THE ANTIBODY RESPONSE IN CONVENTIONAL AND GERMFREE ANIMALS TO HEATED AND UNHEATED WHOLE MILK

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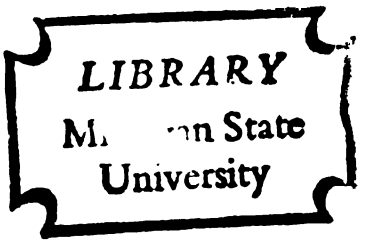

ABSTRACT

To attempt to determine if severely heated whole milk had retained immunogenicity, conventional and germ-free animals were sensitized with whole milk autoclaved for 30 minutes at 121 C. Rabbits were immunized with one of the five antigens listed below: 1) unheated whole milk (UHWM), 2) whole milk heated for 15 minutes at 121 C (WM15), 3) whole milk heated for 30 minutes at 121 C (WM30), 4) beta lactoglobulin (BLG), and 5) alpha lactalbumin (ALA). Conventional and germfree rats were also immunized with WM30 with and without Freund's adjuvant or Bordetella pertussis vaccine.

The interfacial precipitation test, and passive cutaneous anaphylaxis (PCA) in guinea pigs and rats were used to examine all sera for antibody. It was determined with both the interfacial and PCA tests that WM30 had retained immunogenicity for rabbits. Antibodies were not detected in the rat sera.

Antisera produced to the WM30 antigen reacted equally well with the WM30, WM15, and UHWM antigens. The WM30 antisera reacted positively with BLG, but negatively with ALA for both the interfacial and PCA tests, suggesting that BLG is a more heat stable antigen than ALA.

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By

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INTRODUCTION

Because of its availability, inexpensiveness, and nutritive value, bovine milk has become a commonly used food for the human newborn. Bovine milk is not without fault, however, and being a foreign protein to human beings it can induce the production of antibodies which in turn can initiate a hypersensitive state in the human infant. Following the recognition of milk hypersensitivity, several disease syndromes were described in association with serum antibody titers developing in response to bovine milk proteins, including the anaphylactic "cot-death" syndrome described by Parish et al. (1960).

Soon after the clinical recognition of milk hypersensitivity, "heat-denatured" milk products became popular as a substitute for native bovine milk in the diet of the hypersensitive infant. Yet, these "heat-denatured" milk products often failed to alleviate the symptoms of milk allergy. Considerable controversy arose concerning the issue of whether milk could actually be heat denatured to the extent that the protein fractions were incapable of antigenically stimulating the human infant.

It was expected that because of the relatively undeveloped gastrointestinal environment of the germ-free animal, and the absence of intestinal micro-organisms, that the germfree animal would lend itself to a study involving the use of whole milk as an antigen. Therefore, the experimental work reported in this thesis was based on the belief that the germ-free rat might be a suitable animal to use for studying orally administered milk as an antigen.

LITERATURE REVIEW

Milk As An Antigen

Hypersensitivity to bovine milk proteins in children was considered a possibility as early as 1916, when Schloss and Worthen reported their suspicion of permeation of the intestinal tracts of children by undigested milk protein fractions. Another early consideration was that of Park, who in 1920 described a clinical case of hypersensitivity to cow's milk in a young child.

Anderson and Schloss (1923) suggested that cow's milk was capable of inducing an allergic state that was in some way associated with nutritional disturbances in the human infant, noting that the blood of most marasmic infants contained precipitins to bovine milk. Marasmus in infancy is complicated by diarrhea, derangement of the intestinal bacterial flora, decreased secretion of digestive enzymes, and other symptoms of general chronic gastroenteritis. Conditions such as these in the infantile intestine would seemingly facilitate abnormal absorption of unaltered proteins (Gruskay and Cooke, 1955).

In an attempt to correlate clinical milk

hypersensitivity with the presence of antibodies. Anderson, Schloss, and Myers (1925) selected nine children below fed human milk and not known to have been exposed to bovine milk and subsequently fed these children bovine milk in place of human milk. All nine soon developed demonstrable precipitins to bovine milk. Lippard, Schloss, and Johnson (1930), using complement fixation, demonstrated the presence of undigested cow's milk proteins in the blood streams of normal infants soon after a bottle feeding. Lippard (1939) suggested that the individual immune response might differ in those children showing clinical symptoms of milk allergy.

