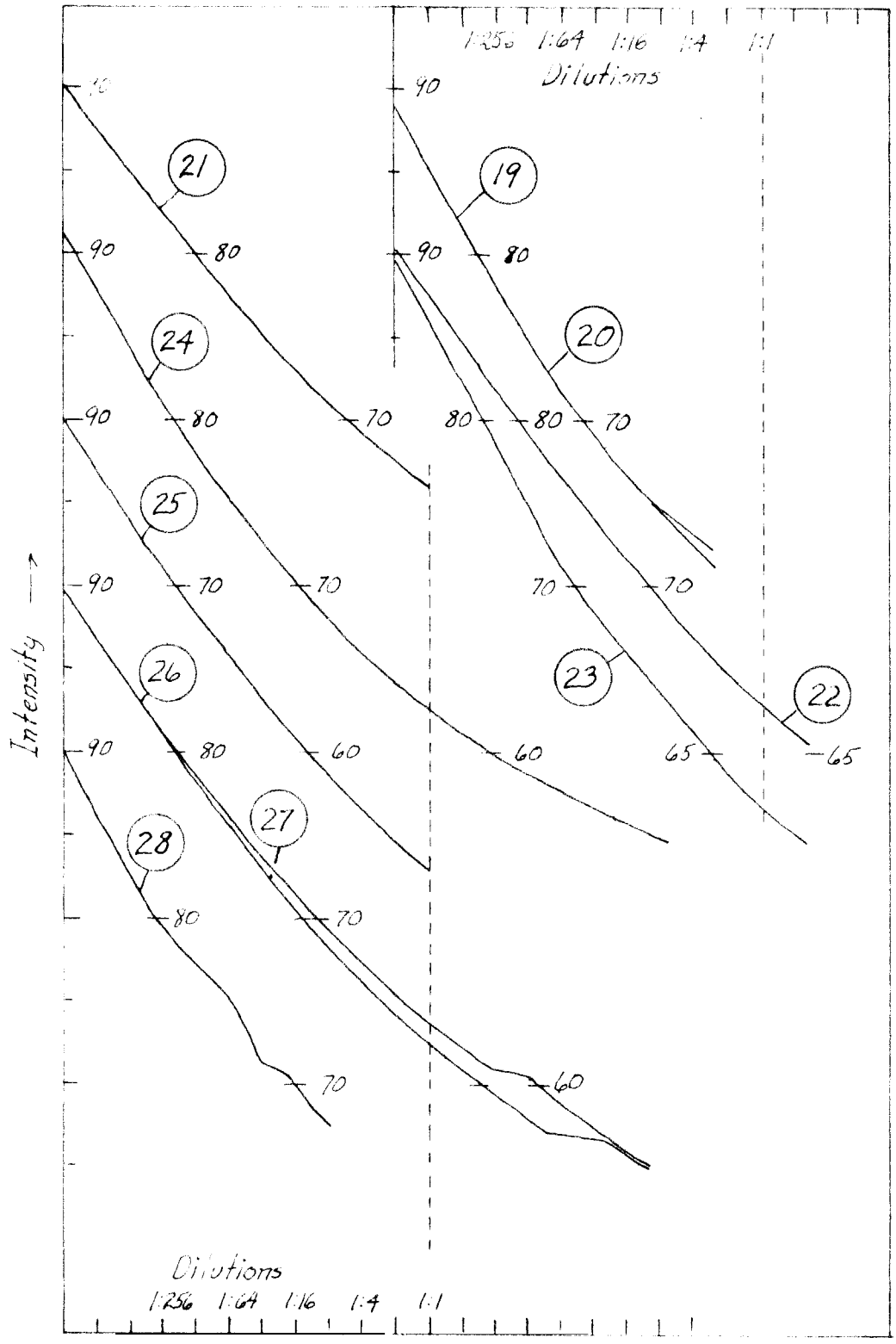

1:256 1:64 1:16 1:4 1:1
Dilutions
Intensity
21
19
24
20
25
23
22
26
27
28
Dilutions
1:256 1:64 1:16 1:4 1:1

Figure 12

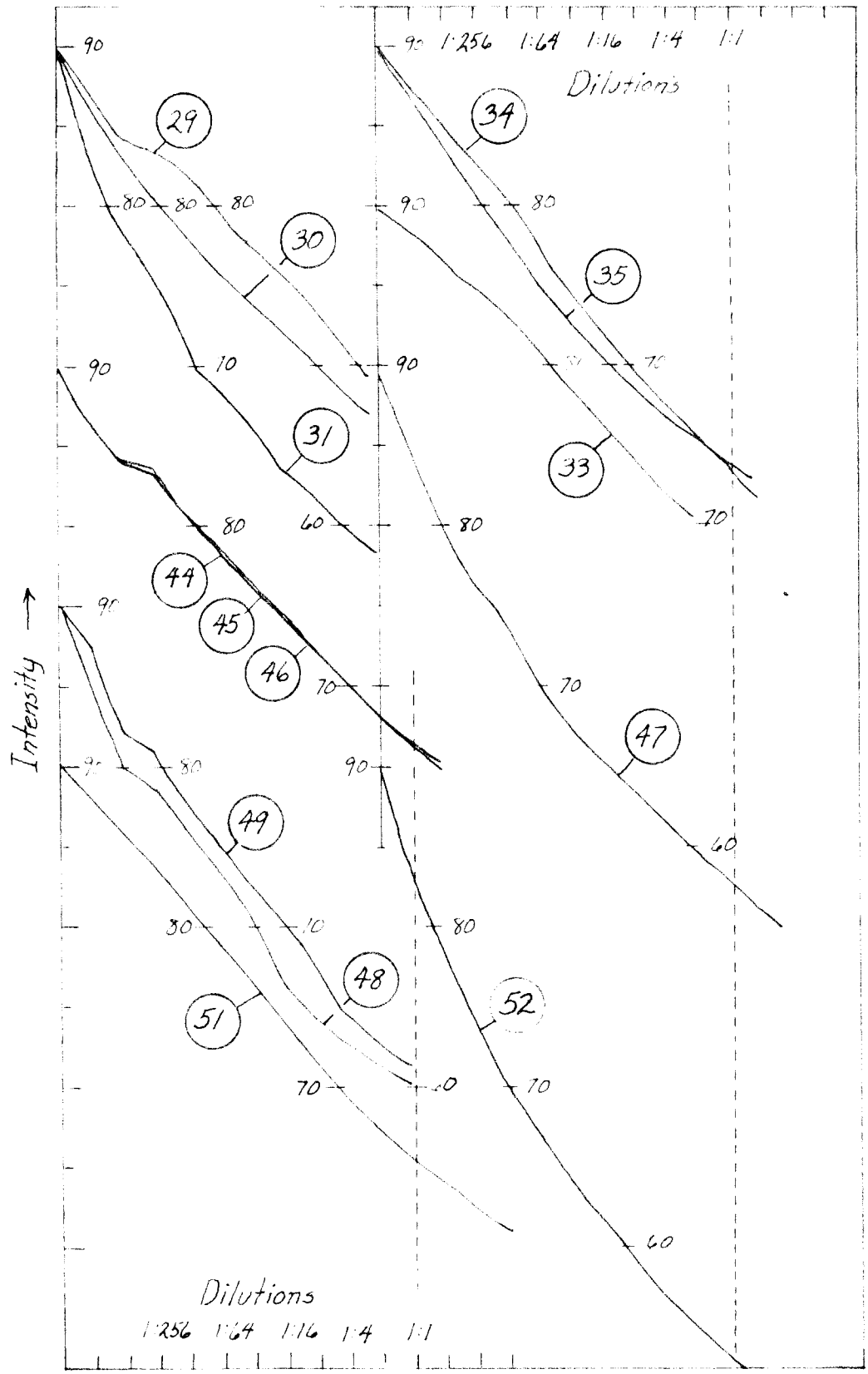

Intensity
Dilutions
1:256 1:64 1:16 1:4 1:1
29
30
31
34
35
33
44
45
46
47
49
48
51
52
90
80
70
60
10

Figure 13

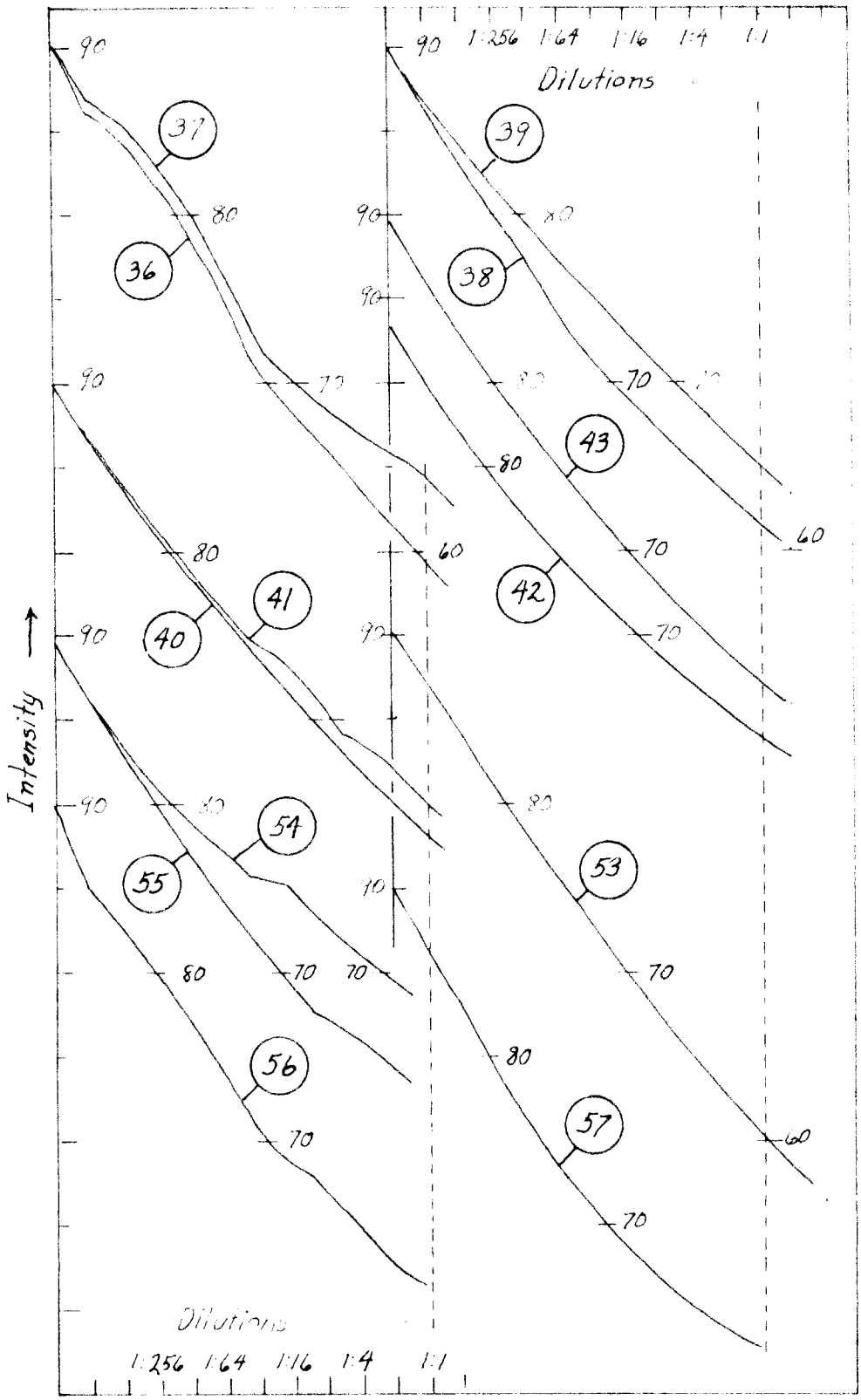

Intensity
Dilutions
1:256 1:64 1:16 1:4 1:1
90
80
70
60
36
37
38
39
40
41
42
43
53
54
55
56
57

Figure 14

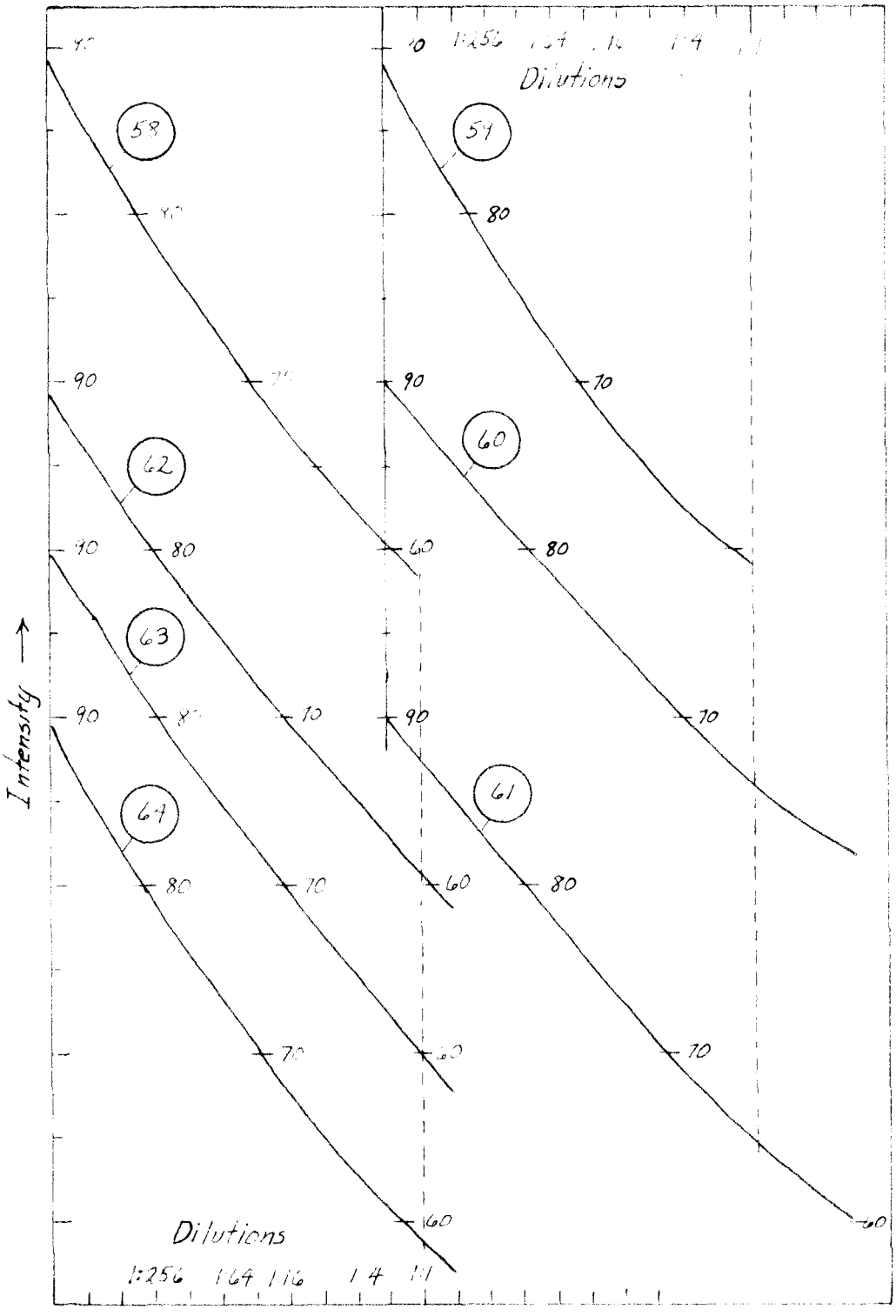

Dilutions
1:256 1:64 1:16 1:4 1:1
58
54
62
60
63
61
64
90
80
70
60
Intensity
Dilutions
1:256 1:64 1:16 1:4 1:1

Figure 15

Description of Results It is scarcely possible to grade the various runs objectively for slight turbidity reaction where the data are based on I_s90 values alone. The reasons for this will be discussed more fully later. Attention is invited to those runs in which noticeable deviation occur from the normal dilution curve indicated by a positive symbol in the tabulated results. For purposes of discussion deviations will be termed "reactions", deferring the question of their significance to the discussion.

Series A, Experiment 1. (Runs 1 to 5) A reaction is indicated for the mixture of splenic extract 5 and serum 1, in Run 1, when readings were taken two minutes after each addition. In Run 2 the same reagents were diluted to half their concentration. No reaction appeared when only two minutes was allowed between each addition and reading. In Run 3, however, equilibration was permitted before reading, regardless of the time this required. About 5 minutes usually elapsed between readings, and a slight reaction is indicated in the curve. In Run 4 the serum 1 was reacted against tissue extract 4 of an unrelated animal, and no reaction is in evidence. Serum 5 titrated with extract 1 (Run 5) shows a reaction comparable to that of Run 3. The importance of allowing sufficient equilibration was brought out in this experiment. The portion of spleen available for preparation of the testing extract in these partially splenectomized animals was insufficient for the required control runs.

Experiment 2. (Runs 11 to 17) A 20 tube $\log_2$ dilution series of lung extract 9 gave a reaction with its specific antiserum 8 in Run 11, and with its autologous serum in Run 12. The effect was observed of diluting only the serum 1:2 in Run 15, and 1:4 in Run 16 and Run 17. The concentration of extract required for the initiation of the reaction appears to be less with greater antiserum dilution, and at the same time the reaction plateau appears to be broadened.

