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REGULATION OF THE IMMUNE RESPONSE: FUNCTION OF PROPERTIES OTHER THAN MOLECULAR SIZE IN THE INDUCTION OF IMMUNITY AND IMMUNOLOGIC TOLERANCE TO BOVINE SERUM ALBUMIN IN BALB MICE.

The University of Michigan, Ph.D., 1974
Health Sciences, immunology

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DEDICATION

Over the years, there have been many people, including family, friends and teachers, whose interest and encouragement have led to the initiation of this work and whose constant encouragement has enabled its completion. It is therefore fitting that this dissertation should be dedicated to all of them.

It is also appropriate to single out one person, among all of those who have provided encouragement and support, whose own efforts were involved and who sacrificed much in time, effort and disappointment to see the work completed.

I should like, in particular, to dedicate this dissertation to my wife, Marlen, who urged me to begin it; who was willing to leave home, friends and family to see it begun; and who was willing to devote her own time and energy to help with its completion.

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LIST OF ABBREVIATIONS

abs NRS- absorbed normal rabbit serum

ag- antigen

BPA- bovine plasma albumin

B-cell- bursal-equivalent or bone marrow derived lymphocyte

BGG- bovine gamma globulin

BMA- bovine mercaptoalbumin

BSA- bovine serum albumin

C D- circular dichroism

DEAE- diethylaminoethyl

DNP- dinitrophenyl

DTNB- dithionitrobenzene

E A- ovalbumin

EDTA- ethylenedinitrolotetraacetic acid

FγG- fowl gamma globulin

F_{ab}- portion of immunoglobulin molecule after enzymatic digestion containing one antigen binding site

F_c- portion of immunoglobulin molecule after enzymatic digestion not containing antigen binding sites

Fr. V- Cohn fraction V albumin preparation

HA- hemagglutination

HGG- human gamma globulin

HSA- human serum albumin

H S monomer- heat-stable monomer

hy- hyperimmune

IgG- Immunoglobulin G

IgM- Immunoglobulin M

IgT- Immunoglobulin T

i.p.- intraperitoneally

Ir_1- immune response gene

i.v.- intravenously

LDH- lactic dehydrogenase

$\bar{M}$- mean of pooled data

M_1- mean of experiment number 1

M_2- mean of experiment number 2

NRS- normal rabbit serum

PCA- passive cutaneous anaphylaxis

PAMEG- synthetic polymeric antigen

PBS- phosphate buffered saline

RAT-PGA- L-tyrosyl azobenzarsonate-poly glutamic acid

-SH- mercaptide group

S-S- disulfide bond

SRBC- sheep erythrocytes (red blood cells)

T-cell- thymus derived lymphocyte

2-me- 2-mercaptoethanol

2-mer- 2-mercaptoethanol resistant

$\bar{V}$- variance of pooled data

V_1- variance of data from experiment number 1

V_2- variance of data from experiment number 2

Xtal- crystalline albumin

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I. INTRODUCTION

Immunological unresponsiveness became a topic of viable theoretical interest with the publication of the Indirect Template Theory of Burnet and Fenner (1949), who postulated the operation of a "self recognition unit" to explain the general lack of immune responsiveness to self antigens. The demonstration of the persistence of pneumococcal antigens in the tissues of specifically unresponsive hosts (Felton, 1949) and the subsequent demonstration that massive doses of soluble protein antigens could also induce a state of acquired immunological unresponsiveness (Hanan and Oyama, 1954; Dixon and Maurer, 1955; Cinader and Dubert, 1956; Smith and Bridges, 1958) brought this topic into sharp focus, stimulated considerable refinements in the theoretical approach to Immunology and in part helped to usher in the Clonal Selection Theory of Acquired Immunity (Burnet, 1959).

The 24 years elapsed since 1949 have witnessed a considerable accumulation of immunological knowledge, including that derived from a not inconsiderable effort devoted to the identification, characterization and treatment of human diseases having associated immunopathological components (Grabar and Miescher, 1965; Westphal et al., 1968; W.H.O. Technical Report No. 402, 1968; W.H.O. Technical Report No. 448, 1970; Sherman, 1970; Kagan and Stiechem, 1971; Samter et al., 1971; Uhr and Landy, 1971). Recently, the attempt to

apply accumulated knowledge to the treatment of immunological disorders has resulted in the emergence of a new clinical discipline, Clinical Immunology. The Clinical Immunologist, according to one definition (Freedman, 1971), is concerned with the elimination of undesirable immune reactions in patients having a wide spectrum of disorders with adverse immunological features; investigation and management of patients with immune deficiency disorders; and production of protective immunity in individuals exposed or potentially exposed to infectious agents.

The practice of clinical immunology is presently limited (Vaughn, 1972) by a paucity of effective laboratory diagnostic aids; incomplete understanding of the mechanisms of the immunological defects; and the un-availability of suitable methodologies for intervention in most cases (Uhr and Landy, 1971).

In their comprehensive work on immunological diseases, Samter, et al. have discussed 54 sets of pathological conditions which are wholly or in part resultant from or associated with either an inappropriate or an insufficient immune response. With the possible exceptions of the immune deficiency disorders, which involve the deletion of one or more components of the system, and the malignancies of the system, which may effectively suppress one or more components, the majority of these diseases may be viewed as being associated with some defect or maladjustment of the regulatory mechanism governing the immune response. Thus, the immuno-

pathologic defect associated with cancer is thought to be a state of acquired immunological unresponsiveness (Hellstrom and Hellstrom, 1970), and an effective form of treatment would be something that released the tolerance; while at the other end of the spectrum, diseases such as rheumatoid arthritis or acquired hemolytic anemia involve an inappropriate response, and an effective form of treatment would be the induction of a state of specific immunological unresponsiveness. From this viewpoint, understanding the disease mechanism requires that the series of regulatory steps involved in the particular response must be identified and a control point established. Such knowledge would provide a base from which to develop effective methods of intervention and, if necessary, to design more effective laboratory diagnostic aids.

Acquired immunological unresponsiveness as a topic of study is essentially a problem in immunoregulation. As such, it provides an excellent model system from which to develop knowledge concerning generally applicable regulatory mechanisms. Knowledge of the physico-chemical and biological properties of the mechanisms that permit the immune system either to ignore or respond to a particular antigen is essential if significant progress is to be made in the control of immunopathological disorders.

The studies described here are intended to further explore the role of the molecular properties of antigens in the regulation of the immune response by comparing the antigenicity (immunogenicity) of several synthetic and nat-

urally occurring forms of albumin found in Cohn fraction V and crystalline albumin.

II. HISTORICAL REVIEW

Definition: Immunological unresponsiveness (tolerance) is most broadly defined (Allison in Bach and Good, 1972) as "the situation in which an animal, following exposure to an antigen, fails to manifest an immune response to that antigen but is able to respond normally to other antigens". This definition is operational rather then mechanistic, and the decision as to whether or not a given experimental situation can be considered immunologic tolerance depends on the sensitivity of methods used to detect an immune response, what is meant by an immune response, and how strictly one interprets the definition. For example, Weigle (1967) graded the spectrum of possible humoral immune responses into four catagories which he termed as follows: (1) unresponsiveness (tolerance); the inability to make a detectable immune response to an antigenic challenge; (2) Hyporesponsiveness (partial tolerance); ability to make only a subnormal response to an antigenic challenge; (3) responsiveness (normal); ability to make a normal immune response to an antigenic challenge; (4) hyperresponsiveness (either a response in a previously stimulated animal or an enhanced primary response); ability to make either a secondary response or an enhanced primary response to an antigenic challenge. These definitions are, once again, operational and relative, depending as they do on a knowledge of what a normal response consists of and being at the mercy of experimental variables in terms of the sensitivities and

specificities of the assay employed.

Another approach to the definition of immunologic tolerance (W.H.O. Technical Report No. 448, 1970) demands that immunologically competent cells become unable to initiate synthesis of a restricted range of antibodies, or comparable recognition units in the case of a cellular immunity, as a direct result of exposure to antigens. However, there is considerable evidence, especially with respect to tumor antigen systems (Hellstrom and Hellstrom, 1970), certain transplantation antigens and protein marker antigens (Jacobson and Herzenberg, 1970) and other antigen systems (Allison et al., 1971 a,b; Katz and Benacerraf, 1972) that this point of view may not be correct. In any event, it remains dependent on the operational definitions in that the identification of tolerance depends on the sensitivity and specificity of assay methods used. Since, under many sets of experimental conditions, it has not been possible to eliminate, but only to reduce the response to antigen, the measure of tolerance depends once again on a definition of "normal".

The detection of immunologic tolerance in situations involving an assay for antibody production requires that an antigen dose and regimen known to induce a humoral immune response be applied to a group of animals or a cell suspension and that these be evaluated at a standard time by the assay methods chosen in order to establish a normal value for the immune response. Other sets of animals or cell suspensions, identical in every way possible to the control set

are pre-treated by the toleragenic regimen and then given the immunogenic regimen under the same conditions, except for pre-treatment, as the controls. Any depression of the humoral response compared to control values is by definition immunologic tolerance. It is essential that any depression observed be great enough to be distinguished from normal variance attributable to experimental error and that the assay technic chosen be sensitive enough to detect a depression in the response.

Numerous assay methods have been used to detect immunological tolerance. These include measurements of antigen clearance rates (Dixon and Maurer, 1955 a,b); direct assay of antibody titers (Cinader and Dubert, 1955), assay of antibody-mediated bacterial flagellar immobilization (Nossal and Austin, 1966); measurement of spleen focus-forming units (Claman et al., 1966 a,b); assay of plaque-forming cells (Miller and Mitchell, 1968); and assay of rosette-forming cells (Howard et al., 1969). Detection of tolerance in the presence of a detectable immune response has been accomplished by studies on antigen clearance rates in whole animals (Dixon and Maurer, 1955 a,b; Dresser, 1962); comparison of immune responses by Farr binding studies (Farr, 1958) after treatment with the immunizing regimen (Mitchison, 1964); and by the use of adoptive transfer studies in which spleen cells from toleragen-treated animals are transferred to immunoincompetent antigen-treated recipients and responses in the donor

animal compared to those in the recipient animal (Miller and Mitchell, 1968; Porter, 1969).

Antigens and Unresponsiveness: Bovine serum (plasma) albumin is considered to be the prototype of a class of weak antigens which are characterized by the tendency to produce very poor and sporadic immunity unless a suitable adjuvant is used (Weiser et al., 1941; Wheeler et al., 1950; Cameron, 1956), and which have been shown to simultaneously induce both unresponsiveness and immunity (Mitchison, 1964, 1965, 1968; Dresser and Mitchison, 1968; Porter, 1969). Its salient feature is that when presented in a highly pure form, it purportedly retains immunogenicity, albeit weakly. In this respect, BSA is distinguished from the non-immunogenic antigens exemplified by gamma globulin which, when presented in a highly pure state, induce tolerance but cannot immunize; although they do so if presented with an adjuvant or in a crude form, yet retain their antigenic reactivity as evidenced by the ability to react with antiserum (Dresser, 1963; Claman, 1963; Gery and Waksman, 1967; Dvorak et al., 1966). A third class, highly immunogenic antigens, is also distinguished by the property of inducing high levels of immunity without the requirement for adjuvants, although it has been shown (Nossal and Austin, 1966; Nossal, 1966) that one variety, monomeric flagellin, can simultaneously immunize and induce tolerance.

With either BSA or flagellin, a state of specific unresponsiveness can be induced in normal animals either at dosages below the smallest required to induce an

immune response (Mitchison, 1964, 1965, 1968; Nossal and Austin, 1966; Feldmann, 1971 a,b) or at massive dosages, considerably in excess of amounts normally required to induce an immune response (Hanan and Oyama, 1954; Dixon and Maurer, 1955 a,b; Smith and Bridges, 1958; Terres and Hughes, 1959; Sercarz and Coons, 1959, 1963; Mitchison, 1964, 1968). The ability to induce tolerance with sub-immunogenic doses in normally responsive animals has been generally limited to the gamma globulins and serum albumins. However, factors such as age (Mitchison, 1965; Weigle, 1967), strain differences in the ability to respond (Fink and Quinn, 1953; Ipsen, 1959; Sobey, Risner and Mc Grath, 1966; Thorbecke and Benacerraf, 1967; Golub and Weigle, 1967; Playfair, 1968; Staples and Talal, 1969; Mc Devitt and Benacerraf, 1969; Green and Benacerraf, 1971), or the use of immunosuppressive agents (Taliaferro, 1957; Goh, Miller and Diamond, 1961; Nachtigal and Feldman, 1964; Mitchison, 1968) can influence the outcome. By the latter approach (Schwartz and Damashek, 1963; Mitchison, 1968), it has been possible to demonstrate the induction of low-zone tolerance with other antigens, including bovine ribonuclease, lysozyme, ovalbumin, and diptheria toxoid.

The induction of tolerance can be prevented by the use of adjuvants or the administration of an immunogenic form of the antigen within a limited time after the administration of the toleragenic dose of antigen (Dresser, 1961; Mitchison, 1964; Golub and Weigle, 1967; Pinckard et al., 1967 a,b). Once induced, tolerance of either the high-zone or low-

zone variety may terminate spontaneously after a few weeks, the animal becoming able to respond with near normal levels of antibody after challenge (Smith and Bridges, 1958; Terres and Hughes, 1959; Sercarz and Coons, 1963; Mitchison, 1964, 1965, 1968); or the animal may remain tolerant for a prolonged period without deliberate efforts to maintain the tolerant state, such as continued maintenance doses of antigen (Smith and Bridges, 1958; Humphrey, 1964 a,b; Thorbecke and Benacerraf, 1967).

Recovery from tolerance has been observed to follow several courses, including: (1) spontaneous production of antibody (Sercarz and Coons, 1963); (2) partial recovery marked by antibody levels after stimulation which are lower than normal (Claman and Talmage, 1963) and appear to be of restricted specificity (Humphrey, 1964 a,b) but of normal affinity (Dietrich and Grey, 1964); (3) complete recovery with "overshoot" (Mitchison, 1964, 1965, 1968; Kraft and Rothberg, 1969), in which the recovered animal eventually produces hyperimmune levels of antibody greater than those produced by normal animals receiving antigen and adjuvant, and which may have greater than normal affinities; or (4) recovery of a normal immune function indistinguishable from that of the normal animal (Benjamin and Weigle, 1970).

It has been shown that tolerance can be deliberately broken. The recovery occurs earlier in the forced animals than in unforced controls. Three models have been employed

in "breaking" tolerance to heterologous proteins, including: (1) "breaking" tolerance to native proteins by administration of the same protein conjugated to an artificial haptenic group (Cinader and Dubert, 1955, 1956; Weigle, 1962); (2) "breaking" tolerance to a native protein by immunization with a phylogenetically and serologically related protein (Weigle, 1961), which requires that the degree of relatedness not be too close or too distant and which can be blocked by simultaneous administration of the toleragen (Porter, 1962; Humphrey, 1964 a,b; Weigle, 1964); (3) "breaking" tolerance to a hapten-conjugated protein by injection of the hapten conjugated to a different carrier protein (Coe and Salvin, 1963; Weigle, 1965 a,b,c; Brownstone et al., 1966).

Upon recovery from tolerance, whether spontaneous or induced, the immunologically recovered animal intially forms antibody which appears to be of limited specificity and lower than normal affinity. With time however, the range of specificities, the affinity, and even the quantity produced eventually reach normal values (Weigle, 1965 a,b,c; Linscott and Weigle, 1965 a,b; Weigle et al., 1971).

On the basis of the studies cited and similar behavior in animals responding to secondary immunization with hapten-protein conjugates in delayed hypersensitivity systems, it has been postulated (Mitchison, 1966, Nossal, 1971) that carrier function requires the participation of a larger portion of the molecule than is required for hapten func-

tion. However, single hapten experiments (Boyden and Sorkin, 1962; Nachtigal and Feldman, 1964; Weigle, 1965 a,b,c; Brownstone et al., 1966; Alkan et al., 1971) have suggested that carrier recognition involves sites not much larger than the inidvidual hapten (Dresser and Mitchison, 1968) or perhaps even smaller (Alkan et al., 1971).

From the experiments of Coe and Salvin (1963), and of Weigle (1965b) on hapten-carrier specificity, it appears that tolerance involves the carrier function in preference to the hapten function (Weigle, 1966). Several lines of evidence tend to support this observation. It is now recognized that immunological reactions are generally dependent on two populations of lymphocytes having distinct functions, the so-called thymus-dependent (T) and bursal equivalent (B) lymphocytes (Claman et al., 1966 a,b; Miller and Mitchell, 1967, 1968, 1969, 1970; Roitt et al., 1969 a,b; Bach and Good, 1972). It has been shown that restoration of an immune response in irradiated and thymectomized animals requires the administration of thymus-dependent (T) cells (Miller and Mitchell, 1967, 1969; Mitchell and Miller, 1968; Miller et al., 1971; Taylor, 1968, 1969; Chiller et al., 1970). Similarly, it has been recognized that recovery from tolerance can be prevented by thymectomy (Claman and Talmage, 1963; Taylor, 1964). It has also been shown that the induction of tolerance to BSA involves the production of tolerant T cells (Taylor, 1968, 1969); although Chiller et al. (1970)

and Weigle et al. (1971) have demonstrated tolerance induction in both T and B cell populations using disaggregated HGG, with the T cells susceptible to low doses of toleragen and the B cells requiring high doses. It is also apparent, especially from the report of Weigle et al. (1971), that the kinetics of tolerance induction and maintenance in the two populations are quite different, with T cells more easily tolerized and remaining tolerant longer (>100 days for T cells vs. 49 days for B cells in HGG induced tolerance in mice). It has also been shown that T cells respond to antigenic stimulation by entering a cycle of cell division (Davies et al., 1966; Davies, 1969; Gershon et al., 1968) and the mitotic activity arising as a result of immunization is closely associated with carrier function (Claman et al., 1966 a,b; Davies et al., 1967), but they do not appear to form antibody (Claman, et al., 1966 a,b; Davies et al., 1967). Rather, antibody formation seems to be the major function of the B cell population (Cain, et al., 1968; Mitchell and Miller, 1968; Jacobson et al., 1970).

How a potential immunogen might interact with cells of the immune system to trigger an immune response has obviously been the subject of much interest. One view, essential to the support of clonal selection concepts and variously entitled the "unitarian" or "accurate sample" hypothesis (Erlich, 1900; Burnet, 1959: Weigle, 1966; Dresser and Mitchison, 1968; Jerne, 1971), requires that the potential antibody-forming cell have available for interaction with

antigen a sample of the antibody which it is capable of producing, preferably at the cell surface. Indirect evidence in support of this concept was provided by specific adsorption onto antigen-coated columns of antigen-reactive cells from primed (Wigzell and Andersson, 1969) and unprimed (Biozzi *et al.*, 1968) spleen cell populations. That the spleen cells could in fact adsorb antigen was demonstrated by Naor and Sulitzneau (1967) who showed by autoradiography that mouse spleen cells could bind ^{125}I-BSA *in vivo* and *in vitro*. Later, Ada and Byrt (1969) demonstrated that adsorption of highly radioactive ^{125}I-flagellin would result in a specific state of unresponsiveness in spleen cells treated *in vitro* and transferred into syngeneic irradiated hosts. Davie, *et al.* (1970, 1971 a,b,c) demonstrated by the use of hapten-carrier-coated columns that the spleen cells responding to this antigen with mitotic activity were carrier specific in that they could be adsorbed onto columns treated with hapten and homologous carrier but not to columns treated with hapten alone or with hapten bound to a heterologous carrier. Additionally, it was found that these cells were capable of some hapten discrimination in that columns treated with homologous carrier bound to heterologous hapten were less efficient adsorbants of carrier-specific cells than columns coated with homologous carrier bound to homologous hapten. It was also possible to remove antibody-forming cells by passage over hapten-carrier treated columns. In this case however, hapten, carrier, or

hapten-carrier combinations appeared to work equally well as adsorbants. Supportive evidence that the carrier-specific, mitotically active cells are in fact T cells has been provided recently by Kettman and Dutton (1971) who showed that treatment of spleen cells with anti-kappa-chain serum inhibited the response against trinitrophenyl-conjugated erythrocytes. It was found that the response could be restored by adding thoracic-duct or thymic lymphocytes from erythrocyte-primed but not normal or hapten-primed animals. Numerous experiments, reviewed by Paul (1970) and by Miller et al. (1971) indicate that memory resides in both T and B cell populations, and that a transfer of genetic information between the two populations is an unlikely mechanism to explain the specificity and differential characteristics of memory and function.

Evidence that the receptors are immunoglobulins has been provided by blocking of receptor function with low levels of anti-immunoglobulin antiserum; by inhibition of immune function by cytolysis of antibody-forming cells and their precursors by treatment with anti-immunoglobulin serum and complement; and by isolation of immunoglobulin receptors from lymphocyte membranes (Davie, et al., 1971 a,b,c; Vitetta et al., 1971; Torgianni and Roitt, 1969; Greaves, 1970; Greaves and Moller, 1970 a,b; Greaves and Hogg, 1971). The evidence supporting the existence of immunoglobulin

antigen receptors appears to be quite strong for B cell populations, but there is disagreement as to whether T cells have such receptors. Kettman and Dutton (1971) were able to block T cell function with anti-kappa serum, which suggests that immunoglobulin receptors, or at least immunoglobulins, are significant for T cell function; and Greaves, (1970) reported an association between theta antigen (T-cell specific) and inactivation of antigen-dependent functions by treatment with polyvalent anti-immunoglobulin serum. Raff et al. (1970 a,b) Reithmuller and Reiber (1971a,b) have also reported the binding of anti-immunoglobulin antibodies to T cells, and have shown that the amount bound is at least 10 times less than is bound by B cells, while Cone and Marchalonis (1972) have reported the isolation of an IgM-like globulin monomer, which they term IgT, from the surfaces of theta-bearing mouse lymphocytes. However, Mc Devitt and Benacerraf (1969) postulate the operation of an Immune-response (Ir) gene, the product of which appears unassociated with antibodies, in T cells.

The relative roles and the nature of the interdependence of T and B cells in the initiation of a primary response and the subsequent activation of a secondary immune response are far from clear. It is well established that B cells contain membrane-bound antigen sensitive immunoglobulin receptors and can be induced eventually to form antibody by binding the sensitive receptors with antigen. However, the means by which B cells may interact with T cells, and indeed the

role of the T cells in the activation of an antibody response is less well defined. Several hypotheses have been offered in explanation of the T and B cell interactions, both in the induction of an immune response and in the induction of the tolerant state (Dresser and Mitchison, 1968; Thorbecke and Benacerraf, 1967; Burnet, 1968; Mitchison, 1969; Bretcher and Cohn, 1970; Feldman and Nossal, 1972; Bretcher, 1973). Mitchison (1969) and Bretcher and Cohn (1970) have proposed that T cells, through their receptors, concentrate antigen for presentation to B cells which are then able to complete the cycle of maturation leading to antibody formation. In support of this view are the helper function studies of Rajewski et al. (1966, 1967a,b 1969, 1971), in which it was shown that carrier "educated" T cells markedly enhanced the ability of an animal to show an immune response in artificial hapten-carrier systems. An enhanced response was only observed when the hapten had been covalently linked to the carrier, at least in the case of the primary response. Further support came from the observation of Chou et al. (1966) that BSA administered to rabbits in solution was far less immunogenic than BSA adsorbed onto thymus-derived lymphocytes. Opposing their view is the observation of Dutton et al. (1971) that the immune response cannot be stimulated by the addition of large doses of antigen in vitro in the absence of T cells. Burnet (1968) considered that the mechanism might be indirect, in that T cells, reacting to carrier stimulation, might secrete a soluble mediator substance which would then act on B cells in the presence of antigen to enable the latter to undergo

the maturation steps leading to antibody formation. Again, helper function experiments provide supportive as well as contradictory evidence. Priming requires that hapten and carrier be present on the same structure. Thus, if a soluble chemical mediator does function, it must function only at distances sufficiently short to permit the antigen to simultaneously bind T-cell and B-cell receptors (Dutton et al., 1971). After priming however, carrier alone or hapten bound to a second carrier to which the animal had been previously immunized will permit a secondary response (Rajewski, 1966; Rajewski et al., 1969; Dutton et al., 1971).

An additional phenomenon of interest is antigenic competition, the inhibition of response to one antigen by simultaneous or prior treatment with a second, which has also been studied in hapten-carrier systems. It has recently been found (Gershon and Kondo, 1971 a,b; Fauci and Johnson, 1971) that antigenic competition is an immunosuppressive phenomenon which is both carrier specific and T-cell dependent, behaving just as do the antigens of the Rajewski system. Competitive suppression has been overcome in experimental models similar to those described by Weigle (1967) for the "breaking" of tolerance.

The similarity of the findings in these antigen systems and their associated phenomena strongly suggest that some of the processes of primary immunization, secondary response, antigenic competition and immunological tolerance are, at least with BSA and certain other antigens, dependent on

sets of cellular functions of the T-cells; these processes are quite important to, but possibly independent of, the B-cell response. It is important to emphasize that although tolerance can be induced in both B-and T-cell populations, the T-cells are most immediately and most easily made tolerant. As a practical consequence, animals made tolerant to HGG (Weigle et al., 1971) and possibly other T-dependent antigens can be expected to have a fully competent B-cell population able to make normal amounts of antibodies of the normal range of specificities, except that they cannot be induced to do so because of some alteration in the T-cell population. Recent findings regarding the complexity of the T-cell population with regard to subpopulations of cells with possibly differentiated functions (Raff and Cantor, 1971; Mitchell et al., 1971) and recent suggestions (Howard et al., 1969; Herzenberg, 1970; Allison et al., 1971 a,b; Claman and Thorbecke, 1971; Cudowitz and Haskill, 1971; Katz et al., 1973; Rich and Pierce, 1973; Phillips and Wegman, 1973; Cohen and Werkerle, 1973) that T-cell tolerance may involve the participation of cells capable of specific B cell inhibition, leave the mechanisms of the T-cell-B-cell interaction very much unknown.

It also seems likely that some subtle but not completely defined properties of the antigenic molecules associated with their carrier function are intimately involved with the T-cell processes. Tolerance induction, activation of the secondary response and antigenic competition all

depend on carrier function. In his work on helper function, Rajewski (1966) discovered that the lactic dehydrogenase (LDH) isozymes represented a natural hapten-carrier system which could be studied by the same technics used for the study of artificial haptens grafted onto protein carriers. Here, it was found that LDH-I, a tetramer of a single polypeptide chain (B_4) was non-immunogenic in rabbits although it did have antigenic determinants. LDH-V, a tetramer of an alternate peptide (A_4) was found to be immunogenic, but did not share antigenic determinants with LDH-I. LDH-III, a mixed tetramer (A_2B_2) was also an immunogen, and shared antigenic determinants with both LDH-I and LDH-V. The LDH-V possessed carrier properties in that it could activate a secondary response to itself and the full complement of determinants on LDH-III, and could induce tolerance. LDH-I could do neither alone, but if mildly acetylated under carefully controlled conditions such that fewer than 3 acetyl groups were introduced and the enzyme retained its usual enzymic activity and rate constant, the modified LDH-I became a good immunogen and acquired the necessary carrier properties. The acetylated LDH retained all of its original antigenic determinants, but acquired new specificities which cross-reacted with those of LDH-V. If Mitchison (1966) was correct in the suggestion that carrier function requires a larger portion of the molecule than hapten function does, it must be assumed that acetylation of the LDH-I caused subtle changes in the tertiary structure of the molecule which

enabled it to then fulfill the carrier function. However, recently Alkan et al. (1971) have argued that the low molecular weight portion (RAT, MW 400) of a synthetic hapten-carrier antigen L-tyrosyl azobenzarsonate-poly glutamic acid (RAT-PGA) served as the carrier for the high molecular weight portion (PGA MW 35,000) while Godfrey and Baer (1971) found that the immunogenicity of BSA-hapten antigens depended directly on the chemical reactivity of the hapten moiety with the BSA carrier even though the extent of hapten conjugation was quite low and additionally, Hanna et al. (1972), found that the mode of hapten and carrier linking was exqusitely important to the immunogenicity of the product. Previously, Havas (1969) found that the induction of tolerance to hapten-protein antigens was inversely related to the immunogenicity of the carrier. Numerous earlier studies on denatured or chemically modified serum proteins, as well as more contemporary work (Martin et al., 1943; Maurer, 1959; Maurer et al., 1957; Habeeb, 1969; Peters and Goetzl, 1969) have also shown altered immunogenicity and antigenic reactivity as a result of chemical or physical modifications of serum proteins. This altered immunogenicity may be related to changes in the carrier function of the protein, which have nothing to do with its physical deminsions.

Physico-Chemical Properties of Antigens Associated with the Induction of Immunity or Tolerance: The physico-chemical properties of protein antigens which permit them to express toleragenic or immunogenic behavior have been studied. Several lines of evidence suggest that protein solutions which contain aggregates of the protein are more immunogenic than those which contain no aggregate or greatly reduced amounts of aggregate, while these latter seem to more easily induce tolerance.

Thus, Dresser (1961, 1962) originally observed that bovine gamma globulin (BGG) which had been centrifuged at 20,000 x g for 15 minutes failed to immunize CBA mice at a normally immunogenic dose and rendered the mice unable to respond to a later dose of BGG in Freund's complete adjuvant. Claman (1963) confirmed this observation and found that injection of the pelleted material obtained from the bottoms of the centrifuge tubes did not induce tolerance. The immunogenicity of this material, however, was not directly tested. Additional studies of this type are those of Dresser and Gowland (1964), who failed to induce tolerance with equine gamma globulin in rabbits but did obtain tolerance with hare globulin; Gamble (1966), who immunized mice with human gamma globulin containing various amounts of heat-aggregated globulin and obtained an elevation in the anti-HGG titers which was proportional to the amount of aggregate added; Golub and Weigle (1967), who showed that

BGG cleared of aggregates by either the Dresser method or ammonium sulfate precipitation made CBA strain mice tolerant, but not A strain or balb/c mice; and Sassen et al. (1968) who used sucrose density centrifugation to remove aggregates from heat-denatured HGG and found that all of the particles with sedimentation constants $s_{20,w}$>10S were excellent immunogens of equivalent potency in the range of 10^{-3} to 10^{-7} grams per mouse in mice, whereas supernatants of either heated or unheated HGG obtained after centrifugation failed to immunize unprimed mice but would immunize primed mice.

Pinckard et al., (1967a,b) extended this model to the study of BSA in rabbits by comparing the supernatant obtained after centrifugation to whole albumin as an immunogen in New Zealand strain rabbits. Whole BSA and supernatant were found to induce comparable levels of primary immunity in those rabbits which responded, but the use of whole albumin resulted in the immunization of a larger proportion of rabbits than the supernatant albumin.

Experiments employing "biologically processed" antigens have led to similar conclusions. Dresser (1961) inoculated radioiodine-labelled BGG into mice and separated two pools of radio-labelled protein which were antigentically alike by gel diffusion and were separable by starch gel electrophoresis. Only one of the components could be isolated from the sera of tolerant mice. There re-

mains some doubt as to which of the two compounds was the immunogenic component. Dresser indicated his belief that it was an IgG subtype, but later thought it to be IgA, while Gery and Waksman (1967) and Kong and Weiger (1970) on the basis of gel precipitation and immunoelectrophoretic studies considered it to be a beta globulin, possibly IgA, but of uncertain identity. In any event, this component which is lost on animal passage has not been equated with aggregated protein even though aggregated proteins are very rapidly eliminated from the circulation (Biro and Garcia, 1965. Golub and Weigle (1969) compared "biologically processed" HGG to ammonium sulfate fractionated HGG and found the "biologically processed" antigen was able to induce tolerance at one tenth of the dose required of sulfate fractionated globulin. Both preparations should have been equally free of aggregates, so that the dose difference must indicate that the composition of the "biologically processed" globulin was different from that of the sulfate fractionated globulin. The use of biological processing was extended to the study of serum albumin by Mitchison (1964), who found that "biologically processed" BSA was as immunogenic in CBA mice as were either Cohn fraction V or crystalline albumins. Frei, Benacerraf and Thorbecke (1965) however, found that rabbits immunized with serum from other rabbits which had been given massive doses of BSA 48 hours previously became tolerant to the BSA. Serum from BSA-treated rabbits did not render mice

tolerant to the BSA, unfortunately. This experiment was done on the model of Dresser's 1962 experiment, and it was assumed that animal passage served to remove aggregates from the BSA leaving only a monomeric protein which could induce tolerance but was poorly immunogenic. The experiment was repeated (Frei et al., 1968) using larger doses of antigen and it was found that at doses above 7mg the "processed" and "unprocessed" BSA were equally effective antigens in rabbits, while at doses below 7mg the "biologically processed" albumin appeared to immunize a smaller number of rabbits than the "unprocessed" crystalline albumin. Those animals which did not respond to the initial treatment with "processed" albumin generally failed to respond to later doses of albumin accompanied by endotoxin as an adjuvant. However, as in the experiments of Pinckard et al., (1967a,b), the most consistent immunizations were achieved with 7.0 mg of "biologically processed" albumin and endotoxin, while doses of monomeric albumin obtained by column chromatography were considerably less efficient immunogens under the same conditions. More recently, Porter (1970) was able to induce tolerance with "biologically processed" BSA in balb mice. The processed antigen, although a toleragen in normal mice, proved to be an exceptionally good immunogen in primed mice.

A more direct approach has been the aggregation of serum protein antigens by chemical or physical methods.

Biro and Garcia (1965) heated Cohn fraction II HGG at 63C for 15 minutes to produce aggregates which were then separated by ultracentrifugation. Multiple doses of washed aggregates induced high levels of circulating anti-HGG antibody whereas the supernatants after centrifugation of either heated or unheated globulin did not, nor could the supernatant-treated rabbits be immunized by subsequent injections of aggregated HGG. Those animals which had been immunized with aggregates were able to mount a powerful secondary response when treated with either heated or unheated supernatant globulin. Gamble (1966) found that adding aggregated BGG to unheated Cohn fraction II BGG markedly enhanced the immunogenicity of the Cohn preparation, with the degree of enhancement related to the amount of aggregate added. Golub and Weigle (1967) used the heat and ammonium sulfate fractionation technic of Christian (1958) to produce aggregate-rich and aggregate-free globulin fractions. Administration of either untreated HGG or aggregate-rich fractions of HGG resulted in immunization of both rabbits and mice. The aggregate-free portion failed to immunize any of the test animals. Treatment of the aggregate-free fractions with carbodiimide reagent to produce aggregates resulted in an immunogenic preparation. Since the clearance rates were less precipitous than those resulting from immunization with heat-treated aggregates, it might be that a state of partial tolerance was induced.

That the globulin remaining after heat treatment differs from the aggregated globulin has been amply demonstrated.

Morse (1965) found that 11 of 18 IgG myeloma proteins studied could not be aggregated. Of those that could be, seven aggregated completely (all of the protein was found in the aggregates) and four partially (some of the protein remained in the supernate). Augener and Grey (1970) studied a larger set of myeloma proteins more thoroughly and found that the ability to aggregate was variable and not correlated with isotypic or allotypic markers, light chain type or the carbohydrate content. Further, aggregation did not inhibit the ability to fix complement or to bind to tissue in those subtypes that possessed these properties. It was additionally found by enzymatic and 2-mercaptoethanol cleavage studies that the bulk of aggregates were formed by linking in the F_{ab} portion of the immunoglobulin molecule, although some few IgG_3 and IgG_4 globulins were able to link in both the F_{ab} and F_c regions. In a review of the studies of the molecular size and conformation of immunoglobulins, Dorrington and Tanford (1970) have presented considerable evidence to indicate that the conformational changes introduced by heat aggregation or reaction with antigen are largely similar. Thus, the increased immunogenicity of aggregated globulin may be due either to the exposure of determinants essential to the carrier function or to the activation of biological properties peculiar to globulins and intimately associated with regulation of the immune response rather than to some effect of physical size, as has been suggested (Dresser and Mitchison, 1968).

More direct evidence for this point of view is provided by the work of Henney and Ishizaka (1968) and of Kochawa et al., (1967) who employed several different methods of physical alteration including heat, alkali treatment, bisdiazotization and adsorption onto polystyrene beads to show the common appearance of a new, species-specific antigenic determinant in the F_c piece which appeared to be the same regardless of the method of treatment used.