Quite a wide range of clinical symptoms have been reported in association with cow's milk allergy. Some of these are vomiting, diarrhea, abdominal pain, asthma, rhinitis, failure to thrive, chronic cough, otitis media, melena, colic, irritability, eczema, hemoptysis, anemia, cyanosis, shock, upper respiratory symptoms, and death (DuBois, Schloss, and Anderson, 1925; Cruskay and Cooke, 1955; Bachman and Dees, 1957a; Crawford, Kerrigan, and Arnold, 1958; Gunther et al., 1960; Parish et al., 1960; Heiner, Sears, and Knicker, 1962; Huntington and Jarzynka, 1962; Gill and Coombs, 1963; Goldman et al., 1963a; Nelson, Greene, and Stroup, 1963; Nelson, 1964; Sewell et al., 1963; Buckley and

Does, 1966).

Numerous investigators have correlated data from serological tests with the various symptoms of cow's milk allergy. Further, specific diseases or disease conditions such as atopic dermatitis, celiac disease, Aldrich's syndrome, Loeffler's syndrome, pulmonary hemosiderosis, pneumonia, Hurler's syndrome, congenital heart disease, ulcerative colitis, mongoloidism, and the cot-death syndrome have been discussed on the basis of clinical and laboratory data as being associated with hypersensitivity to milk (Krivit and Good, 1959; Gunther et al., 1960; Heiner and Soars, 1960; Heiner et al., 1962; Parish et al., 1960; Truelove, 1961; Huntington and Jarzynka, 1962; Gill and Coombs, 1963; Peterson and Good, 1963; Nelson, 1964; Rothberg and Farr, 1965; Wilken, 1965; Boyd, 1966). Some of these are more relevant than others with respect to the current investigation, and only a few will be reviewed.

Dubois, Schloss, and Anderson (1925) indicated an association between cutaneous hypersensitivity and milk precipitins in the sera of normal and marasmic infants. Freedman (1961), however, disagreed that cow's milk exerts any influence on the course of infantile eczema.

Heiner et al.(1962) and Immonen (1965) attempted

to correlate a high incidence of precipitins to cow's milk and wheat gluten with the malabsorption syndrome of celiac disease. Sewell et al. (1963) noted that withdrawal of milk and all milk products can be a valuable therapeutic measure for treatment of intractable diarrhea associated with some gastrointestinal disorders. After noting that many ulcerative colitis patients improve considerably when cow's milk is excluded from the diet, Truelove (1961) reintroduced milk into the diet of several of these patients. In every instance this was followed by a frank attack of the disease. According to Wilken (1965), seven times as many ulcerative colitis patients have significant antibody titers to milk proteins as do normal control subjects. No speculation was made as to whether the absorption of milk proteins was a cause or a result of ulcerative colitis.

Using the hemagglutination technique, Peterson and Good (1963) reported that 67% of 230 patients with such conditions as chronic recurrent pneumonia, Aldrich's syndrome, Hurler's syndrome, and congenital heart disease had a significant antibody titer to cow's milk. Rothberg and Farr (1965) agreed that precipitins to milk proteins appear more frequently in the sera of children with chronic pulmonary disease than in normal controls.

Nelson (1964) found an unusually high incidence of hemagglutinins and precipitins to milk proteins in the sera of mongoloid children. Mongoloids characteristically have a high incidence of pneumonia, upper respiratory symptoms, and iron deficiency anemia.

Wilson, Heiner, and Lahey (1962) detected higher levels of milk precipitins in patients with iron deficiency anemia than in normal control populations. It was not possible to state whether these precipitins were a cause or result of the iron deficiency anemia, although the suggestion was made that one form of anemia might be of a secondary nature, depending on primary sensitization with cow's milk.