Experiment 3. (Runs 8 to 10) In Run 8 serum 8 taken after immunization is titrated with its specific kidney extract 9. The fluctuations appearing in the course of the curve might scarcely be regarded as evidence of developing turbidity, if it were not for the fact that wherever such fluctations were observed throughout these experiments, it was later apparent by naked eye inspection that slight precipitation had occurred in the tube. In Run 9 an autologous combination (serum 8 and kidney 8) and another immune combination (serum 9 and kidney 8) in Run 10 gave no indication of reaction.

Experiment 4. (Runs 13 to 22) Extremely high turbidity developed when spleen extract (Run 13) or liver extract (Runs 18 and 19) were reacted against their specific antisera. Similar high turbidity developed when liver extract was reacted against the autologous serum (Run 20). The turbidity was sufficiently high to exceed the scale excursion of the instrument. When the same liver extract was first reacted against an equal volume of autologous serum and clarified by filtration, its titration (Runs 21 and 22) with the specific antiserum

resulted in curves showing multiple fluctuations, and slight visible precipitates. This observation suggested the possibility of absorbing any non-specific precipitating activity from tissue extracts by reaction with autologous serum prior to testing against immune sera.

Experiment 5. (Runs 23 to 41) Cecum was selected for the provision of adequate tissue extract. This choice was based on the fact that the cecum of the adult rabbit provides from 7 to 10 grams of easily suspended cells which are predominantly lymphocytes. It was found, in addition, that the dense precipitate previously noted for liver extract, when it was reacted with autologous serum, did not appear when cecum was so reacted, (Runs 23, 24, and 27). Nevertheless tissue extracts mixed with autologous sera were used in this experiment. Of 16 runs made with various sera and the two cecal extracts, three runs of the immune combinations showed some reaction (Runs 33, 37, and 39), but two of the runs in which serum taken before immunization was reacted with tissue extracts also showed significant reactions (Runs 29 and 43).

Experiment 6. (Runs 44 to 49) Reaction occurred in Run 47, and with less certainty in Runs 48 and 49, which are immune combinations of cecal extracts and their specific antisera. Similar reaction, however, also occurred in Run 45 and perhaps in Run 44, which were control runs on non-immune sera.

Considering the results of Series A as a whole, it would appear that tissue extracts reacted with certain sera in well defined, though

variable, proportion zones. These reactions were not, however, confined to the combinations in which immune recipient sera were titrated with tissue extracts from animals against which the recipients had been immunized. Reactions were noted for non-immune sera as well. If attention is confined to the regions of the titration curves in which additions of extract were taken from doubling-dilution series, certain barely significant deviations appear in Runs 11, 15, 16, 17, 37, 39, 33, and 49. These are immune combinations. In only one case, Run 45, is deviation to be noted in this region for a non-immune combination. Adjustment of the instrument necessary for dealing with the greater variations in intensity encountered in higher concentration zones of the titration curve does not provide optimum sensitivity for recording the slight deviations appearing in lower concentration zones. In Series B the titrations were confined to additions from the dilution series of the extracts and the dual photocell instrument recorded the results with greater sensitivity.

Series B The dilution range of tissue extracts explored by Bollag was given particular attention. The dual photomultiplier photometer permitted the recording of the intensity of the scattered light with greater sensitivity and stability than that attainable in the previous experiments. Deviations from the normal dilution curve, where these appeared at all, were extremely slight. The runs in which deviations are to be noted are marked in the tabulated results with the positive symbol in the appropriate column. In 21 runs on 12 serum samples,

slight deviations from the normal are noted. Each of these is a run in which immune serum was titrated with its specific tissue extract. No such deviations were observed for the controls or the combinations in which cross-reactivity was sought. The deviations are of the magnitude noted by Bollag (43), and they were repeatable in every instance where duplicate runs were made. It was found that the serum samples used in these runs lost their activity after 1, 2, or at most 5 repeated tests, within a few hours after preparation. Upon storage at -18^{o} the serum samples also lost their activity. The deviations marked as indicative of a reaction are so slight that confidence in their significance must be reserved. The fact remains that they were repeatable, even when fresh dilution series were used to remove any doubts that they might have been due to titration errors. Their magnitude significantly exceeds the $\pm 1.0\%$ instrumental error generally observed for the apparatus.

A limited test of the antigenicity of the aqueous extracts of rabbit kidney was carried out. Two rabbits were injected every other day with 5 ml. each of 1:10 kidney extract, distributing the dose in the subcutaneous connective tissue over the back. Eight injections were given to each animal. On the 16th day after the first injection the animals were grafted with 6 pinch grafts taken from the donor of the kidney extract. Six days after the grafts were placed, inspection and biopsy revealed a complete immune breakdown of 4 grafts, partial

breakdown of 4 grafts, and beginning breakdown of the 4 remaining grafts. Normal rabbit homografts placed on non-immune animals have a median survival time of about 10 days. The result indicates that the kidney aqueous extract was of moderate antigenicity. It is to be noted that the extracts had been frozen at -18°C. for storage during the course of the immunization. Apparently freezing and thawing of the aqueous extracts did not obliterate their antigenicity.

Discussion

One problem in evaluating the results obtained here concerns the relation of variations in the scattered intensity at 90° (I_s90) to changes in the dispersity of the reaction mixture. A precise solution cannot be given from present light scattering theory. It is instructive, however, to examine the theoretical behavior of a model aggregating system composed of spheres having dimensions and physical properties similar to the larger molecules present in the reaction mixture. This model is considered in the Appendix, and from the conclusions drawn there some general statements can be made about the direction in which the observed parameters may be expected to change if aggregation reactions take place. The effect of aggregation on the scattered intensities at various angles is more obvious in the case of small molecules than it is for larger ones. It is the latter that are of particular interest in these experiments.

There is some justification for the assumption that if antigens

and their specific antibodies are present in the reagents used here, they are probably large molecules with lengths in the range of 10^2 to 10^3 Angstrom units. Bollag found that the reactive molecules he encountered were non-dialysable through cellophane membranes with pore sizes of 50 - 70 Angstrom units (39). All known antibodies certainly fall within the stated size range, as do those substances which are known to be complete antigens. The possibility cannot be excluded, however, that the specific groups involved in transplantation immunity may be simple haptens of small dimensions (less than 10^2 Angstrom units).

When tissue extract is added to serum under the conditions of the present experiments, the resulting scattering intensity at any angle θ for the mixture in the absence of interaction will be:

$$I_m\theta = \frac{I_s\theta\, V_s + I_e\theta V_e}{V_m} \qquad (1)$$

where $I_s\theta$ is the intensity of the serum at θ, V_s is the volume of serum, $I_e\theta$ is the intensity of the extract at θ, V_e is the volume of extract added and V_m is the final volume of the mixture. The observed titration curve should coincide with that calculated from Equation 1, in the absence of reactions resulting from the mixture. In the present experiments $\theta = 90^\circ$. Equation 1 will hold for low concentrations of the scattering elements, but as the concentration increases there is a reduction in the intensity of the light scattered at 90° due to secondary scattering (44). The dependence of scattered light

at 90° on the concentration of scattering elements is shown in Figure 26 (Appendix), with the derivation.

In the present experiments I_s90 either increased or decreased as extract was added to the serum, depending on the relative initial turbidities of each reagent. If the serum was more turbid than the full strength extract the I_s90 decreased with each addition. If the added extract was the more turbid solution, I_s90 for the mixture increased. According to Figure 26, the reduction in scattered light due to secondary absorption will give increasingly lower values of I_s90 for rising turbidity curves, whereas increasingly higher values for I_s90 will be obtained for falling turbidity curves.

In Figures 5 and 6 the curves calculated by Equation 1 differ from the observed curves in the manner to be expected if the secondary scattering was influencing these results. In other runs where the calculated curves are presented (Figs. 7, 8 and 9) the observed values are greater than the calculated values of the scattered light intensity for curves of increasing turbidity. In order to correctly interpret these results, it must be noted that the numerical values for the intensities of different runs are not comparable. In these experiments no "absolute" turbidity standard was used. The initial value of I_s90 for the tissue extract used in computing the calculated curve is its relative intensity compared on the same "scale" as the particular serum sample to which it was added. The initial values for the reagents used in an individual run are not necessarily

comparable with the same values recorded in a different run since different "scales" may have been used. The fact that the absolute intensity range of some runs may be quite different from that in other runs probably explains the fact that the secondary scattering effect is more noticeable in some runs than in others.

The secondary scattering effect decreases the values of the measured intensity for increasing turbidity curves. For this reason it may be even more valid to regard a curve in which the measured values for the intensity exceeds these calculated as evidence for a reaction.

Run 8 (Fig. 2), runs 21 and 22 (Fig. 4), runs 33, 33-R1 and 33-R2 (Fig. 7) show rather large and somewhat repeatable fluctuations in I_s90 values over the course of the curves. There are other examples of similar behavior to be found among the results. It was commonly observed that visible particles developed in the mixtures in which such fluctuations were noticeable. It was apparent in these cases that the I_s90 values never increased beyond a certain point in proportion to the precipitation.

In the Appendix, the behavior of I_s90 for a model aggregating system of spherical molecules is examined. It is pointed out that when the particle size becomes large compared with the wavelength of the light, I_s90 passes through at least two maxima before it decreases as optical resolution is reached. It is possible that the fluctuations observed in the above mixtures are to be explained in

the same terms as the model. It is obvious that I_s90 measurements alone are useful only in the range of small particle sizes. A more satisfactory arrangement for the kinetic measurement of aggregation would include some provision for measurements at angles closer to the emergent beam in addition to measurements at 90^o.

The objective of the experiments was to examine the tissue extract-serum system for immune reactivity. Bollag reported that such reactivity was the invariable consequence of immunization with homologous tissues in rabbits (39). It is possible that the conditions involved in his experiments were not realized here. Whereas some similar reactions were observed in the ranges of concentration covered by the dilution series in the B series of experiments, these were so variable and the stability of the reagents so poor that they do not provide unqualified support for Bollag's conclusions. There is no indication that the magnitude of the reactions between serum and tissue extracts in the wide range of concentrations studied here is sufficiently great to encourage the view that the antibody response against homologous tissues involves the formation of precipitating antibody against cellular antigens. On the contrary, no reactions, however slight, were found for the I-J and K-L rabbit pairs when these were known to be immune against skin grafts, and the antigenic extracts had been shown to immunize rabbits against skin grafts. It can only be concluded from these latter experiments that serum antibodies are not demonstrated to be invariably involved in the response.

From the present experience with these systems, some limitations can be pointed out which might well be taken into account in future investigations of a similar nature. Commercial instruments now available do not provide a comparison of the ratio of the scattered light at any angle of measurement with the incident light intensity. When kinetic studies are undertaken, a photocell monitoring the incident light would provide unambiguous assurance that the deviations resulting from slight reactions were not the result of light source fluctuations. The same assurance is not unequivocally given by comparing the ratio between the scattered light and the transmitted light, since it is only at low turbidities that the latter relationship serves for the purpose.

A more serious limitation of the apparatus now available is the fact that only one combination can be tested at a time. Since the reagents are unstable, there is no assurance that comparisons are valid between an experimental and a control run carried out at different times. Criteria for the validity of such comparisons should include the condition that the reactions be carried out simultaneously. With improvements in technique and instrumentation suggested from the experience reported here, there is little doubt that light scattering will provide a more sensitive means than any other now foreseen for the kinetic study of aggregation reactions.

PART III

A Reaction Between Immune Cells and Antigenic Tissues *in Vivo*

Introduction

Weaver, Algire, and Prehn devised practical diffusion chambers for the cultivation of tissues *in vivo* (28). These chambers are composed of filter membranes cemented to a lucite framework. Cells cannot enter or leave the chamber, but fluids are freely exchanged through the membranes which have a pore size of about 0.3 micron. The chambers are placed within the peritoneal cavity of mouse recipients, and under these conditions cells within them will survive indefinitely. This prolonged survival is evidence that the recipient is unable to destroy homologous tissues within the chamber. The failure of the chamber recipient to act against the graft is not the result of inability of the homologous tissues to immunize the recipient. If immunity is first established against a homologous donor by transplantation, then the tissue of this donor still survives in the chamber. If, however, spleen cells from an immunized animal are included in the chamber with the tissue of the immunizing strain, the entire culture suffers destruction. Apparently, the immune spleen cells not only destroy the antigenic tissue within the chamber, but in the process they are also destroyed. The recipient of the chamber can be a mouse of any strain, immune or non-immune; it does not specifically alter the reaction

occurring within the chamber. The host may contribute non-specific factors such as complement which are necessary for the reaction to proceed.

Weaver and his colleagues have confirmed the above results in several hundred observations, but in view of the significance of the results an independent observation of the phenomena was desired. In the following experiment a slightly different design was used. Mice of different strains than those used by the previous investigators were also selected.

Since spleen cell suspensions can be standardized quite accurately, these were used for the antigenic tissue cells as well as for the immune cells. The suspensions were mixed together before placing them in the chambers, and an accurately measured drop was placed in the center of the chamber. The suspension fluid was allowed to drain through the membrane, thereby preventing cells from flowing to the edges of the chambers. These precautions were improvements on the technique used in the previous experiments.

Materials and Methods

Chambers Two closely fitting concentric rings, stamped out of Lucite sheet 0.5 mm. in thickness, formed the framework of the chambers. The dimensions of these rings were: outer ring, 17.5 mm. outside diameter, 14 mm. inside diameter; inner ring, 13.9 mm. outside diameter, 10 mm. inside diameter. Circular Millipore HA filter membranes, 150 microns

in thickness, having the same outside diameters as each ring, were cemented on the rings with Lucite cement. Before use the chamber halves were sterilized by soaking in 50% ethanol and rinsed in sterile distilled water. For use, the small ring was placed on cotton moistened with saline in a Petri dish, with the surface to which the membrane was attached facing upwards. A measured drop of the cell suspension was then discharged from a micro-pipette on the center of the membrane. The suspending medium was allowed to drain through the membrane, leaving the cells resting on the surface of the latter. Immediately, the outer ring with its membrane facing upwards was lowered over the small ring. By gentle pressure the rings were snapped together, and the assembly inverted. The surface of the ring assembly was dried with a cotton swab, and Lucite cement of a high viscosity was carefully applied around the joint between the two rings. The cement was allowed to dry, but care was taken to keep the membranes moistened with the suspending medium, so that the cells did not become dry.

Insertion of chambers into recipients After the cement had dried for at least 15 minutes, each chamber was inserted through a midline incision into the peritoneal cavity of the recipient. Care was taken to place the chamber among the folds of the ileum. The incision was then closed with a few fine sutures, and the anesthetized recipients were allowed to recover.

Immunization of spleen donors. Partial splenectomy was performed by simple ligation of the posterio-ventral tip of the spleen. About one-third of the spleen was removed. After pressing the spleen fragment through a sieve, the coarse suspension was taken up in a syringe and injected into the peritoneal cavity of the recipient.

Preparation of spleen cell suspensions. Fourteen days following the injection of spleen for immunization, the recipients, together with the donors, were anesthetized with 0.60 mgs. of Membutal per 10 grams of body weight. Blood samples were withdrawn by cardiac puncture into citrated saline for later use in standardizing the spleen cell suspensions. The spleen were then removed from all of the animals, and placed on 60 mesh stainless steel sieves in individual tubes containing 2 ml. of the suspending medium. Gey's balanced salt solution, to which an equal volume of pooled mouse serum had been added, was used as the medium, but in this initial suspension the serum was omitted. The cells were suspended by pipetting. After the large clumps had settled, 0.2 ml. of each suspension was drawn from the top of the tubes, and diluted 1:10, by adding 9 parts of Gey's solution containing an equal part of pooled serum. The suspensions were maintained in an ice bath while samples of each were withdrawn and the cell content determined in a hemacytometer chamber. The red and white cells were counted separately. Since the immune spleen cell suspensions had been found previously to contain more white cells than red cells, it was necessary to add red cells from the blood sample taken

earlier. Counting and adjustment of the suspensions was continued until they each contained equal numbers of red and white cells, and the total concentration of cells was 10,000 /mm^3. The sampling and counting errors may have been as great as ± 5%.

Equal proportions of the suspensions were mixed in the combinations outlined in Table 4. The procedure involved in this design is described in the following section. The suspensions were added from the mixed combinations to the chambers by measuring out 10 mm^3 of each mixture on the smaller ring as previously described. Care was taken in the pipetting to ensure that the suspensions were well mixed; since sharply pointed micro-pipettes were used, little error accompanied the discharge of the droplet on the membrane. Each chamber thus contained approximately 100,000 cells.

Procedure NMRI inbred albino (A) and agouti (W) mice were used in the experiment. Two A strain mice were designated A, C. Two W strain mice were designated B, D. B was immunized against A, and C was immunized against D, by partial spleen transplantation. After 14 days the spleens were removed from the four animals and the combinations prepared as shown in Table 4. Half of the chambers containing each combination were placed in W strain recipients and the other half were placed in A strain animals.