Heat aggregated BSA was studied indirectly by Frei et al., (1965), who added aggregates to "biologically processed" albumin and found that rabbits treated with this preparation were more consistently immunized than rabbits treated with the "processed" albumin alone. Those animals which were treated with either preparation were less responsive than untreated rabbits to subsequent challenge with alum precipitated BSA, although some of the rabbits which had been treated with the "processed" albumin alone developed extremely high anti-albumin titres after challenge, whereas none of the rabbits treated with aggregate-containing albumin did. Hirata and Campbell (1965), Hirata and Sussdorf (1966) and Draper and Hirata (1968) attempted a more direct study of aggregated BSA. Here, aggregates were prepared by heating albumin at low pH to produce a gel which was then converted by trituration to particles of 1 to 5 u average diameter. The stability of the preparation was good, but some dissociation into monomer occurred on standing and whenever serum was added. Experiments in

rabbits showed that at low doses (0.1 mg/kg) more rabbits developed a detectable primary response on immunization with the aggregated albumin than with a crystalline albumin preparation. As the dose was increased however, this difference disappeared and the crystalline albumin produced superior primary and secondary titers, although the kinetics were quite different. At low doses, the insoluble antigen produced highly variable kinetic patterns, some animals showing a 7-day peak, others a 14-day peak and one a typical biphasic curve with peaks at 7 and 14 days. The crystalline preparation always produced a typical biphasic primary curve. This behavior was also observed in the secondary response, wherein the response to insoluble albumin peaked at 7 days and the response to crystalline albumin peaked at 14 days. By 2-mercaptoethanol inactivation studies, it was found that the antibody response to the insolubilized albumin was mostly IgM and that against the crystalline antigen was IgG. In line with these findings, it was also shown that secondary titers against the crystalline albumin reached considerably higher levels than those against the insolubilized albumin. There are two important considerations in the interpretation of these experiments. First, it is possible that the insolubilized albumin was made from a different lot of albumin than was used for the crystalline albumin control, and second, crystalline albumin preparations usually have a small amount of dimeric or otherwise aggregated material which may have been sufficient to influence

the outcome of the experiment.

One of the basic assumptions in the design and interpretation of these experiments involved the lack of any detectable antigenic differences between the aggregated and non-aggregated albumins. However, there are definite differences. Maurer (1959) studied bovine albumin denatured by several technics, including heat and acid. These latter methods were found to result in an albumin which was non-immunogenic in rabbits unless injected with Freund's complete adjuvant. The various forms differed in the amount of antibody production stimulated and in the degree of reciprocal cross-reactivity in quantitative precipitin studies. It was also found that lines of partial identity were formed in agar gel diffusion plates. More recently, Wolberg et al., (1970) have shown that aggregated and soluble forms of heat-denatured albumin differ antigenically from untreated crystalline albumin, specifically in the ability to induce, early in the response, low levels of transient antibodies which differ in specificity from those produced against the crystalline albumin. Further, both the soluble and aggregated forms of heated albumin were found to be more efficient sensitizing agents for use in passive hemagglutination, in that smaller amounts were required to sensitize the red cells.

Attempts to correlate and interpret the significance of the different experiments are hampered by the variety of animals, immunization schedules, bleeding schedules, and

methods of evaluation used. However, some distinctions can be clearly made. In the gamma globulin system, inclusion of aggregates very clearly improves the success of primary immunization and results in an increase in antibody titer by orders of magnitude. These effects are seen over a wide range of dosage, and inclusion of the aggregates makes it possible to immunize animals with minute doses of protein which would otherwise be non-immunogenic or toleragenic. In experiments with serum albumin, any effect produced by the inclusion of aggregates is observable only at low, marginally immunogenic doses. The addition of aggregate may possibly improve the success of achieving immunization but does not increase the antibody titers of animals successfully immunized. "Biological processing" experiments, while clear-cut in the globulin system have not been clear-cut in the albumin system, and in neither system has the full effect of "biological processing" on the protein antigen been described. Studies on 2-mercaptoethanol sensitivity of the antibodies formed against aggregate-containing and aggregate-free antigens reveal the following similarities. In both the albumin and globulin systems, animals respond with a greater amount of early IgM antibody during the primary response when aggregates are present than when they are absent (Draper and Hirata, 1968; Borel et al., 1968). In the gamma globulin system, removal of the aggregates from the immunizing antigen results in a loss of precipitating IgG production, although the animals still produce antibodies capable of eliciting

a PCA reaction. This type of analysis was not done in experiments with the BSA system, and the effect of aggregates on the ratio of IgG isotypes is not clear, although in the experiment of Hirata and Sussdorf (1966) and of Draper and Hirata (1968) the quantity (in mg) of antibody protein per ml of serum at the maturation of the responses was not significantly different for rabbits treated with aggregated BSA compared with soluble BSA.

Aggregation of protein antigens does not always exert a positive effect on immunogenicity. For example, flagellin, a monomeric protein (MW approx. 38,000) isolated from bacterial flagella was found to be an excellent antigen capable of inducing high levels of IgG in rats. Whole flagella or polymerized flagellin are equivalent to flagellin antigentically but usually induce lower levels of antibody than the flagellin monomer (Nossal and Austin, 1966; Nossal, 1966,1971). On the other hand, whole flagella and polymerized flagellin induce high levels of IgM whereas the monomeric flagellin induces barely detectable amounts, if any. Hemocyanin is another protein which appears to be as immunogenic in the unaggregated state as it is in the normally aggregated state (Dvorak et al., 1966; Weigle, 1967), while the LDH isozymes which occur as tetramers are poorly antigenic at best and one form of the enzyme is not immunogenic at all in rabbits (Rajewski, 1966).

Numerous workers have shown a requirement for macrophages in the induction of a primary immune response (Fishman, 1961; Fishman and Adler, 1963; Mosier, 1967), although others

have argued against this requirement (Ada and Hanna, 1971). Numerous experiments have shown, however, that immunization with antigen bound to viable peritoneal-exudate or spleen cells markedly enhances the immunogenicity of the antigen in that only minute amounts will induce a potent antibody response (Chou et al., 1966; Unanue and Askonas, 1968; Mitchison, 1969; Porter, 1970; Schmidtke and Unanue, 1971). The soluble and aggregated antigens are equally effective once bound to the macrophage, although aggregated protein seems to be more rapidly taken up than soluble protein and seems to remain more firmly bound to the macrophage membrane than does the monomer.

Aside from any differential effects of macrophage uptake, there is no real understanding of why an aggregated protein might be a better immunogen than its unaggregated counterpart. Golub and Weigle (1969) have postulated that the process of aggregation might cause new specificities to appear, which then impart improved immunogenicity to the molecule; or that aggregation might increase immunogenicity by increasing the number of determinants per particle. Basing their reasoning on the argument that carrier function required a larger part of the molecule than hapten function, and on the assumption that the aggregation effect was general, they opted for the latter hypothesis although, as was shown in the work of Henney and Ishizaka (1968), the former is definitely a viable alternative. Nossal (1971) described

experiments with dinitrophenylated-flagellin polymers in which the ratio of DNP to flagellin in the polymer can varied over exteremely wide ranges, thus permitting a critical evaluation of the effect of determinant density on the immunogenicity of this protein system in vitro. It was found that polymeric antigens containing 0.1 DNP per flagellin unit could neither immunize nor tolerize at any dose tested, whereas larger amounts of DNP caused the complex to become immunogenic at lower and lower doses as the number of DNP residues increased until finally microgram amounts would induce tolerance. None of the doses tested would immunize. From these findings it was postulated that the trigger signal was keyed to the number and pattern of antigen molecules bound to the surface receptors. Binding of too few failed to trigger any response, binding of an intermediate number in a restricted variety of patterns triggered an immune response and binding of a large number or an incorrect pattern resulted in tolerance. Thus polymerization of an antigen as a general phenomenon could serve the very practical purpose of increasing the number of determinants to some critical density to enhance the immunogenic capacity of the antigen. Interestingly, flagellin monomers were neither immunogenic nor toleragenic in the experimental system, which had been designed to contain only macrophages and B lymphocytes. It was readily admitted that the model would not work in a T-cell containing system or in vivo, where monomeric proteins will induce both tolerance and immunity.

An additional point of concern in the interpretation of

the experimental data is the general observation that either "biologically processed" or the supernatant from centrifuged protein have been found to be excellent agents for stimulating secondary responses. If tolerance and secondary immunization are both carrier dependent phenomena, and if changes in the physical state of the antigen alone can alter the carrier function, then it seems unreasonable that molecules which have been deprived of the carrier function would be equal to or superior to the carrier enhanced forms of the antigen as has been shown (Porter, 1970; Draper and Hirata, 1968). An observation which seems germane is that of Porter (1970) who found that of the several albumin preparations he examined for primary immunogenicity, only the Cohn fraction V and not the crystalline albumin preparations could induce high titred regular primary responses in balb mice as evaluated by passive hemagglutination studies. A similar observation was made by Martin et al., (1943) who noted that crystalline BSA was a much poorer immunogen in rabbits than the Cohn fraction V albumin as evaluated by quantitative precipitation studies. In the light of these observations, and in consideration of the interpretations placed on the results of hapten-carrier studies with respect to the effect of subtle alterations of the protein and the effect of these alterations on carrier function, it seemed reasonable that there might be systematic variations in albumin preparations other than the state of aggregation that could affect the structure and immunologic properties of the protein.

Heterogeneity of Albumins: A review of the chemical literature indicates that bovine and other mammalian albumins are indeed heterogeneous in composition. While such preparations usually contain low levels of non-albumin contaminants (Cohn in Putnam, 1960), the molecular hetergeneity of the albumins themselves has been demonstrated in several different ways. An early observation is that of Leutscher (1939) who studied the pH dependence of protein mobilities in moving boundry electrophoresis and noted that preparations of either horse or human albumin which had produced only single Schleiren peaks when migrated at pH 7.15 showed two such peaks at pH 4.0. This finding was confirmed by Alberty (1949) who found two boundaries in human albumin and three in bovine albumin. Longsworth and Jacobsen (1949), in studies of the effect of ionic strength on the isoelectric points of proteins noted that the Schleiren patterns of bovine and human albumins in low ionic strength solutions were not homogeneous. Saifer and Corey (1954) studied the effect of ionic strength on the multiple boundary phenomenon at low pH, and found that reduction of the ionic strength of the buffer solution to values between 0.03 and 0.06 enabled them to distinguish up to three boundaries in solutions of crystalline human albumin. The two fastest boundaries were found to vary in proportion as a function of ionic strength and pH which later led Aoki and Foster (1957) to postulate the operation of a pH and ionic strength-dependent conforma-

tional transition. On application of their method to bovine, porcine and human albumins, Saifer and Corey (1955), extended the number of boundaries detectable in BSA to four and found that the number and relative positions of the boundaries were characteristic for a given species of albumin. Raacke (1956) studied Cohn fraction V bovine albumin by starch-gel electrophoresis and was able to detect two components when the experiment was performed in pH 7.0 phosphate buffer but not when other buffer systems were used. Somewhat later, Ressler (1960) employed a two dimensional starch-gel immunoelectrophoretic technic to detect at least three electrophoretically distinct but immunologically similar albumins in Cohn fraction V human albumin. The following year Saifer, Robin and Ventrice (1961) made use of the improved resolution obtainable with urea starch-gel electrophoresis in a discontinuous buffer system to examine Cohn fraction V preparations of rabbit, porcine, bovine, and human albumins. Additionally, an electrophoretically isolated human albumin preparation was examined. Four to five different components could be detected in the fraction V preparations, but only a single component in the electrophoretically isolated material. Because starch-gel electrophoresis has the property of separating proteins of similar mobility according to their relative molecular weights (Smithies, 1959), the authors considered that the multiple bands produced from the fraction V albumins represented aggregates formed during the isolation procedure rather than native forms of albumin. Subsequently,

Pedersen (1962) was able to fractionate crystalline fraction V bovine albumin into monomeric, dimeric and trimeric portions by chromatography in G-150 and G-200 Sephadexes. He was able to detect in addition a small amount of monomer which was different from the bulk of the monomer in terms of its electrophoretic mobility and which was difficult to separate from the dimeric fraction. Additionally, it was noted that the monomeric albumin was always eluted in a skew pattern which indicated further heterogeneity in this albumin fraction.

In 1947, Hughes found that approximately 70% of the protein contained in Cohn fraction V human albumin preparations could be precipitated with methyl mercuric salts. The monomeric protein isolated after removal of the mercuric salts with cysteine was shown to contain one free sulfhydryl group per mole, and was termed mercaptoalbumin. The remaining 30% of the albumin was found not to contain any free sulfhydryl groups when similarly tested. Hughes and Dinzis (1953) later reported the separation of mercaptoalbumin and non-mercaptoalbumin from bovine albumin as well. Benesch and Benesch (1948, 1955) and Benesch, Lardy and Benesch (1955) were able to show by titration against silver ion that crystalline preparations of both bovine and human albumins contained 0.68 moles of -SH per mole of albumin, in agreement with Hughes' earlier data.

In 1956, Sober et al., applied the newly developed DEAE cellulose of Peterson and Sober (1956) to the separation of

individual proteins from whole serum. Human and horse serum samples were studied, and it was shown that protein with the electrophoretic mobility of albumin eluted from the ion exchange column in a large fraction of the effluent volume. Twenty seven different experiments were done, and the reproducibility was found to be excellent. Boman and Westlund (1956) used Dowex-2 resin to separate crystalline human albumin into at least four components, the precise composition depending on the buffer system employed. No effort was made to identify the different fractions, but rechromatographing the isolated fractions did not result in further changes in the elution patterns, and the fractions eluted at their original volumes indicating that they were not artifacts produced in the column material but were distinct protein entities. Tiselius, Hjertin and Levin also in 1956 used columns of calcium hydroxylapitate to study the chromatographic behavior of crystalline bovine albumin. Three fractions were obtained by the use of stepwise gradient elution. Concentration and rechromatography of the fractions indicated that they were chromatographically homogeneous and eluted under the same conditions as they had orginally. Analytical ultracentrifugation of the fractions showed that one consisted entirely of monomeric albumin while the other two seemed to be composed of equal parts of monomer and dimer. In 1961, Hartley, Peterson, and Sober examined the heterogeneity of crystalline bovine albumin with respect to molecular weight, free-SH content, and chromatographic

behavior on DEAE cellulose. Using a phosphate buffer gradient at pH 7.0, these workers were able to classify numerous components in order of their elution from the DEAE column. The following table adapted from Hartley et al. (1961) summarizes their findings with respect to the varities of albumins found:

Early non-SH albumin (5-10%)
Mercaptoalbumin (50-75%)
Late non-SH albumins
  a) reduced by 2-mercaptoethanol (10-20%)
  b) not reduced by 2-mercaptoethanol (10-20%)

Very late monomer (color?) (5%)

Dimers (5-8%)
  a) 2-mercaptoethanol resistant (early and late)
  b) 2-mercatoethanol sensitive (early and late)

High polymers (<1%)

Further studies of the non-SH albumins have been done by several workers. King, Yphantis and Craig (1960) developed a method for separating -SH albumin from non-SH albumins by means of counter-current distribution. Attempts to study the amino terminal amino acids after dinitrophenylation and performic acid oxidation revealed the presence of additional cystine and glutamic acid residues in the non-SH albumins which were not found in the mercaptoalbumin (King, 1960). This suggested that the free mercaptide group of the mercaptoalbumin had been blocked by formation of a mixed disulfide with either cystine or glutathione. This hypothesis was tested by reacting -SH albumin in excess cystine and oxidized glutathione, with the quantitative formation of cystine-S-S-albumin

and limited formation of glutathionyl-S-S-albumins. It was suggested that the cystinyl albumin could be formed by a disulfide exchange reaction between mercaptoalbumin and cystine or oxidized glutathione present in normal serum. Andersson (1966) employed 2-mercaptoethanol reduction of the disulfides at alkaline pH followed by cation exchange chromatography to isolate cystine and gluthione from the non-SH albumins in preparations of bovine albumin. Reduction by 0.2M 2-mercaptoethanol in pH 7.7 bicarbonate buffer was used to split the disulfide bridge without detectably disturbing the native configuration of the protein, while an increase in pH to 9.2 and incorporation of 1% sodium dodecyl sulfate permitted reduction of disulfide bonds in portions of the molecule not accessible otherwise. By this approach, two types of mixed disulfide were detected, one easily reduced and the other not. Studies on the relative proportions of mercaptoalbumin and the various non-mercaptoalbumins in albumins prepared by various methods were also done. The following tables provide a summary of the findings:

Relative proportions of various molecular species of albumin in different preparations.

Albumin Type	Cohn Fr.V	Sulfate precip.	Elect. fresh(3)	Elect. 1 day
mercaptoalbumin	46-49%	61-68%*	70%	45%
cysteinylalbumin (1)	18-21%	NR	0	23%
cysteinylalbumin (2)	17-20%	NR	9%	17%
glutathionylalbumin (1)	<2%	NR	0	2%
glutathionylalbumin (2)	8- 9%	NR	9%	9%
reducible dimers	1/3	NR	0	NR
non-reducible dimers	2/3	NR	0	NR
total dimer content	12-13%	5-8%	0	<2%

(1) Reducible by 0.2 M 2-mercaptoethanol in pH 7.7 buffer. Considered not native. Values vary greatly among samples.

(2) Required treatment with pH 9.2 buffer containing 1% sodium lauryl sulfate for reduction by 0.2 M 2-mercaptoethanol. Considered native.

* Estimated by dividing the value obtained for denatured protein by 1.5 to correct for the higher value obtained on denaturation.

NR = not reported

(3) Albumin isolated by moving boundary electrophoresis from fresh or one day old serum.

Relative compositions of non-SH albumin

	Cohn Fr. V	Sulfate ppt.
cysteinylalbumin (1)	34-37%	9%
cysteinylalbumin (2)	39-41%	48%
glutathionylalbumin (1)	2- 4%	1%
glutathionylalbumin (2)	13-14%	14%
unaccounted for	4-12%	28%

It is quite apparent from these data that the composition of bovine albumin can vary considerably according to the method of preparation. It is particularly noteworthy that albumin isolated electrophoretically from fresh serum lacks some of the monomeric and dimeric forms found in albumins prepared in more conventional ways, and differs markedly in the relative proportions of the remaining forms. The earlier reports of Benesch et al., in which higher values were reported for titrable -SH groups in crystalline fraction V bovine albumin than are reported here by Andersson for ordinary fraction V albumin would lead to the expectation that the non-mercaptoalbumin fractions of these two preparations may differ as well.

There is also some indication that bovine mercaptoalbumin, at least after being subjected to preparative manipulations, may be heterogenous in composition. Keller and Block (1959) studied a commercially prepared crystalline bovine mercaptoalbumin by chromatography on DEAE-SF cellulose using stepwise elution at pH 6.8. They were able to isolate 8 fractions by this procedure and analysed three of these by ultracentrifugation. The first two fractions were found to be entirely composed of monomer ($s_{20,w}$4.3S) while a later fraction was found to be composed of 51% monomer, 43% dimer ($s_{20,w}$6.7S) and 6% of higher molecular weight materials. Additional information has recently been presented by Janatova, Fuller and Hunter (1968) who examined both a crystalline bovine plasma albumin preparation (BPA) and a bovine

mercaptoalbumin (BMA) obtained from a different source. These workers employed column chromatography on DEAE Sephadex to separate the albumins into -SH and non-SH albumin fractions and chromatography on Sephadex G-150 to separate monomers, dimers and higher order materials. The BMA, when originally prepared, contained 1.0 mole of -SH per mole of protein. After freeze-drying and storage for a number of years, the aged BMA preparation was found to contain only 0.8 moles of -SH per mole of protein. Mercaptoalbumin freshly isolated from BPA by chromatography was found to behave similarly after freeze-drying, while whole BPA was said to be more resistant to -SH loss during the freeze-drying process. Similar behavior was seen with chromatographically isolated fractions of whole BPA and aged BMA during attempts to regenerate free -SH groups in the non-mercaptoalbumins by reduction with thioglycollate. An early non-mercaptoalbumin monomer (fraction B1) could be reduced, but the later non-mercaptoalbumin monomer (fraction B2) was resistent. The conditions obtaining during the preparation and storage of the BMA precluded the formation of mixed disulfides with small thiols such as cysteine or glutathione, and the lower efficiency of mercaptide recovery achieved compared with whole BPA required the conclusion that the non-SH albumins produced from mercaptoalbumin on lyophilization differed from those postulated by King and found by Andersson. Comparison of the elution profiles of BPA and aged BMA on DEAE Sephadex suggests strongly that this

new type of non-SH albumin found in aged BMA must also occur in fresh BPA. An additional area studied by these workers was the nature of the dimeric forms of albumin occurring in both the BPA and BMA preparations. Two different dimer fractions were identified; one which contained approximately one -SH per mole but could not be reduced by thioglycollate under conditions where albumin S-S bond reduction did not occur, and one which contained very little free -SH but could be reduced by thioglycollate (50% yield) to mercaptoalbumin monomer.

That albumin preparations contain bound fatty acids has long been recognized. Cohn et al. (1940) noted that the albumin produced by their low temperature ethanol method contained a yellow lipid pigment which they removed by dry ether treatment in a Soxhlet extractor. Later Cohn et al. (1946) compared the amounts of ether-extractable lipids contained in fraction V albumin and in the albumin crystallized from this fraction, and found that the fraction V albumin contained less fatty acid than the crystalline preparation. Willians and Foster (1960) noted that extraction with lipid solvents at acid pH failed to remove all of the fatty acid, since reducing the pH of previously extracted albumin below pH 2 resulted in the separation of a material which proved to be stearic acid and which therefore must have been tightly bound to the protein. Subsequently, Leonard and Foster (1961) reported that removal of the bound fatty acids left the albumin far more susceptible to aggregation,

heat, variations in pH, and changes in the ionic strength of the solution. Goodman (1958) studied the binding of long chain fatty acids to human albumin and was able to postulate three different classes of binding sites. It was indicated in this report that the mean fasting level of 500u moles per liter in normal serum would correspond to less than 1 mole of fatty acid per mole of albumin and that less than 0.01% of the fatty acid in serum was not bound to protein. Grossman et al. (1955) studied the distribution of fatty acids in human and rat sera by the use of ^{14}C tracers and paper electrophoresis. It was reported that at least a portion of the label migrated with the albumin band. Thus it would appear that "native" albumin consists of both fatty acid-containing and fatty acid-free forms in addition to the various -SH and non-SH forms that have been identified. It would seem too that the conditions of preparation can effect the amount of fatty acid which is still present in the purified albumin preparation. Chen (1967) reviewed the methods available for the extraction of fatty acid from albumin and noted that these generally failed to completely remove the fatty acid and led to measurable heterogeneity in the protein preparations. In the course of his investigations, 26 different albumins representing both Cohn fraction V and crystalline preparations of bovine, human, canine, equine, rabbit and porcine origin were tested for lipid content. Considerable variation was seen among the samples tested and, in general, crystalline preparations contained larger amounts of fatty acid

than did fraction V preparations although there were individual exceptions.

In view of the excellent agreement among the findings of Hartley et al., Andersson and Janitova et al., it seems reasonable to combine them in order to arrive at a tentative classification of the various forms of albumin which may be encountered in the usual fraction V or crystalline albumin preparation and may prove to be of interest immunologically:

Macrochemical heterogeneity of albumin in Cohn fraction V and crystalline preparations.

Form of Albumin	Relative Proportion
mercaptoalbumin	45-75%
non-mercaptoalbumin	20-50%
a) mixed disulfides	
1. easily reduced	10-20%
2. not easily reduced	10-20%
b) non-disulfides	10%
1. reducible	
2. not reducible	
pigment-bearing monomer	<5%
dimeric albumins	5-14%
a) -SH bearing, not reducible	
b) disulfide dimer, reducible	
c) non-disulfide dimer, reducible	
d) non-SH bearing, not reducible	
higher order aggregates	<1%

The relative proportions of these various components are known to vary according to the method of preparation and, as has been shown by Finlayson *et al.* (1965), the conditions of storage. Most of the forms, including all of the dimeric and higher order aggregates, as well as at least some of the non-SH monomers are thought to arise as artifacts of preparation. Of the forms thus far identified, only the monomeric mercaptoalbumin and the not easily reduced disulfide monomers have been isolated from freshly drawn serum albumin, where they seem to occur in the ratio of 3 parts of mercaptoalbumin to 1 part of non-mercaptoalbumin.

It has been assumed that animal passage serves to remove any aggregated forms from administered albumin. It may be that the process also results in the removal of some forms of monomeric albumin and an adjustment in the ratios of the various remaining forms to approximate those occurring in the native state. It is also quite possible, judging from the experiments of Pedersen (1962) in which more than one albumin monomer was identified by exclusion chromatography that differences in the physical properties of the various forms of monomer such as hydrodynamic volume might allow sufficient separation by gel filtration or ultracentrifugation to change the composition of resultant protein fractions from that of the original albumin preparation. Such altered composition may effect the outcome of biological experiments designed to elucidate the functions of the various components of the albumin preparation.

III. MATERIALS AND METHODS

Proteins used as antigens

Albumins: Bovine serum albumin (BSA) was the principal substance used in this study. Although some preliminary experiments were done with albumin from several commercial sources, most of the work was done with a Cohn fraction V preparation (Fr. V), Pentex lot 66 (Pentex Division of Miles Laboratories; Kankakee, Ill.) and a crystalline preparation (Xtal), Armour lot G71812 (Metrix division of Armour Pharmaceuticals; Kankakee, Ill.).

Other Proteins: In addition to the serum albumins, whole bovine serum (Flow Laboratories, Inc.; Rockville, Md.); Cohn fraction II bovine gamma globulin (BGG) (Armour lot A30702); Cohn fraction III-0 bovine beta-lipoproteins (Nutritional Biochemicals Corp.; Cleveland, Ohio lot 59999); Cohn fraction IV-4 bovine alpha- and beta-globulins (NBC lot 8638); Cohn fraction IV-1 bovine alpha-globulin (NBC lot 8169; and crystalline ovalbumin (Worthington Biochemicals, Inc.; Freehold, N. J. lot 558-9) were also used.

Solubilization of Proteins: For most purposes, including the preparation of immunizing antigens, proteins were dissolved in pH 7.2, 0.10 M phosphate buffered saline (PBS) initially prepared as a 10X stock solution as follows: 89 gm of NaCl per L were added to a mixture of 802 ml 0.1 M Na_2HPO_4 (MW 142) and 198 ml 0.1 M KH_2PO_4 (MW 137). The pH was then adjusted, if necessary, by adding either 0.1 M KH_2PO_4 or 0.1 M Na_2HPO_4 as required to bring a 1:10 dilution of this solution to pH 7.2.

Other solvents were used in conjunction with particular procedures, and are described with the procedures.

Dilutions: The principal use of protein solutions was in dose-response assays of immunological priming and tolerance. For these purposes, the proteins were made up at 6 mg/ml in pH 7.2 PBS, and serial dilutions prepared in volumetric glassware. The protein concentration was determined on triplicate samples of each dilution either by direct optical density measurements at 280 nm or by the Lowry method (1951).

Sterilization of Protein Solutions: All protein-containing solutions were filter sterilized through 0.22 um membrane filters (Millipore type GSWP047; Millipore Corp.; Bedford, Mass.) before use and prior to storage.

Storage of Proteins: When possible, proteins were obtained in crystalline or powder form and stored at 4C until needed. Once solubilized, the solutions were either used immediately or divided into aliquots and stored at -20C in tightly capped glass containers until immediately before use.

Fractionation of albumin solutions

By Exclusion Chromatography: Polymeric, dimeric, and monomeric albumins were isolated chromatographically from the commercially prepared albumins either directly or after heat treatment at pH 6.4, 65C for 15 minutes to increase the yield of aggregates. Exclusion chromatography for the separation of albumins by molecular size was done on Sephadex G-150 (Pharmacia Fine Chemicals, Inc.; Piscataway, N. J.). The Sephadex gel was prepared by soaking 60 grams of the dry powder in 3L of distilled water, stirred magnetically, for 72 hours at room temperature. It was then washed over a Buchner funnel and re-hydrated in two changes (3L each) of pH 7.2, 0.10 M phosphate-buffered saline (PBS).

The washed gel was then packed into two K 25/100 Pharmacia chromatography columns equipped with reverse-flow adapters. When packed and assembled, the two columns were transferred into a cold room (5C), connected in series, and attached to a PBS reservoir through a Buchler Polystaltic pump (model 2-6100, Buchler Instruments, Inc.; Fort Lee, N. J.) which was adjusted to a flow-rate of 25 ml per hour, with the columns operated in the reverse-flow configuration. Column effluent was collected in either 2.2 or 5.0 ml fractions on a Gilson model V-10 volumetric fraction collector (Gilson Medical Electronics, Inc.; Middleton, Wis.). 50 ml of a 5 gm % solution of albumin could be conveniently fractionated in this manner.

By Ion Exchange Chromatography: Mercaptoalbumin and non-mercaptoalbumin monomers were obtained by ion exchange chroma-

tography on A-50 diethyl aminoethyl (DEAE) Sephadex (rated exchange capacity of 3.5 ± 0.5 meq/gm.).

The Sephadex was prepared by suspending 35 gm of the dry powder in 3L of distilled water at room temperature and stirring magnetically overnight. The following day, the hydrated gel was washed three times at hourly intervals (3L per wash) in distilled water over a Buchner funnel, then suspended in 3L of 0.5 M HCl and stirred magnetically for one hour. The HCl was washed out over a Buchner funnel and the Sephadex washed once in 3L of distilled water. The Sephadex was then stirred in 3L of 0.5 M KOH for one hour to convert it to the hydroxide form and washed again in distilled water. Phosphoric acid (85%) was then stirred slowly into the Sephadex in distilled water until the pH reached 7.00 ± 0.05. When the pH became stable, the Sephadex was washed in one change of 0.05 M, pH 7.00 ± 0.05 potassium phosphate buffer (59.88 gm KH_2PO_4 MW 136.09 and 127.73 gm K_2HPO_4 MW 174.18 in 20 L distilled water) and permitted to stir overnight in 3L of the same buffer.

The following day, the phosphated A-50 DEAE Sephadex was packed into a K 25/45 Pharmacia chromatography column, transferred to a cold room (5C), and connected to a reservoir of 0.05 M pH 7.0 phosphate buffer. The head pressure was adjusted by raising the reservoir flask until a flow rate of about 25 ml per hour was achieved. Approximately 1 L of buffer was permitted to flow through the column, after which the pH of the column effluent was tested and found to equal that of the buffer in the reservoir (pH 7.00 ± 0.05).

Concentration of Column Effluents: Effluents, after pooling, were concentrated and transferred to PBS, when required, by either a combination of pervaporation and dialysis against PBS at 5C or a combination of ultrafiltration on Amicon PM-30 membranes using an Amicon model 50 filtration apparatus (Amicon Corp., Bedford, Mass.) or on Millipore Pellicon PSAC 04710 membranes mounted in a Millipore 47 mm Sterifil apparatus, followed by dialysis against PBS at 5C. The Amicon filter apparatus was operated at 5 lb/sq in air pressure and the Millipore apparatus was operated at 15 lb/sq in negative air pressure. Following dialysis, the protein concentrations were adjusted to approximately 10 mg per ml or less, filtered through 0.22 nm Millipore filters, divided into 4 or 5 ml portions and stored at -20C until needed.

Chemical Study of Albumin Antigens

Estimation of Protein Concentration: Protein concentrations were estimated by comparing the values obtained by either direct optical density measurements at 280 nm or by a modification of the Lowry method (1951) against values obtained with a set of weighed standards prepared from NBC lot 5776 crystalline bovine albumin. Standards of 0.010, 0.025, 0.050, 0.10, 0.25, 0.500, and 1.00 mg/ml were used. Either a Beckman model Acta III (Beckman Instruments, Inc.; Fullerton, Calif.) or a Turner model 350 spectrophotometer (G. K. Turner Asso., Inc.; Palo Alto, Calif.) was used for all protein determinations.

The modified Lowry procedure was the standard procedure employed in the Department of Epidemiology as follows:

1. Reagent A; 2% Na_2CO_3 w/v in 0.1 M NaOH.
2. Reagent B; 0.5% $CuSO_4 \cdot 5H_2O$ w/v in 1% w/v sodium tartrate ($Na_2C_4H_4O_6$).
3. Reagent C; Folin-Ciocalteau phenol reagent purchased as a 2N stock (Harelco; Phila. P. A.).
4. Reagent D; 50 ml of reagent A + 1 ml of reagent B prepared fresh on the day of use.
5. Reagent E; 1:1 dilution of reagent C and glass-distilled water prepared fresh on the day of use.

One milliliter of the protein to be tested was mixed with 5.0 ml of reagent D and allowed to stand 10 minutes. Following this, 0.5 ml of reagent E were added, the solution mixed and allowed to stand an additional 30 minutes. The solution was again mixed and the optical density read at 660 nm. The procedure so modified was capable of detecting proteins in the

concentration range of 0.010 to 1.00 mg/ml.

In practice, triplicate samples of each weighed protein standard were tested in the same experiment with triplicate samples of the unknown protein. The concentration of the unknown was estimated by comparing the mean optical density of the three determinations against a linear plot of the means of the weighed standards. Variation among the triplicate samples was usually within 10% but was occasionally higher.

Estimation of Dimer-aggregate Content: Polyacrylamide disc-gel electrophoresis, essentially as described by Ornstein (1964) and Davis (1964), was used for the detection of dimeric and higher ordered aggregates of albumin. The modification described by Peters (1970), was employed because of the greater resolution of aggregated forms of albumin obtainable. Electrophoresis was performed vertically in a cylindrical, leucite, double-tank apparatus of one liter buffer capacity, capable of mounting eight gel tubes in an octagonal pattern, with the gels located equidistant (5 cm) from centrally placed platinum electrodes. Two such tanks were available and could be connected in parallel to give a capacity of 16 gels in one run. Power was supplied by a Shandon Vokam type 2541 power supply (Shandon Scientific Co.; London, England) operated at a constant current of 2.5 mA per gel. The gels were run in Pyrex tubing, 68 mm in length by 5 mm inside diameter, which was coated with Photoflo 200 solution (Eastman Kodak Co.; Rochester, N. Y.).

Stock solutions were prepared as follows:

1. pH 8.3 Tris-glycine buffer; 6.00 gm Tris hydroxymethyl aminomethane (Sigma Chemical Co.; St. Louis,

Mo.), 28.80 gm glycine (Eastman), sufficient brom phenyl blue (Allied Chemical Corp.; New York, N. Y.) to give a blue tint, and water to make 1 L. The buffer stock was diluted 1:10 for use at pH 8.3.

2. Stock A (pH 9.2 buffer); 48.00 ml 1N HCl, 38.30 gm Tris, 0.46 gm N, N, N', N', tetramethyl-ethylene-diamine (temed, Eastman), and water to make 100 ml.
3. Stock B (pH 6.9 buffer); 25.6 ml of 1N H_3PO_4, 5.7 gm Tris, and water to make 100 ml.
4. Stock C; 2.0 gm N, N, methylene-bisacrylamide (Eastman), 38.0 gm acryl-acrylamide (Eastman), and water to make 100 ml.
5. Stock D (catalyst); 0.28 gm ammonium persulfate, (J. T. Baker Co.; Phillipsburg, N. J.). This reagent must be prepared fresh weekly.
6. Stock E (riboflavin catalyst); 4.0 gm riboflavin (Sigma), and water to make 100 ml.
7. Stock G; 40.0 gm acryl-acrylamide and water to make 100 ml.

All solutions were stored at 5C until needed except the ammonium persulfate catalyst (stock D) which was prepared fresh as needed.

A two part gel was prepared from stock solutions as follows:

1. Running gel; 1.5 ml stock G, 1.5 ml stock A, 3.0 ml stock D, and 5.5 ml distilled water to make 12 ml. This solution was mixed thoroughly and dispensed at 1 ml each into the gel tubes held vertically in salvaged vacutainer-type serum

stoppers which fit snugly over the ends of the tubing. The gels were overlaid with a small amount of distilled water to flatten the meniscus, then permitted to stand until solidified (about 15 minutes). The tubes were then shaken to remove the water layer and returned to their standing position.

2. Stacking gel; 0.5 ml stock B, 0.5 ml stock C, 0.5 ml stock E, and 2.5 ml of distilled water to make 4 ml of gel. This was mixed well and dispensed at 0.2 ml per tube. Again, a small quantity of distilled water was overlaid on the gel to flatten the meniscus. The stacking gel was solidified by exposing the tubes, held vertically, to the light from a Wood's lamp (Mineralight, Ultra-Violet Products, Inc.; South Pasadena, Calif.) until the yellow color of the riboflavin catalyst had disappeared (about 15 minutes). The water overlay was then shaken from the tubes and these were mounted in the tank. Sufficient pH 8.3 Tris-glycine buffer was added to cover the ends of the gel tubes.

Albumin samples were prepared by adding 20 microliters (λ) of protein solution, preferably at 10 mg/ml, to 80 λ of 20% sucrose (Baker) in Tris-glycine buffer. 20 λ of this were then overlaid on the surface of the stacking gel by pipetting through the buffer in the tank, and electrophoresis carried out for 1.5 hours at 5C. At the completion of the run, gels were hydrauli-

cally removed from the gel tubes, taking care to keep the stacking gel layer intact, and transferred to 150 X 10 mm flat-bottomed glass storage tubes.