The cot-death syndrome is one of the most highly publicized disease states to be attributed to milk hypersensitivity (Gunther et al., 1960; Parish, Barrett, and Coombs, 1960; Parish et al., 1960; Gill and Coombs, 1963; Boyd, 1966). The term crib-death, or cot-death, is used when the infant is found dead after a night's sleep, with no apparent symptomatology or prior disease condition. Neither the parent, physician, nor pathologist can find valid reason for the death, the only consistent lesions being pulmonary edema and congestion. The pathologist's report often cites acute interstitial pneumonia or mechanical asphyxia as being the cause of death (Huntington and Jarzynka, 1962). These cot

deaths may account for over 20% of the mortality in children under one year (Johnstone and Lawy, 1966). Extensive bacteriological and virological studies have been conducted on autopsy samples of lung tissue, but the vast majority of those attempts have been unsuccessful. Johnstone and Lawy (1966), however, have reported some success in isolation attempts, and believe that many of these deaths are due to lower respiratory tract infections. Peterson and Good (1963) failed to detect significant levels of precipitins in serum samples from six cases of the crib-death syndrome, indicating that the hypothesis of Parish, as mentioned in the introduction, may not always apply.

Before further consideration is given to the way in which milk acts to induce hypersensitivity, the composition of milk in regard to its antigenicity will be briefly mentioned.

Milk can be defined as the lacteal secretion of the mammary gland intended for the nourishment of young. To meet this requirement, milk must be, and indeed is, one of the most complete foods available. The general composition of bovine milk is as follows: water 87.20%, fat 3.80%, carbohydrates 4.95%, protein 3.35%, and minerals 0.70% (Herrington, 1948). These figures vary considerably with the breed, species, and the time elapsed since the start of lactation within the

individual.

From the standpoint of antigonicity in heterologous species only the protein fraction is significant. Kon and Cowie (1961) break down the nitrogenous compononts of bovine milk in the manner described in Figure 1.