Table 4

Design of Tissue Chamber Experiment

Procedure

1. Immunization

Donor of spleen		Recipient of spleen
A (A strain)	injected into	B (W strain)
D (W strain)	injected into	C (A strain)

2. Spleen cell suspensions prepared from A, B, C, D, 10,000 cell/mm^3.

3. Equal parts of above suspensions mixed as below and 10 mm^3. added to each chamber.

Combination Designation	Number of Chambers	Cell Suspensions in Mixture	Nature of Combination*
M	4	B plus C	Reciprocal, immune
N	2	A plus B	Unilateral, immune
O	2	D plus C	Unilateral, immune
P	2	C plus A	Control, same strains
Q	2	B plus D	Control, same strains
R	4	A plus D	Control, different strains

*Reciprocal combination Each of the tissues donors is immune to the other.

Unilateral combination Only one tissue donor is immune to the other.

Seventy-two hours after the chambers were implanted, they were removed. The outer surfaces of the chambers were cleaned well by swabbing with cotton moistened with saline. This removed inflammatory cells which had collected at the outer surfaces of the membranes. After fixation, the chambers were washed and stained with Erlich's acid hematoxylin, differentiated in a saturated solution of picric acid, and washed in alkaline water. After dehydration in alcohol, the chambers were cleared in cedarwood oil, and the plastic rings were cut free and discarded. The filter membranes were then mounted on glass slides for examination.

Results

Examination of the chamber preparations revealed a marked difference between immune (MNO) and non-immune (PQR) combinations. Without exception, the non-immune cultures showed groups of surviving cells, some of which were actually dividing as shown by mitotic figures. The immune cultures on the other hand showed relatively few or no surviving cells. In both groups pycnotic nuclear remnants were present along with some cellular debris. Erythrocytes were also present in each case. The striking difference was the complete absence of mitotic figures in the experimental (MNO) group. Classification of the surviving cells in the PQR group was not attempted. They were mostly mononuclear cells with large, pale-staining nuclei. The appearance of the cultures is shown in Figures 16 and 17. The

Explanation of Figures

Figure 16

Immune spleen cells combined with antigenic spleen cells in diffusion chambers have resulted in the marked destruction of the culture. The remains consist of few scattered pycnotic lymphocytes, erythrocytes and cellular debris.

Figure 17

Non-immune spleen cells combined with antigenic spleen cells, or immune cells combined with autologous spleen cells, show surviving lymphocytes and cells with large pale-staining nuclei. Numerous mitoses were observed in these cultures.

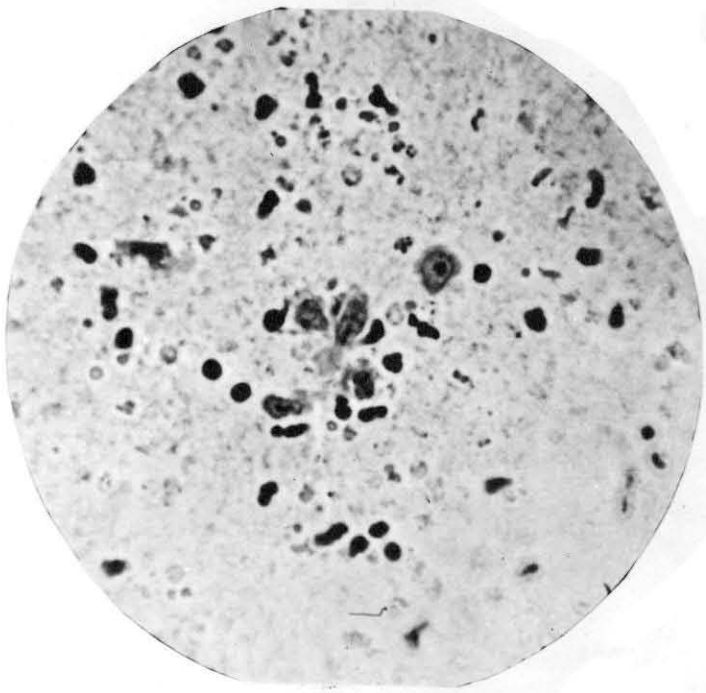


Figure 16

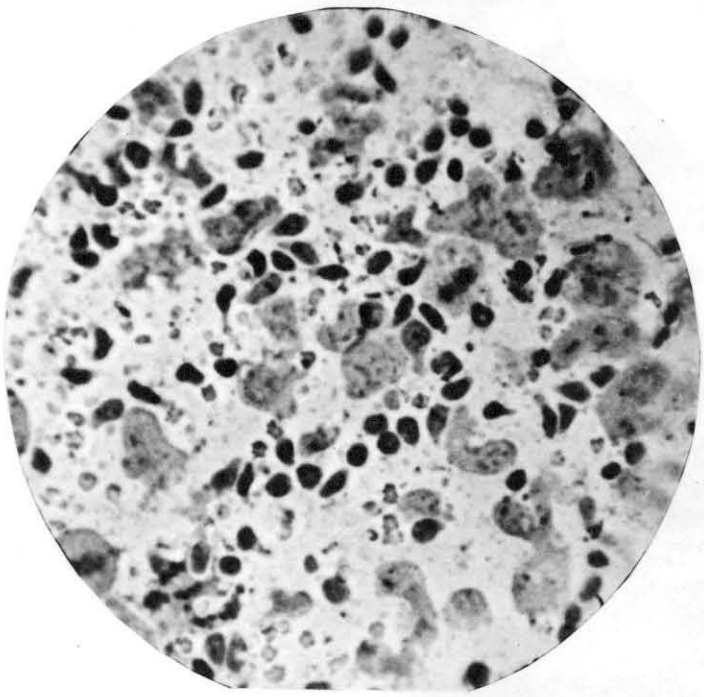


Figure 17

figures selected for comparison display the best survival attained in each of the two groups.

Discussion The experiment demonstrates the vigorous mutual destruction which may result from the association of immune and antigenic cells under appropriate conditions. The results obtained here for two strain combinations confirm and extend the results consistently obtained by Weaver, Algire and Prehn (28) for three additional strain combinations. There can be little doubt that cytolysis in these cases is the result of a specific event; it occurs only when immune cells are challenged with antigenic cells.

The basis for the specificity of the reaction is probably none other than that responsible for the specificity of other sorts of antigen-antibody reactions. Antigens and antibodies are postulated here for the reaction between the cells. These substances may be liberated into the surrounding medium where they react, or they may remain attached to the cells. The substances will have complementary combining sites which express the specificity through integrated weak bonding forces when the combining sites come into contact (45). There is little justification to invoke other "recognition" mechanisms than that mentioned, because at present no other "forces" are known to exist which might fulfil the requirements.

No reservation is withheld in the use of the term "antibody" to refer to the specific molecular configuration postulated here as the agent of transplantation immunity. This term is preferred to other

terms vaguely identifying immune activity, because no basis other than a molecular one is recognized for this or any other specific cellular activity.

PART IV

Reactions Between Immune Cells and Antigenic Tissue Extracts *in Vitro*

Introduction

Billingham, Brent and Medawar (18) have shown that transplantation antigens can be prepared in solutions of physiological salt concentration, providing calcium ions are absent. Immune spleen and lymph node cells presumably have reactive sites for the transplantation antigens, under appropriate conditions. The unequivocal demonstration of a reaction between immune cells and transplantation antigens *in vitro* would constitute a major advance toward understanding the homograft reaction. Information available from parallel studies of bacterial hypersensitivity provided little encouragement that such demonstration would be a casual task. It is possible to detect as little as 10^{-8} mg. of diphtheria toxin, but investigations have failed to reveal a reaction between the toxin and cells which transfer sensitivity against the toxin (46). In the present studies transplantation antigens are available only as crude extracts, they are unstable, and moreover, the assay methods suffer by comparison with the case just mentioned. On the other hand, there was no reason to suppose that the reactions of interest in transplantation immunity were identical with those occurring in bacterial hypersensitivity, and

this fact invited the attempts to be described.

General Methods

Animals Highly inbred strains of mice were used in this study. Strains A albino, CBA agouti, C57BL/10 and B10.D2 were obtained from Dr. G. D. Snell, of the Roscoe Jackson Memorial Laboratory, Bar Harbor, Maine. The first two named strains were used as one combination for the exchange of grafts. The latter two strains are believed to be co-isogenic except for an allelic difference at the H-2 locus. These were used as the second strain combination. For convenience the C57BL/10 strains are designated here as D strain (having the $H\text{-}2^B$ allele) and E strain ($H\text{-}2^D$). The median survival times (MST) for grafts exchanged between A and CBA strains, and those exchanged between the E and D strains, were previously determined by Dr. Leslie Brent, according to the procedure worked out in Medawar's laboratory (6). The MST for CBA skin grafted to A strain recipients is 10 days; for the reverse combination, the MST is 11 days. E strain grafts on D strain recipients have a MST of 9.0 days, whereas the grafts in the reverse combination have an MST of 10 days. Since the strains are highly inbred, each member of the strain accepts grafts permanently from another member of the same strain.

Immunization of Lymph Node Cell Donors The methods were those developed in Medawar's laboratory (5). Only mice whose pelts reveal inactive hair growth were selected for skin donors. The donors were

anesthetized with 0.65 mg. Nembutal per 10 gm. body weight by intraperitoneal injection. They were pinned out, belly down, on an operaing board. The hair was removed as closely as possible with a hair clipper. The skin was cleansed and disinfected with Tincture of Zephiran, 1:1000. Pinches of skin were elevated, and circular grafts about 8 mm. in diameter were removed by excision with a curved scalpel. Following excision, the grafts were placed in sterile Petri dishes on filter paper moistened with saline. The grafts were turned with their dermal side uppermost, and the panniculus muscle and subcutaneous connective tissue were removed as cleanly as possible by scraping with a scalpel. They were then flattened, dermal side down, on the filter paper and stored in the cold until used.

Recipients were grafted on each lateral surface of the thorax, for purposes of immunization. This ensured that both pairs of axillary lymph node were activated. For purposes of testing the state of immunity, only one side was grafted. The recipients were anesthetized and held stretched on the board by elastic bands tightened about the legs. The hair was clipped from a site on each side, and the skin was cleansed and disinfected. The skin was then removed down to the level of the panniculus carnosis muscle by clipping it away with curved scissors. A site was prepared large enough to provide a clear zone of about 2 mm. around each graft. The grafts were flattened into place, and covered with Vaseline-impregnated tulle. A girth of plaster bandage around the thorax was finally provided to protect the grafts and

hold them tightly against their beds.

For inspection and biopsy of test grafts, the plaster bandage and tulle were removed. The cuticle was carefully removed with sharply pointed forceps, if it had not come off with the tulle. Visual observation of a surviving graft will show a dry matte epithelium. A moist surface with an easily peeled epithelium or one lacking the epithelium indicates destruction. Upon exposure to air, the dermis lacking epithelium will quickly harden into a red scab-like surface. Grading the extent of destruction was performed by estimating the percentage of surviving epithelium. If the result was questionable, the entire graft was removed for microscopic evaluation from stained sections.

Preparation of Antigenic Extracts* The spleens were removed from the primary immune recipients on the tenth day after grafting. These were pressed through a 40 mesh stainless steel sieve into buffered normal saline (.85% NaCl buffered at pH 7.4 with .01M mono- and di-sodium phosphates). Depending on the number of spleens and their weight, the sieved tissue was taken up in 2 ml. of buffered normal saline per 100 mg. spleen. Tissue fragments were broken up into individual cells and small clumps by pipetting. After suspension, the cells were sedimented at 1000 RCF for 5 minutes. The supernatant was removed and discarded, and the sedimented cells were resuspended

---

* cf. Billingham, Brent and Medawar (18).

in 0.5 ml. saline per 100 mgs. spleen. Three volumes of distilled water was added to each volume of the saline suspension. The aqueous suspension was centrifuged again at 1000 RCF for 5 minutes, the supernatant drawn off and discarded, and 2 ml. of distilled water added for each 100 mgs. of spleen initially present. After thorough resuspension in the water, the suspension was homogenized by exposure to ultrasound for about 2 minutes. Ultrasound treatment was carried out with 16 kilocycle vibrations produced by a magneto-striction transducer. An exponential horn having a flat circular tip, 7 mm. in diameter was lowered into contact with the surface of the suspension. Equipment was not available to measure the intensity of the sound. Homogenization was simply continued until dispersal of cellular material was complete.

After homogenization the antigenic solutions were standardized for equal concentration by measurement of their transmission in a Klett photoelectric colorimeter. After adjusting the concentration where required, 1.5 M NaCl solution was added to the aqueous suspension in an amount sufficient to bring the final concentration to 0.15 M NaCl. At this concentration of sodium chloride the desoxyribonucleoprotein precipitate immediately appeared, and it was removed by centrifugation at 2500 RCF for 10 minutes. The supernatant extract, which was antigenic, was decanted and saved. The residue was discarded.

Labeling with 5-dimethylamino-1-naphthalene sulfonyl chloride (DAN)*

Two mgs. of DAN was dissolved in 1.5 ml. of absolute ethanol. One-tenth ml. of the alcoholic solution was added to 1 ml. of buffered normal saline. Without delay the cloudy mixture was added to 4 ml. of the antigenic solution containing the extract from 200 mgs. of spleen. The final mixture was maintained at 4°C. for at least five hours before using. Throughout the preparation of antigenic solutions, the temperature was maintained below 5°C. by the use of ice baths.

Preparation of Lymph Node Cell Suspensions The pairs of axillary lymph nodes, from each axilla, were excised and sieved through a 40 mesh stainless steel sieve into citrated saline solution (0.1 M NaCl, 0.15 M trisodium citrate pH 7.4). The tissue fragments were dispersed into individual cells and small clumps by pipetting the tissue. The suspension was centrifuged at 1000 RCF for 5 minutes, the supernatant removed and discarded, and the sedimented cells were carefully resuspended in citrated saline solution. Large cell clumps were allowed to settle out, and the suspended cells were removed. Cell suspensions were standardized in the Klett photometer as required. All cell preparations were maintained at ice bath temperature during handling.

---

*Lot 3107, MP 72 - 73°C., Calif. Foundation for Biochemical Research, Los Angeles, Calif.

Measurement of Fluorescence The fluorescence was determined in the Brice-Phoenix light scattering photometer. This instrument is diagramed in Figure 24 and described in Part I. The apparatus was modified from its arrangement for the light scattering experiments. Ultraviolet transmitting filters (Corning No. 5840) were placed at positions F2. A yellow transmitting filter was placed at F3. With the filters used for transmission of the ultraviolet light, the predominant wavelength was the 365 millimicron line of the mercury arc source. The fluorescent label is brilliantly excited by this radiation. The yellow filter had its cut-off point well above the upper limit of transmission of the ultraviolet transmitting filter, so that only the fluorescent light will be admitted to the photomultiplier P2. The galvanometer was used for measurement rather than the recorder, because it was about three times as sensitive. The units of fluorescence intensity recorded in the results are relative galvanometer readings at a constant level of sensitivity.

The Biological Effectiveness of DAN Labeled Antigenic Extracts

At the outset it was necessary to determine the effects of reacting antigenic extracts with the fluorescent label. The sulfonyl chloride end-group is unstable in aqueous suspension, reacting with free amino groups to form the sulfamido linkage. Combination with the antigenic molecules might lead to their denaturation and a loss of antigenic activity.

Fifteen spleens were removed from A strain mice. These were

processed to provide antigenic aqueous extracts according to the procedure previously outlined. The final extract was divided into two 5 ml. portions. To one portion was added 0.2 mgs. DAN suspended in 1 ml. of buffered normal saline. The other portion received 1 ml. lacking the dye, but containing the dye solvent, 0.1 ml. absolute ethanol. After 4 hours standing at 4°C., each portion was injected intraperitoneally into 3 CBA strain mice. Three days after injection, the mice were given test grafts of A strain skin. Six days later, the grafts were inspected. All grafts showed advanced breakdown, thereby indicating that the procedure had not seriously affected the antigenicity of the extract. Normal homografts on previously unimmunized animals show no such epithelial destruction six days after grafting.

The Uptake of DAN Labeled Antigenic Substances by Immune Lymph Node Cells

Procedure In each of five experiments, immune lymph node cell suspensions of two strains were prepared. In each suspension the cells of 16 axillary nodes from 4 animals were suspended in 10 ml. of citrated normal saline. Corresponding antigenic solutions were prepared by extracting two spleens from each strain and diluting the resulting extract to 10 ml. Square glass light-scattering cells (30 x 30 x 60 mm.) were used for reaction vessels and the measurement of fluorescence. Fifteen ml. of solution was required to fill these cells sufficiently for the meniscus to be well above the light path.

The four possible combinations of the two cell suspensions and the two antigenic extracts were studied. Lymph node cells of each strain were reacted with their autogenous antigenic extracts, and the antigenic extracts of the opposite strain, against which the cell donors had been previously immunized. It was necessary to measure the initial fluorescence of each of the antigenic extracts and cell suspensions, before their mixture. In order to accomplish this, 5 ml. of each of the four ingredients was suspended in 10 ml. of citrated saline in a light-scattering cell, and the initial fluorescence was measured. The remaining 5 ml. of each required ingredient was then mixed into the vessels, and the reaction was allowed to proceed at 4°C. The mixtures were stirred at 15 minute intervals to insure adequate mixing, and after 90 minutes, the contents of each vessel was emptied into a 50 ml. centrifuge tube. The suspensions were then centrifuged at 1100 RCF for 5 minutes, and the supernatant was poured off. The cells were resuspended in 15 ml. of citrated normal saline, and again centrifuged. After pouring off the supernatant, the cells were resuspended in 15 ml. of citrated normal saline, transferred back into the light-scattering vessels, and their final fluorescence determined.