Staining was accomplished by covering the gel for 15 minutes with a solution composed of 0.5 gm amido black 10B (Harelco) in 100 ml of 7% glacial acetic acid. The gels were cleared of excess stain by immersing them in as many changes of a solution consisting of 500 ml absolute methanol, 100 ml glacial acetic acid and water to make 1 L as were required to produce blue-black protein bands on a water-clear background. Following the clearing step, the gels were covered with distilled water and the storage tubes sealed with number 000 stoppers.

The ratio of dimer-aggregate to total protein was estimated from areas under densitometric plots prepared from the stained polyacrylamide gels at 593 nm using a Beckman Acta III spectrophotometer equipped with a densitometer attachment.

Estimation of Lipid Content: Protein-bound lipids were estimated by a modification of the titrimetric method of Dole (1956). 1.0 ml of a 30 to 50 mg/ml solution of albumin was extracted by shaking, vigorously, for one minute in a 15 ml screw-capped culture tube with 5.0 ml of an extraction mixture consisting of 40 parts isopropyl alcohol, 10 parts n-heptane, and one part 1N H_2SO_4. After shaking, the tube containing the mixture was permitted to stand undisturbed for 10 minutes. Following this, 2 ml n-heptane were added, the mixture shaken vigorously for one minute, and 3 ml H_2O were added. The mixture was permitted to stand until the heptane and aqueous phases separated. 3.0 ml of the upper (heptane) phase were transferred

to a clean 15ml screw-capped culture tube, which contained 1.0 ml of a titration mixture composed of one part thymol blue stock (0.1 gm% thymol blue in distilled water) and nine parts 95% ethanol. This mixture was titrated to a yellow-green endpoint with NaOH freshly diluted 1:100 in distilled water from a saturated stock solution. The NaOH was dispensed to the nearest 0.5 λ from a 25λ Hamilton Syringe 702-N equipped with a sequential dispenser no. PB 600 (The Hamilton Co.; Whittier, Calif.) into the titration mixture stirred continuously on a vortex mixer (Scientific Industries, Inc.; Springfield, Mass.). A blank titration was made against a distilled water sample treated identically with the albumin sample, and a set of palmitic acid standards (Hormel lot Jo-5A, Hormel Institute; Austin, Minn.) in n-heptane at 0.800, 0.600, 0.400, 0.200, 0.100, and 0.050 u eqn/L were concurrently titrated. The amount of bound lipid in the albumin sample, expressed as u eqn palmitate/L, was estimated from a standard curve prepared from these titrations. The mean of three determinations was used to arrive at each value, and the volume of NaOH needed was usually within ± 0.5λ for any three triplicate samples, The variation in values for the same material tested on different days was less than 10%.

Estimation of Mercaptoalbumin Content: Free sulfhydryl was determined by the method of Ellman (1959) as modified by Janatova et al., (1968). 1.5 ml of an albumin solution containing between 4 and 10 mg protein per ml were mixed with 0.4 ml of 0.01 M DTNB (5, 5' dithiobis 2-nitrobenzoic acid, MW 396.4, Grade A Cal Biochem; Los Angeles, Calif.) in 0.037 M pH 8.00 potassium phosphate buffer (final pH of the DTNB stock was 6.65);

and 0.1 ml of 0.25 M EDTA (ethylene dinitrolotetraacetic acid disodium salt, MW 372.25; Eastman) in 0.0074 M phosphate buffer, pH 8.10. A reagent blank was prepared by substituting 1.5 ml of 0.0074 M pH 8.10 phosphate buffer for the albumin solution, and an albumin blank was prepared by substituting 0.4 ml of 0.037 M pH 8.00 phosphate buffer for the DTNB solution. All three of the mixtures were permitted to stand 45 minutes at room temperature, then were read on a Beckman Acta III spectrophotometer at 412 nm using a 1 ml cell with a 1.0 cm light path.

Absorbance of the albumin blank was measured against distilled water, and the absorbance of the test solution was measured against the reagent blank. The absorbance of the albumin blank was then subtracted from that of the test solution to yield the absorbance due to the p-nitrothiophenol ion generated through reaction of the DTNB with the available thiol group of the albumin. The extinction coefficient (ε) was taken as $\varepsilon_{412} = 13{,}000$ $M^{-1}cm^{-1}$ in order to calculate the ratio of available sulfhydryl to albumin according to the relation:

$$\text{Molar ratio of -SH/Protein} = 6.765\ A^{1cm}_{412}/(\text{mg protein/ml})$$

where A is the measured absorbance.

Circular Dichroism Measurements: These were performed with the kind assistance of Dr. E. J. Hunter of the Biophysics Research Division , Institute of Science and Technology, The University of Michigan. Measurements were performed using a Durrum-Jasco UV5 scanning spectropolarimiter equipped to make circular dichroism measurements and further modified by addition of a Sproul Scientific SS-20 CD modification. Measurements were made at 25C on 0.025 to 0.15% protein solutions in pH 7.2,

0.10 M PBS. An optical quartz cell of 1.000 cm optical path length was used. Because the measurements were made only to compare different albumin preparations and not to conduct more detailed structural analysis, scale factors were not recorded.

Dichroic absorption differences were measured in the near ultraviolet (360 to 250 nm) and all reported CD data were corrected for solvent base-line non-ideality and the base line and protein dichroisms were matched at 450 nm; a spectral region where no absorption occurs.

Additionally, it was necessary to correct measurements for deviations in protein concentration from the desired 0.025 and 0.15% concentrations. All measurements were made in duplicate. The variance between duplicate readings on the same sample was not more than 0.2 cm on the chart recording.

Alteration of Antigens

Extraction of Lipids: Lipids were extracted by the low pH-charcoal method of Chen (1967). This method is rapid, efficient (up to 99% of bound lipids can be removed) and reportedly causes minimal denaturation of the protein compared to traditionally used solvent extraction methods. To 50 ml of a 10 gm % solution of albumin in water were added 2.5 gm of activated charcoal (Norit A lot N-6502, Sigma Chemical Co.) which had previously been washed over a Buchner funnel and dried overnight in a 50C oven. The pH of this mixture was then adjusted by the addition of 0.2 M HCl, to 3.0 ± 0.01 as determined on a Beckman Expanded Scale pH meter equipped with a Beckman No. 8903 combination electrode. The albumin-charcoal mixture was then transferred to an ice bath and stirred magnetically for one hour. Charcoal was removed by filtration over a one inch bed of Cellite 525 (Sigma) in the cold (5C), followed by filtration through a 0.22 nm Millipore filter. Immediately after removal of the charcoal, and before filtration, the albumin was titrated to pH 7.0 ± 0.01 with 0.2 M KOH. The albumin so prepared was then adjusted to a protein concentration of 3.6 gm % and dispensed into 14 ml portions preparatory to further treatment.

Preparation of Lipid-albumin Conjugates: Palmitic acid (Hormel lot JO-5A) was solubilized by boiling 250 mg with 25 ml of 1 M KOH for 30 minutes. The solution was cooled and titrated to pH 7.6 with 1M HCl and then diluted to 50 ml with distilled water to make a stable 5 mg/ml aqueous suspension. Complexes of potassium palmitate and BSA were then

prepared by adding .38 ml of the pH 7.6 potassium palmitate into 14 ml of the 36 mg/ml lipid-free albumin previously prepared to make a 1 meq/meq conjugate, or 7.6 ml of palmitate to 14 ml of 36 mg/ml albumin to make a 20 meq/meq conjugate. The lipid-treated albumins were then mixed gently on a vortex mixer until all of the palmitate had been absorbed as evidenced by clearing of the initially turbid material (approximately 20 minutes were required).

All of the albumin solutions, whether lipid-conjugated or not, were dialyzed against PBS overnight in individual containers, using two changes of buffer. Following dialysis, the albumins were filtered through 0.22 nm Millipore filters, dispensed in 1 ml portions, and frozen at -20C until needed.

Preparation of Heated Albumin Antigens: It was not found practical to isolate the quantity of soluble aggregates needed from the commercially prepared albumins. However, a high yield of soluble aggregate was obtained by first adjusting 5 gm % solutions of albumin in distilled water to pH 6.4 with 0.1 M HCl and then heating in a 65C waterbath for 15 minutes with constant mixing.

Mice

Source and Care: Balb mice used in these experiments were from colonies maintained individually by Dr. R. J. Porter, Department of Epidemiology, School of Public Health; Dr. A. G. Johnson, Department of Microbiology, School of Medicine; and Dr. W. H. Murphy, Jr., Department of Microbiology, School of Medicine, The University of Michigan. All were derived from the mice initially obtained by Dr. W. H. Murphy, Jr.

These mice were maintained by continuous brother-sister mating according to procedures described by Green (1966). Adult mice including both breeder stocks and weanling mice were maintained on Purina Laboratory Chow (Ralston Purina Co.; St. Louis, Mo.) and water ad libitum. Pregnant females and their subsequent litters were maintained on Purina Mouse Breeder Chow and water ad libitum. Terramycin (Charles Pfizer Co.; New York, N. Y.) was added to the drinking water of mice in the Porter Colony at the ratio of one level teaspoon per gallon of water. Young mice were weaned at the age of approximately 4 weeks, with the young from litters born over a 4 to 5 day span combined into groups of 20 to 30 mice of a single sex.

Immunization: Unless otherwise stated, antigens were always diluted in pH 7.2 PBS so that 0.5 ml contained the desired dosage.

Mice were given primary immunizations intravenously (i.v.) via the tail vein, using a 1 ml tuberculin syringe equipped with a 26 gauge disposable needle.

The mice were prepared for primary injection by warming

them in a large container heated by a 100 watt light, and then transferred to a warm mouse-holder for injection.

Secondary immunizations were given intraperitoneally (i.p.) 6 weeks following primary immunization.

Bleeding and Storage of Serum: Bleeding was done 4 and 7 days following the primary and one or two days before, and 7 days following, the secondary immunization. Occasionally, other intervals were used in addition to these. The mice were bled from the tail vein after prewarming them in a container heated by a 100 watt light, then transferring them immediately to a warm mouse-holder and nicking a lateral tail vein with a razor blade. Blood was collected by permitting about 15 drops to accumulate in a 2 ml conical centrifuge tube. This procedure could be carried out several times on each mouse without apparent ill effect. Blood samples were permitted to stand at room temperature for 2 hours, the clots rimmed, and then were transferred to a 5C cold room for several hours. They were then centrifuged in a Sorval GLC-1 centrifuge (Ivan Sorvall, Inc.; Norwalk, Conn.), equipped with an 80 place swinging-bucket rotor, at 1800 rpm for 10 minutes. The serum was transferred to sterile 13 X 75 mm glass test tubes which were then sealed with 000 stoppers and stored at -20C until needed.

Control Sera

Preparation of Specific Sera: Pooled sera were prepared in mice against hen's egg ovalbumin, bovine gamma globulin (heated at 56C, 30 minutes), whole bovine albumin, polymerized albumin, and several varieties of monomeric albumin isolated from bovine albumin. Groups of 10 to 15 mice were given two doses of 3 mg protein in PBS i.p. separated by a 6 to 8 week interval and bled 7 days after the first injection for primary immune serum or 7 days after the second injection for secondary immune serum. This procedure was found not to result in high titer antisera against certain of the bovine albumins. For these, the albumin was combined with Freund's complete adjuvant (Difco; Detroit, Mich.) for the first dose which was given as 0.5 mg protein in 0.2 ml adjuvant mixture subcutaneously. A second dose of 3 mg protein in PBS was given i.p. 8 weeks following, and the mice bled 7 days later. Such sera are referred to as hyperimmune (hy). The control sera used were all of high titer having passive hemagglutination titers in the range of 10,000 to ≥20,000.

Anti-Bovine Serum: Antiserum prepared in rabbits against bovine serum was obtained from the Sylvana Co. (Millburn, N. J.) and used untreated for immunoelectrophoresis or double diffusion studies. Use of this antiserum for passive hemagglutination studies required that it be absorbed for 30 minutes at room temperature with each of two doses of 1 ml sheep erythrocytes per ml of serum.

Other Sera: Antisera prepared in rabbits and mice against bovine serum albumin, bovine gamma globulin and bovine serum,

obtained through the courtesy of Dr. A. G. Johnson and Dr. Ihn Han of the Department of Microbiology, were also used.

Immunological Assays

Immunoelectrophoresis: Immunoelectrophoresis was used to evaluate the level of contamination in the antigen preparations. The procedure was carried out using 0.6% agarose and a pH 8.6 barbital-lactate buffer as employed by Francis, Ling and Krasteff (1965). 0.6% agarose (Biorad Laboratories, Inc.; Richmond, Calif.) was prepared by adding 0.6 gm of agarose per 100 ml of a solution containing 2 parts of a buffer (no. 2) consisting of 1.6 gm barbital, 10.51 gm sodium barbital and 1.54 gm calcium lactate per L with 1 part of a buffer (no. 1) consisting of 1.38 gm barbital, 8.76 gm sodium barbital and 0.38 gm calcium lactate per L. After heating, the agarose solution was pipetted onto clean 3" X 1" plain glass microscope slides at 2.5 ml per slide, cooled and a set of 2 wells and a trough were punched using a jig constructed for that purpose. Seven slides were mounted in a Shandon combination electrophoresis tank connected to a Shandon Vokam power supply. Paper electrodes made from Whatman no. 1 filter paper were laid flat over the edges of the slides and the buffer tank filled with approximately 1 L of buffer no. 1.

Samples of antigen were pipetted into the appropriate wells, and electrophoresis carried out for 1 hour at a constant current of 4-5 mA per slide.

The slides were transferred to a moist chamber at room temperature and sufficient antiserum added to fill the center trough. After overnight incubation, the slides were soaked in three 24-hour changes of distilled water and dried under filter paper. Once dry, the slides were stained by immersing

for 1 minute in the amido black 10B staining solution previously described and cleared by rinsing in the clearing solution for 1 to 2 minutes as needed to produce blue-black protein bands on a clear background. Following this, the slides were soaked for 1 hour in a 5% v/v of glycerol in distilled water to retard cracking and peeling, then dried and stored until the results could be recorded photographically.

Double Diffusion in Gel: Ouchterlony double diffusion on immunoplate pattern B slides (Hyland Division of Travenol Laboratories; Costa Mesa, Calif.) was used to study antigens of the various albumin preparations. Antigens were pipetted into the peripheral wells and antiserum into the central well of each set (approximately 10 λ). The immunoplates were then incubated overnight at room temperature, washed as described above, mounted on 3" X 1" plain glass microscope slides, then dried, stained and cleared as also described above.

Antigens were compared after first determining the dilution of antigen that resulted in a precipitin band located midway between the antigen and antiserum wells (approximately the equivalence point).

Passive Hemagglutination: Sera of individual mice were assayed for antibody by a semi-micro modification of the passive hemagglutination (H A) method of Boyden (1951) and Stavitsky (1954) as described by Porter (1960, 1964). All of the sera were initially diluted 1:10 in a diluent composed of 2% absorbed normal rabbit serum (abs NRS) in 0.15 M potassium/sodium phosphate-buffered saline. The normal rabbit serum (Flow Laboratories; Rockville, Md.) was inactivated at 56C for

30 minutes, then absorbed with an equal volume of washed sheep erythrocytes (SRBC) for 30 minutes at room temperature to remove antibodies reactive with the unmodified SRBC. After absorption, the abs NRS was filtered through a 0.22 nm Millipore filter and stored at -20C in 5 to 10 ml portions until needed.

The test sera that had initially been diluted 1:10 were then serially diluted in plexiglass microtiter plates (Cooke Engineering Co.; Alexandria, Va.) using an automatic serial diluting machine. The final volume of diluted serum in each well was 0.10 ml. A volume of 0.025 ml of a suspension of tanned, antigen-coated SRBC was added to each serum dilution and the plates shaken for 30 seconds on a modified Kahn shaker. The finished plates were then covered to reduce evaporation and permitted to stand undisturbed overnight before reading. Titers were read and expressed as the reciprocal of the last 2-fold serum dilution showing at least a one plus agglutination pattern according to Stavitsky (1954). Note was also taken of the pattern of hemagglutination as this could indicate a primary or secondary type of response (Bauer and Stavitsky, 1961; Porter, 1962; and Benedict et al., 1962). Unless otherwise noted, results are reported as reciprocal geometric mean titers of 3 or 4 mice per mean. In order to facilitate computation of mean values, reciprocal titers of <10 were treated as 5.

Pooled primary and secondary mouse anti-BSA, anti-BGG, anti EA and non-immune sera were included as controls.

A non-specific control consisting of a 1:10 dilution of each serum and 0.025 ml of tanned SRBC without antigen was also included.

For routine assays, tanned SRBC were sensitized with Pentex lot 66 Fr. V bovine albumin at the level of 1.0 mg of BSA per ml of a 1:50 dilution of SRBC. Some control studies were done with proteins other than the lot 66 Pentex albumin. Antigens were always coated onto the SRBC at the ratio of 1 mg protein per ml of 1:50 SRBC as used for the standard antigen.

2-Mercaptoethanol (2-ME) Treatment of Serum: IgM hemagglutination activity is destroyed by treatment with 0.1 M 2-ME, while IgG activity is not (Deutch and Morton, 1957; Grubb and Swahn, 1958; Benedict et al., 1963). 0.2 ml of serum diluted 1:10 in 2% abs NRS diluent were mixed with 0.2 ml of 0.2 M 2-ME in 2% abs NRS diluent to give a 1:20 dilution of serum in 0.1 M 2-ME. The 2-ME-treated sera were incubated at 37C for 45 minutes, then diluted and prepared for H A as normally, except that the first serum dilution was 1:20 and the final dilution 1:40,000. Using this procedure, primary titers were reduced to <20 (control serum) and secondary titers were reduced by one dilution from the original titer (control serum).

Tolerance Assay: The method employed was patterned after that of Mitchison (1964) but differed in several respects. In the present experiments, only two injections of antigens were given. The first or "test" dose was given to

groups of 4 mice each i.v. in several dilutions in the range of 0.000 to 3.00 mg. Six weeks following the test antigen, a single dose of 3.00 mg of Pentex lot 66 fraction V albumin without adjuvant was given i.p. as a "reference" antigen. Passive H A titers measured on sera taken 7 days after the reference antigen dose were used to evaluate the immune status of the mice. For this purpose, use was made of the reciprocal titers, ratios of the logarithms of the reciprocal mean titers of mice given a test dose and mice given only the reference dose and the 2-ME sensitivity of the antibody formed.

Mice previously exposed to antigen that responded with 2-ME resistant antibody (2-MER) and had titers which were at oeast as great as the titers of mice in the same experiment given only the reference dose of antigen (ratio of test titer to reference titer ≥ 1) were considered primed. Mice previously exposed to antigen that failed to produce titers at least as great as the titers of reference mice (ratio of test titer to reference titer <1), whether or not 2-MER antibody was present, were considered tolerant, no distinction being made between partial and complete tolerance. Since the H A test has a precision no better than $\pm$ one dilution between replicate samples, mice were not considered tolerant unless the mean titer of a group of 3 or 4 mice was less than 0.5 of the mean titer of the reference mice. When converted to logarithms as described in the following section, this ratio became approximately 0.8.

Statistical Treatment of Data

Calculation of Means and Standard Deviations: Groups of 4 mice of a single age and sex were used with each dilution of each antigen tested. Because animals or individual serum samples were occasionally lost, data were not always available for all 4 mice in each group. However, no data are given unless samples from at least 3 mice per mean were used.

An unprimed (reference) control group, and a group consisting of mice treated with the reference antigen as a test dose as well were always included in each assay. The reciprocals of individual titers were converted to their logarithms for the calculation of the mean and standard deviation of each set according to methods described by Schor (1968). These were then converted back to their arithmetic values, resulting in a geometric mean and a weighted standard deviation which is always expressed as the range of $\pm$ one standard deviation. The results of duplicate experiments are reported for each antigen tested.

Pooling of Data: For the purpose of graphic comparison of antigens, the averaged data from duplicate dose-response experiments were first converted to logarithms and normalized to the means of the reference groups, then pooled according to methods described by Finney (1964) to obtain a mean and standard deviation for the pooled data as follows:

Let $\bar{M}$ be the weighted mean of the pooled experiments.

Let $\bar{V}$ be the variance of the pooled data.

Let M_1 and M_2 be the means, respectively, of the duplicate experiments.

Let V_1 and V_2 be the variances, respectively, of the duplicate experiments

$$\overline{M} = \left(\frac{M_1}{V_1} + \frac{M_2}{V_2}\right)\left(\frac{V_1 V_2}{V_1 + V_2}\right)$$

The standard deviation (S. D.) of the pooled mean ($\overline{M}$) is then given by:

$$\text{S.D.} = \sqrt{\overline{V}} = \sqrt{\frac{V_1 V_2}{V_1 + V_2}}$$

IV. EXPERIMENTAL RESULTS

Introduction: Studies on the relationship between physicochemical properties and the property of inducing a state of specific acquired immunological unresponsiveness using protein antigens of biological origin have recently involved almost exclusively the comparison of aggregate-containing and non-aggregated proteins. The weight of evidence derived from numerous studies indicates that aggregate-containing proteins more easily immunize and less easily tolerize the recipient animals than their aggregate-free counterparts. However, there are several protein antigens, such as turkey gamma globulin in mice, the hemocyanins, and flagellin, in which removal of aggregates does not reduce immunogenicity nor confer the capacity for tolerance induction. Similarly, there exists at least one protein, the B chain of porcine lactic dehydrogenase, in which aggregation does not confer the property of immunogenicity. These points, together with observations indicating that there is a common structural change associated with the aggregation of those mammalian globulin antigens that do show increased immunogenicity on aggregation, suggest that there may be some property of the protein antigens operating independently of the state of aggregation which is capable of influencing the ability of the protein to induce specific immunological unresponsiveness.

Review of the chemical literature shows that bovine albumin as it is usually available from commercial sources is subject to numerous systematic differences beyond the monomer-aggregate variation. Among these are differences in the amount of mercaptoalbumin and of at least three non-mercaptoalbumin monomers; differences in the pH profile of a pH dependent conformational transition; and variations in the amount of bound substances, particularly bound lipids. None of these variables, with the exception of lipid binding, has been evaluated in terms of possible immunological effects. Nor has there been any information available on the influence of minor structural alterations which do not affect the state of aggregation nor the apparent antigenic specificity but may influence the conformation of the protein.

The observation of Porter (1970) and the earlier observation of Martin et al. (1943) regarding the superior ability of some Cohn fraction V albumins compared to crystalline albumin to induce a high-titer antibody response in the absence of adjuvant might be explained on the basis of differences in aggregate content. However, the two types of albumin differ in the composition and physicochemical behavior of their monomeric components as well. One wonders if these differences, in the absence of measurable differences in aggregate content, might account for the differential immunological behavior of the Cohn fraction V and crystalline albumin.

The work presently described is intended to test the following five points: (1) whether or not aggregated forms of bovine albumin are, per se, more immunogenic and less toleragenic than monomeric albumin in balb mice; (2) whether or not the immunologic properties of monomeric albumin isolated from a highly immunogenic preparation of bovine albumin differ from those of the monomeric albumin isolated from a poorly immunogenic preparation; (3) in what way the addition or removal of lipid influences the immunologic behavior of the albumin; (4) whether or not it is possible to either isolate or prepare samples of monomeric bovine albumin that differ in their toleragenic or immunogenic properties; and (5) whether or not, within the technical limits of the methods employed, it is possible to correlate changes in the behavior of the monomeric albumins with some change in properties independent of differences in the state of aggregation or in antigenic specificity.

Since Cohn fraction V and crystalline albumins differ markedly and quite consistently from one another in their relative proportions of non-mercaptoalbumin and bound lipid, and since a quantity of highly immunogenic Cohn fraction V albumin was on hand, it was reasoned that a parallel study of this albumin and a crystalline albumin would permit a determination of whether quantitative and possible qualitative differences in the albumin sub-types contribute to differences in immunogenicity.

Validation of Principal Antigens and Immunological Assay Method Employed

Comparison of Unaltered Fr. V and Xtal Albumins: Two lots of albumin were chosen for detailed study. A Cohn fraction V preparation (Pentex lot 66) was chosen for its known property of stimulating high HA titers in mice. A crystalline albumin (Armour lot G71812) was chosen as a representative crystalline albumin of lower immunogenicity.

The albumins differed in the quantity of aggregate, bound lipid and mercaptoalbumin contained as revealed by polyacrylamide gel electrophoresis, titration of lipid by the Dole method, and determination of free -SH by the modified Ellman method.

A study of the antigenic determinants was begun by immunizing groups of balb mice with two 3 mg doses, separated by a 6 week interval, of either Fr. V or Xtal albumin in PBS. Pooled sera, taken by tail vein bleeding 7 days after the first injection (primary) and 7 days after the second injection (secondary), were examined by passive HA using SRBC coated with either the Fr. V or Xtal albumin.

Table 1 shows representative results. Mice raised higher levels of antibodies against Fr. V than against the Xtal albumin, as expected. Additionally, a strong primary response against Fr. V albumin was observed which was not detected with Xtal-coated test cells, and a low-grade primary response against the Xtal albumin was detected by

TABLE 1: COMPARISON OF A COHN FRACTION V AND A CRYSTALLINE ALBUMIN AS IMMUNIZING AND TEST ANTIGENS.

Antiserum prepared against:	Molar Ratios			Reciprocal mean H A titers at 7-days against test cells coated with:	
	SH	FFA	Response	Fr.V Albumin	Xtal Albumin
Cohn Fr. V (Pentex Lot 66)	0.45	0.24	Primary	320	<10
			Secondary	10,000	5,000
			Secondary 2-MER	5,000	2,560
Crystalline (Armour Lot G71812)	0.68	1.00	Primary	20	<10
			Secondary	640	320
			Secondary 2-MER	160	80

Fr. V-coated test cells but not by Xtal-coated test cells. There was also a one dilution difference in titers of secondary sera with Fr. V-coated cells compared to Xtal-coated cells. This difference is not significant in itself, since this degree of variation is within the precision of the test. However, the difference appeared consistently whenever these pairs of antigen-coated cells and antisera were run together. The efficiency with which proteins coated onto SRBC may detect antibodies is partly determined by the amount of protein coated onto the cells, and there is an optimum ratio which may vary among different protein preparations. Hence, it is possible that the differences in titer are simply a result of the lower efficiency of the Xtal albumin as a test antigen at the 1 mg/ml ratio. However, it may be that there are either qualititative or quantitative differences in the antigens in Fr. V compared with Xtal albumin.

It is well known that Cohn fraction V and crystalline albumin are contaminated with non-albumin serum proteins. According to Cohn et al., (1947) their fraction V preparation contained 1.2% of non-albumin protein as estimated by immune precipitation reactions, and their crystalline albumin, after one crystallization, contained 0.4% of the non-albumin protein. Foster (in Putnam, 1960) has indicated that fraction V albumins are generally found to contain about 1% of non-albumin protein, including proteins normally found in Cohn fractions III and IV. Information

provided by The Research Products Division of Miles Laboratory, Inc., indicates that Pentex fraction V albumin usually contains variable amounts of fraction III and fraction IV proteins.

To test the level of non-albumin contaminants, immunoelectrophoresis was conducted on duplicate samples of the Fr. V and Xtal albumins at 100 mg/ml. Albumin was placed in the upper well and undiluted calf serum in the lower well, and electrophoresis was carried out for one hour at room temperature. Hyperimmune mouse anti-Fr. V albumin was placed in the center well of one set, and hyperimmune mouse anti-Xtal albumin in the center well of the duplicate set. The slides were then incubated overnight, which provided sufficient time to develop dense precipitate bands.

Figure 1 shows the results obtained. Under these experimental conditions, one non-albumin precipitin band was detected in the undiluted calf serum with hyperimmune anti-Fr. V albumin antiserum but not with the hyperimmune anti-Xtal albumin antiserum.

A further check was made by conducting Ouchterlony double diffusion studies to compare the antigenic content in 10 mg/ml samples of Xtal, Fr. V, Cohn fractions IV-4, IV-1, III-0 and II (BGG), and calf serum diluted 1:50 in 0.85% NaCl. The antigen solutions were compared using rabbit anti-bovine serum, mouse anti-BGG, mouse hyperimmune anti-albumin prepared against Fr. V albumin, and mouse hyperimmune serum prepared against Xtal albumin

FIGURE 1: IMMUNOELECTROPHORETIC COMPARISON OF FR. V AND XTAL ALBUMIN; DETECTION OF NON-ALBUMIN SERUM PROTEIN CONTAMINANTS.

1. Hyperimmune mouse anti-Fr. V albumin; Fr. V albumin at 100 mg/ml in the upper well, undiluted calf serum in the lower well.

2. Hyperimmune mouse anti-Fr. V albumin; Xtal albumin at 100 mg/ml in the upper well, undiluted calf serum in the lower well.

3. Hyperimmune mouse anti-Xtal albumin; Fr. V albumin at 100 mg/ml in the upper well, undiluted calf serum in the lower well.

4. Hyperimmune mouse anti-Xtal albumin; Xtal albumin at 100 mg/ml in the upper well, undiluted calf serum in the lower well.



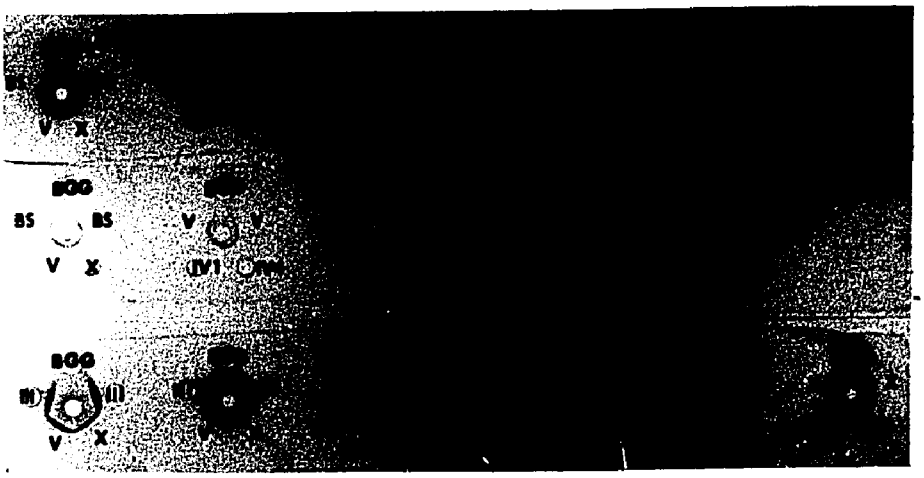
The results shown in figure 2 indicate that both the Fr. V and Xtal albumins contain a non-albumin serum protein found in normal bovine serum and also in BGG and in fractions III-0, IV-1 and IV-4. The contaminant does not appear to be BGG, however, since bands of identity did not form between any component in the bovine serum and any component in BGG, Fr. V or Xtal simultaneously when either anti-bovine serum or anti-albumin antisera were used. Aside from the presence of detectable levels of non-albumin contaminants, there appeared to be no gross antigenic differences between Fr. V and Xtal albumin which could be detected by double diffusion. There remains, however, the question of the differences in the primary response as determined by HA. These could reflect a response to non-albumin contaminants in the Fr. V albumin which are present, if at all, at a lower level in the Xtal albumin. On the other hand, they might represent an antigenic difference in the albumin; this seems unlikely, since antibodies are found against the non-albumin contaminant in anti-Fr. V antisera.

In addition to the non-albumin proteins shared by the Fr. V and Xtal albumin preparations, it is clear in figure 2 that the non-albumin serum protein fractions share an antigen in common with the Fr. V and Xtal. The lines of identity, with absence of spurs, produced in the several combinations indicate that the contaminant is a single, shared substance, most probably albumin, rather than a series of cross-reacting substances.

FIGURE 2: EXAMINATION OF CROSS-CONTAMINATED SERUM PROTEIN PREPARATIONS BY OUCHTERLONY DOUBLE DIFFUSION IN GEL.

Center wells charged as follows: (1),(5a) hyperimmune mouse anti-Fr. V albumin; (2), (5b) hyperimmune mouse anti-Xtal albumin; (3), (4), (6) rabbit anti-bovine serum; (5c) mouse anti-bovine gamma globulin.

Symbols: (BGG), Cohn fraction II bovine gamma globulin at 10 mg/ml; (BS), Calf serum diluted 1:50 in 0.85% NaCl; (V), Fr. V albumin at 10 mg/ml; (X), Xtal albumin at 10 mg/ml; (IV-1), Cohn fraction IV-1 bovine alpha globulin at 10 mg/ml; (IV-4), Cohn fraction IV-4 bovine alpha and beta globulins at 10 mg/ml; (III), Cohn fraction III-0 bovine beta lipoproteins at 10 mg/ml.


1
2
3
4
5
6
V X
BS BS
V X
V X

To test this point, the Fr. V albumin, which should contain the highest level of non-albumin protein compared to Xtal albumin, and the fraction II BGG, representing the opposite end of the protein spectrum, were compared against fractions IV-4, IV-1 and III-0 by immunoelectrophoresis, using hyperimmune mouse anti-Fr. V antiserum and mouse hyperimmune anti-BGG. Results are shown in figure 3. It is seen from the positions of the precipitin bands that each of the proteins III-0 and IV-1 contains both albumin and globulin contaminants. The IV-4, however, contains only the albumin contaminant.

Selectivity of the Hemagglutination Assay: It was shown in the work of Boyden et al. (1951) that proteins displayed wide differences in the efficiency with which they could be coated onto tanned SRBC as demonstrated by the differences in HA titers obtained with homologous antisera when comparing cells coated with varying amounts of protein. Moreover, it is possible, though unlikely, that the albumin used in the present experiment may be altered in a manner that could result in non-specific cross-reactivity with antibody to non-albumin proteins.

It was reasoned that if proteins were denatured in a manner to yield non-specific cross-reactivity, or if non-albumin proteins were more efficient coating substances than albumins under the conditions chosen (50 mg protein/ml packed cells) then titers of anti-albumin serum and anti-

FIGURE 3: EXAMINATION OF CROSS-CONTAMINATED SERUM PROTEINS BY IMMUNOELECTROPHORESIS; IDENTIFICATION OF ALBUMIN IN NON-ALBUMIN PREPARATIONS.

1-3. Hyperimmune mouse anti-Fr. V albumin. Upper wells all contain Fr. V at 10 mg/ml. Lower wells contain respectively, at 10 mg/ml, (slide 1), Cohn Fr. IV-4 bovine alpha and beta globulins; (2) Fr. IV-1 alpha globulins; and (3) Fr. III-0 beta lipoproteins.

4-6 Mouse anti-BGG. Upper wells all contain BGG at 10 mg/ml. Lower wells contain respectively, at 10 mg/ml, (slide 4), Fr. IV-1; (5), Fr. IV-4; (6), Fr. III-0.


1
2
3
4
5
6

globulin serum using cells coated with Cohn fractions V, IV-4, IV-1, III-0 and II, as well as ovalbumin, an unrelated protein, should be uniformly high or show no clear pattern. However, if cross reaction were merely due to the cross contamination of the serum protein fractions, then there should be no reaction of the anti-bovine protein antisera with ovalbumin-coated cells. Moreover, if there were no preferential adsorption of particular serum proteins onto the tanned SRBC, and if mice did not show a preferential response to particular contaminating proteins present in the immunizing antigens, then the titers detected in mouse anti-bovine albumin by HA using SRBC coated with bovine serum proteins other than the albumin fraction should bear a quantitative relationship to (1) the amount of albumin in the serum protein fraction used to coat the cells; and (2) the amount of that particular protein present in the albumin fraction used to immunize the mice. The same argument holds in the case of anti-bovine globulin or any other serum protein.

The relative concentration of albumin in the Cohn protein fractions can be ranked in the order II < III-0 < IV-1 < IV-4 < V, while relative concentrations of globulin can be ranked in the reverse order. The same is true for the intervening fractions, so that fraction II should contain as contaminants more of the fraction III-0 proteins than of the fraction V; while fraction V should have more of the fraction IV-4 proteins than of the fraction II

proteins (Pennell in Putnam, 1960). Thus, if spurious protein effects are not effectively interfering with the specificity of the HA assay, then the titers of mouse anti-albumin serum with SRBC coated with the various serum proteins should follow the order II < III-0 < IV-1 < IV-4 < V, while the titers of mouse anti-bovine globulin serum with these cells should follow the inverse order. Examination of table 2 shows that this latter prediction holds. Therefore, the test is selective for effects due to albumin antigens and the presence of non-albumin contaminants, although detectable, did not unduly influence the HA titers in the albumin-anti-albumin system.