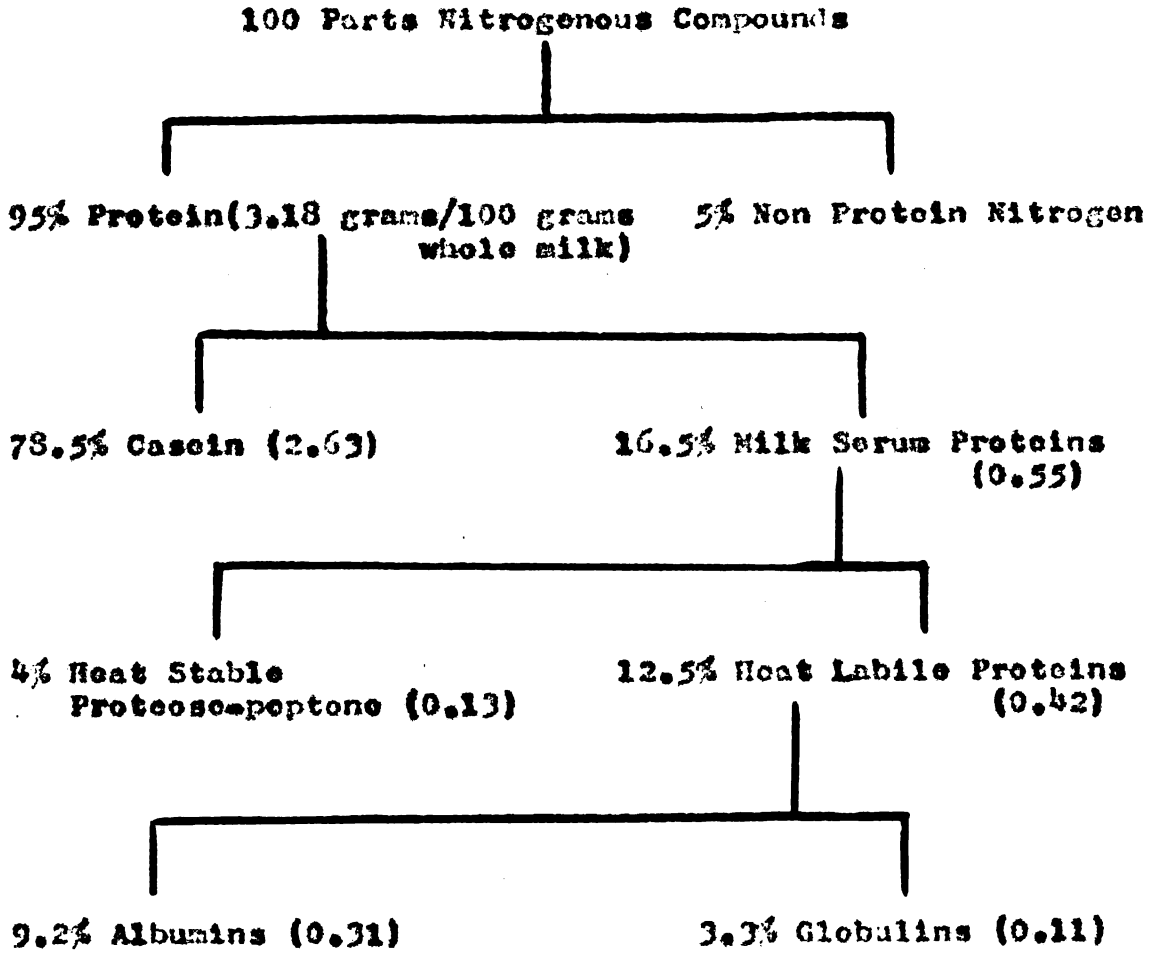

Figure 1. Nitrogenous compononts of bovine milk.

The major fractions of bovine milk proteins and some of their characteristics are listed according to Jenness et al. (1956) in Table 1.

Table 1. Major fractions of bovine milk proteins.

Protein	Fraction from which isolated	Approx. % of milk proteins	Molec. wt.
alpha casein	casein	45-63	?
beta casein	casein	19-23	24,000
gamma casein	casein	3-7	?
beta lactoglobulin	lactalbumin	7-12	35,000
alpha lactalbumin	lactalbumin	2-15	15,100
blood serum albumin	lactalbumin	0.7-1.3	65,000
euglobulin	lactoglobulin	0.8-1.7	180,000
pseudoeuglobulin	lactoglobulin	0.6-1.4	180,000

Originally the milk proteins were separated only on differences in solubility, and were classified as casein, lactalbumin, and lactoglobulin fractions (Jenness et al., 1956). A fourth fraction, designated "proteose-peptone," was isolated by Rowland (1938).

Casein is the protein fraction in milk precipitated by acidifying raw skim milk to pH 4.6-4.7. It comprises 82% of the proteins, and consists of a mixture of five principal components: 1) alpha-1-casein, 2) alpha-2-casein, 3) beta casein, 4) gamma casein, and 5) delta casein (Fries, 1959). These components exist in milk as complex particles containing calcium and phosphate (Jenness et al., 1956; Brunner et al., 1960;

Kon and Cowie, 1961).

Approximately 17% of the protein fraction of skim milk is the whey or milk serum proteins. As opposed to casein, this fraction is soluble when milk is acidified to pH 4.6-4.7. An additional one percent of the total protein is present in whole milk only, as the lipoprotein designated as fat globule membrane protein (Jenness et al., 1956; Fries, 1959; Brunner et al., 1960).

The milk serum proteins are divided into heat stable and heat labile fractions on the basis of whether or not the protein can be rendered acid precipitable at pH 4.6 by previous boiling for 20 minutes. The "proteose-peptone" fraction is that part that is not rendered acid precipitable by prior boiling, and is thus termed heat stable (Jenness et al., 1956; Brunner et al., 1960; Kon and Cowie, 1961). The heat labile group of proteins which is rendered precipitable at pH 4.6 by previous boiling is further classified into two fractions, the albumins and the globulins.

One group of the heat labile milk serum proteins, the albumins, represent the most significant antigens from the standpoint of the individual who is allergic to milk. The principal components of the albumin fraction are alpha lactalbumin, beta-1-lactoglobulin, beta-2-lactoglobulin, serum albumin, and peroxidase (Fries, 1959). The globulins, although of major importance

from the standpoint of immunity within the individual, are of minor significance as an antigen, and are represented as the gamma globulins pseudoeuglobulin and euglobulin (Jenness et al., 1956; Fries, 1959).

Electrophoretic analyses have revealed at least twelve distinct antigenic factors associated with bovine milk proteins (Hanson and Johanson, 1959). Six of these are also related to bovine blood serum proteins (Hanson, 1959).

Due to its antigenic complexity, Fries (1959) mentions that milk should not be treated as a single allergen, but rather as a complex of many allergens. The three proteins that are most significant as allergens are alpha lactalbumin, beta lactoglobulin, and casein. Of lesser importance is bovine serum albumin.