Results The results of five duplicate experiments are shown in Table 5 in the chronological order in which they were performed. The first two experiments invited the conclusion that immune lymph node cells were taking up fluorescent labeled substances from their specific antigenic solutions in a highly selective manner. It was the result

Table 5

Results of Fluorescence Uptake Experiments

1. DAN 9				
Antigen	(A)	A	(CBA)	CBA
Cells	A	(CBA)	CBA	(A)
Uptake	6.2	37.2	10.3	26.8
2. DAN 11				
Antigen	(A)	A	(CBA)	CBA
Cells	A	(CBA)	CBA	(A)
Uptake	0	26.5	4.0	35.5
Agglutination*	4+	+	5+	+
3. DAN 13				
Antigen	D	(E)	(D)	E
Cells	(D)	D	E	(E)
Uptake	32	6.5	10.2	15.2
Agglutination	+	4+	2+	-
4. DAN 14				
Antigen	D	(E)	E	(D)
Cells	(D)	D	(E)	E
Uptake	33.5	13	30	13.5
Agglutination	2+	3+	2+	5+
5. DAN 17				
Antigen	A	(C)	C	(A)
Cells	(A)	A	(C)	C
Uptake	36	14.5	27.5	12.5
Agglutination	2+	4+	2+	5+

Parentheses set off the ingredient first diluted and measured, Where cells are first diluted, these are underlined. Where reading is underlined, this is the highest reading of the experimental and control pair.
*Agglutination after addition of autologous serum.

to be expected for a specific reaction between immune cells and their corresponding antigens. However, when the next two experiments (Nos. 3 and 4) were performed on the D-E strain combination, the results were reversed. The only difference between the procedures was the order in which the cells and antigens were arranged in the light-scattering vessels for their initial dilution and reading. The ingredient first placed in the vessels and diluted for the initial reading is enclosed in parentheses in Table 5. After the reading, the remaining ingredient was added as indicated. Experiment 5 was carried out by the same arrangement as experiments 3 and 4, except that the CBA - A strain combination was used rather than D and E strains. The results immediately suggested that the difference in uptake of fluorescence, which behaved so oddly in the first four experiments, could be correlated with the dilution sequence rather than the origin of the materials. Attention is directed to the fact that the uptake is greatest for the cell suspensions diluted for measurement prior to the addition of antigenic extract. The uptake is least where the antigenic reagents were diluted for measurement prior to the addition of cells. This curious effect of dilution on the cells could hardly have been anticipated a priori. The procedural design, which had been laid out for convenience in measuring control values of the initial fluorescence, clearly defeated the initial objective of the experiments. However, its employment had led to the observation of a quite unexpected phenomenon, which may not lack

significance in designing future experiments.

The effect of adding serum to the above cell suspensions following the reaction with antigen provides further evidence for uptake. In the second experiment 0.75 ml. of serum, autologous to the cells in each case, was added following the final fluorescence measurements. The mixtures were allowed to stand overnight at 4°C. The rationale for this procedure was that immune lymph node cells which had presumably taken up antigen, might then be affected by co-factors in serum such as complement. The appearance of the suspensions 9 hours after the addition of the serum is shown in Figure 18. Upon gentle agitation it was noticed that the cells which had taken up the most fluorescent materials suspended easily, but the cells which had not taken up the fluorescence were strongly agglutinated. In the remaining experiments (3, 4 and 5), serum was added to suspensions with exactly the same result. In each case the agglutination of cells seemed to bear an inverse relationship to the fluorescence uptake. It must be noted that lymph node cells normally agglutinate when suspended in serum without previous treatment. The anomalous effect noted here is the absence of agglutination in the serum-containing suspensions which had taken up substances from the antigenic extracts.

Figure 18

Vessels Nos. 2 and 4 show the normal agglutination of lymph node cells following the addition of serum. Agglutination does not appear in vessels Nos. 1 and 3. The cells have been experimentally treated as outlined in text, page 92.

Discussion The increased uptake of fluorescent labeled substances by cell suspensions diluted in a large volume of citrated saline before the addition of antigenic extracts may be explained in the following way. Citrate ions remove calcium ions from the cell surface, releasing bound protein. Equilibrium will be established for this reversible dissociation, and the number of binding sites exposed on the cell surfaces will be proportional to the dilution volume and the concentration of citrate. Cells diluted in 15 ml. of citrated medium will have a greater portion of their surface binding sites exposed than will cells diluted out in only 5 ml. of citrate saline. If the volumes are now made equal by adding the labeled antigenic extracts in the required volume of citrated medium, the equilibrium will shift, binding the protein in solution to the exposed cell surface sites. It will be assumed that fluorescent protein molecules in the added extracts and the original cell bound protein have an equal probability for binding on cell surface sites in the presence of calcium. If it is further assumed that more of the labeled molecules than unlabeled protein are present in the added extract, more labeled molecules will be bound to the cells in those suspensions which had been diluted at first in the greater volume of citrated medium. These assumptions are not unreasonable. The explanation simply involves competition of labeled and unlabeled molecules for a limited

number of combining sites on the cells.

At the outset it was assumed that if calcium ion activity was not reduced, antigen and other proteins might be taken up by the cells in a non-specific manner, obscuring a slight reaction between the antigen and specific sites within the cells. The fixation of proteins by calcium in the cell cortex is a well-known phenomenon (47). Most cells adsorb proteins on their surfaces, if these are present in the suspension medium, by means of calcium-complex formation. Furthermore, the antigens in the aqueous extracts used here are precipitated in the presence of calcium.

Citrate was used as a means of reducing calcium ions, and this was not expected to interfere with specific reactions of the sort involved in antigen-antibody combination. The conditions were obviously not those intended to reproduce in vivo conditions, nor does it appear that they were optimal for the experiment. A lesser concentration of citrate might have been more desirable than that used.

The experiment suffers further from the impurity of the antigenic extract. In fact, it is not improbable that only trace amounts of the antigen molecules are only present in such extracts.

It is suggested that the antigen in vivo may form a gel in the presence of calcium, having quite different properties than the amorphous flocculent precipitate formed in vitro. Calcium ions are strongly hydrated and they will cause swelling of gels and even the transition to a sol (48).

In extra cellular spaces, the antigen will be deposited in contact with ground substance which may further modify its behavior.

Future experiments may profit from the present experience; as conditions are varied, it is not improbable that a reaction between immune cells and the antigenic solutions will be demonstrated.

Biological Effectiveness of Antigenic Extracts After Absorption With Immune Lymph Node Cells

Procedure A direct biological test was undertaken of the ability of immune lymph node cells to react with antigenic extracts. Six A strain mice were immunized against CBA strain tissues by skin grafts. The six spleens and twenty-four axillary lymph nodes were removed on the tenth day after grafting, when graft destruction was complete. Antigenic extracts were prepared from the spleen of four A and four CBA strain mice. The immune lymph node cells and splenic cells were pooled and divided into two 2 ml. portions. To one lot, antigenic extract from the four A strain spleens was added in 3.5 ml. solution volume. The other lot of immune cells received a similar quantity of the CBA antigenic extract. The mixtures were allowed to react for 90 minutes at 4°C. They were then centrifuged to sediment the cells. The antigenic supernatant solutions were removed without loss, and recentrifuged to remove any remaining cells. The sedimented immune cells were resuspended in 3 ml. normal citrate saline. One ml. portions of the A strain cell suspensions were injected into six A strain

mice, three of which received the cells that had reacted with autologous A strain antigen, the other three mice receiving the cells that had reacted with their specific CBA strain antigen.

One of the supernatant solutions contained 5 ml. of A strain antigenic extract, and the other 5 ml. of CBA strain antigenic extract, Each of these solutions was divided between two mice of the opposite strain in intraperitoneal injections in order to test for any loss of antigenicity which may have occurred in the reaction.

Results Three days after injection of the cells and antigenic extracts, the recipients were each grafted with skin from donors of the opposite strain. Six days later the grafts were inspected, and the results recorded. The immune cells, which had been reacted with the antigenic extracts, proved able to transfer adoptive immunity; the grafts on all six of the recipients were completely destroyed. The A strain antigenic extract that had been reacted with A strain immune cells as a control had effectively immunized the two CBA mice into which it was injected; the grafts were both completely broken down. On the other hand, the CBA strain antigenic extract which had been reacted with A strain anti-CBA immune cells, had not induced immunity in its A strain recipients. Apparently the latter antigens had been removed by absorption or rendered inactive in the presence of the immune cells.

The experiment next described provided more reliable control of the initial activity of the antigenic extracts than the previous design.

This was achieved by reacting equal portions of the same antigenic preparation with non-immune nodes for the control, and specifically immune nodes for the experimental group.

Procedure Six E strain mice were immunized against D strain by grafting. On the tenth day after grafting lymph nodes and spleens were removed and a cell suspension was prepared. A similar cell suspension was prepared from the nodes and spleens of six non-immune E strain mice. Twelve D line spleens were processed to provide 8 ml. of antigenic extract. This was divided into two equal portions of 4 ml. each. The cells in the above suspensions were packed by centrifugation and the supernatant was discarded. The packed non-immune cells were resuspended in one lot of the antigenic extract, and the packed immune cells were resuspended in the remaining lot. The volume of packed cells in each lot was identical (0.5 ml.). After one hour at 4°C., the cells were sedimented by centrifugation at 2500 RCF. The supernatant solutions were removed, and the cells were discarded. Each 4 ml. lot of the recovered antigenic extracts was divided into two equal parts, and injected intraperitoneally into two E strain recipients. Three days later the animals were all grafted with D strain skin.

Results Inspection six days later revealed that one of the grafts on an animal receiving antigenic extract that had been reacted with non-immune cells was completely broken down, and the other was about 25% destroyed. On the other hand, both grafts on the experimental animals which had received antigenic extract reacted against the immune

cells showed 100% survival. There could be little doubt that the activity of the antigenic extract had been reduced by its reaction with the immune lymph node and spleen cells, as compared with its reaction with non-immune cells.

Discussion The results of the previous experiments suggest, but do not conclusively prove that immune spleen and lymph node cells react specifically with their corresponding antigens. They have demonstrated some of the difficulties involved in attacking the problem, and continuation of the antigen absorption experiments offers promise of settling the question. The significance of the results thus far obtained must be qualified. Isogenic mice are remarkably uniform in their response to homografts. Billingham, Brent, Medawar, and Sparrow (6) may be consulted for a statistical evaluation of homograft survival times and of the tempo of homograft destruction. The standard deviation for survival times of these strains is about one day. In the absence of previous immunity, the probability that the destruction of a graft will commence on or before the sixth day is negligible. Where epithelial breakdown is in an advanced stage on the sixth day, there can be little doubt of the existence of previous immunity because first set homografts require about four days from the beginning of epithelial destruction to completion. On the other hand, the failure of an antigenic preparation, injected before grafting, to induce immunity can be explained through loss of activity in the preparation, faulty injection technique, or merely a delayed response

to the antigenic stimulus. Consequently, a large series of animals would be required for confidence in the results.

Biological Effectiveness of Immune Lymph Node Cells Labeled With DAN

Adoptive immunity is brought about when immune lymph node cells are injected into a non-immune recipient of the same inbred strain. It is necessary to inject a massive dose of cells, not later than the time of test grafting, for optimum results (24).

It was thought that DAN might offer some advantages over other forms of labeling in tracing the fate of the injected cells. The sulfamido linkage is a stable covalent attachment. Labeling occurs rapidly in vitro, an advantage where minimum modification of activity is of importance. Unlike other fluorescent labels, DAN is a compact molecule, with inactive methyl groups hindering its polar group, a fact that may explain its failure to modify drastically those molecules to which it becomes attached (49). Furthermore, as it will be pointed out in the next section, the long fluorescent lifetime permits the use of depolarization analysis. Labeling viable cells with DAN, which forms a covalent attachment to exposed amino groups, is a quite different approach from simply allowing cells to absorb fluorescent dyes, such as acriflavine etc.

Procedure Twenty-six axillary lymph nodes were removed from E strain mice which had been grafted ten days previously with D strain skin on only one side. The nodes were sieved into 4 ml. of citrated saline, pipetted to break up cell clumps, and the labeling solution was added.

The labeling solution contained 0.33 mg. of DAN and 0.1 ml. of absolute ethanol in 1 ml. of citrate saline. After ten minutes the suspension was centrifuged at 1000 RCF to sediment the cells, and the supernatant was withdrawn and discarded. The cells were not visibly affected by the brief exposure to the label, and upon examination in the fluorescence microscope they appeared to be brilliantly labeled. Nuclei of some cells were markedly fluorescent. The cells were injected into two E strain mice. Each mouse received the equivalent of 13 nodes, compared to the 8 nodes generally required to bring about adoptive immunity under other circumstances. Three days later the mice were grafted with D strain skin, and six days following the grafting the mice were examined.

Results Both grafts were at least fifty percent destroyed, which indicated the existence of definite immunity. This result is highly significant, and it is concluded that labeling cells with DAN under the conditions employed here does not eradicate their ability to induce adoptive immunity.

The fate of labeled cells upon injection has thus far been studied in only a preliminary manner. It has been determined that such cells are very quickly removed from the circulation after intravenous injection. Two hours after the injection of labeled non-immune lymph node and spleen cells, only a few cells could be identified in the buffy coat from blood samples. Microscopic examination of the spleen, liver, kidney, lung, and lymph nodes revealed that the majority of

injected cells had been removed from the circulation by the spleen. Examination eighteen hours after injection failed to reveal any remaining fluorescent cells in the buffy coat from a blood sample.

PART V

Fluorescence Polarization as a Cytological Method

Fluorescent labels have been used previously in locating labeled molecules in tissues. Under certain conditions, the number of labeled molecules in a tissue preparation can be estimated from the intensity of the fluorescent light. However, the potential usefulness of fluorescent labeling in cytological preparations may extend far beyond this limited application. Some physical properties of the fluorescent molecules and their environment are expressed through polarization of the emitted light. Observation of fluorescent labeled cells during the present study of their immunological properties suggested that polarization analysis might be profitably exploited in approaching many cytological problems. Previous applications of polarization analysis have been limited to the study of molecules in solution. The application to be discussed here concerns the association of fluorescent molecules within the structure and substance of tissue cells.

The polarization of fluorescent light depends on the tumbling motion of labeled molecules. The velocity of tumbling will depend on the size of the molecule, its shape and freedom of motion. The fluorescent label provides a chronometer for measuring the velocity of the motion. When used for this purpose it becomes a tool of considerable versatility.

The theoretical basis of polarization phenomena will be briefly reviewed here, and initial observations which indicate the feasibility of measuring the polarization of fluorescent light from cells in suspension will be described. Afterwards, some suggestions for future employment of the method will be discussed.

Theoretical Perrin (50) was the first to consider polarization as an expression of the relaxation times of molecules in solution, but Singleterry and Weinberger (51), Weber (52, 53), and Steiner and McAlister (49) have developed polarization analysis to a method of practical utility. The principle of the method depends on the fact that fluorescent molecules have finite excited lifetimes between the absorption and emission of light. The lifetimes of many organic molecules are of the order 10^{-8} seconds. Fortunately, the velocity of relaxation for high molecular weight substances is sufficiently high so that appreciable displacement will occur during such a period of time.

Absorption and emission of light takes place through oscillators having fixed geometrical positions within the fluorescent residue. In a randomly disposed collection of molecules only oscillators which are oriented parallel to the plane of vibration of the incident light will absorb energy. A fraction of the absorbed energy will be emitted some time later through the oscillators of emission, and the emission will be a plane polarized ray having an axis of vibration parallel to the emission

oscillator. In view of the length of time between the absorption and the emission, the place of emission may have become randomly oriented with respect to its position during absorption, if the molecule is free to move in space.

If a solution of fluorescent molecules is illuminated with a parallel beam of unpolarized exciting light, the horizontal and vertical intensities (I_h, I_v) of the light emitted perpendicular to the incident beam describe the polarization, which is defined as,

$$p = \frac{(I_v - I_h)}{(I_v + I_h)} \quad . \qquad (2)$$

The extent of the polarization will be a function of the harmonic mean of the rotational and translational displacements of the fluorescing oscillators during their excited lifetimes. If the orientation of the molecules changes little between the absorption and emission events, the polarization will be maximal. In other words, the orientation existing at the time of absorption has been preserved, since the molecules have undergone little rotation.

The simplest case to be considered is for spherical molecules. If the oscillator is rigidly bound to the sphere, and unpolarized exciting radiation is used, the following equation will hold;

$$\left(\frac{1}{p} + \frac{1}{3}\right) \Big/ \left(\frac{1}{p_o} + \frac{1}{3}\right) = 1 + \frac{R'T}{\eta V}\tau_o = 1 + \frac{3\tau_o}{\rho_o} \qquad (3)$$

where R' is the gas constant, T is the absolute temperature, η is the

solvent viscosity, V is the molecular volume, and τ_0 is the excited lifetime of the fluorescent residue. The constant ρ_0 is the value of polarization for an infinite relaxation time, and it is characteristic of the fluorescent group when no displacement occurs ($\eta = \infty$, or $T=0^{\circ}$).