Effect of Lipid: It has been reported (Jones and Fleischer, 1936; Agarwal and Neter, 1971) that the addition or removal of lipids can profoundly affect the immunologic behavior of serum proteins. It has also been recognized that albumin preparations may vary considerably in lipid content. In general, however, crystalline albumins (Cohn et al., 1946; Chen, 1967) contain more lipid than Fr. V albumins. The results of the Jones and Fleischer study suggested that there was no direct relationship between the amount of lipid present and the immune response against the albumins tested.

The use of organic solvents for extracting lipids, however, could have serious effects on the proteins, making distinction between the lipid effect and the protein denaturation effect impossible.

TABLE 2: SELECTIVITY OF THE HEMAGGLUTINATION ASSAY: COMPARISON OF COHN-FRACTIONATED BOVINE SERUM PROTEINS IN RELATION TO LEVELS OF CROSS CONTAMINATION.

Antisera	Reciprocal H A titers against cells coated with:						
	E.A.	BGG	III-0	IV-I	IV-4	Fr.V	Xtal
NMS	>10	<10	<10	<10	<10	<10	<10
Anti E.A.	5,000	<10	<10	<10	<10	<10	<10
Anti BGG Hy[a]	<10	≥20,000	≥20,000	2,560	640	<10	<10
Anti Fr.V BSA, 1°	<10	<10	<10	<10	10	320	320
Anti BSA Fr.V, 2°	<10	20	2,560	5,000	5,000	10,000	≥20,000
Anti BSA Fr.V, Hy[a]	<10	80	10,000	10,000	≥20,000	≥20,000	≥20,000
Anti BSA Xtal Hy[a]	<10	20	2,560	5,000	5,000	5,000	10,000
Absorbed rabbit anti-bovine serum	<10	1,280	10,000	5,000	2,560	1,280	1,280

a. Hyperimmune

The studies of Agarwal and Neter did not involve extraction of lipids and were concerned with lipid effects outside the normal physiological range of lipid binding.

Accordingly, a direct test of the lipid effect was thought to be desirable, and the availability of a lipid extraction method that is rapid and efficient and reportedly causes little denaturation of the albumins made such a direct test feasible. The Fr. V and Xtal albumins, which were found to contain 0.24 and 1.0 meq of lipid/meq of albumin respectively, were treated to extract lipids and then reconstituted with known amounts of palmitic acid. Groups of 4 mice were immunized with either Fr. V, Xtal, or lipid-extracted albumins, given Fr. V albumin 6 weeks later, and HA titers determined. The results are shown in tables 3 a,b.

The Fr. V originally had very little bound lipid (0.24 meq/meq). Treatment to remove lipid resulted in a preparation which was no less immunogenic, and replacement of lipid at 1 meq/meq failed to alter the immunogenicity of the albumin, but replacement at 20 meq/meq resulted in a much lower titer than before, in agreement with the findings of Agarwal and Neter.

The Xtal albumin had a larger quantity of lipid (1 meq/meq). The variability of responsiveness seen with this albumin made evaluation difficult. However, treatment to remove the lipid did not appear to have altered the immunogenicity of the albumin, nor did replacement of the lipid at the level of either 1 or 20 meq/meq.

TABLE 3a: COMPARATIVE PRIMING CAPACITIES OF COHN FRACTION V AND CRYSTALLINE ALBUMIN FOLLOWING REMOVAL AND REPLACEMENT OF BOUND FATTY ACIDS. EXPERIMENT I.

Priming Albumin[a]	Fatty Acid Removed	Meq. Palmitate added per meq. albumin	Reciprocal mean 7-day secondary titer[c]
Cohn Fr.V (Pentex Lot 66, 0.24 meq. lipid bound per meq. albumin)	no	0.0	6,200 (4,400-8,800)
	yes	0.0	7,400 (5,000-10,000)
	yes	1.0	5,000 (2,200-14,500)
	yes	20.0	900 (640-1,280)
Ceystalline (Armour Lot G71812, 1.0 meq. lipid bound per meq. albumin)	no	0.0	1,280 (760-2,200)
	yes	0.0	225 (135-380)
	yes	1.0	270 (135-540)
	yes	20.0	760 (225-1,100)
Unprimed control[b]	no	0.0	380 (225-640)

a. Mice received 3.0 mg. i.v. albumin treated as indicated and reinjected 6 weeks later with 3.0 mg. i.p. untreated Fraction V albumin.
b. Unprimed controls received no initial dose of albumin but received 3.0 mg i.p. Fraction V albumin at the same time as the primed mice. The titer expressed is a primary titer.
c. Mean ± one standard deviation.

TABLE 3b: COMPARATIVE PRIMING CAPACITIES OF COHN FRACTION V AND CRYSTALLINE ALBUMIN FOLLOWING REMOVAL AND REPLACEMENT OF BOUND FATTY ACIDS. EXPERIMENT II.

Priming Albumin[a]	Fatty Acid Removed	Meq. Palmitate added per meq. albumin	Reciprocal mean 7-day secondary titer[c]
Cohn Fr.V (Pentex Lot 66, 0.24 meq. lipid per meq. albumin)	no	0.0	1,800 (760-4,400)
	yes	0.0	2,200 (900-5,000)
	yes	1.0	2,200 (900-5,000)
	yes	20.0	380 (270-540)
Crystalline (Armour Lot G71812, 1.0 meq. lipid bound per meq. albumin)	no	0.0	640
	yes	0.0	640
	yes	1.0	320
	yes	20.0	320
Unprimed control[b]	no	0.0	1,280

a. See Table 3a.
b. See Table 3a.
c. Mean ± one standard deviation. Standard deviations are omitted where the variation between replicate sera was immeasurable.

Albumin is capable of binding as much as 5 meq of lipid per meq of albumin without incurring irreversible structural effects (Goodman, 1958). Above this level, however, the albumin undergoes changes in electrophoretic mobility, optical properties and enthalpy which do not revert to normal values after removal of the lipids.

The immunological behavior of the Fr. V and Xtal albumins was not correlated with the presence or absence of lipid per se. In the case of Fr. V, however, the manipulations involved in removal or addition of lipids appeared to influence immunological behavior. This was not so with Xtal albumin.

The Xtal albumin, during the manufacturing process, has been stabilized by the addition of a small amount of n-decanol (Cohn et al., 1947). It may be that the presence of the bound decanol prevents the changes in structure necessary for altered immunogenicity. Attempts to prepare decanol conjugates of the Fr. V albumin in order to study this point were not successful, owing to the generation of large quantities of aggregates and a high level of toxicity to the mice.

Preparation of Isolated Monomers and Polymers: Exclusion chromatography on Sephadex G-150 was used to separate monomers, dimers and higher order aggregates (figures 4 and 5). After one passage, each fraction was contaminated with traces of the others. Hence, the monomeric fractions were concentrated and passed through the

FIGURE 4: SEPARATION OF MONOMERS, DIMERS AND POLYMERS FROM FR. V ALBUMIN BY EXCLUSION CHROMATOGRAPHY ON G-150 SEPHADEX.

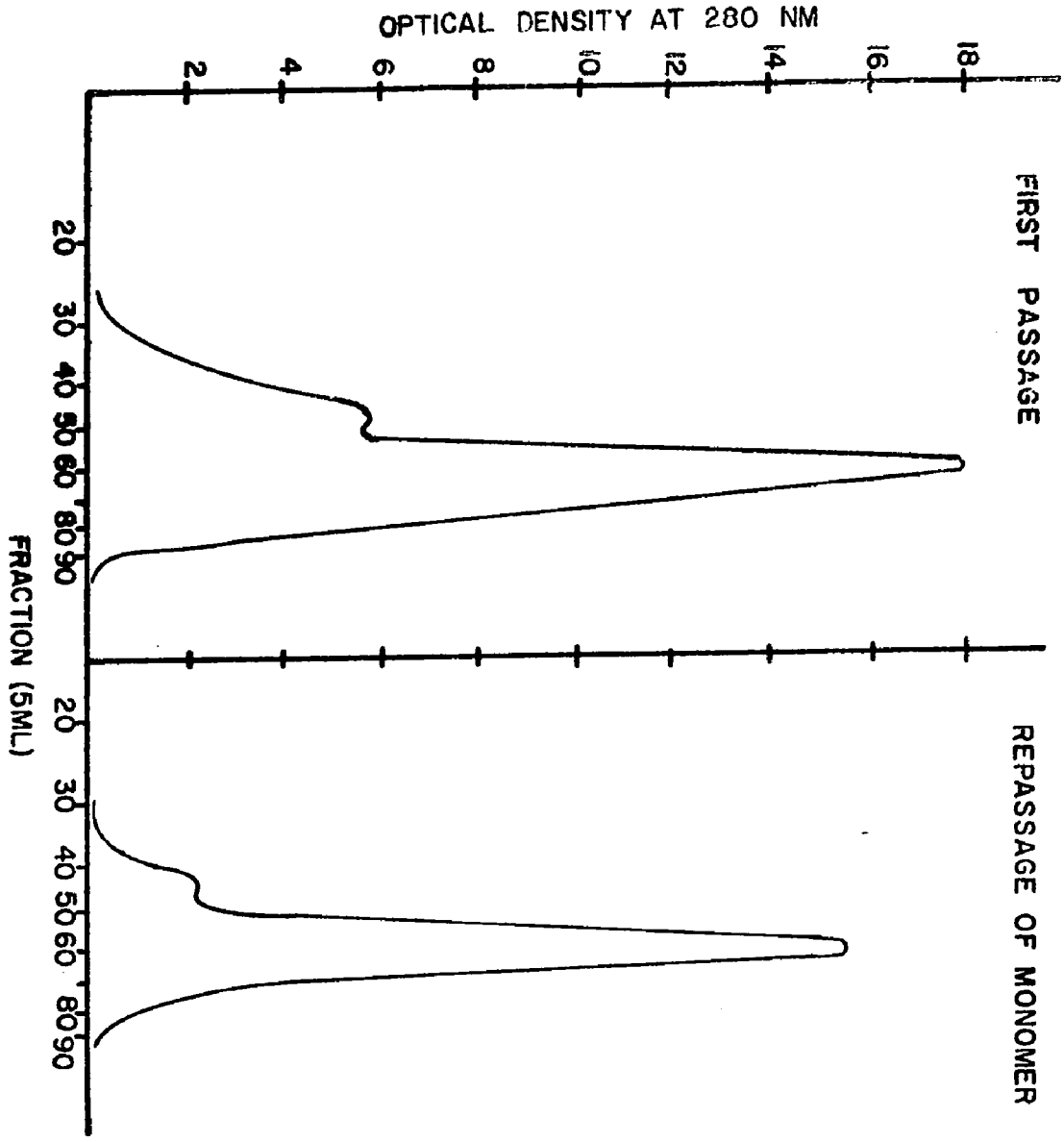

OPTICAL DENSITY AT 280 NM
2
4
6
8
10
12
14
16
18
FIRST PASSAGE
REPASSAGE OF MONOMER
FRACTION (5ML.)
20
30
40
50
60
80
90

FIGURE 5: SEPARATION OF MONOMERS, DIMERS AND POLYMERS FROM XTAL ALBUMIN BY EXCLUSION CHROMATOGRAPHY ON G-150 SEPHADEX.

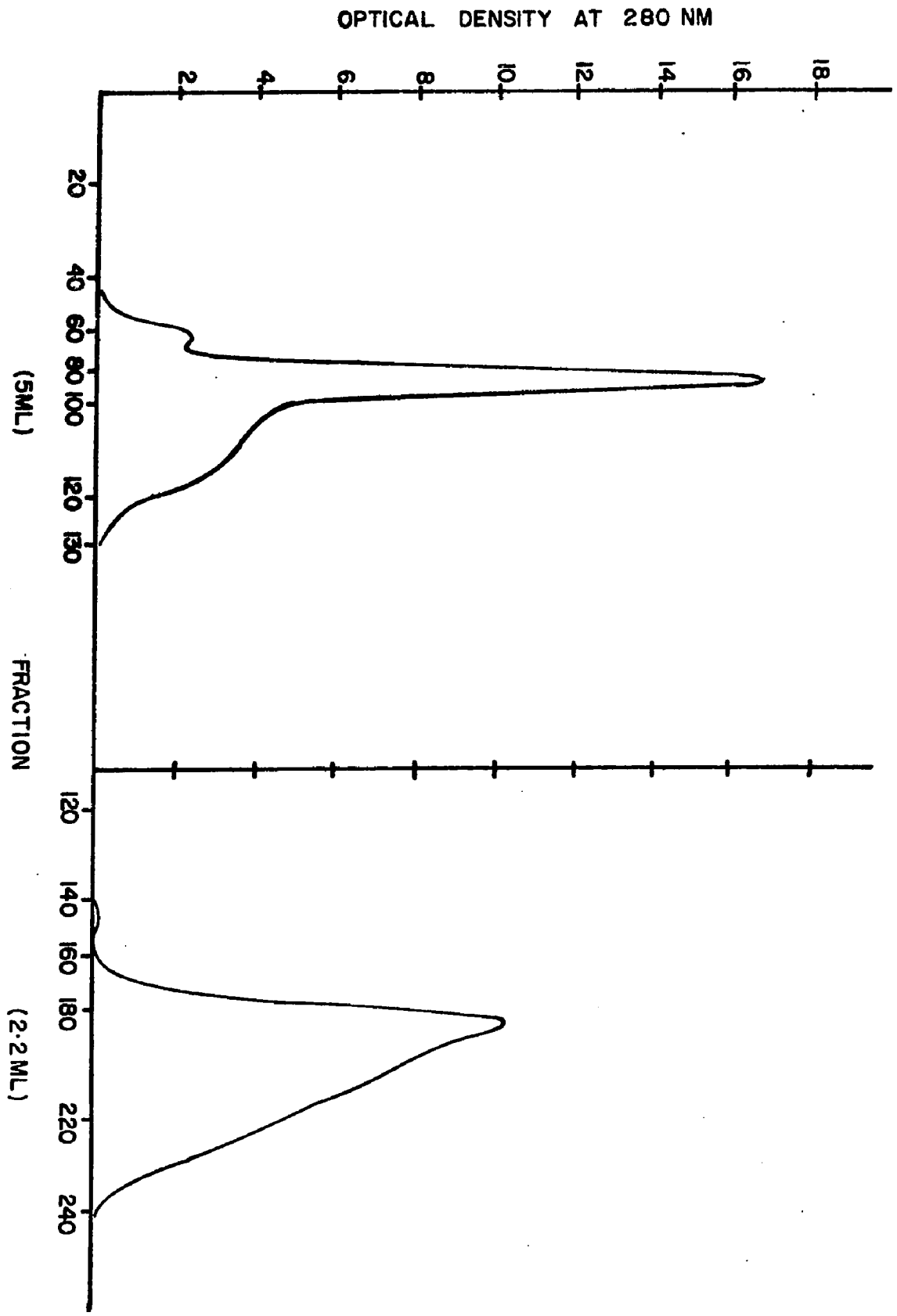
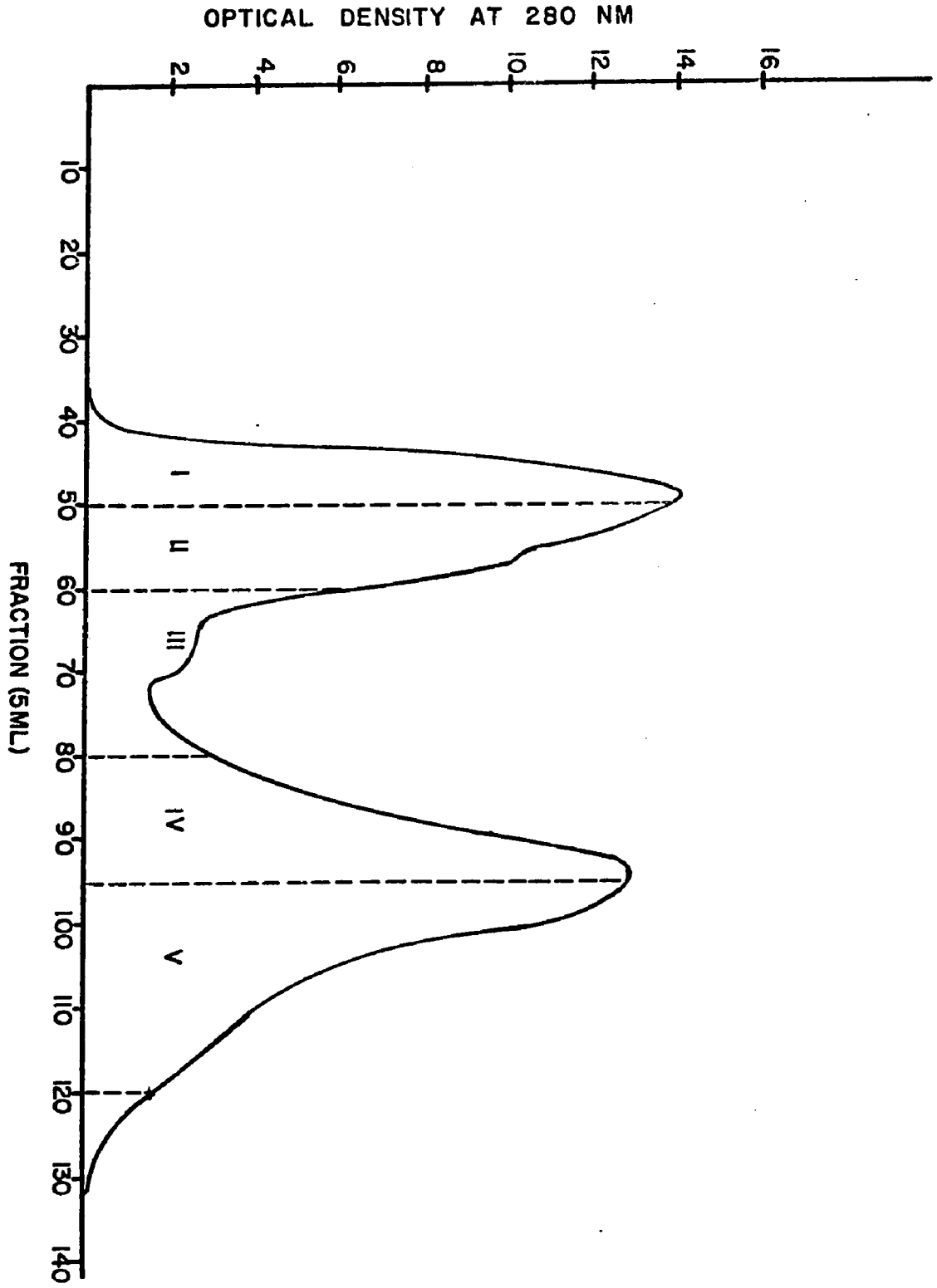

OPTICAL DENSITY AT 280 NM
2
4
6
8
10
12
14
16
18
20
40
60
80
100
120
130
(5ML)
FRACTION
120
140
160
180
220
240
(2·2 ML)

FIGURE 6: SEPARATION OF FRACTIONS FROM HEATED XTAL ALBUMIN BY EXCLUSION CHROMATOGRAPHY ON G-150 SEPHADEX.


OPTICAL DENSITY AT 280 NM
2
4
6
8
10
12
14
16
FRACTION (5ML)
10
20
30
40
50
60
70
80
90
100
110
120
130
140
I
II
III
IV
V

Sephadex a second time. The low yields of dimeric and polymeric albumins, together with their high level of cross-contamination (shown in figure 7), made further purification impractical.

Since the yield of higher order aggregates was extremely low, the aggregate content of the albumin was enriched by heating under conditions of controlled temperature and pH. The intent of the heating process was to form soluble aggregates, and heating at pH 6.8 and 65C for 15 minutes was found to give satisfactory results. Under these conditions, the Fr. V albumin was converted quantitatively to soluble aggregates, while the Xtal was converted to a mixture of forms, including a large quantity of heat-stable monomer (H S monomer). The heated Xtal was separated into 5 fractions on G-150 Sephadex (figure 6). Fractions 37-50 were combined as pool I, 51-60 as pool II, 61-80 as pool III, 81-95 as pool IV, and 96-120 as pool V.

Polyacrylamide disc-gel patterns of the final material are shown in figure 7. The monomeric albumin still contained a trace of dimer, detectable at the limit of resolution for the method and therefore representing an estimated concentration of between 0.1 to 0.3 ug/mg of albumin.

Also shown in figure 7 are the polyacrylamide patterns for the heated Fr. V and Xtal pools. The Fr. V contained only a slight trace of monomer. The heated Xtal pools all contained monomer, which was present in trace quantities in pools I and II, was approximately equal in concentration

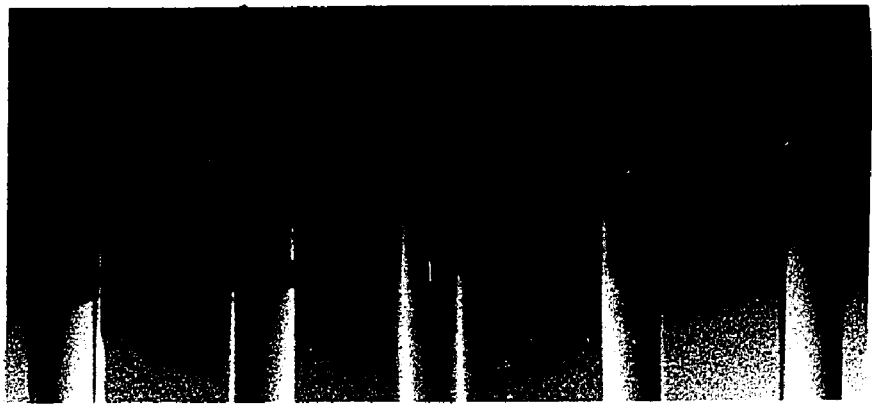
FIGURE 7: EVALUATION OF ALBUMINS BY POLYACRYLAMIDE DISC-GEL ELECTROPHORESIS; RELATIVE CONTENTS OF MONOMER, DIMER AND HIGHER AGGREGATES (POLYMER) IN POOLED COLUMN ELUATES.

a. Fr. V pools; (1) whole albumin, (2) monomer pool, (3) polymer pool, (4) heat-polymerized albumin.

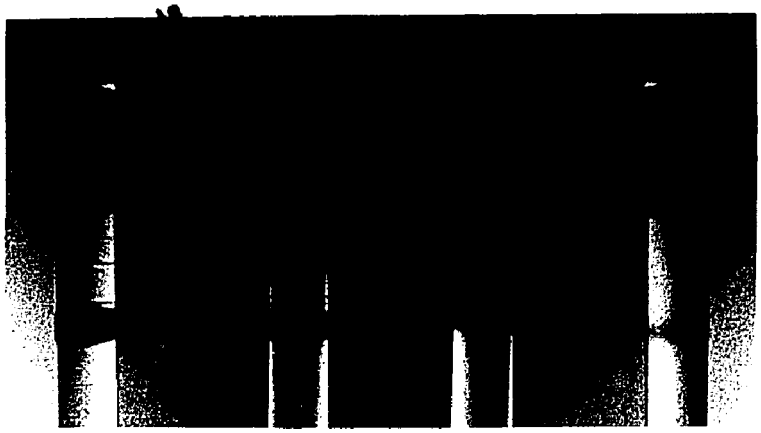
b. Xtal pools, (1) whole albumin, (2) monomer pool, (3) dimer pool, (4) polymer pool.

c. Heated Xtal albumin; (1) whole albumin, (2) pool I polymer, (3) pool II polymer, (4) pool III mixed fractions, (5) pool IV monomer-dimer, (6) pool V HS monomer.

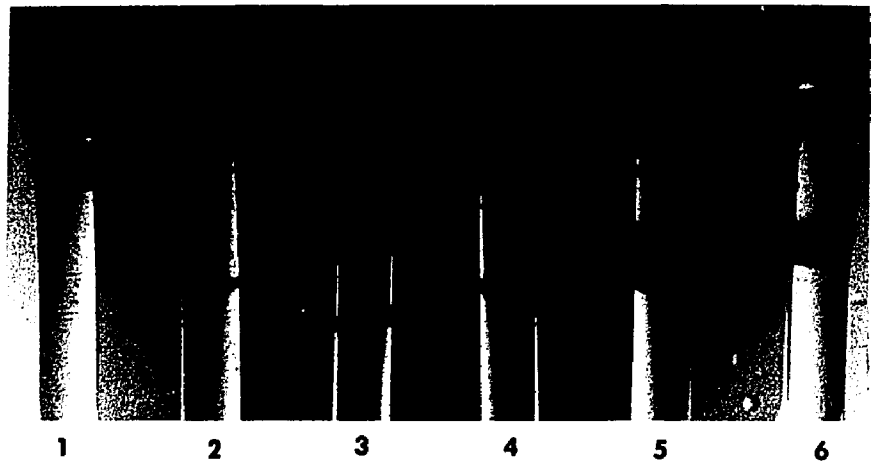
a



b



c

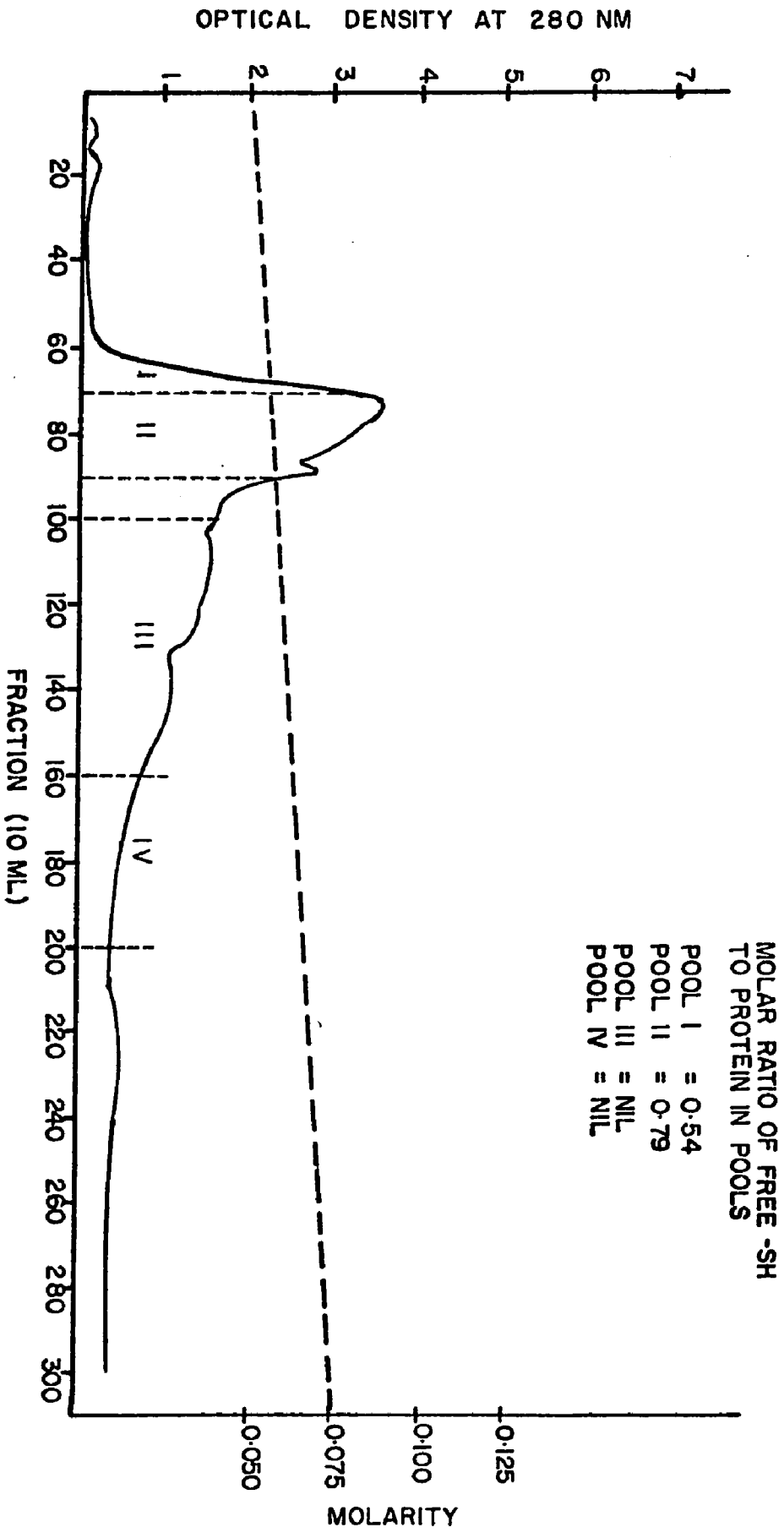


to the other forms in pool III, and constituted the major fraction in pools IV and V. Pool V contained monomer and only a trace of dimer, which, again was detectable at the limit of resolution for the method, and therefore represents a concentration of approximately 0.1 to 0.3 ug/mg of monomer.

Preparation of Mercaptoalbumin and Non-mercaptoalbumin Monomers by Ion Exchange Chromatography on DEAE-Sephadex: Numerous methods, reviewed earlier, have been devised to separate and identify chemically different forms of monomeric albumin. The method described by Janatova, Fuller and Hunter (1968) was employed to segregate various forms of albumin monomers in both Fr. V and Xtal albumin. Sulfhydryl determinations and polyacrylamide gel electrophoresis done on every 10th fraction of column effluent were used to segregate the effluent into dimer-free pools of monomeric albumin which were sulfhydryl-rich or sulfhydryl-poor. Further distinction could be made among the sulfhydryl-poor albumins according to whether these albumins eluted in the early or late fractions.

Figure 8 depicts the elution pattern of the Fr. V albumin. It can be seen that the composition of this albumin differed from that of the Xtal albumin (figure 9). The early peak, containing the mercaptoalbumin, was lower, and the later peaks were higher. Additionally, there were two small peaks appearing just before fraction 20, which seems to be non-albumin protein contaminants. Sulfhydryl

FIGURE 8: SEPARATION OF MERCAPTOALBUMIN AND NON-MERCAPTOALBUMIN MONOMERS FROM FR. V ALBUMIN BY ION EXCHANGE CHROMATOGRAPHY ON DEAE-SEPHADEX.


OPTICAL DENSITY AT 280 NM
1
2
3
4
5
6
7
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
I
II
III
IV
FRACTION (10 ML)
0·050
0·075
0·100
0·125
MOLARITY
MOLAR RATIO OF FREE -SH TO PROTEIN IN POOLS
POOL I = 0·54
POOL II = 0·79
POOL III = NIL
POOL IV = NIL

determinations indicated that the percentage of mercaptoalbumin even in the early fractions was low, ranging from 47 to 88% between fractions 50 and 80.

Since the mercaptoalbumin content was low, it was possible to separate four varities of albumin. These included an early non-mercaptoalbumin (pool I, 54% mercaptoalbumin); mercaptoalbumin (pool II, 79%) and two pools of late non-mercaptoalbumin (pools III and IV, mercaptoalbumin undetectable).

Figure 9 depicts the elution patterns of the Xtal albumin. All of the fractions between 40 and 70 contained more than 60% mercaptoalbumin. The sulfhydryl values dropped to levels below the sensitivity of the Ellman method by fraction 130.

Three pools were made of this albumin; pool I, mercaptoalbumin; pool II, mixed mercaptoalbumins and non-mercaptoalbumins; pool III, late non-mercaptoalbumins. The mercaptoalbumin pool contained a small amount of an early non-mercaptoalbumin which was not present in sufficient quantity for practical isolation.

Pools I and III from the Xtal albumin and pools I and IV from the Fr. V albumin were the principal materials studied. Figure 10 shows polyacrylamide disc gels indicating no detectable contamination of the monomers with dimer or higher order aggregates.

The mobilities of the Fr. V pools appeared to increase in the direction of elution from the chromatography column.

FIGURE 9: SEPARATION OF MERCAPTOALBUMIN AND NON-MERCAPTO-ALBUMIN MONOMERS FROM XTAL ALBUMIN BY ION EXCHANGE CHROMATOGRAPHY ON DEAE-SEPHADEX.

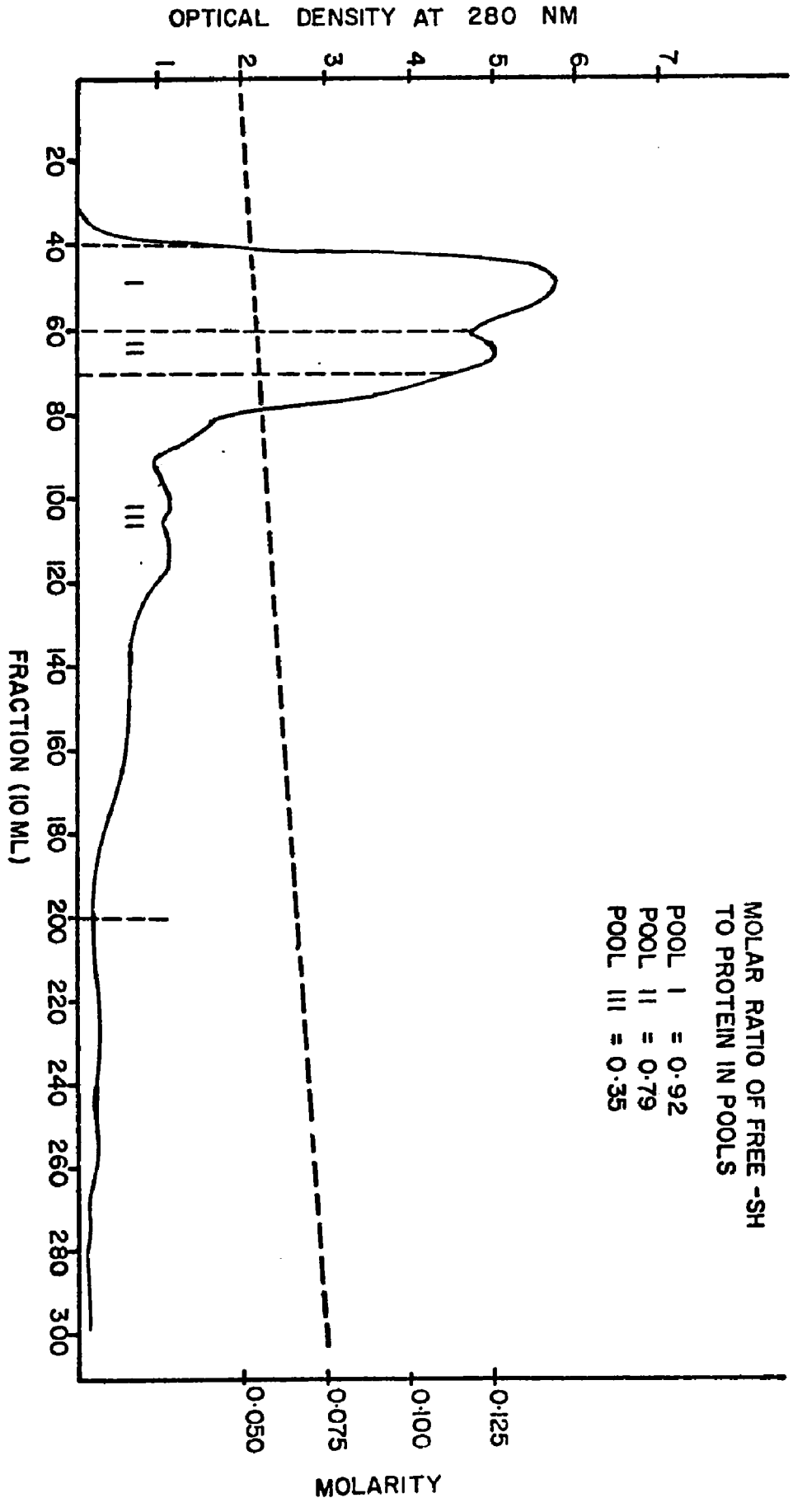
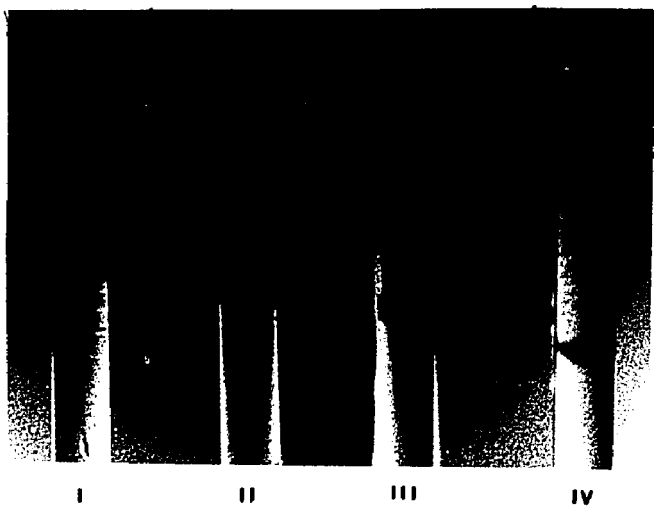

OPTICAL DENSITY AT 280 NM
1
2
3
4
5
6
7
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
FRACTION (10 ML)
I
II
III
0·050
0·075
0·100
0·125
MOLARITY
MOLAR RATIO OF FREE -SH TO PROTEIN IN POOLS
POOL I = 0·92
POOL II = 0·79
POOL III = 0·35

FIGURE 10: POLYACRYLAMIDE DISC GEL ELECTROPHORESIS PATTERNS OF MONOMERIC ALBUMINS ISOLATED BY ION EXCHANGE CHROMATOGRAPHY ON DEAE-SEPHADEX.