Most pediatricians agree that some infants who are hypersensitive to cow's milk can tolerate goat's milk while others cannot (Hill, 1939; Saperstein, 1960). One reason for this is that the casein fraction is immunologically almost identical between the species, but that the alpha lactalbumin, beta lactoglobulin, and serum albumin are species specific (Wells, 1911; Hill, 1939; Fries, 1959; Lee, 1965). Thus, those individuals hypersensitive to the caseins of bovine milk could probably not tolerate goat's milk, while those individuals who are hypersensitive to the heat labile milk

serum proteins would often be able to incorporate goat's milk into their diet without difficulty.

It is generally assumed that an antigen must be introduced beyond the epithelial tissues of an animal before antibody production will be stimulated (Carpenter, 1965). Milk has been injected into neonates for experimental purposes, however, it is obvious that a more subtle manner of sensitization must be involved in the process of natural induction of hypersensitivity (Hodder, 1873; Hedenstadt and Heijkensjold, 1965). At least three methods for natural sensitization to milk proteins have been considered in the past, and these will be discussed.

The first possible route for sensitization, suggested by Ratner (1928), involves the passive transfer of milk protein antigen across the placenta from the mother to the fetus. It is known that during pregnancy many women have a psychic craving for certain foods, and the excessive consumption of these foods, if they happen to be milk or milk products, could thus lead to excessive absorption of milk protein antigens (Ratner, 1957). These protein antigens would then be transmitted to the fetus via the common circulatory system, and thus antigenically stimulate the fetus to produce antibody (Ratner and Greenburgh, 1932; Ratner, Crawford, and Flynn, 1956). It would seem that the

validity of this theory would depend on whether the human fetus could be declared immunologically competent. Passive maternal-fetal circulatory transmission of maternally produced antibody has also been considered (Gunther et al., 1960).

The second possible route of sensitization is aspiration of gastric contents, which in the non-breast fed infant would usually consist of bovine milk (Parish et al., 1960). Milk proteins present in the lung might then be absorbed across the alveolar membrane into the interstitial tissue and circulation with resulting antigenic stimulation (Nelson, 1964). In support of this explanation are Peterson and Good's (1963) findings of substantially higher milk protein antibody titers in children with anatomically defective swallowing mechanisms than in normal children.

The third and probably predominant route of sensitization to milk proteins is intestinal absorption. Early investigations by Mendel and Rockwood (1904) were interpreted as indicating that proteins could be absorbed intact from the intestine. They tied off a section of the intestinal tract, and demonstrated significant reduction of a protein substance, edestin, that had been placed within the segment.

Borgstrom et al. (1957) demonstrated that under normal conditions up to ten percent of the dietary

protein reaches the bottom of the small intestine without being digested. Mills et al. (1923) and Ratner and Gruehl (1934) believe that under normal conditions absorption of unaltered protein occurs with great regularity, and that proteins may enter the blood stream from any part of the bowel, including the rectum.

Three criteria have been proposed for oral sensitization with milk proteins: 1) the protein fractions must be in a native state, soluble, dialyzable, and of low molecular weight, 2) the intestinal tract must be particularly permeable at the time the food is ingested, and 3) the quantity ingested must be sufficient for absorption to take place (Ratner, 1937). Holland et al. (1962) suggested that the incidence of milk precipitins in the blood of infants could be associated with increased exposure to antigen, increased permeability of the gut to protein, or variations in host response to physiologic quantities of antigen.

Park (1961) and Nelson (1964) have suggested the need for a genetic predisposition to hypersensitiveness before sensitization to milk proteins can occur.

Two lines of defense against transmission of unaltered proteins across the intestinal wall are that protein foods are normally hydrolyzed by enzymatic digestion in the gastrointestinal tract, and that the intestinal wall is usually thought to be impermeable to

colloids (Ratner, 1957). The failure of these defenses may result from alteration of the intestinal mucosa, permitting increased absorption of undigested protein into the circulation (Nelson, 1964). These defenses may fail due to pathologic conditions, developmental anomalies, malnutrition, convalescence from diarrhea, following gastrointestinal disturbances, or increased permeability in early infancy (Gruskay and Cooke, 1955; Ammonen, 1965). With ulcerative colitis patients, increased absorption of antigenic protein from the gut might occur for at least two separate reasons: depressed proteolytic activity in the upper gastrointestinal tract, or more facile absorption of undigested protein by the inflamed colonic mucosa (Taylor and Truelove, 1961).