The emitted rays from a number of different fluorescent molecules in a large collection or from different oscillators on the same molecule will be independent of each other. The intensities of the radiation will be additive, and the observed polarization will indicate an average value for the relaxation time of the collection. The polarization will be independent of the intensity of the exciting beam and the fluorescent efficiency or degree of quenching of the fluorescent residue. The accuracy of the measurement depends on the constancy of the excited lifetime. Steiner and McAlister (49) have found that the value of the excited lifetime of DAN, the fluorescent label used in the present studies does not change over a wide range of pH or ionic strength. For practical purposes this is most important, since it means that the chronometric standard will be unaffected by environmental conditions.

Polarization measurements indicate the relaxation times of the fluorescent residues. If the residue is rigidly bound to another molecule, the polarization will indicate the relaxation time of the larger kinetic unit, if there are no internal rotational degrees of freedom. A similar reservation must be made concerning the influence of viscosity. The polarization values will vary with changes in the viscosity of the medium, if the viscosity in the neighborhood of the

kinetic unit is continuous with the viscosity of the medium.

Procedure The demonstration of fluorescence polarization to be described here concerns the reaction of DAN with a spleen cell suspension.

Two mouse spleens (A strain) were sieved into 3 ml. citrated saline. The tissue fragments were broken up by pipetting, centrifuged and resuspended in 3 ml. normal citrate saline. 0.5 mgs. of DAN dissolved in 0.1 ml. of absolute ethanol was added to 1 ml. of normal citrate saline, and this was added with stirring to the cell suspension. After 10 minutes standing, the cells were centrifuged, washed in 3 ml. of normal citrate saline, and diluted into 15 ml. total volume of citrate saline in light-scattering cuvettes. The intensities of fluorescent emission were measured using the Brice-Phoenix light scattering photometer, modified as in the previous section for the measurement of fluorescence. However, a polaroid filter was inserted at the position marked POL (Figure 24). It was determined that the photocell was indifferent to the polarity of light striking its cathode. With the polaroid in place the fluorescent intensity was measured for vertical and horizontal orientation of the polaroid filter. After the initial measurement, the cells were centrifuged, the supernatant solution was removed and set aside for reading. The cells were resuspended in 15 ml. of citrate saline and read again. Three such washings were performed in all. The

readings given in Table 6 were taken at 25°C.

Table 6

Polarization of Fluorescence of Cells and Supernatants

Suspension	P	Supernatant	P
1	.0250	1	
2	.0682	2	.0198
3	.0841	3	.0189
4	.109	4	.0231

After the last reading the influence of temperature on the polarization was recorded between the range of 15°C. and 25°C. (Figure 19).

Discussion The value of polarization for the cell suspension (Table 6) increases after each successive extraction from the lowest value of 0.0250 to 0.109. Successive supernatants do not show an appreciable change in their polarization. The fluorescence extracted from the cells in each wash is probably due to unreacted label or labeled small molecules as shown by the slight polarization.

The readings describe the removal of small fluorescent molecules having short relaxation times from the cells, leaving more tightly bound residues. The latter are probably immobilized partially

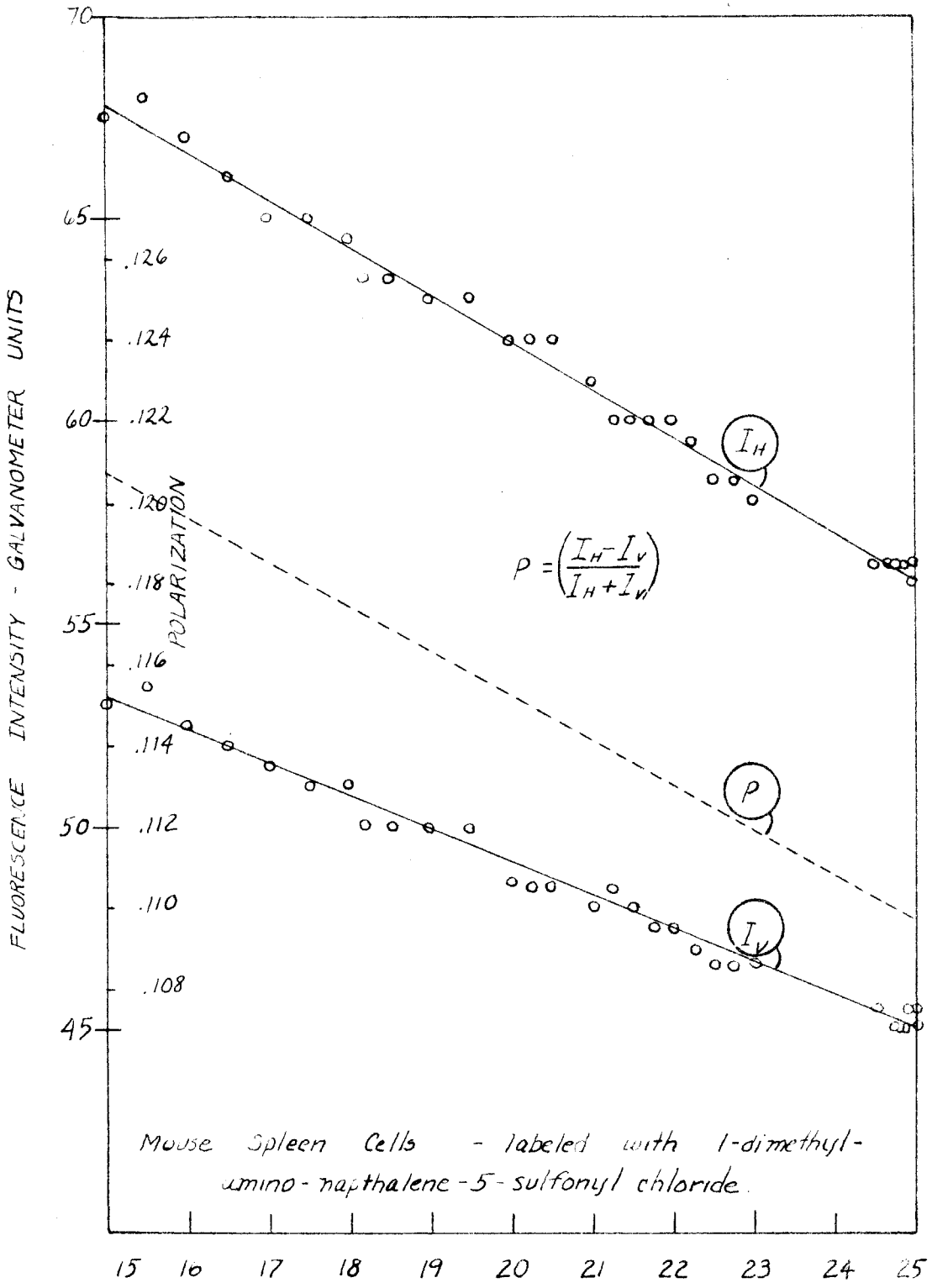

FLUORESCENCE INTENSITY - GALVANOMETER UNITS
POLARIZATION
70
65
60
55
50
45
.126
.124
.122
.120
.118
.116
.114
.112
.110
.108
I_H
$P = \left(\frac{I_H - I_V}{I_H + I_V}\right)$
P
I_V
Mouse Spleen Cells - labeled with 1-dimethyl-amino-napthalene-5-sulfonyl chloride.
15
16
17
18
19
20
21
22
23
24
25
TEMPERATURE CENTIGRADE

Figure 19

by their binding to the cell framework and partially by the higher viscosity of the intracellular environment.

At higher temperatures the polarization of the cell bound fluorescence decreases (Figure 19). The cells are stationary during the short excited lifetime of the label (1.28×10^{-8} sec.). The temperature will little affect their motion. However, the viscosity of the fluid constituents within the cell will probably be decreased with rising temperature. In addition, the thermal motion of fluorescent residues which are loosely bound to the firmer structure of the cells by freely rotating chains will be increased at higher temperatures. These effects will combine to provide the decrease in the observed polarization values as the temperature is increased.

The experiment is only intended as an example of the remarkable description provided by polarization of the partition of the label by the cells. If the fluorescent residue had been attached to another molecule as its label, the binding of this molecule by the cell would have been described in the same manner.

Additional variables might be introduced to add more to the description. By experimentally altering the viscosity of the suspending medium, using a solute for this purpose to which the cells are impermeable, fluorescent molecules attached to the outer surface might be identified and the rigidity of their linkage evaluated.

In addition to describing the uptake and binding of fluorescent molecules by cells, the problem of intracellular viscosity may be

approachable by the same method of analysis. Previous investigations of intracellular viscosity have been unsatisfactory. Crick and Hughes (54) allowed cells to phagocytize magnetic particles, which were then subjected to external fields of force. Their analysis was hampered by the large size of the particles which were less influenced by the microscopic viscosity of the cytoplasm, than by macroscopic structure within the cytoplasm. Employment of fluorescent molecules having well defined relaxation times could be used in place of the magnetic particles to evaluate the viscosity of the intracellular medium. Careful selection of molecules for this purpose would be necessary to eliminate those which are altered by the environment.

PART VI

A Simplified Method for the Demonstration of Mouse Isohemagglutinins

Hemagglutinating antibodies are demonstrable in the serum of mice following immunization with homologous tissues. The role of these isohemagglutinins in transplantation immunity was discussed in the introductory review. The antibodies involved in the response appear to be heterogeneous, falling into various orders of reactivity, depending on the methods used for their demonstration (16, 17, 55, 56, 57).

Rarely will agglutination occur in saline-diluted mixtures of antisera and cells. For this reason, the antibodies involved are described as "incomplete". It is necessary to use various enhancing media to bring about the agglutination. The only suitable method of demonstrating the agglutination has been that devised by Gorer and Mikulska, (57). This procedure requires the addition of human serum and dextran as diluents for the cells and antisera. For the most satisfactory results, the human serum must be procured from donors who have not handled mice (16), but who have some debilitating disease which has resulted in an increased sedimentation rate (55). In addition to these extraordinary requirements, the serum must be absorbed with mouse red blood cells prior to use.

Certain dextrans, the fluids from psuedomucinous ovarian cysts, or Wharton's jelly are required in addition to the human serum (55). Variations in the results obtained by those who have become sufficiently emboldened to use the technique either reflect the understandable variation in the enhancing reagents, or variability and instability of the antibodies and antigens involved. It is sometimes difficult to decide between these alternative explanations in the absence of other means of demonstrating the reactions. In spite of the alchemical nature of the enhancing reagents, the procedure has served in analysis of the H-2 alleles in mice and the cellular antigens associated with this locus, and the results have been in general agreement with those resulting from tumor transplantation analysis.

The hyaluronic acids of human umbilical cord and bovine vitreous humor have recently become available in highly purified form*. The potassium salts in the lyophilized state are stable. In the course of the present study these preparations were tested for their ability to enhance the activity of mouse isohemagglutinins. Various iso-immune sera from four strains of mice available (C 57 BL/10, B.10/H2-D, CBA, and A) were tested. Of these combinations, C 57 BL/10 anti-B.10/H2-D was found to react with B.10/H2-D and A cells, at titers of 1:64 and

*Potassium hyaluronate from bovine vitreous humor , Lot No. 216-4, and potassium hyaluronate from human umbilical cord, Lot Nos. 28-1 and 172-1, were obtained from the Wyeth Institute for Applied Biochemistry, Philadelphia, through the courtesy of Dr. Joseph Seifter, Director.

1:128 respectively, following a first set skin graft. Hyperimmune CBA anti-A serum was found to react with A cells at a dilution of 1/1280. The following procedure was used. One drop of a one percent suspension of washed cells in buffered saline (0.85% NaCl buffered at pH 7.4 with 0.01M sodium phosphate buffer) was added to one drop of the dilution of serum in buffered saline. After mixing, one drop of a solution containing 5 mg./ml. of potassium hyaluronate from human umbilical cord or bovine vitreous humor was added to the tube. Test tubes (8 x 75 mm.) were used for the tests. After addition of the hyaluronate solution, the mixture was shaken and incubated at 37°C. for 15 to 60 minutes. The strength of agglutination seemed to increase only slightly with incubation. After incubation, the tubes were centrifuged at 1000 RCF. in an angle centrifuge, and then read after gentle shaking. The agglutinated clumps of cells could be dispersed by shaking, but upon allowing the dispersed cells to sediment, they reagglutinated to a lesser extent forming a sedimentation pattern quite different from the normal, non-agglutinated controls. Of the two hyaluronates tested, that from umbilical cord gave better results with the C 57 Bl/10 antiserum, whereas the vitreous humor preparation gave slightly better results with the CBA antiserum. When Glaxo dextran, a preparation recommended by Gorer, was used in addition to the hyaluronate by adding one drop of a 2% solution to the tubes, false agglutinations were obtained. These clumps of cells had a stringy

consistency, even in control tubes containing no antiserum. False positive agglutinations in control tubes which contained normal non-immune serum were not noted at the concentration of hyaluronate used here. Higher concentrations than 10 mg. per ml. were not tested, because a limited supply of the hyaluronate was at hand. The strength and titers of the agglutinations fell off as the concentration of hyaluronate was reduced below 5 mg./ml. Two samples of umbilical cord hyaluronate were tested, one of which had been stored four years longer than the other. Both of these samples gave identical results.

The titers obtained for the antisera cannot be directly compared with those obtained with the Gorer technique, because a different method for determining the end point is used. With the Gorer technique, the cells are not centrifuged following incubation, but are removed with a pipette and placed on a glass microscope slide, which is then rocked gently.

The simplicity and speed of the present technique, and the reproducibility of the hyaluronates used here recommend the method for further study. Only saline and purified hyaluronate are required in the system, evading the extreme variability in dextrans from different sources and the time-consuming and expensive absorptions of human serum involved in the Gorer method. Considerably more extensive study of the two systems in parallel tests would be required, however, before substitution of the present procedure for the Gorer technique could be

recommended for routine immuno-genetic work with mice. Gorer has noted, for example, that certain antisera will agglutinate the red cells of particular strains in saline media, while they fail to produce visible agglutination in others. Sometimes, however, the latter cells, negative in saline, will agglutinate in the "enhanced" system, and will absorb the antibodies with which the saline agglutinated cells react. Experiences of this sort, multiplied in Gorer's extensive experiences, indicate that any abbreviated method suggested as a replacement for his technique must prove successful with many diverse combinations of antisera and cells, before it can be accepted for routine use.

PART VII

Relation of the Immunological Properties of Elastin to Its Structure

The survival of elastin in homo- and heterologous arterial transplants invited attention to the structural basis for this remarkable immunological tolerance. Elastin persists in foreign recipients for years, a property not known to be shared by any other native proteins (58).

Two features of elastin set it apart from most other proteins. The substance is highly resistant to chemical and enzymatic digestion in vitro (59), and it possesses unusual rubber-like elasticity (60, 61). However, elastin is not totally unsusceptible to enzymatic digestion in vivo, a fact well supported in the literature on arterial pathology. Either of the above properties of elastin might explain its tolerance by a foreign recipient. The hypothesis chosen here as the more probable explanation is that elastin lacks immunological specificity by virtue of its elastic structure. In the following study this hypothesis has been explored.

Structural Requirements for Elasticity Rubber-like substances are characterized by their thermodynamic behavior (60). Extension of these substances involves no volume change, and heat is liberated.

If a strip of such a material is maintained at constant length, and the retractile force is measured as the ambient temperature is increased, the force will increase. It can be shown that little internal energy change of the system is associated with the change in force, but the latter is the result of greater thermal motion of the molecules. Extension of a rubber-like substance accordingly involves a decrease in entropy.

The structure proposed for a rubber-like substance must account for the thermodynamic observations. The structure must allow for extension of the molecular network, without the distortion of valence bonds. A network of flexible linear macromolecules, anastomosing at intervals, is the generally accepted picture of such a system. Folding of the segments between the junctions with neighboring chains is permitted by free rotation about single bonds, and such rotation must be permissible in acceptable structures. The relaxed network of the sort just described will assume the most probable configuration, i.e., maximal configurational entropy. Thermal motion will bring about random disorder of the segments between the junctions. When the network is extended, the chains are extended proportionally; they are consequently brought into parallel arrays at high extensions. Since reversible behavior is required, there must be little opportunity for strong interaction between neighboring chains. The

structural requirements just set forth clearly restrict the order of specificity possible for a rubber-like protein to that residing in the sequence of amino acids along the polypeptide chains or to limited crystalline or ordered regions of an imperfectly elastic network.

Structure of the Aortic Wall Previous electron microscopic study of the aortic wall had revealed some details of its fine structure (62, 63). The tunica media contains the most elastin. The elastic lamellae of the media encircle the wall in concentric sheets. These sheets are almost wholly elastin. Elastin is an amorphous substance, having no fibrillar structure at electron microscopic resolution. In the process of formation, elastin is apparently deposited in a fibrous network of collagen. As the result, some collagenous fibers are entrapped within the elastin sheets. Smooth muscle cells are arranged in rows, between the concentric elastin lamellae. These cells fill the spaces within a collagenous and elastin network which connects concentric lamellae. The tunica intima consists of irregularly disposed sheets of elastin and the tunica adventitia consists largely of collagenous fibers.