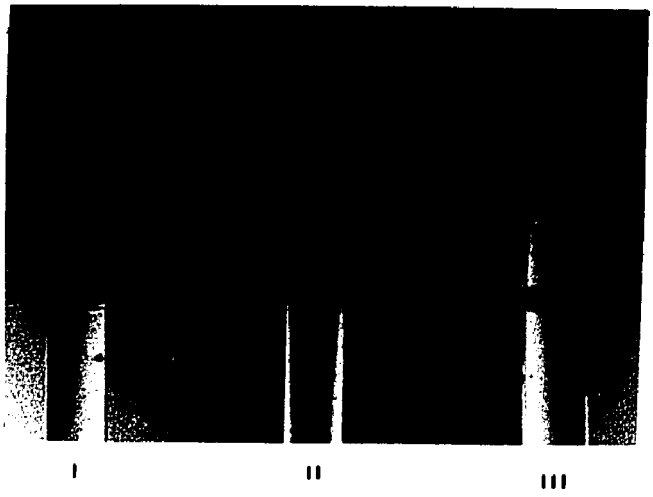
a. Fr. V pools

b. Xtal pools

a



b



Those of the Xtal preparations did not. This apparent difference in mobility may not be real, however, since the lengths of stacking gel and even the current across each gel may not have been precisely the same.

Antigenic Study of Albumins: Antisera were prepared in mice against Fr. V DEAE pools I and IV; Xtal DEAE pools I and III; and unfractionated Xtal albumin, using Freund's complete adjuvant to insure high-titered antisera. Antisera against Fr. V and Xtal polymers were also prepared in mice without the use of adjuvants.

Figure 11 displays the results of immunoelectrophoresis conducted at pH 8.6 according to the method described by Francis, Ling and Krasteff (1965). Monomers isolated by exclusion chromatography, and DEAE fractionated monomer pools were compared with whole bovine serum using rabbit anti-bovine serum antibody (figure 11 a,b). The bovine serum was used undiluted, and the albumin fractions were used in their available concentrations, which ranged from 6 to 11.5 mg/ml. All showed only single bands against the rabbit anti-bovine serum antibodies. However, the Fr. V DEAE pool I showed a peculiar mobility.

A second approach was taken in which Fr. V albumin at 100 mg/ml concentration was compared with undiluted bovine serum using antisera prepared against Fr. V DEAE pools I and IV and Xtal DEAE pools I and III. Multiple bands are seen with the Fr. V albumin when antisera against Fr. V DEAE pools I and IV (figure 11c) were used but when antisera against Xtal DEAE pools I and III were used only single

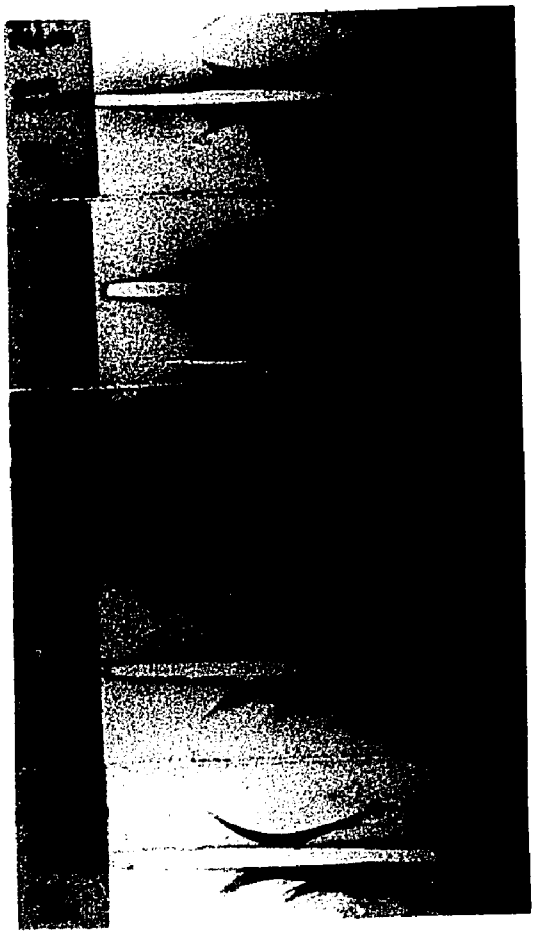
FIGURE 11: IMMUNOELECTROPHORETIC PATTERNS OF MONOMERIC ALBUMINS OBTAINED BY ION EXCHANGE CHROMATOGRAPHY.

a. Rabbit anti-bovine serum; top wells contain (1) Fr. V monomer, (2) Fr. V pool I, (3) Fr. V pool II, (4) Fr. V pool III, (5) Fr. V pool IV. Bottom wells contain bovine serum.

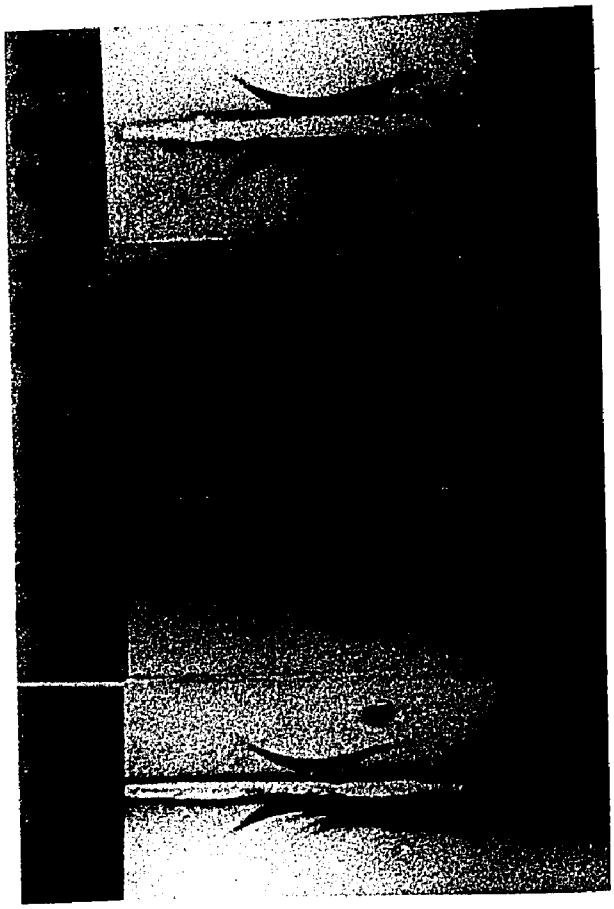
b. Rabbit anti-bovine serum; top wells contain (1) Xtal monomer, (2) Xtal pool I, (3) Xtal pool II, (4) Xtal pool III. Bottom wells contain bovine serum.

c_1 Hyperimmune mouse anti-Fr. V pool I; top well contains whole Fr. V albumin, bottom well contains bovine serum.

c_2 Hyperimmune mouse anti-Fr. V pool IV; top well contains whole Fr. V albumin, botton well contains bovine serum.

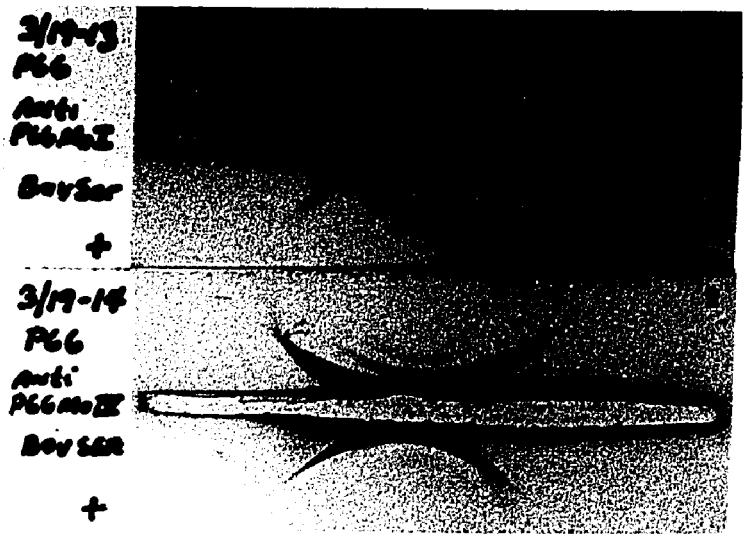


a



b

c


3/19-13
PLG
Anti
PLG Mo IgE
Boy Ser
+
3/19-14
PLG
Anti
PLG Mo IgE
Boy Ser
+

bands were observed. The positions of the bands are consistent with the behavior of a slowly diffusing form of albumin; hence, they most likely represent dimeric and higher order albumin aggregates. Ouchterlony double diffusion studies (not shown) were also done in an effort to determine if any antigenic differences could be found among the different bovine albumin preparations. Polymers, dimers, monomers and DEAE fractionated monomer pools were compared in all possible combinations against Fr. V antiserum, after determination of the dilution of each antigen that produced a band approximately midway between the antigen anti-serum wells (approximately the equivalence point). All of the Fr. V and Xtal albumin preparations excepting only the Xtal dimer, which was not available, were compared by double diffusion in all possible combinations. In each case only lines of identity were observed indicating that each preparation contained all of the antigenic determinants detectable by the anti-Fr. V antiserum.

Cross Agglutination Study by Passive HA: Since HA was used for most of the work reported, it was of interest to determine if the Fr. V test antigen used could efficiently detect antigens present in each of the forms of albumin studied and if HA could detect antigenic differences among the albumins.

The maximum serum diltuion employed in the HA test in this study was 1:20,000. Several of the titers shown were at least that high, but no attempt was made to deter-

mine endpoints beyond the 1:20,000 dilution.

Tables 4 a,b show the results of this study. Analysis of the data indicated that Fr. V test antigen detected antibody formed against each of the albumins as well as or better than did the homologous antigen. Fr. V monomer, DEAE pool IV from the Fr. V albumin (mono-4, table 4a), and all of the isolated Xtal fractions detected antibodies in anti-Fr. V mono-4 antiserum much less well than unfractionated Fr. V, Fr. V polymer, or unfractionated Xtal albumin, indicating that these forms express antigenic determinants differently from the others. Fr. V mono-4, although it does not appear to express the full complement of albumin antigenic determinants, must contain them, since anti-Fr. V mono-4 antiserum reacted to high titer against both Fr. V and Xtal albumins. The Xtal albumins (table 4b) appeared to react less well than the Fr. V albumins against hyperimmune anti-Xtal albumin. This could indicate either a lower efficiency of cell coating or a slightly different expression of antigenic determinants among the Xtal albumin fractions, which permits the detection in the antisera of slightly different antibody specificities when adjuvant is not used. Similarly, the Fr. V monomer, Fr. V mono-4 and all of the Xtal albumin fractions detected lower antibody titers in anti-Xtal mono-3 antisera than either the whole Fr. V or Xtal albumins.

Comparing the titers in each of the anti-monomeric albumin antisera against cells coated with each of the

TABLE 4a: CROSS-AGGLUTINATION STUDY OF ALBUMIN ANTIGENS AND ANTISERA.

Against Antisera:	Reciprocal H A titers against cells coated with Fr. V albumins						
	Fr.V	Polymer	Monomer	Mono-1	Mono-4	Early DEAE Fraction	BGG
Fr.V	10,000	10,000	5,000	5,000	≥20,000	320	<10
Fr.V Mono-1	10,000	≥20,000	≥20,000	≥20,000	≥20,000	<10	<10
Fr.V Mono-4	10,000	2,560	640	2,560	640	<10	<10
Fr.V Polymer	≥20,000	≥20,000	≥20,000	≥20,000	≥20,000	2,560	<10
Xtal 2^{o}	640	320	160	320	640	<10	<10
Xtal Hy^{a}	10,000	≥20,000	≥20,000	≥20,000	≥20,000	160	<10
Xtal Mono-1	10,000	10,000	1,280	2,560	2,560	160	<10
Xtal Mono-3	≥20,000	≥20,000	2,560	≥20,000	2,560	320	<10
Xtal Polymer	≥20,000	≥20,000	≥20,000	≥20,000	≥20,000	5,000	<10
NMS	<10	<10	<10	<10	>10	<10	<10

a. Hyperimmune

TABLE 4b: CROSS-AGGLUTINATION STUDY OF ALBUMIN ANTIGENS AND ANTISERA.

Against Antisera:	Reciprocal H A titers against Xtal albumins						
	Xtal	Polymer	Monomer	Mono-1	Mono-3	HS Mono	BGG
Fr.V	≥20,000	2,560	2,560	1,280	1,280	2,560	<10
Fr.V Mono-1	≥20,000	2,560	2,560	2,560	1,280	2,560	<10
Fr.V Mono-4	5,000	640	640	640	640	640	<10
Fr.V Polymer	≥20,000	5,000	5,000	10,000	5,000	5,000	<10
Xtal 2[o]	640	320	80	40[a]	40[a]	80	<10
Xtal Hy[b]	≥20,000	10,000	10,000	10,000	10,000	≥20,000	<10
Xtal Mono-1	10,000	2,500	2,560	2,560	1,280	2,560	<10
Xtal Mono-3	≥20,000	5,000	5,000	5,000	5,000	5,000	<10
NMS	<10	<10	<10	<10	<10	<10	<10

a. Atypical agglutination patterns.
b. Hyperimmune serum

different monomeric albumins, it is seen that all showed comparable titers except when Fr. V mono-2-coated cells were used. Here, titers were higher. This may again indicate that all of the albumins contain similar antigens, each capable of stimulating an immune response but not fully expressed in the unmetabolized protein, so that fewer antibodies are detected, or else the equivalence point between antigen and antibody is altered. This could result in a lower titer when the homologous antigen was used to sensitize cells than when some other albumin was used. Such behavior was seen with aggregated and non-aggregated albumin antigens in the experiment of Draper and Hirata (1968).

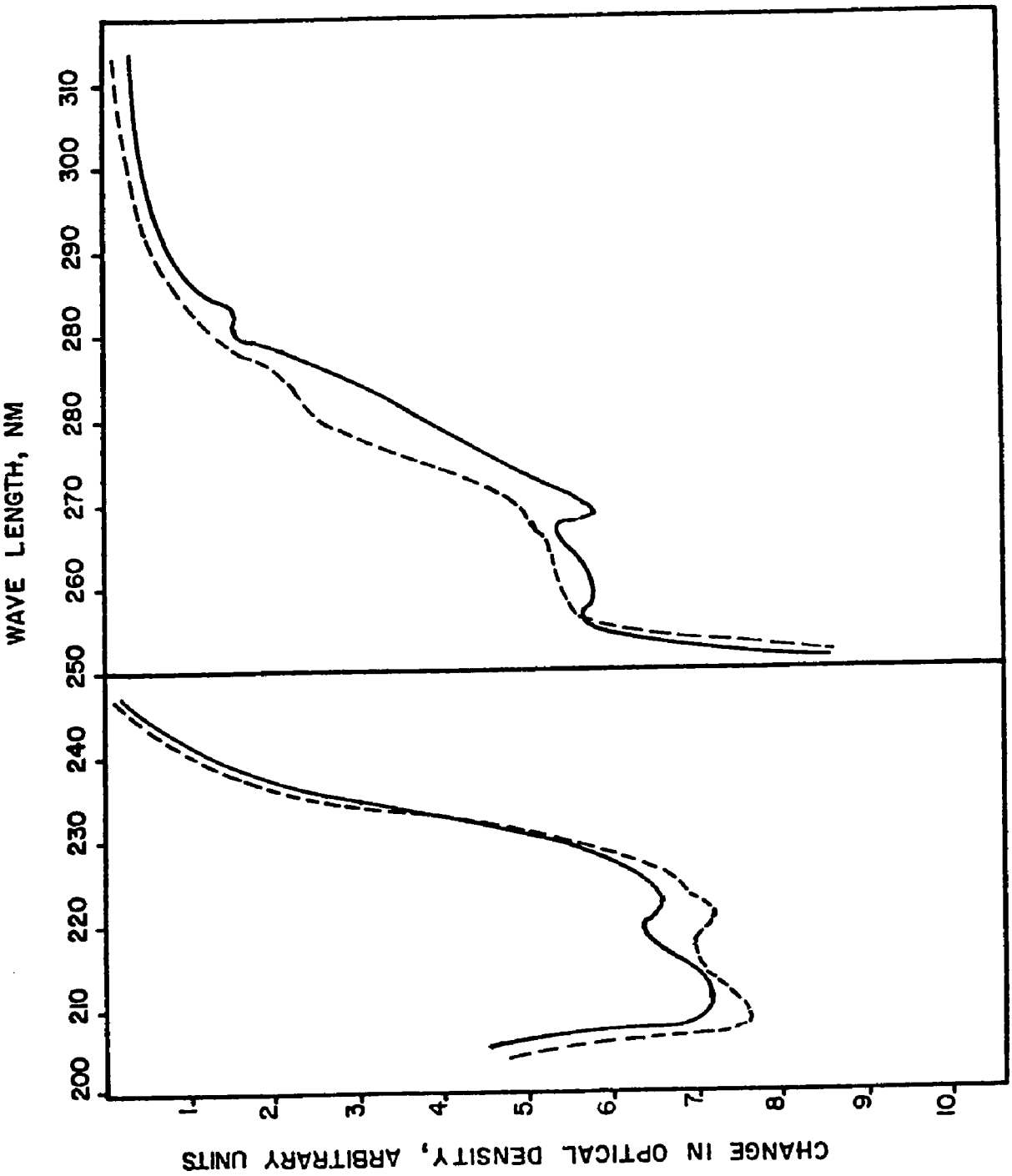
Examination of the results obtained with the early DEAE fraction from Fr. V albumin (small peaks seen in the region of fraction 20) indicates that this material shares antigens with albumin which are detectable at low titer and also shares antigens with BGG detectable at a higher titer. This material most probably contained the non-albumin contaminant observed in Fr. V albumin by immunoelectrophoresis.

Circular Dichroism Studies of "Early" and "Late" DEAE-Sephadex Isolates from Fr. V and Xtal Albumins: Since any differences in immunological behavior among the albumins might be the result of gross structural changes introduced in preparative manipulation of the albumin during either the manufacturing process or the preparation of antigens for experimental use, it was of interest to determine if the

different monomeric forms of albumin isolated for study were alike or different structurally. Measurement of the circular dichroic spectrum has been used with bovine albumins as a sensitive indicator of structural change (Chen, 1967; Noel and Hunter, 1972). DEAE Sephadex pool I from Fr. V and Xtal albumin (termed "Early" monomer) and DEAE Sephadex pool III from Xtal albumin and pool IV from Fr. V albumin (termed "Late" monomer) were studied, since these received the greatest amount of manipulation during the preparative process and since they afforded an opportunity to compare different forms of monomeric albumin from the same and different sources.

Figure 12 represents the results of duplicate determinations on each albumin sample. Within the limits of instrument error, the "Early" and "Late" forms of the albumins displayed identical spectra, hence are shown as a single curve for each albumin. The identity of the "Early" and "Late" monomer spectra indicates that there were no detectable internal differences in structure among the monomeric forms present in each of the albumin preparations. Comparison of the spectra of the Fr. V monomers with those of the Xtal monomers reveals slight differences in the 210-230 nm region and in the 260-280 nm region. These differences are very slight and most probably within the limits of experimental error. The results seen here are consistent with those of Noel and Hunter who found no gross differences among mercaptoalbumin and various forms

FIGURE 12: CIRCULAR DICHROISM SPECTRA OF "EARLY" AND "LATE" MONOMERIC ALBUMINS OBTAINED BY ION EXCHANGE CHROMATOGRAPHY ON DEAE-SEPHADEX. FR. V —— ; XTAL – – – –.


WAVE LENGTH, NM
200
210
220
230
240
250
260
270
280
290
300
310
CHANGE IN OPTICAL DENSITY, ARBITRARY UNITS
1
2
3
4
5
6
7
8
9
10

of non-mercaptoalbumin under conditions of constant pH and ionic strength. They did, however, find changes consistent with perturbation of a tryptophanyl and a cystinyl residue among the different forms in their preparations by examining changes in dichroic absorption with changes in pH and fatty acid content; and by employing fluorescence quenching studies. The differences in structure were associated with changes in the oxidation state of the free -SH group of a cystinyl residue in the albumin. It is not known if such differences exist among monomers from different sources or if such differences are important immunologically.

Dose-Response Studies for the Detection of Immunological Priming and Immunological Tolerance

Studies on Whole Albumin and Fractions obtained by heat treatment and/or Exclusion Chromatography: Studies of the effect of dosage on the immune response to albumin were done with unfractionated albumin, with monomer isolated by chromatography, with polymer and heat-stable monomer isolated by chromatography after heat treatment, and with polymer obtained after heat treatment alone.

Two purposes were to be served by these experiments. The first was to determine if there was any difference among the preparations in the smallest dose capable of priming for a secondary response. This point could be evaluated by studying 2-MER responses. The second purpose was to learn if any of the preparations induced a dose-dependent depression of the immune responses compared to unprimed controls, hence could be considered toleragenic.

Both of these points were accessible through the use of an assay system similar to that described by Mitchison (1964, 1968). In the original method, using CBA mice, the reference system consisted of determing antibody titers by the Farr method on sera taken 4 weeks after immunization with BSA in Freund's complete adjuvant. However, assay of serum taken 10 days after injection of a single dose of fluid BSA was also used, although titers were much lower than when adjuvant was used.

The most efficient induction of immunologic tolerance occurred when small doses of BSA were given 3 times per week over a 10 week period and the animals rested 10 days before challenge. Such treatment resulted in a tolerant state which lasted 10 weeks before recovery occurred, and the titers after challenge of mice given toleragenic doses were seldom more than 10% of the control titers. Single doses of BSA at the toleragenic level also induced a tolerant state, but the depression of titers was not as great, and the responses of treated mice returned to normal after 4 weeks.

Since the titers of both experimental and control mice varied inexplicably among different groups, it was found necessary to include untreated control mice which received only the immunizing regimen in each group and report the responses as ratios of the responses of the treated groups to the control group response.

In the present experiments, balb mice were used, and the reference or challenge dose of antigen was a single dose of fluid Fr. V BSA (Porter, 1969, 1970) without adjuvant, given i.p. This dose regimen usually gave high reciprocal primary HA titers in the range of 80 to 640 after a single dose and secondary reciprocal titers after two doses, spaced 6-8 weeks apart, in the range of 2560 to 10,000. Ocassionally, values outside these ranges were obtained.

Priming and tolerance induction were evaluated after

single graded doses of fluid BSA i.v. via the tail vein 6 weeks prior to challenge. HA responses were determined on sera taken at intervals after the initial dose and on sera taken one day before and 7 days after the challenge or reference dose of antigen. 2-MER titers were determined by HA on 7 day post-challenge sera. To compensate for possible variation between similar experiments, control mice given no initial dose and mice given an initial dose of 3.00 mg Fr. V or Xtal albumin i.v. were included with each set. Tables 5 through 7 depict the results obtained with Fr. V preparations, and tables 8 through 11 depict the results obtained with Xtal preparations.

The Fr. V albumin (tables 5 a,b) gave priming for a secondary response at doses above 0.250 mg, as judged from the appearance of 2-MER antibodies, and was also able to induce a depression of the response compared to the references at doses of 0.100 mg or less. The two experiments shown in tables 5a and 5b do not show complete agreement as to the degree of depression obtained but both show the same qualitative results, in that a depression does occur at primary doses below 0.100 mg. The difference appears to be one of scale between the two experiments.

The polymeric albumin (tables 6 a,b) appeared to behave similarly to the whole Fr. V albumin. The magnitude of responses at doses above 0.100 mg was similar, although priming for a secondary response occurred at dosages of

TABLE 5a: EFFECT OF THE DOSAGE OF AN INITIAL INJECTION OF FRACTION V ALBUMIN ON THE RESPONSE TO REINJECTION OF FRACTION V ALBUMIN (3 mg.) IN BALB MICE. EXPERIMENT I

Initial dose mg. albumin	Reciprocal 7-day H A titers: Mean (± S. D.)		
	Primary	Secondary	Secondary 2-MER
3.00	320 (190-540)	6,200 (3,500-10,000)	3,100 (1,700-5,200)
1.00	320	2,100 (1,500-2,800)	320 (180-590)
0.500	100 (60-160)	760 (540-1,000)	80 (45-300)
0.250	60 (20-190)	800 (580-1,100)	50 (20-160)
0.100	10 (<10-25)	320	<20
0.050	<10	160 (100-260)	<20
0.025	<10	17 (<10-40)	<20
0.010	<10	135 (75-240)	<20
0.001	<10	640 (400-1,000)	<20
0.0005	<10	320	<20
0.000	<10	760 (420-1,280)	<20
Xtal 3.00	80	1,080 (620-1,900)	270 (150-480)

TABLE 5b: EFFECT OF THE DOSAGE OF AN INITIAL INJECTION OF FRACTION V ALBUMIN ON THE RESPONSE TO REINJECTION OF FRACTION V ALBUMIN (3 mg.) IN BALB MICE. EXPERIMENT II

Initial dose mg. albumin	Reciprocal 7-day H A titers: Mean (± S. D.)		
	Primary	Secondary	Secondary 2-MER
3.00	135 (95-225)	3,300 (2,200-5,100)	640 (450-900)
1.00	50 (35-70)	380 (270-640)	320 (220-450)
0.500	25 (18-35)	225 (160-320)	110 (60-220)
0.250	<10	110 (80-160)	40 (<20-95)
0.100	<10	35 (20-160)	<20
0.050	<10	<10	<20
0.025	<10	<10	<20
0.010	<10	<10	<20
0.001	<10	135 (95-225)	<20
0.0005	<10	160	<20
0.000	<10	135 (95-225)	<20
Xtal 3.00	<10	540 (380-760)	<20

TABLE 6a: EFFECT OF THE DOSAGE OF AN INITIAL INJECTION OF HEAT-FORMED POLYMER PREPARED FROM FR. V ALBUMIN ON THE RESPONSE TO A SUBSEQUENT INJECTION OF FR. V ALBUMIN (3 mg.) IN BALB MICE. EXPERIMENT I.

Initial dose, mg. albumin	Reciprocal 7-day H A titers: Mean (± S. D.)		
	Primary	Secondary	Secondary 2-MER
3.00	190 (140-500)	900 (640-1,280)	190 (140-260)
1.00	160	640	40 (35-65)
0.500	70 (40-110)	320	30 (15-50)
0.250	80	380 (170-840)	<20
0.100	30 (<10-110)	380 (210-640)	<20
0.050	<10	225 (120-410)	<20
0.025	<10	15	<20
0.010	<10	380 (205-700)	<20
0.001	<10	640	<20
0.0005	<10	540 (290-1,000)	<20
0.000	<10	380 (280-590)	<20
Fr.V 3.00	235 (160-320)	1,800 (1,280-2,560)	900 (640-1,280)
Xtal 3.00	20 (<10-40)	225 (160-320)	50 (15-150)

TABLE 6b: EFFECT OF THE DOSAGE OF AN INITIAL INJECTION OF HEAT-FORMED POLYMER PREPARED FROM FR. V ALBUMIN ON THE RESPONSE TO A SUBSEQUENT INJECTION OF FR. V ALBUMIN (3 mg.) IN BALB MICE. EXPERIMENT II.

Initial dose, mg. albumin	Reciprocal 7-day H A titers: Mean (± S. D.)		
	Primary	Secondary	Secondary 2-MER
3.00	110 (80-160)	1,500 (820-2,700)	225 (95-540)
1.00	135 (100-180)	900 (640-1,280)	70
0.500	135 (100-180)	450 (320-640)	<20
0.250	40	320 (200-520)	<20
0.100	30 (15-50)	380 (280-540)	20 (<20-60)
0.050	<10 (<10-20)	270 (200-360)	<20
0.025	<10	70 (40-120)	<20
0.010	<10	110 (80-160)	<20
0.001	<10	135 (40-120)	<20
0.0005	<10	380 (280-540)	<20
0.000	<10	380 (210-680)	<20
Fr.V 3.00	160	8,800 (5,000-15,000)	3,700 (1,900-7,000)
Xtal 3.00	10 (<10-20)	640	190 (105-340)

0.500 mg or more as judged by the first appearance of 2-MER antibody. At dosages below 0.500 mg, there was a depression in the response as with the unfractionated albumin, although the degree was not as great. Polymer prepared from Xtal albumin, discussed later, showed similar behavior at low doses. There is a disparity in the secondary titers achieved with the 0.100 mg dose between the two experiments, which again appears to be one of scale.

Monomeric albumin isolated from Fr. V albumin (tables 7 a,b) behaved similarly to the unfractionated Fr. V and the Fr. V polymer at doses above 0.500 mg, giving similar titers as well as priming for a secondary response. There is clearly depression of the response at doses below 0.250-0.500 mg, and all appeared to induce a depression in the response when used at initial dosages below the minimum required for priming. When the initial dose was less than 0.025 mg, all three preparations appeared to lose immunological activity as evaluated by passive HA.

Information on the unfractionated Xtal albumin (tables 8 a,b) again shows priming and progressive increase in titer at doses above 0.500 mg, one dilution higher than with the Fr. V albumin. As with the Fr. V albumin, titers were depressed below control values at dosages below the minimum priming dose. The lowest dose capable of causing a depression appears here to be 0.010 mg, which is slightly lower than that seen with the Fr. V albumin. Also, the highest titer seen at the 3.00 mg dose is well below that of

TABLE 7a: EFFECT OF THE DOSAGE OF AN INITIAL INJECTION OF MONOMERIC ALBUMIN ISOLATED FROM FR. V ALBUMIN ON THE RESPONSE TO A SUBSEQUENT INJECTION OF FR. V ALBUMIN (3 mg.) IN BALB MICE. EXPERIMENT I.

Initial dose, mg. albumin	Reciprocal 7-day H A titers: Mean (± S. D.)		
	Primary	Secondary	Secondary 2-MER
3.00	135 (70-250)	1,800 (1,000-3,500)	1,280 (760-2,150)
1.00	80	1,000 (640-1,600)	65 (20-200)
0.500	50 (25-80)	160	50 (25-100)
0.250	10 (<10-20)	15 (<10-40)	<20
0.100	25 (<10-110)	20 (<10-110)	<20
0.050	<10	<10	<20
0.025	<10	25 (<10-70)	<20
0.010	<10	225 (120-420)	<20
0.001	<10	540 (400-640)	<20
0.0005	<10	510 (300-640)	<20
0.000	<10	380 (240-640)	<20
Fr.V 3.00	450 (320-640)	2,560	1,280
Xtal 3.00	70 (35-125)	760 (400-1,400)	190 (150-250)

TABLE 7b: EFFECT OF THE DOSAGE OF AN INITIAL INJECTION OF MONOMERIC ALBUMIN ISOLATED FROM FR. V ALBUMIN ON THE RESPONSE TO A SUBSEQUENT INJECTION OF FR. V ALBUMIN (3 mg.) IN BALB MICE. EXPERIMENT II.

Initial dose, mg. albumin	Reciprocal 7-day H A titers: Mean (± S. D.)		
	Primary	Secondary	Secondary 2-MER
3.00	270 (190-380)	2,200 (1,100-4,400)	760 (450-1,280)
1.00	160 (100-270)	760 (380-1,500)	270 (160-450)
0.500	80 (50-135)	135 (80-225)	35 (<20-135)
0.250	30 (20-40)	95 (70-135)	<20
0.100	<10	70 (50-95)	<20
0.050	<10	40 (25-70)	<20
0.025	<10	40 (25-70)	<20
0.010	<10	160 (95-270)	<20
0.001	<10	380 (270-540)	<20
0.0005	<10	640	<20
0.000	<10	450 (320-640)	<20
Fr.V 3.00	540 (380-760)	3,100 (1,500-6,200)	1,800 (1,280-2,560)
Xtal 3.00	60 (40-80)	320 (160-640)	135 (70-270)

TABLE 8a: EFFECT OF THE DOSAGE OF AN INITIAL INJECTION OF CRYSTALLINE ALBUMIN ON THE RESPONSE TO A SUBSEQUENT INJECTION OF FR. V ALBUMIN (3 mg.) IN BALB MICE. EXPERIMENT I.

Initial dose, mg. albumin	Reciprocal 7-day H A titers: Mean (± S. D.)		
	Primary	Secondary	Secondary 2-MER
3.00	<10	640 (320-1,280)	110 (80-160)
1.00	<10	225 (10-450)	55 (15-200)
0.500	<10	110 (60-220)	<20 (<20-27)
0.250	<10	15 (10-20)	<20
0.100	<10	20 (10-50)	<20
0.050	<10	35 (15-95)	<20
0.025	<10	160 (135-190)	<20
0.010	<10	95 (80-110)	<20
0.001	<10	135 (110-160)	<20
0.0005	<10	160 (95-225)	<20
0.000	<10	160 (80-320)	<20
Fr. V 3.00	160 (100-225)	2,560 (540-12,200)	900 (160-5,000)

TABLE 8b: EFFECT OF THE DOSAGE OF AN INITIAL INJECTION OF CRYSTALLINE ALBUMIN ON THE RESPONSE TO A SUBSEQUENT INJECTION OF FR. V ALBUMIN (3mg.) IN BALB MICE. EXPERIMENT II.

Initial dose, mg. albumin	Reciprocal 7-day H A titers: Mean (± S. D.)		
	Primary	Secondary	Secondary 2-MER
3.00	10 (<10-15)	450 (320-640)	160
1.00	<10	160 (90-280)	80 (32-220)
0.500	<10	160 (70-380)	35 (<20-50)
0.250	<10	95 (40-230)	<20
0.100	<10	<10	<20
0.050	<10	<10	<20
0.025	<10	<10 (<10-10)	<20
0.010	<10	20 (<10-60)	<20
0.001	<10	110 (60-200)	<20
0.0005	<10	190 (135-270)	<20
0.000	<10	160 (95-270)	<20
fr.V 3.00	160 (100-270)	1,800 (1,280-2,560)	1,280

the Fr. V albumin control in each experiment.

Polymeric Xtal (tables 9 a,b) behaved quite similarly to Fr. V polymer and unfractionated Fr. V albumin (tables 5,6). Again, there is priming for a secondary response with increasingly high titers at dosages above 0.500 mg. The titers do not, however, appear as high as those seen with the Fr. V control in each experiment. The depression in response seen at dosages below the minimum priming dose are more profound than the depressions observed with Fr. V polymer, but are similar to the depressions seen with the unfractionated albumins and the monomeric Fr. V albumin.

Monomeric Xtal albumin behaved quite differently from the albumins examined thus far. While there was evidence from 2-MER titers for priming at dosages of 1.00 mg and 3.00 mg, the highest titers attained were below the titers of the unprimed controls. Moreover, there is not clear evidence for depression in titers at low initial albumin dosages, as was seen with the other albumins. There does appear to be a depression in the dose range of 0.250 to 0.025 mg in the data of table 10a. This is not clear in table 10b, although the titers expressed in this dose range are below control values.

Tables 11 a,b show the results of dose-response experiments done with the heat-stable monomer prepared from Xtal albumin. Here, priming occurred at doses of 0.500 mg or more, in agreement with the results obtained

TABLE 9a: EFFECT OF THE DOSAGE OF AN INITIAL INJECTION OF HEAT-FORMED POLYMER PREPARED FROM CRYSTALLINE ALBUMIN ON THE RESPONSE TO A SUBSEQUENT INJECTION OF FR. V ALBUMIN (3 mg.) IN BALB MICE. EXPERIMENT I

Initial dose, mg. albumin	Reciprocal 7-day H A titers: Mean (± S. D.)		
	Primary	Secondary	Secondary 2-MER
3.00	70 (60-80)	900 (760-1,100)	450 (230-900)
1.00	20 (<10-60)	230 (130-400)	50 (20-130)
0.500	25 (<10-100)	80 (30-210)	<20 (<20-30)
0.250	<10	20 (<10-35)	<20
0.100	<10	10 (<10-30)	<20
0.050	<10	10 (<10-20)	<20
0.025	<10	10 (<10-15)	<20
0.010	<10	10 (<10-15)	<20
0.001	<10	135 (95-180)	<20
0.0005	<10	190 (140-260)	<20
0.000	<10	190 (150-260)	<20
Fr.V 3.00	160 (110-225)	3,700 (3,100-4,400)	900 (500-1,900)
Xtal 3.00	70 (60-80)	270 (170-440)	110 (54-200)

TABLE 9b: EFFECT OF THE DOSAGE OF AN INITIAL INJECTION OF HEAT-FORMED POLYMER PREPARED FROM CRYSTALLINE ALBUMIN ON THE RESPONSE TO A SUBSEQUENT INJECTION OF FR. V ALBUMIN (3 mg.) IN BALB MICE. EXPERIMENT II.