For analysis of the mechanical properties of elastin, a preparation dissected free of the adventitia and the intima was chosen as the most uniform and reproducible sample. The aortas of various animals were examined microscopically. The aorta of the pig was selected as the most suitable for thermodynamic analysis. Pig aortas have

uniformly arranged elastic lamellae, and strips consisting of medial lamellae can be cleanly dissected from them.

Digestion of the Aortic Media Whereas the strips of aortic media consisted largely of elastin, other fibrous proteins were additionally present. Preliminary experiments were carried out to remove these contaminants by chemical and enzymatic digestion. Trypsin digested most of the interlamellar cellular material, leaving a skeleton of collagen and elastin. Such treatment did not affect the elastic properties of the wall, and it only slightly reduced the ultimate strength. Digestion with acetic acid or pepsin had little effect on the elasticity, but since collagen fibers were attacked, the wall was markedly weakened. When medial strips of pig aorta were digested with trypsin buffered at pH 8.5 for 24 hours at 37°C., digested further with 1% acetic acid for 48 hours at 37°C., and then digested with pepsin buffered at pH 4.5 for 24 hours at 37°C., the wall had lost its structural integrity, leaving only undigested elastin. The remaining elastin network would not support its own weight on removal of the digestion solution. It appeared to retain the property of elasticity, insofar as this could be shown for the individual fibers and plates. When these were manipulated on a microscope slide they had spring-like qualities. Digestion with 0.1 N NaOH 45 minutes at 90° did not appreciably affect the elasticity of aortic strips, but it did reduce their strength.

X-ray Diffraction

Elastin fibers were exhaustively purified for X-ray diffraction analysis. Beef aorta was fragmented in a Waring Blendor in 0.85% NaCl solution. After repeated washing with saline, the insoluble residue was digested in 0.1 M NaOH solution for one hour at 90°C. The sediment was washed with distilled water, homogenized again in the Waring Blendor, and subjected to another hour of digestion at 90°C. with 0.1 M NaOH solution. The sediment was washed repeatedly with distilled water, and refluxed with a mixture of equal parts of ether and ethanol to extract lipids. The elastin powder was finally washed with ether and partially dried. Before drying was complete, samples of the powder were rolled into threads between glass slides. Such threads are sufficiently cohesive on further drying to permit mounting for X-ray diffraction analysis.

Philips X-ray diffraction apparatus, provided with a micro-camera was used. The camera had a collimating bore of .002 inches, and an adjustable fiber to film distance. A copper target furnished 35 kV. radiations, which were predominantly K alpha rays after filtering with nickel foil. Exposures ranged between 48 and 72 hours at 25 ma, depending on the sample used and the adjustment of the camera. The camera chamber was exhausted to reduce air scattering. A typical diagram is shown in Figure 20. The distances calculated for the two diffuse rings are 7.05 and 3.78 Angstrom units.

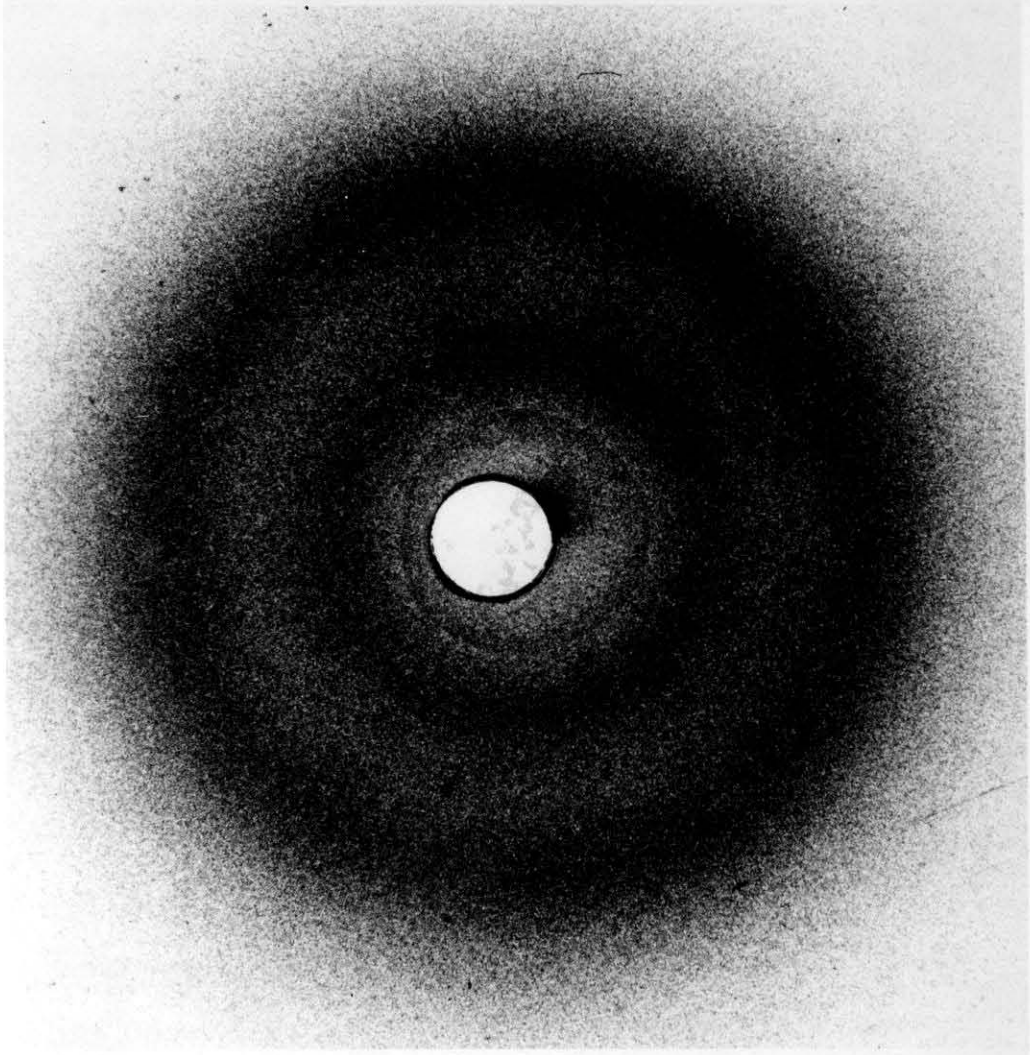


Figure 20

X-ray diffraction pattern of bovine aortic elastin.

The ring distances and relative intensities are most similar to those obtained by Kratky, Schauenstein and Sekora for native silk from Bombyx mori (64). Their pattern for silk 1, following swelling in formic acid, consisted of a ring of medium intensity at 7.0 Angstrom units and one of strong intensity at 3.5 Angstrom units. The distances obtained here were 7.05 Angstrom units for the ring of medium intensity and 3.78 Angstrom units for the strong ring. These are tentatively interpreted as the first and second order reflections for the side chain distance of a fully extended polypeptide. According to Corey and Donohue, the distance expected from calculated bond distances and angles is 7.27 Angstrom units (65). The above patterns give no evidence of orientation along the fiber axis, but the rings are sufficiently sharp to indicate the presence of rather uniform repeating distances along randomly oriented polypeptide chains.

Thermoelastic Behavior

Meyer and Ferri (60) have discussed the expression of the retractile force of a rubber-like elastic system in thermodynamic terms. Accordingly,

$$F = \left(\frac{dU}{dL}\right)_T - T\left(\frac{dS}{dL}\right)_T + P\left(\frac{dV}{dL}\right)_T, \quad (4)$$

where F is the retractile force for a reversible extension, U is the internal energy of the system, S is the entropy, L is the length of the network, and P, T and V are the pressure, volume and temperature.

It can further be shown that

$$\left(\frac{dS}{dL}\right)_T = -\left(\frac{dF}{dT}\right)_L \quad . \qquad (5)$$

The term $P\left(\frac{dV}{dL}\right)_T$ has been shown to be negligible for elastin (61) so that Equation 1 can be reduced to

$$F = \left(\frac{dU}{dL}\right)_T + T\left(\frac{dF}{dT}\right)_L \quad , \qquad (6)$$

where the term $T\left(\frac{dF}{dT}\right)_L$ describes the entropic contribution to the retractile force. The value of $\left(\frac{dF}{dT}\right)_L$ is evaluated from the tangent of the isometric force–temperature curve at any temperature T, and the value of the internal energy term $\left(\frac{dU}{dL}\right)_T$ is obtained by the difference between the entropic term and the retractile force. Note that heat is evolved in stretching a rubber-like system, and the entropy of the system decreases as the retractile force increases.

Thermoelastic Measurements A fiber balance, shown in Figure 21, was constructed for the combined measurement of the force at various extensions and temperatures*. A balance beam has a hook attached to one arm, and an adjustable sliding counterweight attached to the other. The hook dips into the bath. A second, lower hook immersed within the bath is rigidly attached to an adjustable vertical slide. The latter hook can be lowered or elevated with respect to the upper hook, by adjustment of the vertical slide, and its distance below the upper hook

---

* The invaluable assistance of Mr. Frank Ostrander of the Biology Division during the construction of the elastic balance is herewith acknowledged.

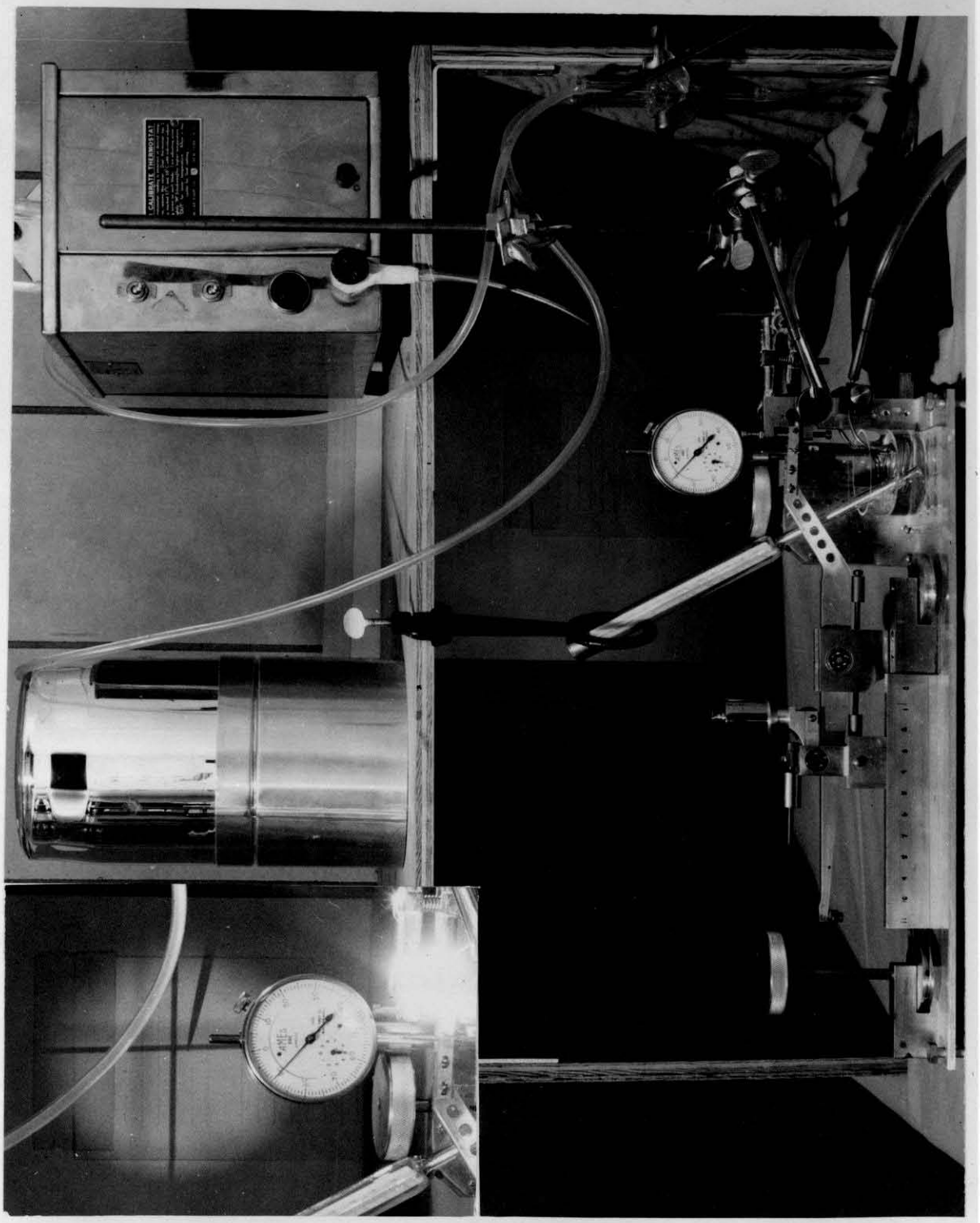


Figure 21

Thermo-elasticity Balance

measured to within .001 inch by means of the dial guage.

The strip of elastic material to be tested is stretched between the upper and lower hooks. The level of the immersion fluid is above the top edge of the strip. Temperature of the fluid is varied by passing either hot or cold water through a coil of hypodermic tubing immersed in the bath. The selection and rate of flow are selected by means of a two-way stopcock leading to the reservoirs on the table above the balance. The temperature within the bath is read on a precision, jacketed thermometer to $\pm .05^{\circ}C$. The condition of balance is indicated by means of a needle attached to the arm of the beam, which may be centered on a reference point formed by two crossed hairs. The magnified images of the needle point and the crossed hairs are projected on the back wall by means of a light beam and projection lens system. Any change in the retractile force of an elastic strip suspended between the two hooks, brought about as the result of varying the temperature or the extension of the strip, will alter the position of the beam, and the needle will move from its centered position. By adjustment of the sliding counterweight, the needle point is brought back to its centered position, and the weight required for the compensation read off of the horizontal scale. The position of the counterweight along the balance beam is indicated on this scale by means of a vernier attached to the yoke from which the counterweight is suspended. Either force-extension or isometric force-temperature

measurements can be carried out with considerable precision.

Isometric force–temperature runs were performed by selecting a given extension of the aortic strip, and increasing the temperature to the maximum (55°C.), while maintaining a constant length. The strip was maintained under the resulting stress until no further relaxation of the force was evident. The temperature was then allowed to fall at the rate of about 1°C. per minute, and the force was read at 0.5°C. temperature intervals. The length and width of strips were measured under a microscope with a calibrated stage. The thickness was measured with a compression micrometer.

Results A force–extension curve and a family of force–temperature curves taken at different extensions are given in Figure 22. The values of the tangents of the force–temperature curves at 10°, 20°, 30°, 40° and 50°C. were determined from the curve. These are the coefficients $\left(\frac{dF}{dT}\right)_L$. Multiplying by the absolute temperature, the values are obtained for the entropic contribution $T\left(\frac{dS}{dL}\right)_T$ to the retractile force (F). The term $\left(\frac{dU}{dL}\right)_T$ is evaluated as the difference between the retractile force F and the entropic term. The resolution of the force into the above terms is given in Figure 23.

Discussion The thermoelastic behavior of only a single strip of pig aorta is presented as an example of the typical results obtained for many random samples of pig aortas. Aortic elasticity varies greatly with the species, age and physiological condition of the animal from

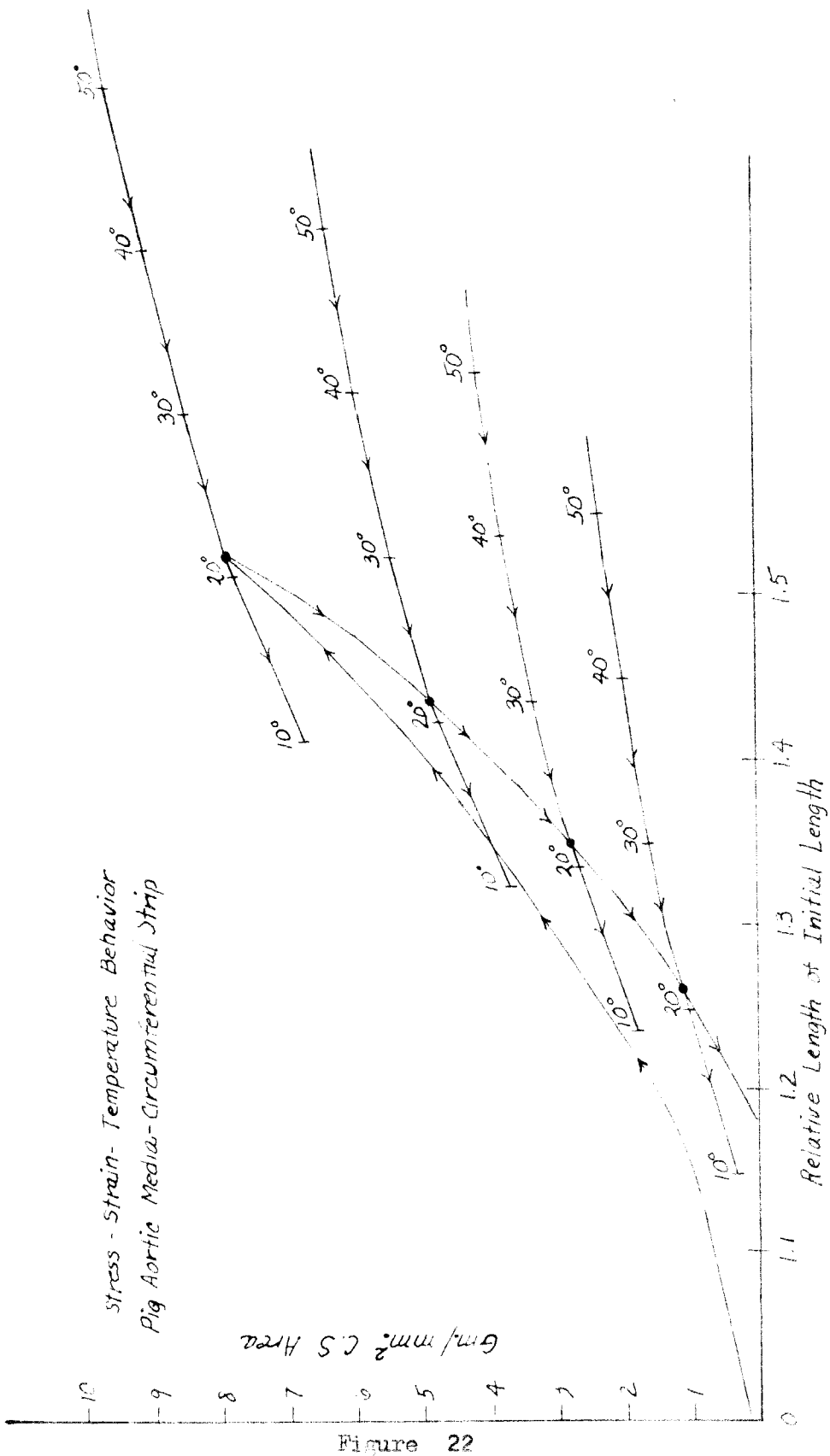

Stress - Strain - Temperature Behavior
Pig Aortic Media - Circumferential Strip
Gm/mm² C.S Area
Relative Length of Initial Length
0
1
2
3
4
5
6
7
8
9
10
1.1
1.2
1.3
1.4
1.5
10°
20°
30°
40°
50°

Figure 22

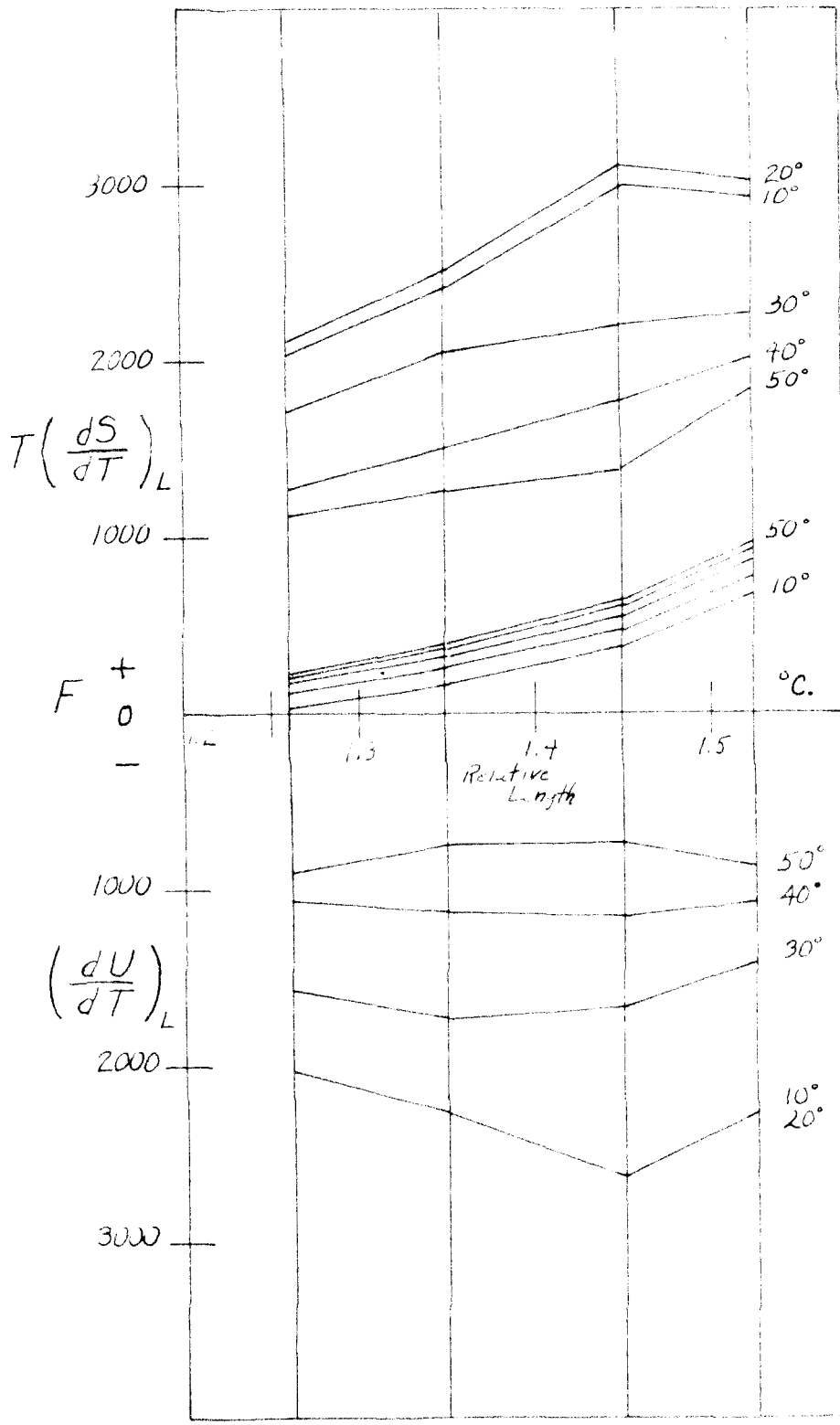

3000
2000
$T\left(\frac{dS}{dT}\right)_L$
1000
F
+
0
−
1000
$\left(\frac{dU}{dT}\right)_L$
2000
3000
20°
10°
30°
40°
50°
50°
10°
°C.
1.3
1.4
1.5
Relative Length
50°
40°
30°
10°
20°

Figure 23

which a sample is taken. Furthermore, the behavior is somewhat variable with different strips taken from the same aorta. The aortic wall is not homogeneous in composition. Comparison of the numerical values of the forces for different strips is hardly justified. However, the derivatives of the force-extension and force-temperature curves vary little for the samples, and the conclusions to be drawn here are based on these.

Certain features of the behavior were brought out by the continuous measurement of the force over broad temperature and extension ranges with the present apparatus. These have apparently been overlooked in previous studies where the measurements were limited. The sharp transition from linear dependence of the force on the temperature to curvilinear dependence between 20°C. and 30°C. was invariably observed, (Fig.22), for pig aortic strips. For the sample figured here, this transition decreases from 26°C. for the lowest extension to 21°C. at the highest extension.

Moderate extension, below 45%, at the lower temperatures, 10°-30°C., involves a reduction in internal energy opposed by a greater decrease in entropy. Above 45% at these temperatures, the extension involves little further decrease in entropy, and the work of extension increases the internal energy of the system. It is a curious fact that nearly ideal rubber-like elasticity is obtained for extension near body temperature. At 40°C. no change in internal energy is involved over the entire range of extension. The

retractile force at this temperature is due only to the change in entropy of the system. Above 40°C. where linear behavior is obtained, the moderate extension involves an increase in internal energy, and at the higher extension the latter decreases.

It might be expected that an elastic network of polypeptide chains would show ideal behavior at moderate extension and higher temperatures; because either of these conditions will minimize the forces which are involved in the immobilization of the chains. More than 90% of the amino acid residues in elastin have non-polar side chains (66). The retractile force of the strips studied here was little affected by changes in pH or ionic strength. The force is affected by less than 2% when the pH is varied through a range from pH4 to pH9. It is apparent that electrostatic interaction of neighboring chains is of little importance. Probably the predominant forces tending to immobilize the chains are van der Waals' forces and hydrogen bonds. Elastin has a high content of the amino acids, valine and proline (66). These residues probably prevent immobilization of the chains by interfering with the close packing required for maximal interaction through van der Waals' forces. Accordingly, chain immobilization must be largely attributed to hydrogen bonding, but this must be limited to the lower temperatures. In view of the low bond energy (5 kcal.) of hydrogen bonds, these will be easily broken at higher temperatures, especially if they are strained by the presence of the bulky R groups. At body

temperature it is improbable that elastin is capable of immobile configurations; thus, it is uniquely adapted for its physiological role.

Immunological Properties of Soluble Elastin Derivatives

Partridge, Adair and Davis (67) prepared derivatives of beef elastin by digestion of highly purified fibers with 0.25 M oxalic acid. The hydrolysis products fell within two number average molecular weight classes: 60–80,000, for alpha elastin, and 6000 for the beta product. From end group analysis, Partridge and Davis (66) concluded that alpha elastin consisted of 17 chains each containing 35 amino acid residues. The amino acid analyses for alpha elastin, beta elastin and the parent fibers were sufficiently similar to encourage the assumption that their structures were similar. To all indications, the chains were long ones – held together by only a few covalent cross linkages. The data obtained from a detailed analysis of the soluble derivatives were in accord with the structure suggested by the X-ray diffraction analysis and the thermoelastic behavior.

Alpha elastin was a sufficiently large fragment of the elastic network to warrant inspection of its antigenic properties. On the basis of the hypothesis advanced at the outset, it would be predicted that fragments of the elastic network would lack antigenicity; they should possess no greater structural immobility than the parent compound. Whereas immunological activity might be thwarted by the insolubility of the intact network, this explanation would not apply

to the soluble derivatives. The fragments should lack specificity, according to the hypothesis, unless this is a property of the amino acid sequences.

Procedure Soluble fractions of bovine aortic elastin were prepared according to the procedure of Partridge, Adair and Davis (67). The aortas were cut into small pieces and minced in the Waring Blendor in excess 1% NaCl solution. Following repeated extraction with the saline, the insoluble material was washed with water to remove the salt. It was then autoclaved at 15 pounds pressure for 45 minutes and washed to remove the gelatin. Autoclaving and washing was continued for three successive cycles, after which little further material could be extracted. The sedimented cake was dehydrated with absolute ethanol. It was extracted repeatedly with a mixture of equal parts of ethanol and ether. After further extraction with ether, the remaining material was dried. The fibrous powder was pulverized in a ball mill, resuspended in water, autoclaved, washed with excess water, extracted again with ethanol-ether, and finally dried from ether.

The white powder obtained from the above procedure was digested by refluxing with 0.25 M oxalic acid at 90°C. Ten grams of the elastin was suspended in 20 ml. of the oxalic acid solution in a boiling flask, provided with a reflux condenser. The flask was immersed in a boiling water bath. At intervals of two to three hours, the contents of the flask were centrifuged, and the supernatant hydrolysate was removed.

A fresh aliquot of oxalic acid was then added to the sediment, and digestion was continued. The time required for complete digestion varied somewhat from batch to batch. After ten hours it was nearly complete. The concentration of the initial material contained in the combined hydrolysates ranged between 5 and 10 percent, depending on the number of changes of oxalic acid required for full digestion of the batch.

The hydrolysate was exhaustively dialysed against distilled water to remove the last traces of oxalic acid. A large part of the hydrolysate was also dialysable. Nitrogen analysis showed that about 70% of the initial nitrogen was lost in the dialysate. After the dialysis, the non-dialysable fraction was a slightly yellow solution of from one to two percent protein.

Immunization Six rabbits received 10 mg. of protein and 4 rabbits received 50 mg. of protein, intravenously, three times weekly for 8 weeks. Serum samples were taken at weekly intervals after the fourth week of immunization. Various dilutions of the serum were tested for precipitation against various dilutions of the protein. Interfaces were formed between the serum and the antigen in tubes having a 2 mm. inside diameter. These were observed several hours for precipitation rings with negative results.

Guinea-pigs were sensitized by two procedures. Twenty-six animals received 5 mg. protein intraperitoneally, and at intervals

of three to four weeks they were challenged with 15 mg. protein injected into the peritoneum. Thirteen animals received 5 mg. protein initially, followed by 5 mg. protein one week later. These were challenged intravenously with 15 mg. protein 30 days later. None of the above guinea-pigs displayed symptoms of anaphylaxis.

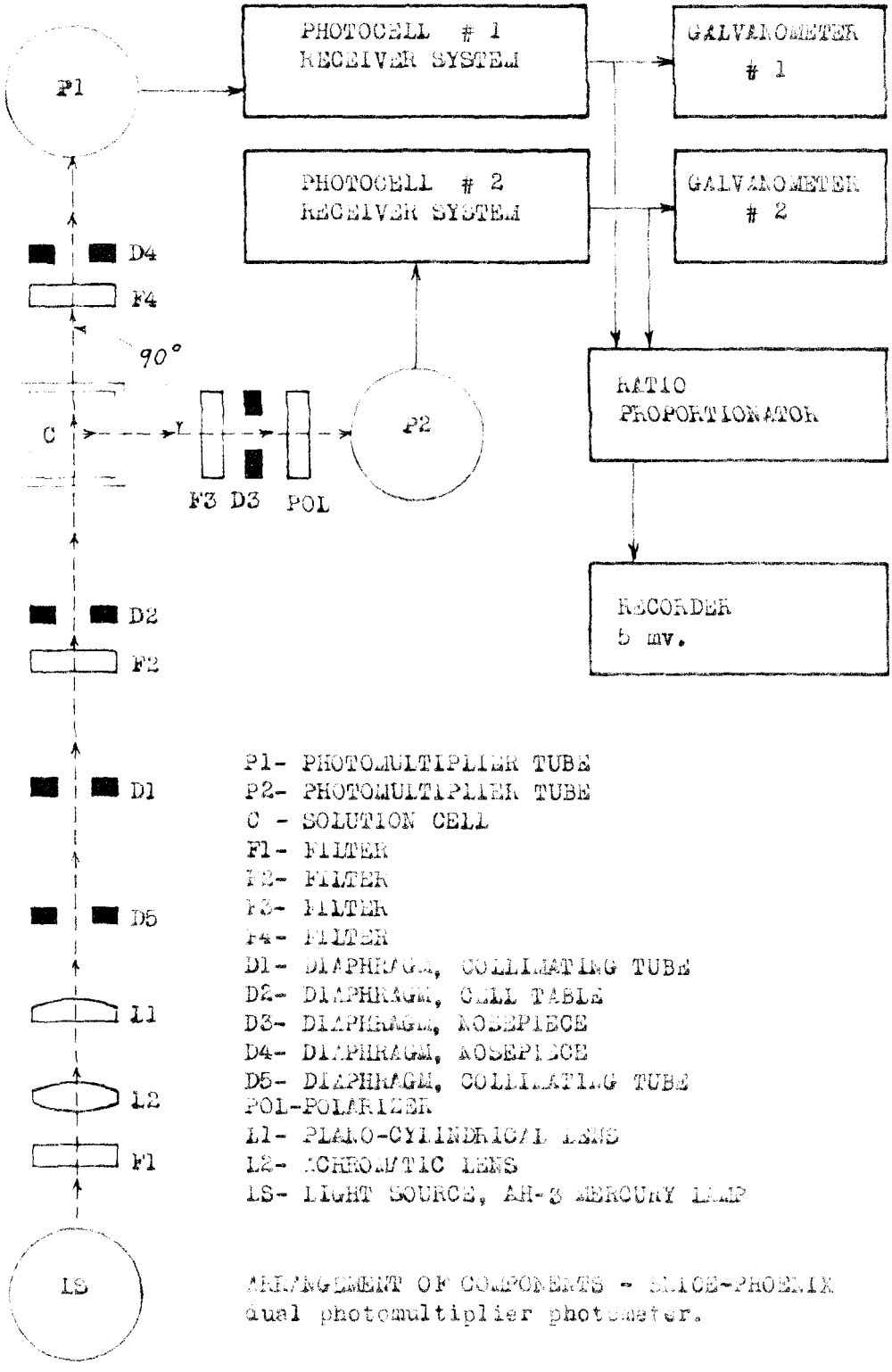
Discussion

The data previously discussed indicate that elastin is composed of a network of polypeptide chains capable of little structural immobility resulting from interaction between the chains. At mammalian body temperature it is doubtful that hydrogen bonding contributes to the rigidity of the chains. Van der Waals' forces between the residues on neighboring chains are probably of somewhat less significance in elastin because of the bulky R groups and proline residues which hinder close packing in the absence of other secondary valence forces between the chains.

The specificity of such a system is probably limited to that within the sequence of amino acids along segments of the chains. Apparently, non-dialysable hydrolytic fragments of the elastin network lack sufficient specificity to induce the formation of precipitating antibodies in rabbits or to bring about anaphylaxis in the guinea-pig. It is tentatively concluded that the hydrolysis products are non-antigenic for the species tested.

Alternative explanations for the absence of antigenicity in elastin can be advanced. It may be argued that the substances are either unsusceptible to enzymatic digestion by the recipient, or that they are degraded and excreted so rapidly that antibody formation is not stimulated. It is considered unlikely that native elastin, which survives heterologous transplantation, and the soluble derivative would each fall within the requirements demanded for the alternative explanations. In view of this, the more probable explanation is thought to be based on the absence of specific lattices in the elastic network.