Initial dose, mg. albumin	Reciprocal 7-day H A titers: Mean (± S. D.)		
	Primary	Secondary	Secondary 2-MER
3.00	60 (40-80)	1,500 (900-2,700)	540 (270-1,100)
1.00	20 (10-40)	380 (290-500)	160 (100-260)
0.500	10 (<10-20)	110 (80-160)	20 (<20-30)
0.250	<10	100 (55-160)	20 (<20-30)
0.100	<10	10 (<10-25)	<20
0.050	<10	20 (<10-60)	<20
0.025	<10	30 (10-80)	<20
0.010	<10	65 (30-130)	<20
0.001	<10	270 (135-540)	<20
0.0005	<10	270 (150-470)	<20
0.000	<10	320 (200-500)	<20
Fr.V 3.00	190 (110-330)	2,200 (1,280-3,100)	1,100 (610-1,900)
Xtal 3.00	15 (10-20)	380 (290-500)	160 (100-260)

TABLE 10a: EFFECT OF THE DOSAGE OF AN INITIAL INJECTION OF MONOMERIC ALBUMIN ISOLATED FROM CRYSTALLINE ALBUMIN ON THE RESPONSE TO A SUBSEQUENT INJECTION OF FR. V ALBUMIN (3 mg.) IN BALB MICE. EXPERIMENT I.

Initial dose, mg. albumin	Reciprocal 7-day H A titers: Mean (± S. D.)		
	Primary	Secondary	Secondary 2-MER
3.00	60 (30-110)	225 (160-320)	30 (20-40)
1.00	25 (10-50)	225 (110-450)	20 (<20-35)
0.500	50 (25-100)	255 (170-350)	<20
0.250	35 (25-50)	40 (25-65)	<20
0.100	10 (<10-15)	35 (25-50)	<20
0.050	<10	70 (30-160)	<20
0.025	<10	100 (50-160)	<20
0.010	<10	160	<20
0.001	<10	380 (225-640)	<20
0.0005	<10	270 (190-380)	<20
0.000	<10	450 (270-640)	<20
Fr.V 3.00	225 (160-320)	1,800 (1,300-2,600)	640 (320-1,280)
Xtal 3.00	30 (15-60)	270 (160-450)	60 (20-160)

TABLE 10b: EFFECT OF THE DOSAGE OF AN INITIAL INJECTION OF MONOMERIC ALBUMIN ISOLATED FROM CRYSTALLINE ALBUMIN ON THE RESPONSE TO A SUBSEQUENT INJECTION OF FR. V ALBUMIN (3 mg.) IN BALB MICE. EXPERIMENT II.

Initial dose, mg. albumin	Reciprocal 7-day H A titers: Mean (± S.D.)		
	Primary	Secondary	Secondary 2-MER
3.00	50 (35-70)	270 (190-380)	95 (50-190)
1.00	50 (35-70)	160 (70-380)	<20
0.500	40 (25-70)	160	<20
0.250	20	110 (80-160)	<20
0.100	<10	225 (160-320)	<20
0.050	<10	320	<20
0.025	<10	190 (135-270)	<20
0.010	<10	540 (380-760)	<20
0.001	<10	540 (320-800)	<20
0.0005	<10	320 (160-640)	<20
0.000	<10	380 (270-540)	<20
Fr.V 3.00	190 (135-270)	2,560 (1,500-4,400)	900 (320-2,500)
Xtal 3.00	10 (<10-20)	540 (380-760)	160 (100-260)

TABLE 11a: EFFECT OF THE DOSAGE OF AN INITIAL INJECTION OF HEAT-STABLE MONOMERIC ALBUMIN PREPARED FROM CRYSTALLINE ALBUMIN ON THE RESPONSE TO A SUBSEQUENT INJECTION OF FR. V ALBUMIN (3 mg.) IN BALB MICE. EXPERIMENT I.

Initial dose, mg. albumin	Reciprocal 7-day titers: Mean (± S. D.)		
	Primary	Secondary	Secondary 2-MER
3.00	70 (40-160)	2,200 (1,500-3,100)	380 (270-540)
1.00	190 (135-270)	1,100 (540-2,000)	95 (40-250)
0.500	80 (50-135)	320 (190-540)	25 (<20-40)
0.250	35 (15-70)	225 (80-640)	<20
0.100	15 (<10-160)	95 (70-135)	<20
0.050	10 (<10-20)	110 (60-225)	<20
0.025	<10	110 (25-540)	<20
0.010	<10	270 (190-760)	<20
0.001	<10	160 (80-320)	<20
0.0005	<10	225 (160-320)	<20
0.000	<10	450 (225-900)	<20
Fr.V 3.00	30 (10-70)	7,400 (4,400-12,200)	1,500 (760-3,100)
Xtal 3.00	<10	1,280 (900-1,800)	380 (190-760)

TABLE 11b: EFFECT OF THE DOSAGE OF AN INITIAL INJECTION OF HEAT-STABLE MONOMERIC ALBUMIN PREPARED FROM CRYSTALLINE ALBUMIN ON THE RESPONSE TO A SUBSEQUENT INJECTION OF FR. V ALBUMIN (3 mg.) IN BALB MICE. EXPERIMENT II.

Initial dose, mg. albumin	Reciprocal 7-day H A titers: Mean (± S. D.)		
	Primary	Secondary	Secondary 2-MER
3.00	NOT AVAILABLE--SEE TEXT	1,500 (1,100-2,200)	110 (80-180)
0.500		540 (380-760)	<20
0.100		190 (135-270)	<20
0.050		190 (125-270)	<20
0.025		320 (190-540)	<20
0.010		640 (380-1,100)	<20
0.001		380 (160-900)	<20
0.0005		450 (320-640)	<20
0.000		640	<20
Fr.V 3.00		5,000 (3,100-8,800)	1,000 (760-1,500)
Xtal 3.00		270 (190-380)	40 (<20-95)

with albumins other than the Xtal monomer. Additionally, the highest titer attained was considerably above that attained with the Xtal control in each experiment, although still below the titer of the Fr. V control.

Primary titers are not presented for the experiment shown in table 11b owing to a technical failure in the HA experiment. A repeat of the HA test using fresh reagents permitted the determination of secondary titers and secondary 2-MER titers, but insufficient primary serum was available to permit a repeat of the HA test for these sera. A third experiment was done in which only primary titers were determined (data not shown). These were in agreement with the primary titers displayed in table 11a.

Although the results obtained with the several preparations of Fr. V and Xtal albumin are quite similar, with the apparent exception of the puzzling disagreement in results between whole Xtal albumin and Xtal monomer, there do appear to be systematic differences. Tables 12 a,b compare the means of the combined 2-MER responses of the experimental pairs. Whole Fr. V albumin and the Fr. V monomer appear to be the most effective priming antigens. The polymers from both Fr. V and Xtal albumin appear to prime well, but at a lower level of effectiveness. The Xtal monomer and whole Xtal albumin were the least effective.

In 1970, Wolberg et al. reported that immunization with heated albumin antigens stimulated early, transient antibodies against non-native antigenic determinants present

TABLE 12a: 2-MER RESPONSES AS INDICATORS OF PRIMING WITH ALBUMINS SEPARATED BY MOLECULAR SIZE AND STABILITY TO HEAT: FR.V ANTIGENS.

Priming dose, mg albumin	Reciprocal Mean 2° 2-MER Titers and Titer-ratios[a] against Fr.V-coated cells					
	Priming Antigen[b]					
	Whole		Monomer		Polymer	
	titer	ratio	titer	ratio	titer	ratio
3.00	1,900	1.0	1,000	0.5	210	0.1
1.00	320	1.0	170	0.5	40	0.1
0.50	95	1.0	40	0.4	50	0.5
0.25	45	1.0	<20	-	<20	-
0.10	<20	-	<20	-	<20	-
<0.10	<20	-	<20	-	<20	-

a. Ratio calculated is the ratio of the 2-MER titer of each indicated albumin to the 2-MER titer of the whole Fr.V albumin at the equivalent priming dose.

b. All mice received a second injection consisting of 3.00 mg Fr.V albumin i.p. 6 weeks following the indicated priming antigen.

on the heated albumin but not on unheated albumin. Table 13 shows early primary responses detected by passive HA using Fr. V-coated cells. It can be seen that the Fr. V monomer and polymer both stimulate such early antibodies, but none of the Xtal preparations tested did so. Although it is not apparent here, the whole Fr. V albumin has stimulated early antibody in other experiments, while the Xtal albumin has never done so.

Comparison of results is facilitated by normalization and pooling of data to simultaneously eliminate scale differences and provide a larger data base from which to compare results. Tables 14, 15, and 16 display the normalized and pooled data from the experiments shown in tables 5 through 11. For clarity, the same data are displayed graphically without superimposed standard deviations in figures 13 through 17. Standard deviations ≤ 0.20 are within the precision of the HA test. The apparent difference in behavior between Xtal and Xtal monomer can be attributed to experimental error (tables 15 and 16, figure 16). It is seen in table 15 that the standard deviations are quite large for the whole Xtal. These result from differences in the titers of individual animals at each dilution tested rather than from differences between experiments. Four titration experiments were done with this albumin, and all show large variations in titer. The control animals in these experiments, given either one or two doses of Fr. V albumin, however, never showed titers

TABLE 12b: 2-MER RESPONSES AS INDICATORS OF PRIMING WITH ALBUMINS SEPARATED BY MOLECULAR SIZE AND STABILITY TO HEAT: XTAL ANTIGENS.

Initial dose, mg albumin	Reciprocal Mean 2^o 2-MER Titers and Titer-ratios[a] against Fr.V-coated Cells							
	Priming Antigen[b]							
	Whole		Monomer		HS Monomer		Polymer	
	titer	ratio	titer	ratio	titer	ratio	titer	ratio
3.00	135	0.1	65	0.03	380	0.2	500	0.3
1.00	70	0.2	<20	-	95	0.3	110	0.3
0.50	25	0.2	<20	-	25	0.3	<20	-
0.25	<20	-	<20	-	<20	-	<20	-
0.10	<20	-	<20	-	<20	-	<20	-
<0.10	<20	-	<20	-	<20	-	<20	-

a. See table 12a.

b. See table 12a.

TABLE 13: EARLY PRIMARY RESPONSES OF MICE IMMUNIZED WITH FR.V AND XTAL ALBUMIN ANTIGENS.

Initial dose, mg albumin	Reciprocal Mean 4-day H A Titers against Fr.V-coated Cells					
	Priming Antigen					
	Whole		Polymers		Monomers[a]	
	Fr.V	Xtal	Fr.V	Xtal	Fr.V	Xtal
3.00	<10	<10	140	<10	70	<10
1.00	<10	<10	60	<10	15	<10
0.50	<10	<10	50	<10	<10	<10
0.25	<10	<10	25	<10	<10	<10
0.10	<10	<10	35	<10	<10	<10
0.05	<10	<10	<10	<10	<10	<10
<0.05	<10	<10	<10	<10	<10	<10
Fr.V 3.00	<10	<10	20	<10	<10	<10
Xtal 3.00	<10	<10	<10	<10	<10	<10

a. No data are included for heat-stable monomeric albumin prepared from Xtal albumin as 4-day sera were not collected for these experiments.

TABLE 14: RELATIVE RESPONSES OF MICE TO FR.V ALBUMIN AFTER AN INITIAL INJECTION OF FR.V MONOMER OR POLYMER: LOGARITHMIC RATIOS OF TITERS IN EXPERIMENTAL MICE TO THOSE IN CONTROL MICE GIVEN NO INITIAL INJECTION.

Initial dose[a]	Monomer	Polymer
3.00	1.40 ± 0.17	1.30 ± 0.20
1.00	1.20 ± 0.17	1.20 ± 0.14
0.500	0.73 ± 0.17	1.01 ± 0.14
0.250	0.53 ± 0.17	0.95 ± 0.26
0.100	0.49 ± 0.17	1.00 ± 0.17
0.050	0.13 ± 0.17	0.89 ± 0.17
0.025	0.34 ± 0.51	0.41 ± 0.30
0.010	0.78 ± 0.24	0.77 ± 0.20
0.001	1.10 ± 0.14	1.01 ± 0.20
0.0005	1.10 ± 0.14	1.02 ± 0.17
0.000	1.00 ± 0.20	1.00 ± 0.17
Fr.V 3.00	1.54 ± 0.20	1.56 ± 0.20
Xtal 3.00	1.11 ± 0.17	1.01 ± 0.14

a. All mice received a second injection consisting of 3.00 mg of Fr.V albumin i.p. 6 weeks after the initial injection of the indicated albumin.

TABLE 15: RELATIVE RESPONSES OF MICE TO FR.V ALBUMIN AFTER AN INITIAL INJECTION OF FR.V OR XTAL ALBUMIN: LOGARITHMIC RATIOS OF TITERS IN EXPERIMENTAL MICE TO THOSE IN CONTROL MICE GIVEN NO INITIAL INJECTION.

Initial dose[a]	Fr.V	Xtal
3.00	1.80 ± 0.14	1.36 ± 0.20
1.00	1.26 ± 0.17	1.16 ± 0.58
0.500	1.02 ± 0.10	0.93 ± 0.41
0.250	0.99 ± 0.14	0.37 ± 0.46
0.100	0.78 ± 0.20	0.26 ± 0.22
0.050	0.31 ± 0.17	0.29 ± 0.24
0.025	0.12 ± 0.14	0.47 ± 0.24
0.010	0.32 ± 0.20	0.81 ± 0.51
0.001	0.98 ± 0.17	0.90 ± 0.36
0.0005	1.00 ± 0.17	1.10 ± 0.33
0.000	1.00 ± 0.17	1.00 ± 0.20
Fr.V 3.00	-- --	2.15 ± 0.14
Xtal 3.00	1.31 ± 0.17	-- --

a. See table 14.

TABLE 16: RELATIVE RESPONSES OF MICE TO FR.V ALBUMIN AFTER AN INITIAL INJECTION OF XTAL MONOMER, POLYMER, OR H S MONOMER: LOGARITHMIC RATIOS OF TITERS IN EXPERIMENTAL MICE TO THOSE IN CONTROL MICE GIVEN NO INITIAL INJECTION.

Initial dose[a]	Monomer	Polymer	H S Monomer
3.00	0.92 ± 0.14	1.58 ± 0.14	1.30 ± 0.14
1.00	0.81 ± 0.30	1.05 ± 0.10	1.20 ± 0.47
0.500	0.92 ± 0.20	0.70 ± 0.10	0.95 ± 0.14
0.250	0.54 ± 0.17	0.62 ± 0.17	0.82 ± 0.17
0.100	0.54 ± 0.14	0.52 ± 0.14	0.65 ± 0.14
0.050	0.44 ± 0.14	0.16 ± 0.17	0.70 ± 0.17
0.025	0.88 ± 0.20	0.28 ± 0.17	0.88 ± 0.17
0.010	0.98 ± 0.14	0.48 ± 0.14	0.90 ± 0.14
0.001	0.92 ± 0.14	0.92 ± 0.14	0.79 ± 0.35
0.0005	0.99 ± 0.17	1.03 ± 0.14	0.78 ± 0.17
0.000	1.00 ± 0.17	1.00 ± 0.10	1.00 ± 0.48
Fr.V 3.00	1.40 ± 0.17	1.71 ± 0.14	1.57 ± 0.22
Xtal 3.00	0.91 ± 0.26	1.05 ± 0.10	0.92 ± 0.14

a. See table 14.

FIGURE 13: RELATIVE RESPONSES OF MICE TO FR. V ALBUMIN AFTER AN INITIAL INJECTION OF WHOLE FR. V o——o; FR. V MONOMER •– –•; OR FR. V POLYMER □—·—□: LOGARITHMIC RATIOS OF TITERS IN EXPERIMENTAL MICE TO THOSE IN CONTROL MICE GIVEN NO INITIAL INJECTION.

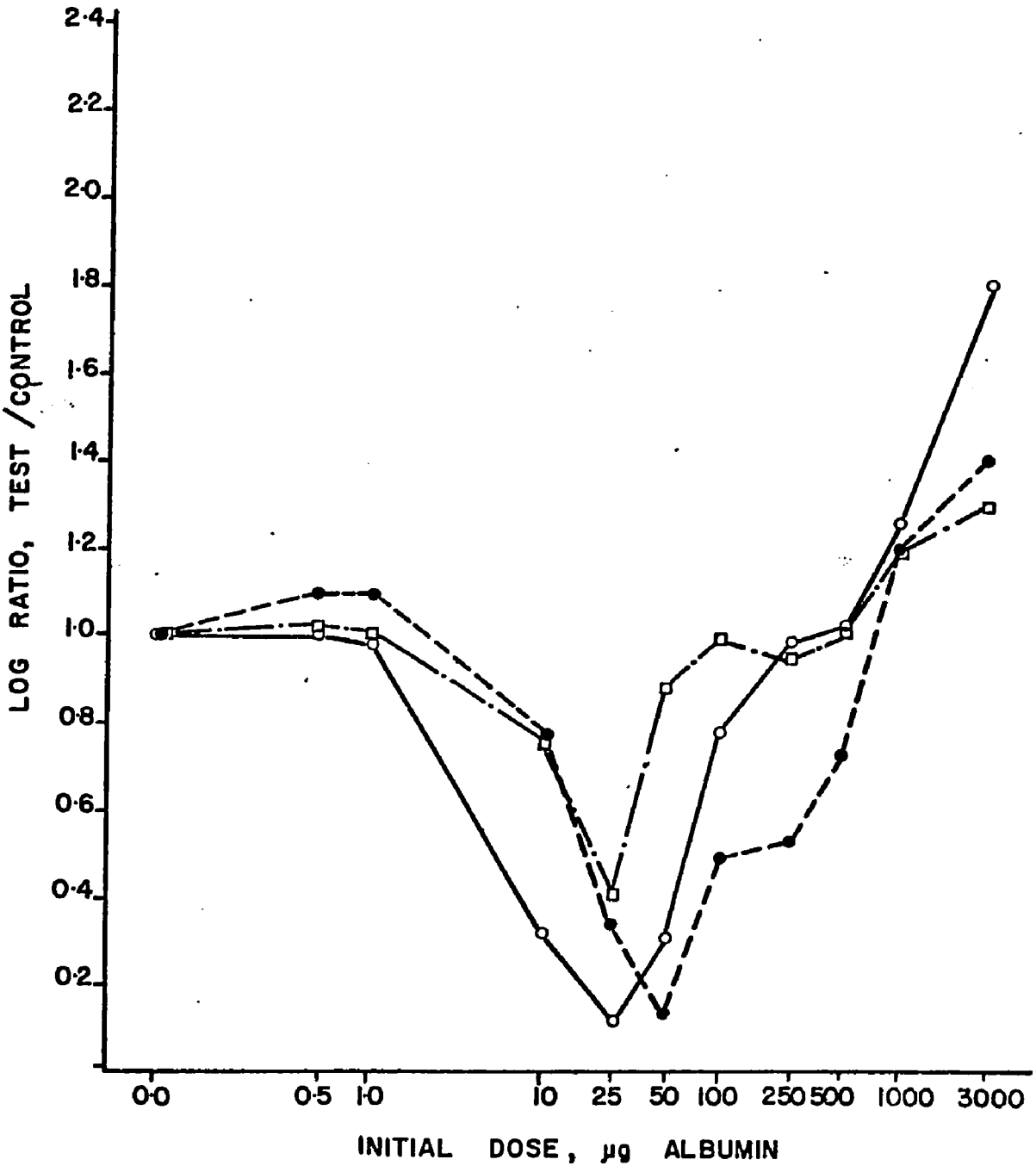

LOG RATIO, TEST /CONTROL
2·4
2·2
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0·0
0·5
1·0
10
25
50
100
250
500
1000
3000
INITIAL DOSE, µg ALBUMIN

FIGURE 14: RELATIVE RESPONSES OF MICE TO FR. V ALBUMIN AFTER AN INITIAL INJECTION OF WHOLE FR. V o—o OR XTAL ALBUMIN •—•: LOGARITHMIC RATIOS OF TITERS IN EXPERIMENTAL MICE TO THOSE IN CONTROL MICE GIVEN NO INITIAL INJECTION.

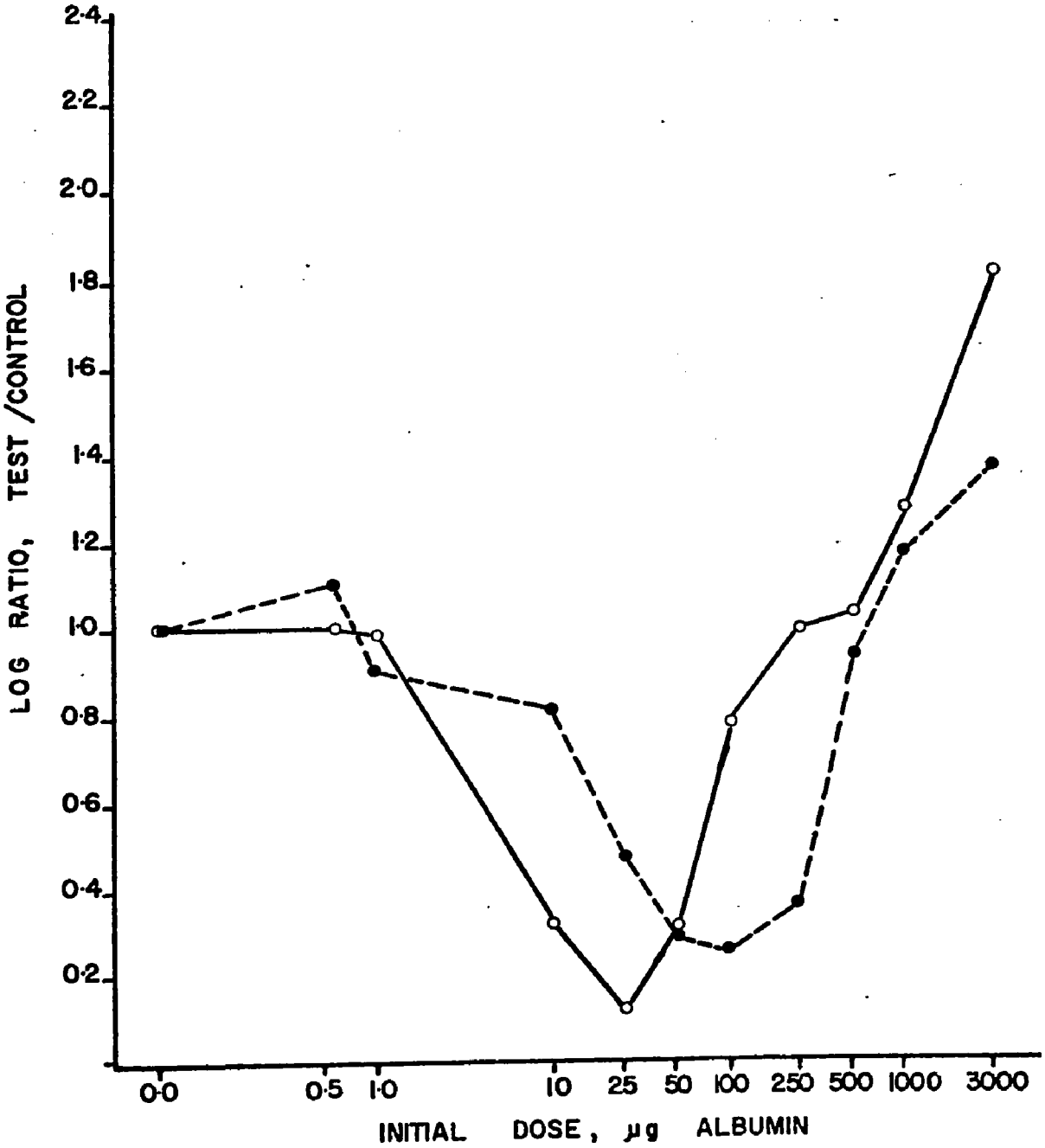

LOG RATIO, TEST /CONTROL
2·4
2·2
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0·0
0·5
1·0
10
25
50
100
250
500
1000
3000
INITIAL DOSE, µg ALBUMIN

FIGURE 15: RELATIVE RESPONSES OF MICE TO FR. V ALBUMIN AFTER AN INITIAL INJECTION OF WHOLE XTAL o——o; XTAL MONOMER •– –•, HS XTAL MONOMER ✡–··–✡OR XTAL POLYMER□—·—□: LOGARITHMIC RATIOS OF TITERS IN EXPERIMENTAL MICE TO THOSE IN CONTROL MICE GIVEN NO INITIAL INJECTION.

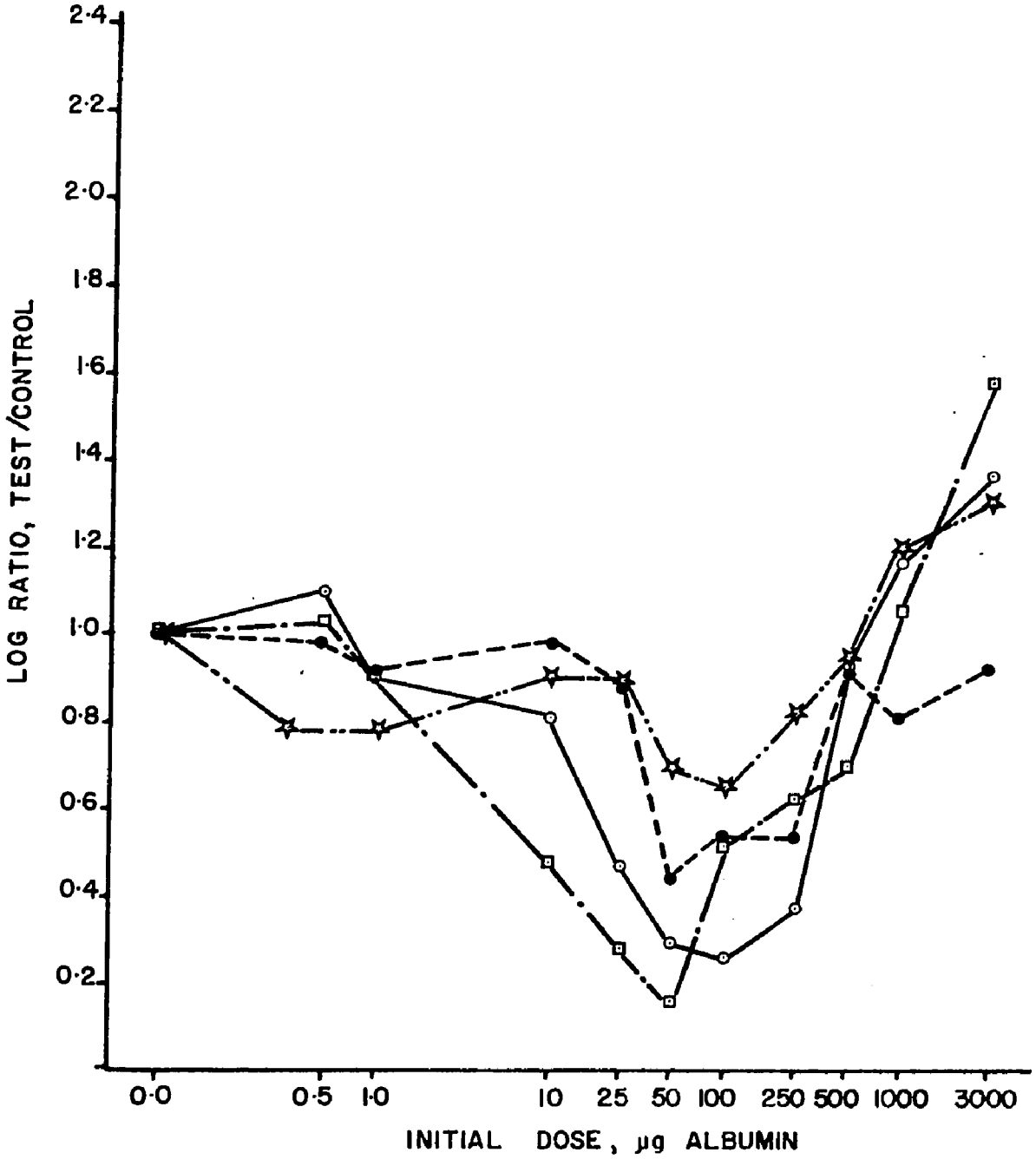

LOG RATIO, TEST/CONTROL
2·4
2·2
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0·0
0·5
1·0
10
25
50
100
250
500
1000
3000
INITIAL DOSE, µg ALBUMIN

FIGURE 16: COMPARISON OF RESPONSES IN MICE TO FR. V ALBUMIN AFTER AN INITIAL INJECTION OF FR. V MONOMER o—o , XTAL MONOMER •– –•, OR HS XTAL MONOMER □—·—□: LOGARITHMIC RATIOS OF TITERS IN EXPERIMENTAL MICE TO THOSE IN CONTROL MICE GIVEN NO INITIAL INJECTION.

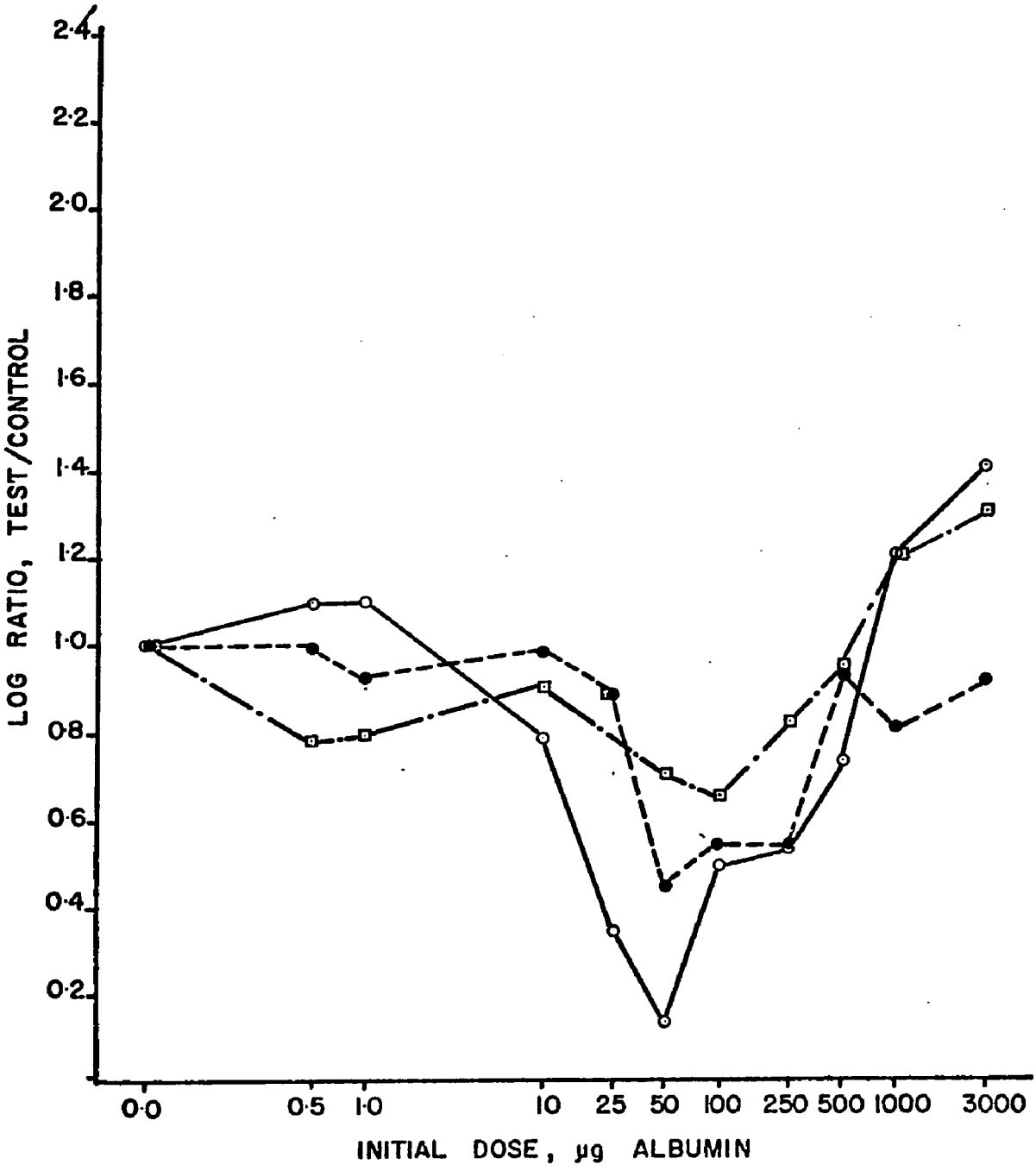
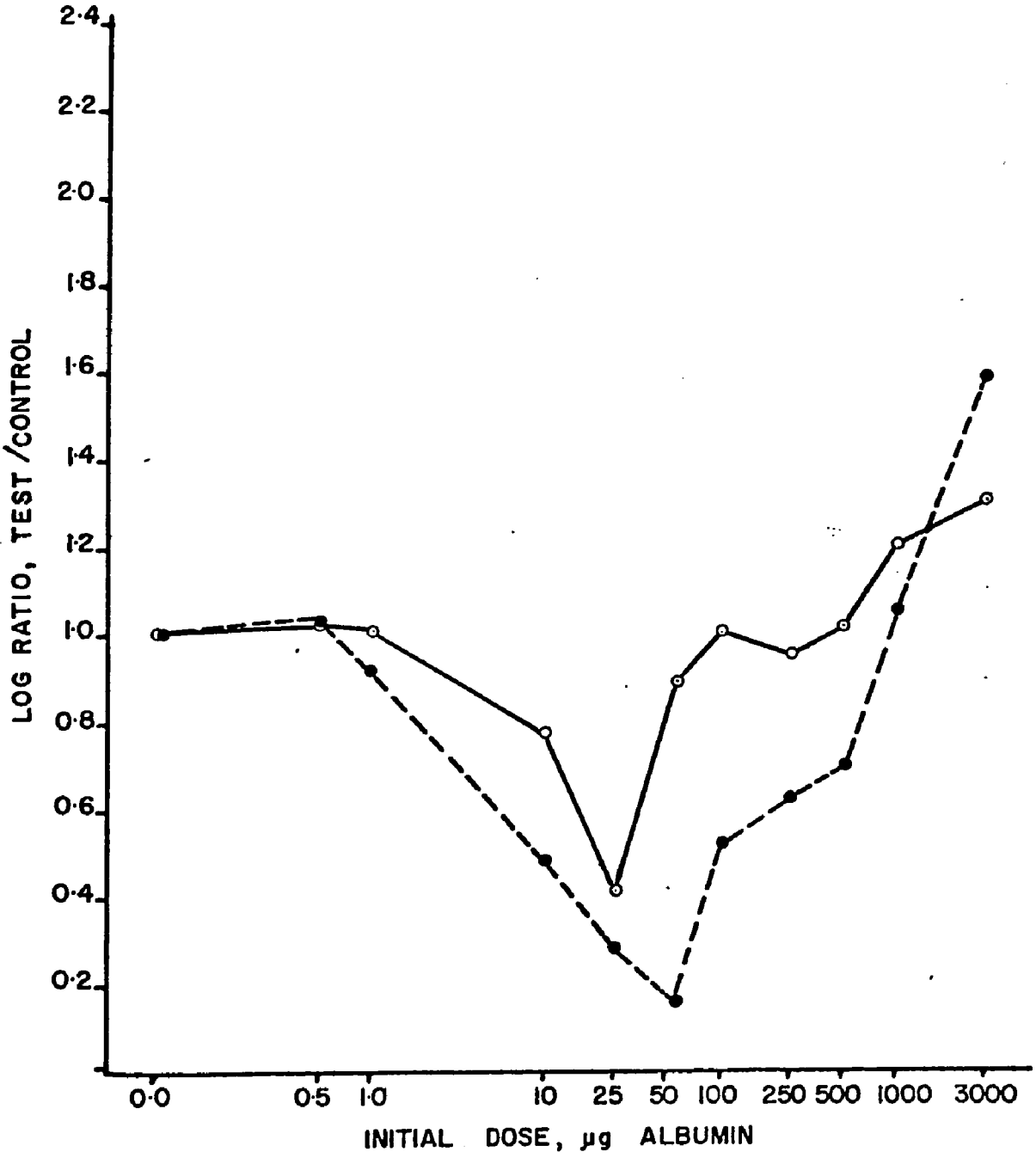

LOG RATIO, TEST/CONTROL
2·4
2·2
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0·0
0·5
1·0
10
25
50
100
250
500
1000
3000
INITIAL DOSE, µg ALBUMIN

FIGURE 17: COMPARISON OF RESPONSES IN MICE TO FR. V ALBUMIN AFTER AN INITIAL INJECTION OF FR. V POLYMER o—o , OR XTAL POLYMER•—•: LOGARITHMIC RATIOS OF TITERS IN EXPERIMENTAL MICE TO TITERS IN CONTROL MICE GIVEN NO INITIAL INJECTION.