A P P E N D I X



P1- PHOTOMULTIPLIER TUBE
P2- PHOTOMULTIPLIER TUBE
C - SOLUTION CELL
F1- FILTER
F2- FILTER
F3- FILTER
F4- FILTER
D1- DIAPHRAGM, COLLIMATING TUBE
D2- DIAPHRAGM, CELL TABLE
D3- DIAPHRAGM, NOSEPIECE
D4- DIAPHRAGM, NOSEPIECE
D5- DIAPHRAGM, COLLIMATING TUBE
POL-POLARIZER
L1- PLANO-CYLINDRICAL LENS
L2- ACHROMATIC LENS
LS- LIGHT SOURCE, AH-3 MERCURY LAMP

ARRANGEMENT OF COMPONENTS - SPICE-PHOENIX dual photomultiplier photometer.

Figure 24

Reduction of scattered intensity at 90° (I_s90) by secondary scattering, (see text page 64)

According to Caspersson (44), if a beam of light is passed through a turbid solution enclosed in a cubic space d^3 (Fig. 25), it will lose part of its energy through scattering and,

$$E_t = E_o - E_s \quad , \tag{7}$$

where E_t is the transmitted energy of the initial beam E_o, and E_s is the scattered light. If the solution is not infinitely dilute, the light scattered at any angle θ will further lose intensity through secondary scattering. Considering the situation for a particle at the point (u,v),

$$I_v = I_o \cdot e^{-kCv} \quad , \tag{8}$$

where I_v is the intensity of the beam incident on the particle, and k is the extinction coefficient. In colorless solutions the absorption of light is negligible and it will be assumed that k is equivalent to the turbidity, i.e. the loss in energy is all due to scattering. C is the concentration of particles along the line v.

$$I_\theta = k'' I_v \quad , \tag{9}$$

where k" is the fraction of energy scattered at an angle θ, and is a function of the angular scattering pattern of the particle which

will be later considered. The observed intensity will be inversely proportional to the square of the distance r, and

$$I_{obs.} = \frac{I_\theta \, e^{-kCu}}{r^2} \quad . \tag{10}$$

Combining the previous equations,

$$I_{obs.} = \frac{k'' \, I_o \, e^{-kC(v+u)}}{r^2} \quad . \tag{11}$$

In order to obtain the total intensity of the scattered beam in the θ direction, the scattered rays of all the particles in the volume d^3 must be considered, and the equation becomes,

$$I_{obs.} = k'k''C \, I_o \cdot \frac{I}{d^2 \, r^2} \int_{v=o}^{v=d} \int_{u=o}^{u=d} e^{-kC(u+v)} du \, dv \, . \tag{12}$$

Upon integration,

$$I_{obs.} = (k'k'' \, C \, I_o) \cdot \frac{1}{k^2 \, C^2 \, d^2 \, r^2} \cdot (e^{-kCd} + 1)^2 \quad , \tag{13}$$

where k' is the proportionality constant relating the number of particles N to the concentration C. $N = k'C$, and since $N < \infty$, the right side of Equation 10 must be multiplied by N/d^3, and subsequently Equation 12 multiplied by $k'C/d^3$. For $\theta = 90^\circ$, Equation 13 describes the intensity measured in the present experiments. The relation of the scattered intensity I_{90} to the relative concentration is shown in Figure 26.

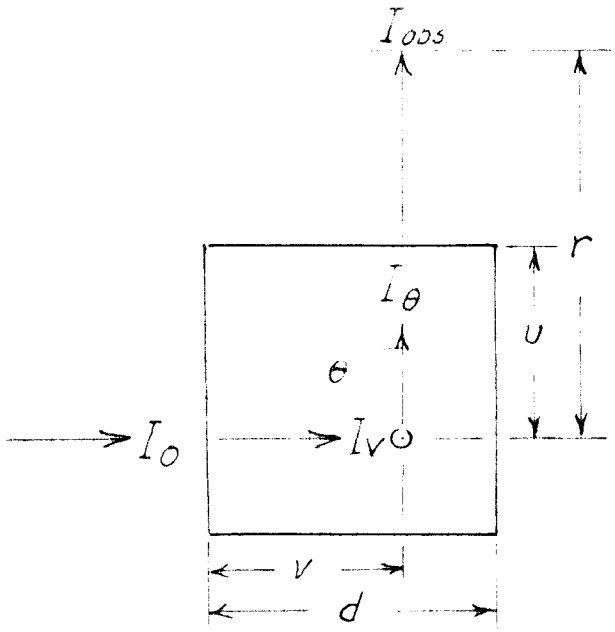


Figure 25

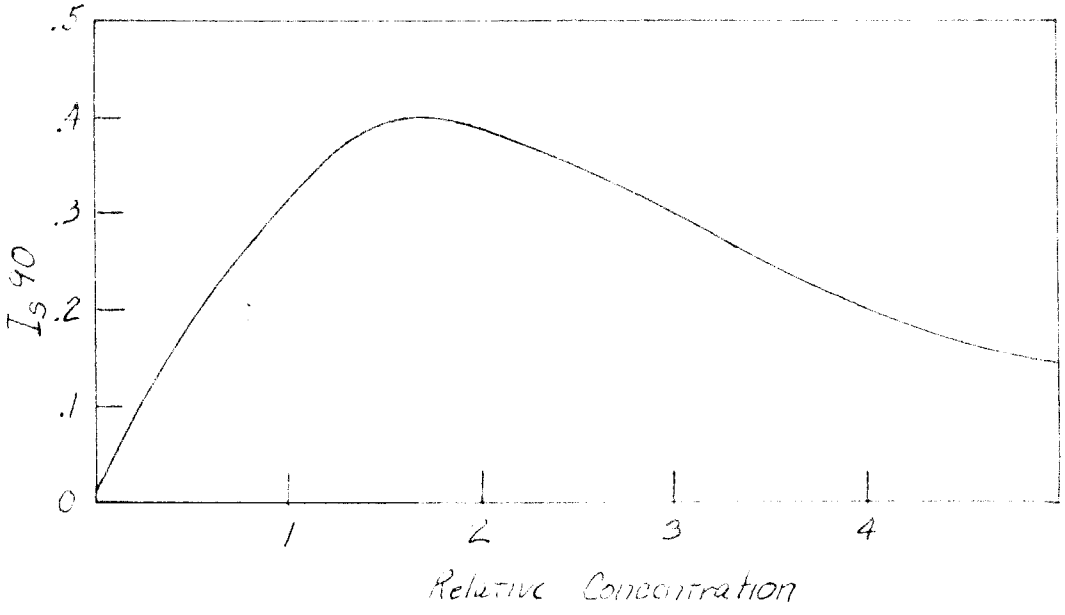


Figure 2[illegible]

The transmitted light at 0^{o}, I_t, follows Beer's law, and

$$I_t = I_o \, e^{-kCd} \quad . \tag{14}$$

Equation 13 describes the attenuation of the lateral and forward scattered light and the transmitted light as these are determined in the present experiments. It is important to point out that the solutions examined in the experiments were of sufficiently low turbidity that the forward scattered light I_s0 never exceeded about 1% of the transmitted light I_t. As a result the variation of I_s0 in the Series A measurements was negligible in the ratio $(I_s0 + I_t)/I_s90$.

Variation in scattered intensity at 90^{o} (I_s90) upon aggregation.

For the purpose of evaluating the results it will be necessary to determine how I_s90 will vary as aggregation takes place in a fixed volume of dispersed phase. The model to be considered is the fixed volume of material distributed in spherical particles of various sizes.

For spheres having a diameter less than about one-tenth the wavelength of incident light (420 mμ) the intensity envelope will have the form,

$$p = 1 + \frac{\cos^2\theta}{2} \tag{15}$$

and $I_o = 2\, I_{90}$. Then, $I_\theta = \frac{I_o\,(1 + \cos^2\theta)}{2}$. (16)

The total scattered energy E_s is obtained by integrating the scattered intensity I_θ over all angles of θ, where θ is the angle between the incident and scattered beams and r is the radius of the sphere of whose surface the integration is performed.

$$E_s = \int_0^{\pi} I_\theta \, 2\pi r^2 \sin\theta \, d\theta \qquad (17)$$

therefore,

$$E_s = \int_0^{\pi} I_o \cdot \frac{1 + \cos^2\theta}{2} \cdot 2\pi r^2 \sin\theta \, d\theta \qquad (18)$$

integrating, $E_s = 8/3\pi I_o = 16/3\pi I_{90}$, and it can be shown for low turbidities that

$$\tau = 16/3\pi (I_{90} r^2/I_t) \qquad (19)$$

where τ is the extinction coefficient or turbidity defined by $I_t = I_0 e^{-\tau d}$, and I_t is the transmitted intensity.

In the Rayleigh scattering range, I_s90 will be directly proportional to the total scattered energy. According to Oster (68), the scattered energy of a single particle will increase as the sixth power of the radius. Since the number of spheres will decrease as $1/r^3$, the scattered energy for the collection and hence I_s90 will increase as the third power of the radius.

As the fixed volume is aggregated into larger spheres with diameters approaching the wavelength, the shape of the scattering envelope is

markedly changed, as is the total scattered energy of the particle. This is the result of interference between assemblies of Rayleigh scattering elements in the same particle. Angular distribution for particles above about 50mμ has been treated by Caspersson (69) whose calculations allow an approximation of I_s90 and extinction coefficient behavior in the size range of ca.75 to 400mμ . Whereas doubling the diameter of spheres in the Rayleigh range results in an eight-fold increase in I_s90, this factor diminishes to 1.1 for 75mμ spheres aggregating to 150mμ spheres. 100mμ spheres aggregating to 200mμ spheres will diminish I_s90 by a factor of 0.975, and this factor will drop to 0.668 for 125mμ spheres aggregating to 250mμ spheres, then increase above 250mμ to a factor greater than unity. Above 300mμ , the total scattered energy drops off markedly. Thus the maximum scattering for a given volume of material dispersed in the form of spheres will be found when the diameter of the spheres is about 300mμ . For particle sizes above 300mμ the scattered energy is proportional to the square of the radius, but since the number of spheres is decreasing as 1/r3 the total scattered energy for the fixed volume system will decrease.

It is of importance to note that the system aggregating at fixed volume will have gone through at least two maxima by the time the particles are within the resolving power of the light, i.e. microscopically visible as precipitates.

REFERENCES

1. Brent,L. Progress in Allergy (1957) 5:in press.

2. Brown,J.B. Surgery (1937) 11:558-563.

3. Padgett,E.C. Skin Grafting (1942) Charles C. Thomas, Springfield. page 13.

4. Converse,J.M. and G.Duchet J. Plast. and Recon. Surg. (1947) 2:342-344.

5. Billingham,R.E. and P.B.Medawar J. Exp. Biol. (1951) 28:385-402.

6. Billingham,R.E., L.Brent, P.B.Medawar and E.M.Sparrow Proc. Roy. Soc. B. (1954) 143:43-58.

7. Loeb,Leo The Biological Basis of Individuality (1945) Charles C. Thomas, Springfield. 711 p.

8. Little,C.C. Biology of the Laboratory Mouse (1941) Blakiston, Phila. pp. 279-309.

9. Snell,G.D. J. Nat. Canc. Inst. (1953) 14:691-700.

10. Barnes,A.D. and P.L.Krohn Trans. Bull. (1956) 3:43.

11. Burnet,F.M. Proc. Roy. Soc. B. (1956) 146:1-92.

12. Medawar,P.B. J. Anat. Lond. (1944) 78:176-199.

13. Medawar,P.B. J. Anat. Lond. (1945) 79:157-176.

14. Billingham,R.E., L.Brent and P.B.Medawar Phil. Trans. Roy. Soc. B. (1956) 239:357-414.

15. Gorer,P.A., S.Lyman and G.D.Snell Proc. Roy. Soc. Lond. B. (1948) 135:499-505.

16. Hoecker,G., S.Counce and P.Smith Proc. Nat. Acad. Sci. (1954) 40:1040-1051.

17. Amos,D.B., P.A.Gorer and Z.B.Mikuska Proc. Roy. Soc. B. (1955) 144:369-380.

18. Billingham,R.E., L.Brent and P.B.Medawar Nature (1956) 178: 514-519.

19. Owen,R.D. and L.Brent Pers. Communication.

20. Snell,G.D. J. Nat. Canc. Inst. (1954) 15:665-675.

21. Billingham,R.E., L.Brent and P.B. Medawar Trans Bull (1956) 3:84-88.

22. Mitchison,N.A. Nature (1953) 171:267-268.

23. Mitchison,N.A. J. Exp. Med. (1955) 102:157-177.

24. Billingham,R.E., L.Brent and P.B.Medawar Proc. Roy. Soc. B. (1954) 143:58-80.

25. Maumenee,A.E. Ann. N.Y. Acad. Sci. (1951) 59:453-461.

26. Billingham,R.E. and T.Boswell Proc. Roy. Soc. Lond. B. (1953) 141:392-406.

27. Algire,G.H., J.M.Weaver and R.T.Prehn J. Nat. Canc. Inst. (1954) 15:493-507.

28. Weaver,J.M., G.H.Algire and R.T.Prehn J. Nat. Canc. Inst. (1955) 15:1737-1767.

29. Egdahl,R.H. and D.M.Hume Ann. N.Y. Acad. Sci. (1957) 64:950-951.

30. Medawar,P.B. Quart. J. Micr. Sci. (1948) 89:239-252.

31. Allgower,M., T.G.Blocker and B.Engley Plas. and Recon. Surg. (1952) 9:1-21.

32. Billingham,R.E. and E.M.Sparrow Brit. J. Exp. Biol. (1954) 31:16-39.

33. Gorer,P.A. J. Path. Bact. (1942) 54:51-65.

34. Amos,D.B. and E.D.Day Ann. N.Y. Acad. Sci. (1957) 64:851-858.

35. Gorer,P.A. and P.O'Gorman Trans. Bull (1956) 3:142-143.

36. Barrett,M.K. and W.H.Hansen and B.F.Spilman Canc. Res. (1951) 11:930-936.

37. Barrett,M.K. and W.H.Hansen Canc. Res. (1953) 13:269-275.

38. Bollag,W.S. Trans. Bull (1956) 3:43-44.

39. Bollag,W.S. Experientia (1956) 12:57.

40. Lawrence,H.S. Ann. N.Y. Acad. Sci. (1957) 64:826-835.

41. Favour,C.B. Ann. N.Y. Acad. Sci. (1957) 64:842-850.

42. Raffel,S. Prog. in Allergy (1954) 4:173-198.

43. Bollag,W.S. Pers. Communication.

44. Caspersson,T. Koll. Zeit. (1933) 65:162-170, 301-307.

45. Campbell,D.H. and N.Bulman Fort. der Chem. org. Naturestoffe (1952) 9:443-478.

46. Pappenheimer,A.M.Jr. J. Imm. (1955) 75:259-264.

47. Chambers,R. Ann. N.Y. Acad. Sci. (1950) 50:817-823.

48. Frey-Wyssling,A. Submicrosopic Morphology of Protoplasm and Its Derivatives (1948) Elsevier, N.Y. page 107.

49. Steiner,R. and A.McAlister J. Coll. Sci. (1957) 12:80-98.

50. Perrin,F. J. Phys. Radium (1926) 7:390.

51. Singleterry,C. and L.A.Weinberger J. Am. Chem. Soc. (1951) 73:4574-4579.

52. Weber,G. Biochem. J. (1952) 51:145-167.

53. Weber,G. Adv. in Prot. Chem. (1953) 8:415-459.

54. Crick,F. and A.Hughes Exp. Cell Res. (1950) 1:37-80.

55. Gorer,P.A. Ann. N.Y. Acad. Sci. (1955) 59:365-373.

56. Amos,D.B. Brit. J. of Cancer (1955) 9:216-221.

57. Gorer,P.A. and Z.B.Mikulska Canc. Research (1954) 14:651-655.

58. Brown,R.B. Trans. and Studies of Coll. of Physicians of Phila. (1956) in press.

59. Lansing,A.I. and E.W.Dempsey Internat. Rev. of Cytology (1954) 3:437-453.

60. Meyer,K.H. and C.Ferri Pflugers Arch. f.d.ges Physiol. (1936) 238:78-90.

61. Lawton,R.W. Circ. Res. (1954) 2:344-353.

62. Berrian,J.H. U.S.Naval Sch. Av. Med. Reports (1953) Proj. NM 001 057.10.02.

63. Berrian,J.H. U.S.Naval Sch. Av. Med. Reports (1953) Proj. NM 001 057.10.03.

64. Kratky,O. E.Schavenstein and A.Sekora Nature (1950) 165:319-320

65. Corey,R.B. and J.Donohue J. Am. Chem. Soc. (1950) 72:2899.

66. Partridge,S.M. and H.F.Davis Biochem.J. (1955) 61:11-30.

67. Partridge,S.M., H.F.Davis and G.S.Adair Biochem.J. (1955) 61:1-11.

68. Oster,G. Chem. Rev. (1948) 43:319-365.

69. Caspersson,T. Koll. Zeit. (1932) 60:151-159.