2·4
2·2
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
LOG RATIO, TEST /CONTROL
0·0
0·5
1·0
10
25
50
100
250
500
1000
3000
INITIAL DOSE, µg ALBUMIN

differing by more than one dilution. Thus, the variability in response appears to be a characteristic of the Xtal albumin.

Graphic presentation of the results makes it clear that all of the albumin antigens tested showed parallel behavior in the dose range tested. All caused at least some depression of the immune response when given at dosages below those necessary to prime. The degree of depression, of course, is quite different among the preparations. Fr. V, Fr. V monomer, and Xtal polymer displayed the greatest ability to depress titers at low dosages yet primed quite strongly and comparably at doses above the priming threshold (tables 12 a,b). Fr. V polymer and Xtal H S monomer effected much less depression of the immune response at low dosages while still priming quite strongly at doses above the priming threshold. Whole Xtal and Xtal monomers neither depressed strongly at low dosages nor primed well at high dosages. The behavior seen (figures 13, 16) indicates that the behavior of the whole albumin is most closely paralleled by the behavior of the isolated monomer. This is not surprising, inasmuch as the monomer is the major component of the commercially prepared albumins.

Three interesting findings are (1) the demonstration of low zone tolerance induction <u>in vivo</u> with polymeric antigen, (2) the demonstration of the existence of monomeric forms of albumin which appear to be at least as effective priming antigens as the polymers and (3) the similarity of the dose

effects, i.e. the loss of immunological effectiveness at dosages below 0.025-0.010 mg and the uniformity of priming at dosages above 0.250-0.500 mg. Certainly the possibility of immunological effects at dosages below 0.010 mg cannot be ignored, nor can the possibility of priming at dosages below 0.250 mg. be ignored, as more sensitive assay methods might well detect such effects.

Study of Monomeric Albumins Isolated by Ion Exchange Chromatography on DEAE Sephadex: Studies on the effect of dosage on the immune response were done with Fr. V and Xtal DEAE pools I (termed "Early" monomer) and with Xtal DEAE pool III and Fr. V DEAE pool IV (termed "Late" monomer). As with the monomeric and polymeric albumins, the purpose of these studies was to learn if there was any difference among the preparations in the smallest dose capable of priming for a secondary response and if the preparations differed in the induction of low-dose immunosuppression. Mitchison-type assays were performed as before, along with 2-mercaptoethanol treatment of secondary sera for the detection of priming. Mice used in these experiments were carefully chosen to be between 6 and 8 weeks of age in order to minimize variations in titer due to age and to take advantage of the increased sensitivity of young adult mice to the tolerizing properties of antigen.

The data in tables 17 a,b describe the behavior of the "Early" monomer isolated from Fr. V albumin, which is the early non-mercaptoalbumin pool. As with the other Fr. V

TABLE 17a: EFFECT OF THE DOSAGE OF AN INITIAL INJECTION OF "EARLY" DEAE SEPHADEX-SEPARATED FR. V MONOMER ON THE RESPONSE TO A SUBSEQUENT INJECTION OF FR. V ALBUMIN (3 mg.) IN BALB MICE. EXPERIMENT I.

Initial dose, mg. albumin	Reciprocal 7-day H A titers: Mean (± S. D.)		
	Primary	Secondary	Secondary 2-MER
3.00	160	7,400 (5,000-10,000)	2,560 (1,500-4,400)
1.00	225 (110-450)	3,700 (2,200-6,200)	760 (1,450-1,280)
0.500	80 (60-135)	1,100 (380-3,100)	380 (135-2,200)
0.250	40	270 (190-380)	<20
0.100	15 (<10-40)	135 (95-190)	<20
0.050	<10	160 (80-320)	<20
0.025	<10	380 (270-540)	<20
0.010	<10	320	<20
0.001	<10	640	<20
0.0005	<10	640 (380-1,100)	<20
0.000	<10	640	<20
Fr.V 3.00	80	2,560 (1,500-1,400)	1,100 (760-1,500)
Xtal 3.00	<10	540 (320-900)	190 (110-320)

TABLE 17b: EFFECT OF THE DOSAGE OF AN INITIAL INJECTION OF "EARLY" DEAE SEPHADEX-SEPARATED FR. V MONOMER ON THE RESPONSE TO A SUBSEQUENT INJECTION OF FR. V ALBUMIN (3 mg.) IN BALB MICE. EXPERIMENT II.

Initial dose, mg. albumin	Reciprocal 7-day H A titers: Mean (± S. D.)		
	Primary	Secondary	Secondary 2-MER
3.00	110 (80-160)	10,000 (6,200-17,000)	2,200 (1,500-3,100)
1.00	160	1,500 (1,100-2,200)	450 (320-640)
0.500	60 (30-110)	320 (190-540)	35 (10-95)
0.250	40 (25-70)	380 (270-540)	<20
0.100	20 (10-40)	135 (80-225)	<20
0.050	<10	160 (95-270)	<20
0.025	<10	270 (190-380)	<20
0.010	<10	320	<20
0.001	<10	640	<20
0.0005	<10	450 (320-640)	<20
0.000	<10	380 (225-640)	<20
Fr.V 3.00	270 (190-450)	2,200 (1,500-3,100)	1,100 (640-1,800)
Xtal 3.00	30 (15-60)	540 (380-760)	70 (30-160)

antigens tested, priming occurred at dosages of 0.500 mg or more. In contrast to results obtained with the whole Fr. V or Fr. V monomer, however, there is little evidence of low-zone depression of the response.

Tables 18 a,b show the results obtained with "Late" Fr. V, the late non-mercaptoalbumin monomer. Here, it is seen that no primary response was induced in either experiment. Additionally, priming did not occur at all below the 3.00 mg dose, and even there, the priming was marginal. Total titers were suppressed, compared to controls, from the 0.025 mg dose to the 3.00 mg dose. The immunosuppression seemed not to occur at dosages below 0.025 mg with this antigen, in agreement with the general lack of immunological effect seen with the other preparations at these low dosages.

Tables 19 a,b show the results obtained with "Early" Xtal, the mercaptoalbumin monomer pool from Xtal albumin. Primary titers for the experiment displayed in table 19b were inordinately high, including the primary titers of the Fr. V control mice. Experiment 19b was done in 12 week old mice rather than the preferred 6 to 8 week old mice, because of a shortage of animals of the proper age. Such older animals are known to respond more vigorously to BSA antigens (Jones, 1972). Since the primary responses in this experiment are inconsistent with the results obtained generally with 6 to 8 week old mice, they are omitted.

Despite this difference, however, the secondary res-

TABLE 18a: EFFECT OF THE DOSAGE OF AN INITIAL INJECTION OF "LATE" DEAE-SEPHADEX-SEPARATED FR. V MONOMER ON THE RESPONSE TO A SUBSEQUENT INJECTION OF FR. V ALBUMIN (3 mg.) IN BALB MICE. EXPERIMENT I.

Initial dose, mg. albumin	Reciprocal 7-day H A titers: Mean (± S. D.)		
	Primary	Secondary	Secondary 2-MER
3.00	<10	110 (80-160)	25 (<20-60)
0.500	<10	25 (10-60)	<20
0.100	<10	10 (<10-20)	<20
0.050	<10	70 (50-95)	<20
0.025	<10	80 (50-135)	<20
0.010	<10	160	<20
0.001	<10	160 (95-270)	<20
0.0005	<10	225 (160-320)	<20
0.000	<10	320	<20
Fr.V 3.00	70 (50-95)	2,560	900 (640-1,280)
Xtal 3.00	<10	110 (60-225)	25 (<20-60)

TABLE 18b: EFFECT OF THE DOSAGE OF AN INITIAL INJECTION OF "LATE" DEAE-SEPHADEX-SEPARATED FR. V MONOMER ON THE RESPONSE TO SUBSEQUENT INJECTION OF FR. V ALBUMIN (3 mg.) IN BALB MICE. EXPERIMENT II.

Initial dose, mg. albumin	Reciprocal 7-day H A titers: Mean (± S. D.)		
	Primary	Secondary	Secondary 2-MER
3.00	<10	190 (95-380)	40 (<20-110)
0.500	<10	135 (95-190)	<20
0.250	<10	80	<20
0.100	<10	20 (10-40)	<20
0.050	<10	20 (10-40)	<20
0.025	<10	95 (70-135)	<20
0.000	<10	640	<20
Fr.V 3.00	135 (60-320)	7,400 (5,000-19,000)	1,500 (760-3,100)
Xtal 3.00	<10	640 (380-1,100)	380 (160-900)

TABLE 19a: EFFECT OF THE DOSAGE OF AN INITIAL INJECTION OF "EARLY" DEAE-SEPHADEX-SEPARATED XTAL MONOMER ON THE RESPONSE TO A SUBSEQUENT INJECTION OF FR.V ALBUMIN (3 mg.) IN BALB MICE. EXPERIMENT I.

Initial dose, mg albumin	Reciprocal 7-day H A titers: Mean (± S. D.)		
	Primary	Secondary	Secondary 2-MER
3.00	35 (<10-135)	225 (160-320)	30 (20-40)
0.500	15 (<10-30)	160	20
0.100	<10	135 (70-270)	<20
0.050	<10	160 (80-270	<20
0.025	<10	160 (80-270)	<20
0.010	<10	190 (135-270)	<20
0.001	<10	380 (270-540)	<20
0.0005	<10	450 (320-690)	<20
0.000	<10	190 (135-270)	<20
Fr.V 3.00	160	≥20,000	2,200 (1,500-3,100)
Xtal 3.00	<10	1.280 (760-2,200)	190 (95-380)

TABLE 19b: EFFECT OF THE DOSAGE OF AN INITIAL INJECTION OF "EARLY" DEAE-SEPHADEX-SEPARATED XTAL MONOMER ON THE RESPONSE TO A SUBSEQUENT INJECTION OF FR.V ALBUMIN (3 mg.) IN BALB MICE. EXPERIMENT II.

Initial dose, mg albumin	Reciprocal 7-day H A titers: Mean (± S. D.)		
	Primary	Secondary	Secondary 2-MER
1.00	NOT AVAILABLE -- SEE TEXT	380 (160-900)	50 (<20-110)
0.500		270 (190-380)	<20
0.250		160 (80-320)	<20
0.100		160	<20
0.050		135 (60-320)	<20
0.025		160 (95-270)	<20
0.010		135 (30-640)	<20
0.001		270 (35-2,200)	<20
0.0005		450 (40-5,000)	<20
0.000		640 (380-1,100)	<20
Fr.V 3.00		13,000 (7,400-20,000)	3,300 (3,100-3,800)
Xtal 3.00		225 (80-640)	225 (80-640)

ponses are in good agreement with those in table 19a, which seems to show that this albumin neither primes well nor suppresses well and is a poorer immunogen than the parent Xtal albumin.

Tables 20 a,b show the results obtained with "Late" Xtal, the late non-mercaptoalbumin monomer pool from Xtal albumin. This albumin was also a poor immunogen but appeared more like the unfractionated Xtal albumin than the mercaptoalbumin fraction did. Again, there is little evidence to indicate any marked toleragenic properties in this antigen.

Tables 21 a,b compare the means of the pooled data on 2-MER responses. The "Early" Fr. V antigen appears to be a superior priming antigen, while the "Late" Fr. V antigen failed to prime at any dosage less than 3.00 mg, at which it primed quite poorly. None of the Xtal fractions primed well. The mercaptoalbumin most closely approximated the behavior of the unfractionated monomer isolated from Xtal albumin.

Table 22 and figures 19 and 20 compare the normalized, pooled data in the same manner as was done in tables 14 to 16 and figures 14 through 18. The "Early" Fr. V monomer shows no clear suppression of the immune response beyond that attributable to experimental error. The "Late" Fr. V monomer clearly shows a depression, with no indication of titers above control values at high dosage.

The responses obtained with "Early" and "Late" Xtal

TABLE 20a: EFFECT OF THE DOSAGE OF AN INITIAL INJECTION OF "LATE" DEAE-SEPHADEX-SEPARATED CRYSTALLINE MONOMER ON THE RESPONSE TO A SUBSEQUENT INJECTION OF FR. V ALBUMIN (3 mg.) IN BALB MICE. EXPERIMENT I.

Initial dose, mg. albumin	Reciprocal 7-day H A titers: Mean (± S. D.)		
	Primary	Secondary	Secondary 2-MER
3.00	80	1,280 (450-3,700)	160 (80-320)
1.00	80	640 (380-1,100)	95 (60-160)
0.500	80	95 (20-450)	20 (<20-50)
0.250	<10	200 (30-900)	<20
0.100	<10	225 (160-320)	<20
0.050	<10	450 (320-640)	<20
0.000	<10	540 (270-1,100)	<20
Fr.V 3.00	160	3,100 (2,200-4,400)	540 (270-1,100)
Xtal 3.00	10 (<10-20)	640 (380-1,100)	80 (50-135)

TABLE 20b: EFFECT OF THE DOSAGE OF AN INITIAL INJECTION OF "LATE" DEAE-SEPHADEX-SEPARATED CRYSTALLINE MONOMER ON THE RESPONSE TO A SUBSEQUENT INJECTION OF FR. V (3 mg.) ALBUMIN IN BALB MICE. EXPERIMENT II.

Initial dose, mg. albumin	Reciprocal 7-day H A titers: Mean (± S. D.)		
	Primary	Secondary	Secondary 2-MER
3.00	160	640 (380-1,100)	160 (95-270)
0.500	30 (10-70)	640 (190-2,200)	40 (<20-135)
0.100	<10	190 (135-270)	<20
0.050	<10	270 (160-450)	<20
0.025	<10	270 (190-380)	<20
0.010	<10	640	<20
0.001	<10	640	<20
0.0005	<10	640 (270-1,500)	<20
0.000	<10	380 (190-760)	<20
Fr.V 3.00	320	≥20,000	5,000 (3,100-8,800)
Xtal 3.00	<10	750 (540-1,100)	190 (95-380)

TABLE 21a: 2-MER RESPONSES AS INDICATORS OF PRIMING WITH MONOMERIC ALBUMINS SEPARATED BY DEAE-SEPHADEX CHROMATOGRAPHY: Fr.V ANTIGENS

Priming dose, mg. albumin	Reciprocal Mean 2° 2-MER Titers and Titer-ratios[a] against Fr.V-coated Cells					
	Priming Antigen[b]					
	Monomer		Early Fraction		Late Fraction	
	titer	ratio	titer	ratio	titer	ratio
3.00	1,000	1.0	2,380	2.4	33	0.03
1.00	170	1.0	600	3.6	-	-
0.50	40	1.0	210	5.2	<20	-
0.25	<20	-	<20	-	<20	-
<0.25	<20	-	<20	-	<20	-

a. Ratio calculated is the ratio of the 2-MER titer of each indicated albumin to the 2-MER titer of the Fr.V monomer at the equivalent priming dose.

b. All mice received a second injection consisting of 3.00 mg Fr.V albumin i.p. 6 weeks following the indicated priming antigen.

TABLE 21b: 2-MER RESPONSES AS INDICATORS OF PRIMING WITH MONOMERIC ALBUMINS SEPARATED BY DEAE-SEPHADEX CHROMATOGRAPHY: XTAL ANTIGENS

Priming dose, mg albumin	Reciprocal Mean 2^o 2-MER Titers and Titer-ratios against Fr.V-coated Cells					
	Priming Antigen[b]					
	Monomer		Early Fraction		Late Fraction	
	titer	ratio	titer	ratio	titer	ratio
3.00	135	0.1	30	0.04	160	0.2
1.00	70	0.4	40	0.20	95	0.6
0.50	25	0.6	<20	-	30	0.8
0.25	<20	-	<20	-	<20	-
<0.25	<20	-	<20	-	<20	-

a. See table 21a.

b. See table 21a.

TABLE 22: RELATIVE RESPONSES OF MICE TO FR.V ALBUMIN AFTER AN INITIAL INJECTION OF "EARLY" OR "LATE" DEAE-SEPHADEX-SEPARATED MONOMER ISOLATED FROM FR.V OR XTAL ALBUMIN: LOGARITHMIC RATIOS OF TITERS IN EXPERIMENTAL MICE TO THOSE IN CONTROL MICE GIVEN NO INITIAL INJECTION

Initial dose[a], mg albumin	Fr.V		Xtal	
	Early	Late	Early	Late
3.00	1.68 ± 0.20	0.71 ± 0.33	1.05 ± 0.42	1.17 ± 0.28
1.00	1.35 ± 0.17	-- --	0.88 ± 0.53	1.04 ± 0.30
0.500	1.00 ± 0.26	0.55 ± 0.17	0.83 ± 0.14	0.86 ± 0.52
0.250	0.88 ± 0.14	0.46 ± 0.17	0.67 ± 0.50	0.75 ± 0.34
0.100	0.63 ± 0.17	0.25 ± 0.17	0.67 ± 0.17	0.80 ± 0.14
0.050	0.73 ± 0.24	0.55 ± 0.20	0.83 ± 0.28	0.91 ± 0.17
0.025	0.88 ± 0.14	0.54 ± 0.14	0.78 ± 0.22	0.90 ± 0.20
0.010	0.90 ± 0.14	0.80 ± 0.32	1.22 ± 0.20	1.14 ± 0.22
0.001	1.07 ± 0.14	0.80 ± 0.32	1.22 ± 0.20	1.14 ± 0.22
0.0005	1.04 ± 0.14	0.90 ± 0.22	1.29 ± 0.14	1.14 ± 0.54
0.000	1.00 ± 0.17	1.00 ± 0.14	1.00 ± 0.17	1.00 ± 0.17
Fr.V 3.00	1.51 ± 0.17	1.60 ± 0.14	1.96 ± 0.14	1.70 ± 0.17
Xtal 3.00	1.03 ± 0.14	0.91 ± 0.22	1.62 ± 0.32	1.14 ± 0.17

a. See table 21a.

FIGURE 18: RELATIVE RESPONSES OF MICE TO FR. V ALBUMIN AFTER AN INITIAL INJECTION OF "EARLY" o—o OR "LATE" •--• FR. V MONOMER ISOLATED BY ION EXCHANGE CHROMATOGRAPHY: LOGARITHMIC RATIOS OF TITERS IN EXPERIMENTAL MICE TO THOSE IN CONTROL MICE GIVEN NO INITIAL INJECTION.

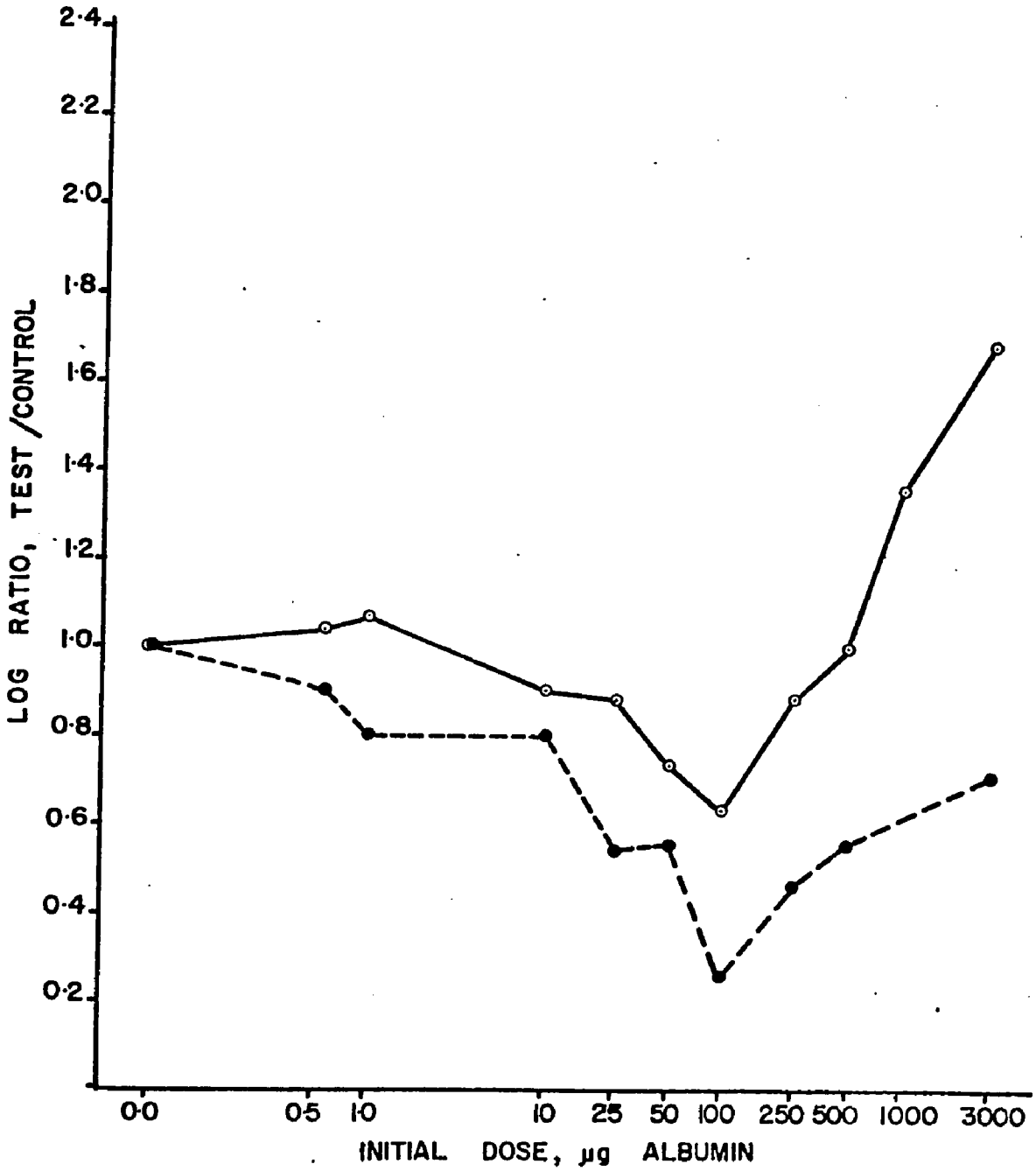
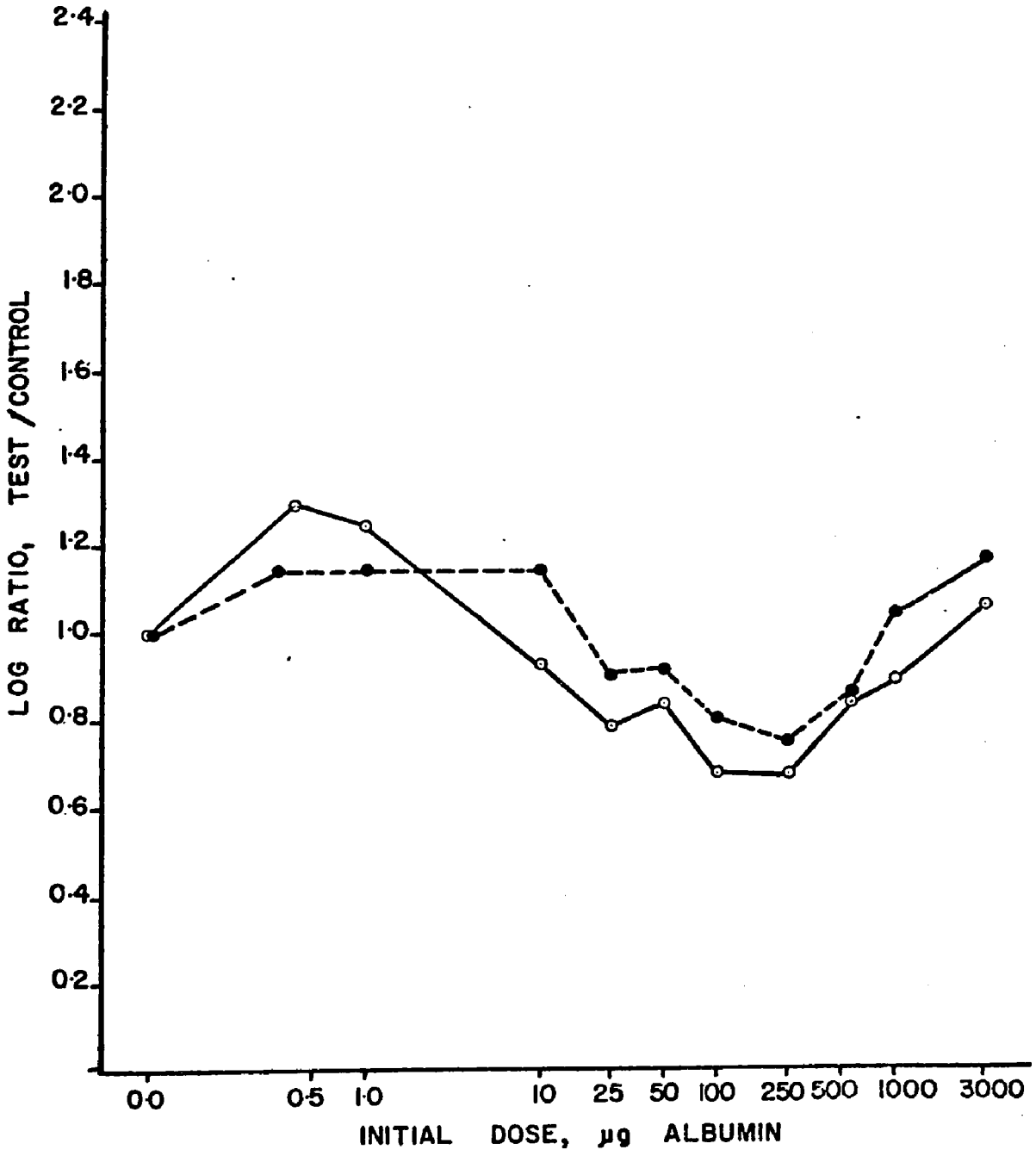

2·4
2·2
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
LOG RATIO, TEST /CONTROL
0·0
0·5
1·0
10
25
50
100
250
500
1000
3000
INITIAL DOSE, µg ALBUMIN

FIGURE 19: RELATIVE RESPONSES OF MICE TO FR. V ALBUMIN AFTER AN INJECTION OF "EARLY" o—o OR "LATE" •—• XTAL MONOMER ISOLATED BY ION EXCHANGE CHROMATOGRAPHY: LOGARITHMIC RATIOS OF TITERS IN EXPERIMENTAL MICE TO THOSE IN CONTROL MICE GIVEN NO INITIAL INJECTION.


2·4
2·2
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
LOG RATIO, TEST / CONTROL
0·0
0·5
1·0
10
25
50
100
250
500
1000
3000
INITIAL DOSE, µg ALBUMIN

monomers were, within the limits of experimental error, identical. Moreover, there is no clear indication of either low dose depression of the response or of titers significently above controls at high dosages. Again, as with the whole Xtal, there is considerable variability in the titers within each group of mice as indicated by the large standard errors.

The outcome of these experiments seems to be the identification of monomeric albumins having different immunologic properties. The most interesting result is the isolation of separate immunogenic and toleragenic forms of monomer, first predicted by Mitchison (1964).

V. DISCUSSION

Objectives of the Study: The experiments were intended to develop immunologically relevant information concerning five aspects of bovine serum albumin antigens, as follows: Firstly, it was of interest to learn whether or not albumin polymers are intrinsically more immunogenic and less toleragenic than monomers. The data comparing polymers to monomers isolated from Fr. V albumin indicate that both substances primed mice equally well and at comparable doses. Moreover, polymer preparations from both Fr. V and Xtal albumin caused immunologic tolerance when given to mice at sub-immunogenic doses. The dose-range for tolerance induction and the degree of tolerance induced by the polymers were comparable to the dose-range for tolerance induction and the degree of tolerance induced by the monomers. Although there was a difference in the degree of immunologic tolerance induced by the two different polymers, such differences also appeared among the monomers. These findings suggest that the extent of aggregation *per se* was not a determinant of immunogenicity or toleragenicity.

Secondly, it was of interest to learn if the immunologic properties of a monomeric albumin isolated from a highly immunogenic bovine albumin preparation differed from those of a monomeric albumin isolated from a poorly immunogenic preparation. Data comparing monomers obtained from Fr. V albumin with those obtained from Xtal albumin

indicate considerable differences in immunologic activity. Fr. V monomer obtained by exclusion chromatography stimulated higher antibody titers at doses above the priming threshold and produced a greater degree of tolerance at doses below the threshold than did the comparable Xtal preparation. The behavior of monomers isolated by ion exchange chromatography from Fr. V and Xtal also differed.

Thirdly, since lipid content varies widely among different samples of albumin, it was of interest to know in what way the addition or removal of lipid might influence the immunologic behavior of the albumin. Results of the study on the immunological effect of lipid binding can be interpreted as indicating no direct effect of the lipid on immunological properties. However, at high molar ratios of bound lipid to albumin, the immunogenicity of the Fr. V albumin was reduced markedly while the immunogenicity of the Xtal albumin, although quite low, remained unchanged. While the effect on albumin structure of lipid removal and replacement was not directly examined in the present work, the effects of the methods used have been studied by other workers (Chen, 1967; Noel and Hunter, 1972) and found to induce only minimal changes in structure under physiological conditions. However, lipid binding at high molar ratios of lipid to albumin (>5 meq/meq) has previously been shown to result in profound changes in optical properties, enthalpy and electrophoretic mobility (Goodman, 1958). The failure of the lipid when bound at 20 meq/meq

to further reduce the quite low immunogenicity of the Xtal albumin suggests; (1) that the immunologically important change introduced on addition of the lipid was already present, or (2) that some property unassociated either with the lipid itself or with lipid-induced structural changes must be responsible for the low immunogenicity of the Xtal albumin compared to the Fr. V albumin. Since changes in structure similar to those associated with binding large amounts of lipid should be accompained by alterations in optical properties that would be detectable by circular dichroism studies, and since circular dichroism spectra of the Fr. V and Xtal albumin monomers were found to be nearly alike, a gross structural difference between the two albumins can be eliminated from consideration.

Fourthly, it was of interest to learn if samples of monomeric albumin differing in immunogenic or toleragenic behavior could be isolated or prepared in the laboratory. The data comparing the immunologic behavior of the monomeric albumins variously prepared by exclusion chromatography, ion exchange chromatography and heat treatment show that the monomers obtained differed widely in their capacity to either immunize or induce tolerance in balb mice. The differences seen were independent of any aggregate content, since preparations equally low in aggregate content were quite different in behavior.

Fifthly, it was of interest to learn whether or not,

within the limits of the methods used, it would be possible to correlate changes in the immunologic behavior of monomeric albumins with some property independent of differences in aggregate content or antigenic specificity. Structural data were not collected on all of the albumins studied. However, circular dichroic absorption (CD) spectra were obtained for four different forms of monomer prepared by ion exchange chromatography. These monomers were quite free of aggregates, although all contained equivalent trace amounts of dimeric albumin in the ratio of, at most, 0.1 to 0.3 ug/mg albumin. Two were relatively inert immunologically, one was a good immunogen but poor toleragen, and one was a poor immunogen but a good toleragen. All four albumins produced similar CD spectra, indicating that any difference in either helix content or conformation in the regions of amino acids capable of differentially absorbing polarized light must be so slight as to be undetectable by this method. Given these findings, one would expect there to be little difference in antigenic specificity among those preparations, and none was found.

Relative Capacities of Aggregated and Monomeric Albumins for Either Stimulating Immunity or Inducing Tolerance: Results of numerous studies have been interpreted as indicating the greater immunogenicity of aggregates or aggregate-containing proteins compared to monomeric forms of the same protein (Weigle, 1973).

Numerous observations consistent with this view have also been reported in the case of bovine serum albumin (Frei et al., 1965, 1968; Pinckard et al., 1967 a,b; Draper and Hirata, 1968; Porter, 1970; Katsura et al., 1972).

The results of the present study both confirm and contradict the interpretation that aggregates are intrinsically superior immunogens. For example, polymers from both the Xtal and Fr. V albumins gave higher primary titers than the unfractionated albumins or isolated monomers at comparable dosages. At dosages above the priming threshold, the polymer prepared from Xtal albumin gave secondary titers that were clearly superior to those of either the unfractionated Xtal albumin or any of the monomers isolated from Xtal albumin, in agreement with the prevailing view. The results obtained with the Fr. V polymer-monomer pair contradict this view, and, indeed, the Xtal monomer after heating was found to be as good an immunogen as either the Xtal or Fr. V polymer, also in disagreement with the currently held view.

Since Fr. V albumin initially contained more aggregate and since heating is known to cause the formation of aggregates in albumin solutions, it might be argued that the high immunogenicities of Fr. V monomer and heat-stable Xtal monomer solutions were derived from the presence of aggregates in these solutions which either were not present or were present at a much lower level in the unfractionated Xtal and Xtal monomer. This argument does not seem tenable for several reasons.

Firstly, although the highly immunogenic Fr. V albumin initially contained more than twice the aggregates present in the poorly immunogenic Xtal albumin, the Fr. V monomer isolated by exclusion chromatography contained much less, yet was more immunogenic than the Xtal albumin and, indeed, was as immunogenic as the unfractionated Fr. V albumin containing a large quantity of aggregate. Similarly, the heat-stable Xtal monomer which had also been isolated chromatographically and contained much less aggregate than the parent Xtal albumin was still a better immunogen than the unfractionated Xtal albumin.

Secondly, monomers eluted from ion exchange columns at high ionic strength ("Late" monomer pools) contained more aggregate then monomers eluting at low ionic strength ("Early" monomer pools), yet the "Early" monomer pool from Fr. V albumin proved to be the most immunogenic preparation isolated, while the "Late" Fr. V monomer, which should have contained more aggregate, albeit in trace amounts, was the least immunogenic preparation.

Thirdly, the polymer solutions were found not to induce an immune response at doses below 0.250 mg. The greatest amount of aggregate present in any of the monomer solutions tested could not have been more than 0.3 ug/mg, or, in a 3 mg dose, less than 1/250th of the minimum dose of aggregate required.

The apparent superiority of aggregates over monomers seen by others might be explained by the variations in

immune responsiveness seen in experimental animals. For example, Sobey et al. (1966) reported that the capacity to respond to bovine serum albumin could be genetically selec-ed in rabbits and mice. The trait was found to segregate in simple mendelian fashion, and separate strains of "high responder" and "low responder" mice were developed. Subsequently, Hardy and Rowley (1968) studied the responses of Sobey "low responder" mice to small doses of bovine serum albumin. Using sensitive isotope binding studies, it was found that these mice responded optimally to doses of less than 500 ug. Doses between 500 ug and 5000 ug, the highest studied, were toleragenic. Heat-aggregated albumin and unheated albumin gave similar results.

Cerottini and Lambert (1969) compared NZB, $(NZBxNZW)_{F_1}$, A/J, CBA/J and balb/CJ mice for the capacity to respond to aggregated or unaggregated bovine serum albumin, aggregated or unaggregated human gamma globulin, sheep erythrocytes, and keyhole-limpet hemocyanin. Using a variety of assay technics, they found strain differences in the responses to each antigen, with a lack of parallelism among the antigens; i.e., a good response to HGG was not predictive of the response to BSA, and an attempt to order the immunogenicities of the antigens by the intensity of responses according to the strain of mouse gave a different order for each strain and a different order for primary and secondary responses.

Golub and Weigle (1969) studied the relationship

between the relative content of aggregate in human gamma globulin solutions and the capacity for tolerance induction in several inbred stains of mice and their F_1 crosses. Several methods were used to remove aggregates, including ultra-centrifugation, ammonium sulfate precipitation and "biological filtration". The strains varied considerably in the ease with which they could be rendered tolerant. CBA/J mice were made tolerant by doses of 0.1 to 10.0 mg of centrifuged globulin, while balb/CJ mice could not be made tolerant with any dose of gamma globulin tested between 5 picograms and 10 milligrams, even after removing aggregates by ammonium sulfate or "biological filtration" technics, which are more effective than centrifugation.

Amkraut, Malley and Begley (1969) studied the responses of rabbits to hemocyanin from three different molluscan and three different arthropod species. No difference was found in the hyperimmune response to aggregated or monomeric hemocyanin, but the titers to mollusc hemocyanins were higher than the titers to arthropod hemocyanins. The authors reasoned that the degree of phylogenetic relationship might govern the intensity of the response, since they considered that rabbits were more closely related phylogenetically to arthropods than to molluscs. However, an effect of aggregation was seen in the primary response, in which aggregated hemocyanins gave higher titers than the monomeric hemocyanins. This effect was partly dose-dependent and showed a "phylogenetic effect"; i.e., the

difference in response to aggregated vs. monomeric hemocyanin was greater in the hemocyanins from arthropods than molluscs.

Rajewski (1966), and Rajewski and Rottlander (1967b) studied the antigenic determinants, immunogenicity and toleragenicity of the family of lactic dehydrogenase enzymes. The enzymes consist of a family of five tetramers formed from two antigentically distinct polypeptide chains, A and B, assembled in all possible combinations. When these were given to rabbits with adjuvant, antibodies were raised which cross-reacted with rabbit LDH enzymes and LDH enzymes from several other species as well. Rabbits could be segregated into two populations; those that responded to LDH-I (B_4) with antibody formation and those that did not. Both populations of rabbits nonetheless responded equally well to LDH-V (A_4). Injection of combinations of the A and B peptides (LDH-II, III or IV) to LDH-I non-responders resulted in a response to determinants on both the A and B peptides. LDH-I non-responder rabbits could be made tolerant to LDH-V and any LDH isozyme containing the A peptide (LDH-II, III, IV, V) by large repeated doses of LDH-V (A_4). Such tolerant animals did not make antibodies against either the A or B peptide when subsequently given an immunogenic dose of LDH-II, III or IV. Attempts to induce a similar tolerance with LDH-I (B_4) failed; i.e., although responses were marginally lower than those of untreated controls, LDH-I non-responder rabbits

given massive doses of LDH-I still responded to the A and B determinants when subsequently immunized with LDH-II, III or IV. All of the antigens used were polymerized to the same degree, hence the differences in immunological behavior must have been related to some other property of the LDH molecule.

While it has been shown that individual animals and strains of inbred animals may respond differently to an antigen presented in different molecular forms, detailed reviews of the available genetic information (Mc Devitt and Benacerraf, 1969; Benacerraf and Mc Devitt, 1972) indicate that the response to many different antigens in many different animals (including BSA in mice) are regulated by one or more immune-response genes which are intimately associated with the genes governing the transplanation antigens of the animal. It is also apparent from the 1972 report that different genes control the response to different antigenic substances. Hence, in attempting to understand the contribution of structural properties of the antigen, one must consider the possibility that what is really being measured is the functioning of one or more genes regulating the immune response. Thus, the interaction between some feature of the antigen and the products of these immune-response genes may be, at least partly, responsible for the immunological behavior of the antigens under study.

Composition of Albumins: Although an immunogenic form of monomer can be obtained from the Armour crystalline

albumin by heating, it is noteworthy that the Pentex fraction V albumin contains a highly immunogenic monomer that can be isolated without heat treatment. Linscott (1963) reported on the variations in composition that may be observed with commercially prepared albumins, particularly with respect to the content of extraneous proteins. The possibility cannot be ignored that the Pentex and Armour albumins may differ in a manner not consistent with their identities as fraction V and crystalline bovine albumin. However, the cross agglutination, immunodiffusion and immunoelectrophoretic studies failed to identify any gross antigenic differences between them that would invalidate the results of the studies on immunological priming and tolerance.

Induction of Low-Zone Tolerance with Polymer: A particularly interesting finding of the present study was the induction of immunologic tolerance with polymeric albumin at subimmunogenic doses. Polymeric protein antigens have been used previously *in vivo* to induce states of immunologic tolerance, but only at a supra-immunogenic dose. For example, Nossal, Ada and Austin (1965) and Nossal and Austin (1966) reported the induction of significant levels of tolerance in newborn rats given large doses of either monomeric flagellin or a polymerized form but not with whole flagella. Dvorak *et al.* (1969) used human serum albumin (HSA) polymers at supra-immunogenic doses to induce tolerance to HSA in adult guinea

pigs. The tolerance could be induced with aggregates isolated from the commercially prepared albumin or with heat-formed aggregates, which were less effective toleragens. The effective toleragenic doses of polymer were the same as those of monomer under the conditions of the study, and were many times higher than the immunizing dose.

Polymeric protein antigens have also been used to induce tolerance in vitro. Armstrong, Diener and Shellam (1969) reported the induction of tolerance in isolated CBA strain mouse B lymphocytes in culture with polymerized, but not with monomeric flagellin. The toleragenic dosage required was 1 ug/ml or more, considerably above the 2 to 20 nanogram dose that induced antibody formation in cultures. Using dinitrophenylated flagellin at various levels of substitution (Feldmann, 1971, 1972) resulted in behavior related to the degree of substitution. Less than 0.1 DNP per flagellin unit led to no anti-DNP antibody formation; 0.1 to 1.0 DNP per flagellin unit led to formation of anti-DNP antibody; 1.5 to 2.5 DNP per flagellin unit led to formation of anti-DNP antibody at low doses of antigen but led to specific DNP tolerance at high doses; and 3 or more DNP per flagellin unit resulted in tolerance to DNP at doses of 1 mg/ml or more but did not stimulate anti-DNP antibody at any dose tested.

Tolerance to polymerized flagellin, whether or not it was DNP-substituted, was B-cell directed and high-zone in nature. However, Diener and Feldmann (1972) re-

ported that the addition of small amounts of anti-flagellin antibody to the system in the range of 1 to 10 bacterial immobilizing units per milliliter of culture fluid permitted the conversion of a 20 nanogram dose of polymerized flagellin from an immunogen to a toleragen. However, this still does not represent low-zone tolerance, since the dose of polymerized flagellin used was in the immunogenic range rather than the subimmunogenic range (< 2ng/ml).

Induction of Low-Zone Tolerance with Monomeric Antigens: Low-zone immunologic tolerance induction with subimmunogenic doses of a fluid protein antigen was first reported by Mitchison (1964,1965) using fraction V bovine serum albumin in CBA strain mice. The protein solution was not treated to remove aggregates. Subsequently, Shellam and Nossal (1968) and Ada and Parish (1968) reported on the induction of low-zone tolerance to flagellin in rats using monomeric flagellin or a cyanogen bromide-derived fragment of the monomer at doses of 1 picogram per gram of body weight in adult rats. Mitchison (1968) also reported the induction of low-zone tolerance to a variety of other proteins in mice, provided that the animals were first immunosuppressed.

In vitro studies using monomeric flagellin and the cyanogen bromide-derived fragment of monomeric flagellin provided interesting results (Diener and Feldmann, 1972). Monomeric flagellin or the cyanogen bromide-derived fragment did not stimulate a primary immune response in vitro, nor

did they induce tolerance *in vitro*. However, when lymphocytes were cultivated in the presence of small amounts of anti-flagellin antibody, tolerance was induced with doses as low as 2 picograms of flagellin or the cyanogen bromide fragment, provided only that the ratio of antibody to antigen was held constant over the entire dose range. Tolerance could be reversed if the cultured lymphoid cells were treated with trypsin within 6 hours after adding the antigen-antibody mixtures. This same tolerance-inducing behavior was achieved with solubilized sheep red blood cell antigens and anti-SRBC antibody and with chicken gamma globulin and anti-chicken gamma globulin antibody. Tolerance induction in this system was dependent on bivalent antibody but not dependent on the Fc portion of the antibody molecule.

It is difficult to visualize what structural features of the antigen govern its capacity to induce tolerance at low doses. It cannot be simple aggregation or its absence, or else the flagellin monomers should be as toleragenic *in vivo* as *in vitro*. Moreover, flagellin polymers, whether aggregated by formation of antigen-antibody complexes or by their normal aggregation mechanism, ought to be equally toleragenic or immunogenic, yet they are not.

The concept of variable epitope density leading to specific membrane effects (Feldmann, 1971 a,b; Nossal, 1971) as a result of single or multipoint binding of antigen may offer an answer, but the experiments of Mitchison (1968), in which it was necessary to immunosuppress mice

before low-zone tolerance could be induced with any of several antigens, suggest the operation of some as yet undefined additional relationship between the antigen and the immune response mechanism of the recipient animal that determines whether a particular antigenic exposure will lead to tolerance, immunity or non-recognition.

Similarity of Dose-Related Effects of Polymeric Albumin Compared to Monomeric Albumin: All of the albumins examined in the present study, whether polymerized or not and whether of Fr. V or Xtal origin, showed similar thresholds for immunologic effect. All of the albumins tested, with the single exception of the "Late" DEAE pool of Fr. V monomer, primed mice for a secondary response when given as an initial injection of 0.250-0.500 mg or more. The "Late" DEAE pool of Fr. V monomer gave marginal priming at 3.00 mg, but not at 0.500 mg or less caused depression of the secondary responses below those of untreated (primary) controls when given as an initial dose less than the minimum dose required for priming. While the precise degree of depression differed among the various albumins, the dose at which maximum depression occurred was approximately 0.025 mg in each case. At dosages below 0.025 mg, less and less depression occurred. At an initial dose of 0.001 mg or less, none of the albumins tested appeared to have any effect on the immune responses of balb mice.

This similarity in dose-response effect with bovine albumin monomer and polymer is quite in contrast with the behavior of flagellin polymer and monomer *in vitro* (Diener and Feldmann, 1972) in which polymer and monomer exhibited their dose effects in widely different dose ranges.

The data on BSA in the present study argues for similar *in vivo* handling of the polymer and monomer. This interpretation is supported by studies on the effect of polymerization of BSA on macrophage uptake and the immunogenicity of macrophage-bound monomeric and polymeric BSA (Kolsch and Mitchison, 1968; Mitchison, 1969; Frisch and Wilson, 1969; Schmidtke and Unanue, 1971; Unanue *et al.*, 1971; Schmidtke and Dixon, 1972) which indicated that, although there was a difference in the rate of uptake of aggregated compared to unaggregated BSA, there was no demonstrable difference in immunogenicity between monomeric and aggregated BSA once bound to macrophages.

Certainly, there is as much albumin in a gram of polymer as in monomer, and both are "processed" in the macrophage (Unanue, 1971). If enzyme cleavage is involved in the "processing" step, as is suggested by the work of Lapresle and Webb (1959); Lapresle *et al.* (1959); Ishizaka, Campbell and Ishizaka (1960); Lapresle and Goldstein (1969); Wright and Rothberg (1971), then the lack of difference is understandable.

However, in one point of view (Leskowitz, 1967) it is argued that immunological tolerance results from a direct interaction between antigen and lymphocytes without

the intervening macrophage "processing" step.

In a study of the effect of molecular size on the distribution of antigen in tolerant animals, Ada and Parish (1968), using monomeric and polymeric flagellin, showed that monomers were diffusely distributed throughout the lymphoid organs of treated rats whereas polymers were found not to be diffusely distributed. In addition, Nakamura et al. (1968) were able to demonstrate that the fraction of injected protein found in the extravascular fluid spaces (lymph) of rabbits was inversely proportional to the logarithm of the effective diffusion radius of the protein molecule. On the basis of these findings, it was argued that monomeric proteins might more readily reach the target cells and induce tolerance than could polymers. It is thus conceivable that monomers would have a better chance of bypassing the macrophage "processing" step and reaching the lymphocytes directly than would the polymers, which might also be taken up faster by macrophages because of their larger size. The data of Kolsch and Mitchison (1968) indicate that only a minute portion of fluid BSA ever reaches the macrophages at any one point in time, but the data of Schmidtke and Unanue (1971) indicated (1) that the degree of uptake of serum albumins by macrophages and the rate of catabolic degradation of the albumin was proportional to the degree of aggregation; (2) that such albumin as was taken up was rapidly catabolized except for a small amount bound at the surface and; (3) that a portion of the albumin taken up was released both degraded and undegraded. Thus, while more polymer than monomer might be "processed", it also seems likely that a considerable amount of the "processed" polymeric

albumin ends up as low molecular weight fragments that would be no different from fragments obtained from monomer. It may be that such rapid degradation has a salutory effect on the activation of an immune response. However, it is difficult to balance this against the *in vitro* observations of Diener and Armstrong (1969) and Diener and Feldmann (1972), in which direct action of antigen at lymphocyte membranes was found to be involved in tolerance induction and that either a chemically aggregated antigen or an antigen-antibody complex was required. If, as the Diener and Feldmann experiments suggest, an aggregated form of antigen is necessary for the induction of tolerance, then there must be some *in vivo* mechanism for converting aggregated antigens and monomeric antigens to the same immunologically functional form. The enzymatically degraded forms of albumin have been shown to contain the native albumin antigenic determinants, and would be the same whether derived from aggregate or monomer. These fragments, together with small amounts of antibody formed early in the response, might serve to form the complex capable of inducing tolerance. Such a mechanism would be compatible with the Schmidtke-Unanue and Diener-Feldmann observations and would account for the similarity seen in the dose effects of BSA in the present study.

Differences Among Monomeric Bovine Serum Albumins in the Capacity to Immunize or Tolerize Balb Mice: One finding of the present study which is of great potential importance in understanding the mechanism by which an antigen signals the host system to respond with an immune

reaction, immunologic tolerance or non-recognition was the identification of different preparations of monomeric albumins which differed in their capacity to immunize or tolerize balb mice. The behavior seen, although different in many respects from the behavior of the LDH isozymes, was conceptually similar, in that the mice responded differently to the different antigens at the same state of aggregation.

The available CD data indicated that all of the monomers, with the possible exception of the heat-stable Xtal monomer, which was not tested, were alike structurally. This does not mean that a more refined approach to the CD study or perhaps a more sensitive molecular probe could not detect structural differences; in fact, structural differences do exist among the albumins that are difficult to detect by CD alone (Noel and Hunter, 1972).

It is well known that treatment of albumin by denaturing technics results in detectable changes in structure, antigenic reactivity in vitro and immunogenic properties in vivo (Erenpreis, Maurer and Sri-Ram, 1957; Maurer, 1959; Wold, 1961; Habeeb, 1967; Peters and Goetzl, 1969).

However, these studies do not yield information on how much structural or chemical difference is required to cause a change in immunological properties, particularly in the capacity of an antigen to induce tolerance or immunity.

Evidence has been accumulating which may shed light

on this question. For example, acetylation of LDH-I under mild conditions (Rajewski, 1966) resulted in a protein that retained its normal enzymatic properties and its original set of antigenic determinant sites, but gained antigenic determinants cross reactive with some of the determinants of LDH-V and was altered electrophoretically. In LDH-I non-responder rabbits, this altered protein was found to be capable of stimulating both an immune response and immunologic tolerance to LDH isozymes bearing the native B peptide, even though the native LDH-I (B_4) itself was not.

More recently, Parish (1972) has reported a series of experiments in which acetoacetylated flagellin antigens were found to induce a state of complete humoral unresponsiveness to the native flagellin in rats, while simultaneously stimulating a strong delayed hypersensitivity response. Differences were found both by quantitative precipitin and electrophoretic studies which were indicative of major structural alterations.

In an earlier observation, Austin and Nossal (1966) found that rats made tolerant to salmonella flagellar antigen fg also failed to respond when subsequently challenged with the serologically unrelated flagellar antigens i and d. The flagellins are a homologous series of proteins, products of the same gene locus, alike in structure and function, and, therefore, expected to differ by only a relatively few amino acid substitutions. Since the antigenic determinant sites have been found to be

restricted to a fragment of the molecule which can be isolated by cyanogen-bromide cleavage (Ada and Parish, 1968) and since this fragment has been used to both immunize and induce tolerance, it can be concluded that some small region of the flagellin molecule which exists independently of the state of aggregation and the antigenic determinant groups was able to interact with the immune system of the rat to effect a toleragenic conversion. Once converted, the immune system did not respond to other flagellins containing that same site, even though their antigenic determinants differed.

The behavior of the flagellin is quite similar to the behavior of hapten-carrier systems in which tolerance to the carrier prevents a response to the hapten unless the hapten is presented on a different carrier (Rajewski et al., 1969; Taylor and Iverson, 1971).

It is also the so-called carrier region that seems to be the target of the Ir gene (Benacerraf and Mc Devitt, 1972), and is responsible for both the induction of tolerance and immunological priming in vivo.

Thus, by inference, whether or not the monomeric forms of albumin isolated in this work differ in their antigenic determinants, they must be different in their carrier properties. Unfortunately, there is not sufficient information at present to determine if the differences in immunologic behavior observed have anything to do with the identifiable chemical differences among the monomers.

The Possibility of an Effect of Antigenic Differences Present but Not Readily Detected: The data comparing albumins

by cross-agglutination and double-diffusion studies failed to reveal any clear differences in antigenic expression among the albumins. There were differences in titers by passive hemagglutination which could indicate either antigenic differences or differences in the efficiency of cell-coating. In any case, the untreated Fr. V and Xtal albumins reacted to high titer with all of the anti-albumin antisera, indicating that any difference would be more likely to be a deletion in some determinant than the appearance of an entirely new determinant.

The double-diffusion studies confirm this interpretation, since only lines of identity were found when comparing the antigens in all possible combinations. However, there were differences in the concentrations of protein needed to give lines at equivalent positions in the agar. This would again suggest quantitative differences rather than the expression of new determinants.

The work of Lapresle and others, previously cited (p. 198), indicated that the normal response to BSA includes the formation of antibodies specific for determinants which are present but not normally expressed on the unaltered protein. The work of Draper and Hirata (1968) indicated that animals immunized with monomers of albumin formed antibodies which reacted with determinants expressed on polymers but not on monomers, in agreement with the findings of Lapresle and others. In addition, Draper and Hirata found that polymeric BSA antigens absorbed out all of the antibody activity directed against monomeric antigens, indicating that the polymers

expressed all of the determinants expressed by the monomers. These results suggest that differences observed among bovine albumin preparations by quantitative precipitation (Sri-Ram and Maurer, 1959; Habeeb, 1969; Peters and Goetzl, 1969) may have been reflective of changes in the proportions of antibodies specific for the different albumin determinant groups, and in the distribution of existing determinants on the various albumins, rather than the creation or destruction of determinants.

It is now well known, from the data of Weigle and others (reviewed in Weigle, 1973), that an existing state of immunologic tolerance can be "broken" by injection of a cross-reacting antigen. It is also apparent from the work of Rajewski (1966) and the work of Parish (1972) that an antigen can be converted from an inert or immunogenic form to a toleragenic form by alterations that introduce antigenic changes, and from the work of Nossal and Austin (1966) that tolerance to a particular antigenic determinant can be induced with a serologically unrelated, albeit chemically related, antigen.

One wonders how alterations in antigenic specificity might be related to the conversion of a substance from an immunogen to a toleragen and _vice versa_. The studies of Brownstone _et al._ (1966); Rajewski and co-workers (1966, 1967 a,b, 1969, 1971); Taylor and Iverson (1971); and Mitchison (1971) on hapten-carrier antigens suggest that tolerance induction _in vivo_ involves a region of the

molecule which is independent of the antigenic determinant (hapten). These studies have also shown that changing one hapten in a double-hapten-protein antigen can alter the response to the second hapten; i.e., one hapten in some way can contribute to the carrier function of the protein to permit or inhibit a response to the other. The result of this is that a state of specific immunologic tolerance to a hapten can be broken either by changing carriers or by changing the second hapten.

In addition to the carrier effect in immunologic tolerance, it has been shown that the properties of the carrier and the particular relationship between the carrier and hapten are important in determining the immunogenic efficiency of an antigen. For example, Mc Donald, Odstrachel and Maurer (1972) found that the antibody response to a synthetic polymeric antigen dubbed PAMEG was much better when PAMEG conjugated with rough pneumococcus as a carrier was used to immunize rabbits, than when a conjugate of PAMEG and BSA was used. Similarly, Hanna, Jarosch and Leskowitz (1972) found that responses to dinitrophenylated proteins were quite sensitive to the method of linking the carrier and hapten; while Alkan, Nitecki and Goodman (1971) found that separating the hapten and carrier in their system by a linkage giving about 10 Å separation resulted in better responses than when the hapten and carrier were more closely linked. These results suggest that some intrinsic property of the carrier combined with the

physical relationship between hapten and carrier were important in governing the immune responses to these antigens.

In the examples cited, changes in immunological behavior have always been accompained by some combination of alterations in physical characteristics, chemical characteristics, or antigenic specificity. In attempting to extrapolate these findings for the purpose of analyzing the immunological behavior of the monomeric albumins isolated in the present study, one would expect to find similar alterations in chemical, physical or antigenic structure. Although there is a hint of difference in serological specificity among the albumin monomers, this is not well documented, and the CD data do not indicate any gross structural change. There are chemical differences among the albumins, however. The highly immunogenic Fr. V monomer is an "Early" non-mercaptoalbumin found in quantity in the Fr. V but not in the Xtal albumin, which also may be assumed to differ in containing bound n-decanol. These differences might be sufficient to result in altered carrier properties capable of converting the immunological properties of the albumin, although that assertion can only be speculative without some direct test, such as interconverting mercaptoalbumin and non-mercaptoalbumin and measuring the change in immunological behavior.

Correlation of the Present Findings with Current Concepts of Regulation in the Immune System: The induction of an immune response is considered to involve three phases of activity including, (1) an afferent phase in which antigen is brought to the lymphoid elements of the immune system in a form which is suitable for inducing a response; (2) a central phase, in which interaction among two or more cell types, antigen, and "mediators" leads to the maturation of antibody-forming and/or "effector" cells; and (3) an efferent phase in which the antibody-forming and/or effector cells react with the antigen, thereby facilitating its removal from the host (Leskowitz, 1967; Hellstrom and Hellstrom, 1970).

It has been established that at least two lymphoid cells must interact in the induction of a humoral antibody response (Claman, Chaperone and Triplett, 1966 a,b) and that interaction of these with "adherent cells" (macrophages) is necessary (Mosier and Coppleson, 1968). It has also been argued that there is a temporal relationship in the three-cell interaction, in which T-cells and macrophages contact antigen earliest, followed by presentation of the antigen, bound either to the T-cell or the macrophage or both, to the B-cell, which then responds with antibody formation (Roitt, et al., 1969 a,b; Moller, 1970).

The specificity of the response is considered to be mediated through restricted expression of antigen-

specific "receptors" at the surfaces of both the T and B lymphocytes (Burnet, 1959; Naor and Sulitzneau, 1967, 1969; Ada and Byrt, 1969; Metzger, 1970; Cone, Sprent, Marchalonis, 1972; Marchalonis, Cone and Atwell, 1972). Following the interaction of anitgen and antigen-specific "receptors" at the cell surface, the lymphocytes undergo a round of cell division leading to their maturation into effector cells capable either of secreting antibody (B-cells) or of collaborating in the induction of a humoral immune response (activated T-cells) (Tannenberg and Malavia, 1968; Feldmann and Basten, 1971).

A condition recognized as immunological unresponsiveness occurs when the immunologic effector cells or substances are not detectable in their usual amounts, or appear unable to function normally (reviewed in Weigle, 1973; Feldman, 1972). An unresponsive state could conceivably be mediated by an afferent mechanism in which properly "processed" antigen fails to reach the lymphoid elements of the system in sufficient quantity. Evidence has been offered in support of such a mechanism (Frisch and Wilson, 1969; Martin, 1966). Unresponsiveness might also involve an efferent mechanism, in which the effector substances cannot reach their targets. Such a mechanism, involving competition between the humoral and cellular branches of the immune response, has been postulated and evidence in support of it cited (Hellstrom and Hellstrom, 1970). A central mechanism of unresponsiveness, in which the antigen

is presented to the lymphoid elements of the system in such a manner as to result in their inactivation has also been postulated (Burnet, 1959), and remains favored (Weigle, 1973). Mechanisms postulated include (1) the destruction or blockage of antigen-specific cells by large quantities of antigen during the maturational stages of the animal (Burnet, 1959; Jerne, 1971); (2) exhaustive differentiation of antibody-forming cells in response to large amounts of antigen (Sterzl, 1966); (3) inactivation of cells as a result of improper presentation of antigen (Leskowitz, 1967); (4) the operation of antigen-specific suppressor cells of the T class which prohibit the activation of antibody-forming cells or of effector lymphocytes (Baker, et al., 1970; Herzenberg, 1970; Gershon and Kondo, 1971 a,b; Phillips and Wegman, 1973; Rich and Pierce, 1973; Cohen and Werkerle, 1973); or (5) the functioning of some form of "feedback" mechanism, in which antigen-antibody complexes somehow serve to regulate the response (Terres and Wollins, 1961; Boynes and Hardwicke, 1968; Tong and Boose, 1970; Moller, 1970; Safford and Tokuda, 1971; St. Sinclair and Chan, 1971; Diener and Feldmann, 1972; Feldmann and Nossal, 1972; Bretscher, 1973).

In each of these arguments, immunocyte (lymphocyte) triggering, whether the result is to be immunity or tolerance, is thought to begin with the interaction of antigen and antigen-specific receptors at the cell surface. Studies on multivalent ligand binding (Taylor,

et al., 1971; Unanue, Karnovsky and Engers, 1973; Ashman and Raff, 1973; Diener and Paethau, 1972) indicated that the multipoint linking of surface immunoglobulins with antigen or anti-immunoglobulin resulted in migration of the complexes toward one pole of the cell. This was followed by internalization of the complexes and subsequent reappearance of cell surface-immunoglobulins at a higher density than before. The rate and character of of migration appeared to vary according to the density of receptors bound and the amount of cross-linking of receptors by antigen or anti-immunoglobulin. High binding-densities and extensive cross-linkage were found to slow or prevent migration and internalization of the complexes. Such behavior was observed with both T-and B-cells, and was thought to reflect an early step in either the activation or tolerization of immunocytes.

These observations are consistent with the observations of Diener and Feldmann (1972) on in vitro triggering with polymers or antigen-antibody complexes, and are consistent with the observation of antibody-antigen complexes in the serum of BSA-tolerant animals (Day and Farr, 1966) and also with the adoptive transfer of tolerance from tolerant to normal mice using cells coated with antigen-antibody complexes (Terman, Minden and Crowle, 1973 a,b).

Whether or not such a direct antigen-membrane interaction is sufficient, in itself, to account for the induction of either an immune response or tolerance is

still open to question. For example, Moller and Kashiwagi (1972) presented evidence for a two-signal mechanism of triggering involving a mitogen and antigen, with the induction of immunity or tolerance in vitro dependent on the dose of mitogen. Additionally, Schrader (1973) was able to trigger the formation of anti-F γ G antibody in vitro using disaggregated fowl gamma globulin (F γ G) in the absence of T-cells, providing the B-cells were first activated with polymerized flagellin. Thus, the role of aggregation in vitro may merely be to act as a mitogenic signal. While this function in vivo may be replaced by something else, aggregates or antigen-antibody complexes might still act as either mitogens or inhibitors of mitogenesis under proper conditions.

An additional point of contention lies in whether or not a simple antigen-membrane interaction is sufficient to account for either the induction of immunity or the induction of tolerance. Evidence has been referred to (p. 209) which indicates the requirement for a round of cell division in both T- and B-cells before these can mature into effector cells. Clear evidence for this requirement is not available in the case of tolerance induction. The demonstration of antigen-induced tolerance without the need for cell division would be strong support for a direct effect of an antigen-membrane interaction. It has been argued (Chiller and Weigle, 1972) that rapid inactivation of antigen-sensitive

cells indicates tolerance induction by a direct antigen-cell interaction, while a lengthy period for induction indicates a requirement for an intervening maturational step. Evidence consistent with both situations has been presented by several workers. For example, Golub and Weigle (1967) and Das and Leskowitz (1970)found that a 4 day period was necessary *in vivo* for the induction of tolerance to serum protein in rabbits and mice. Since *in vivo* kinetic studies have shown that mouse lymphocytes, stimulated by sheep erythrocyte antigens, can divide in a matter of hours (Perkins, Sado and Makinodan, 1969), these findings suggest the need for cell division as part of the cycle leading to tolerance, at least in the whole animal. On the other hand, Diener and Armstrong (1969), using *in vitro* technics to study the effect of flagellin antigens on rat lymphocytes, and Chiller *et al.* (1971), using cell transfer methods to study HGG in mice, were able to show tolerance induction in 6 to 18 hours. This finding suggests that individual cells may be rendered tolerant without the necessity for cell division. However, Diener and Armstrong reported that trypsin treatment of toleragen-treated cells *in vitro* within 4 days of the toleragen treatment permitted full recovery of immunological responsiveness. Additionally, Moller and Kashiwagi (1972) reported the induction of tolerance *in vitro* using antigen and non-specific mitogens, when the mitogens were used in high concentration, suggesting the possibility of some mechanism in addition to the direct action of anitgen on lymphocytes.

A particularly facile explanation of the sequence of

events leading to the induction of immunologic responsiveness or immunologic tolerance is that of Feldmann and Nossal (1972). Here, a model requiring interaction among antigen, activated T-cells, secreted T-cell immunoglobulin, macrophages and B-cells is proposed. In this model activated T-cells secrete a carrier-specific immunoglobulin (IgT) which, when complexed with antigen via carrier determinants, binds to macrophages. If it is accumulated in the proper density on the macrophages to initiate multipoint binding to B-cells, it facilitates B-cell triggering and production of circulating antibody. Tolerance, on the other hand, derives from a stoichiometric imbalance among the several components, resulting in either too little or too much of the IgT "collaborative factor". Too much of the factor could induce tolerance via interaction of IgT-ag complexes directly with B-cells resulting in excessive cross-linking of the B-cell membrance and to cell tolerance. Too little of the factor would result in the failure to trigger adequate numbers of B-cells. It is easy to visualize the functioning of the model in a high-zone tolerance situation, but the explanation for low-zone tolerance appears weak, if the assumption holds that polymerization of antigen-antibody complexes is required to induce either tolerance or immunity. In the situation of underproduction of IgT, it is also difficult to conceive of just why the animal would not respond to a second dose of antigen by

increasing the synthesis of IgT, unless one assumes some metabolic event in the T-cell population that would prevent such a response.

An additional weakness of the model, as it stands, lies in the initiation of the sequence subsiquent to the activation of T-cells. This weakness appears to be due to a failure to trigger isolated T-cells in vitro with either antigen aggregates or antigen-antibody complexes, although induction of tolerance in in vivo triggered T-cells in vitro has been accomplished by these authors with antigen-antibody complexes. If T-cells are activated by the same membrane events required for the activation of B-cells and evidence indicating that they may be has previously been referred to (p. 211), then one must wonder how the necessary aggregation of antigen takes place to permit the triggering event.

Recent evidence (Taylor, 1969; Chiller, Habicht and Weigle, 1971) suggests that low-dose tolerance with serum proteins is essentially T-cell tolerance. Thus, there could be a T-cell-associated metabolic event leading to the underproduction of the IgT "collaborative factor" called for in the Feldmann-Nossal model, but this still requires some antigen-T-cell interaction leading to a toleragenic conversion of the T-cell.

The findings of the present study that both monomeric and aggregated albumin induce low-zone tolerance, and do so at equivalent dosages, suggests a role for an additional step of macrophage processing beyond that required by the

Feldmann-Nossal model. Such macrophage processing would most likely occur prior to contact of antigen with T-cells, and would have something to do with converting the antigen to a form capable of interacting with the T-cell. Although the Feldmann-Nossal model proposes a role for antigen bound to macrophage via an antigen-antibody complex involving cytophilic IgT antibodies, Schmidtke and Unanue (1971) demonstrated that macrophages were capable of binding antigen at their surfaces without the need for sytophilic antibody. These latter authors also studied macrophage-lymphocyte "rosettes" and found them to be examples of macrophage B-lymphocyte conjunctions, making it unlikely that a direct macrophage-T-cell interaction would be an integral part of T-cell triggering.

The findings, cited by Feldmann and Nossal (1972), that T-cells could not be affected in vitro by antigen fragments, monomers or aggregates, although they could be rendered tolerant by antigen-antibody complexes, suggests an in vivo role of antigen-antibody complexes in T-cell triggering. It has been noted that a number of "aggregated" sntigens, including proteins and non-proteins, are capable of stimulating B-cells directly. It has also been observed (Miller and Mitchell, 1969) that IgM responses to T-cell dependent antigens could still be triggered in the absence of T-cells; and that loss of T-cell function interfered more with IgG than with IgM responses (Taylor

and Wortis, 1968). It is therefore suggested that macrophage-bound antigen fragments can interact with B-cells to trigger IgM formation. Complexes of IgM and antigen fragments could then engage in multipoint binding with T-cells to initiate T-cell triggering, thereby stimulating the activated T-cells essential to initiate the Feldmann-Nossal cycle of further B-cell triggering.

In the event that the antigen signal to the T-cells was a toleragenic signal, the T-cells would not be activated, and a quantity of IgT sufficient to permit optimal stimulation of B-cells would not be formed. It is necessary in this regard to distinguish between an insufficient antigenic stimulus and a toleragenic one. Both might have the appearance of low-zone tolerance, since in either case, too few activated T-cells would be formed to permit optimal stimulation of the B-cell population. In the case of an insufficient stimulus, a second, larger dose of antigen should trigger more T-cells and thereby permit a normal response. In the case of a toleragenic stimulus, the T-cells would have been "converted" and would, therefore, be unable to respond to a second dose by converting to the activated T-cells called for in the Feldmann-Nossal model.

Thus, some additional mechanism functioning in the T-cell population is needed to account for low-zone tolerance. In the present experiments, only low-dose tolerance was produced. Such tolerance was optimal when doses of

approximately 0.025 mg of albumin were used, with the depression of the response becoming less noticeable as the dose was increased. Moreover, it was possible to isolate two forms of monomer, one an efficient toleragen at low dosage, the other not. Such a finding cannot be explained by invoking cell-surface phenomena dependent on states of aggregation, since the two forms of albumin were physically similar.

If it is assumed that more than one antigen-dependent step is involved in the activation of a T-cell, then the differential toleragenic and immunogenic properties of the monomeric albumins can be explained. A first step could involve multipoint binding at the membrane in order to activate a mechanism for internalizing the antigen and perhaps triggering the mitotic sequence of the cell. Once internalized, antigen fragments might serve to select and activate some particular gene complex, thereby determining the subsequent function of the cell. For example, Parish (1972) reported that acetoacetylation of flagellin converted this particular protein antigen from an immunogen to a toleragen with respect to antibody formation. T-cells from rats previously exposed to acetoacetylated flagellin did not collaborate with B-cells to permit antibody formation. However, these same T-cells could act as effector cells in cell-mediated immune responses directed against the flagellin. Assuming that this difference in result following triggering with flagellin or acetoacetylated flagellin repre-

sents two alternate pathways of several possible for the antigen directed differentiation of the T-cell, then low dose tolerance becomes explicable. Examples of one type of such a mechanism have been demonstrated in procariotic cells (Jacob and Monod, 1961), where they have been shown to be involved in the regulation of complex multipathway enzyme-substrate systems (Datta, 1969) through the functioning of substrate-dependent proactivators bound at the initiation site for gene transcription. Such mechanisms have also been postulated as essential mechanisms operating in the differentiation and regulation of eucariotic cells (Britten and Davidson, 1969). Positioning of the proactivator (repressor) on the gene prevents transcription. Releasing the repression requires formation of an activator in the form of a complex between the proactivator and its substrate. The activator formed by binding of substrate and proactivator is then released from the initiation site for gene transcription by undergoing an allosteric conversion. Once the activator complex is released, transcription can occur. Regulation of transcription in this type of system occurs in the following manner:

The interaction between proactivator and its substrate occurs by means of weak Van der Waals forces, and is governed by mass action laws. Thus, for a given substrate-proactivator combination, there is a specific dissociation constant K. If the substrate is modified

appropriately, the value of K can be made to decrease, so that at a given concentration, less proactivator and substrate are bound, hence less activator is formed and there is less opportunity for translation of the regulated gene. By increasing the concentration of substrate, more activator complex can be formed, and hence more opportunity for transcription of the gene results. In applying this model to the explanation of tolerance, it is postulated that the carrier region of the antigen molecule would complex with a proactivator. If the fit were good, the gene complex in question would be derepressed and the cell would become activated. If the fit were not good, then at low doses, the activator complex would not form in sufficient quantity, resulting in failure to activate the gene complex. Assuming that the gene complex in question is the one, of perhaps several possible, which must be activated in order to form a Feldmann-Nossal activated T-cell, then failure to activate that particular gene complex in preference to the other alternative gene complexes would result in low-zone or T-cell tolerance. However, by increasing the antigen dose sufficiently, provided the value of the association constant were not too low, it would be possible to activate the T-cells. If the fit between proactivator and substrate were poor enough, no activation would occur at all.

The hypothetical argument presented is, admittedly, highly speculative and unsupported by direct experimental evidence. If methods are found which permit the triggering of isolated T-cells <u>in vitro</u>, it should be possible to

design a direct test of the hypothesis. It may also be possible to derive indirect evidence by examining differences in cell-mediated responses after stimulation of mice with the different forms of albumin. It is not presently known if they would stimulate different cell-mediated immune responses, or if the humoral toleragen would be a good stimulator of the cell-mediated immune response. Such a finding would support the concept of alternate pathways of T-cell activation. However, it would not determine how such pathways are chosen.

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