There is reason to believe that the intestinal environment of the germ free animal is very similar to the intestinal tract of the newborn (Smith, 1965). This similarity is largely due to the lack of an intestinal bacterial flora, which the conventional animal acquires soon after birth. This bacterial flora is probably largely responsible for stimulating histological changes in the small intestine that discourage the further absorption of large molecules (Lagercrantz et al., 1965; Smith, 1966).

Clark (1959) identified orally ingested bovine gamma globulin and ovalbumin in the apical cytoplasm

of the jejunum and ileum of rats and mice that had been fed these proteins. He postulates that the cell takes in these particles by pinocytosis, a process of invagination of the apical cell membrane to form vacuoles containing material from the intestinal lumen. Eighteen days after birth, however, the columnar absorptive cells lost their ability to ingest proteins and colloids, this being the age at which rats and mice lose the ability to absorb antibodies from the intestine in an immunologically intact form (Clark, 1959). Anatomical changes in the columnar absorptive cells accompany these changes in function.

Particles absorbed by the epithelium of the small intestine can be picked up by the intestinal lymphatics, and transported to the blood stream. Ingestion by columnar absorptive cells appears to be a non-selective mechanism for taking in whatever happens to be in the environment. Labelled proteins absorbed in this manner can be recovered in the blood within ninety minutes from the time of oral ingestion (Clark, 1959).

Farr, Dickenson, and Smith (1960) fed bovine serum albumin to rabbits in the drinking water, and found that a 0.0025% solution for thirty days, or a 0.25% solution for one day was able to elicit the production of a sufficient antibody titer to be detectable with the ammonium sulfate technique. Antibody was

detected as early as seven days after the first feeding of a 0.1% solution. Intravenous injection of a non-antigenic dose of 0.006 mg. of bovine serum albumin, however, resulted in a higher blood level of bovine serum albumin after seven days than did the feeding of a 0.25% solution at any time. Thus it is expected that immunization by the oral route is initiated by lymphoid tissue associated with the intestinal tract, where higher concentrations of bovine serum albumin are available (Farr, Dickenson, and Smith, 1960). These sensitized lymphoid cells could then be transferred to other sites of antibody production such as the spleen and lymph nodes (Farr and Dickenson, 1961; Holland et al., 1962). Intestinal permeability is greater in the newborn, and immunologically competent cells of the newborn lie in the gut wall, making them quite accessible to antigens in the gut (Hinkle, Hong, and West, 1961).

Rothberg, Kraft, and Farr (1966,1967) showed that antibodies produced to either orally or parenterally administered bovine serum albumin were qualitatively similar when tested by radioimmunoelectrophoresis, passive hemagglutination, 2-mercaptoethanol treatment, detection of antibody in sucrose density gradient fractions of serum, ammonium sulfate test, antigen precipitating capacity, and density gradient

ultracentrifugation.

Soon after the advent of commercially prepared evaporated milk, it became evident to pediatricians that heat denaturation of milk proteins significantly reduced their antigenicity. This loss of antigenicity was probably due to at least two factors: 1) actual destruction of the antigenic portion of the molecule by heat denaturation, and 2) increased susceptibility to enzymatic breakdown in the gut with consequential reduction in protein available for intestinal absorption.

Denaturation of a protein implies any change in physical and chemical properties from the native state including especially the loss of solubility at the isoelectric point for that protein, but also changes in electrophoretic mobility, and reduction of sulfhydryl groups (Maurer and Heidelberger, 1951; Larson and Rolleri, 1955; Saperstein and Anderson, 1962). These changes can be brought about by a large variety of chemical and physical agents including enzymes, irradiation, acids, alkalies, alcohol, acetone, salts of heavy metals, dyes, heat, light, and pressure (Ratner, 1957; Grogan and Crawford, 1961). The stages of denaturation are not necessarily all or none with many proteins, and some stages of thermal denaturation are reversible.

Denaturation of native proteins often results in loss or diminution of specific immunologic properties

because, due to their physical structure, coagulated proteins are delayed in the gastrointestinal tract, thus giving more time for enzymatic digestion (Lewis and Hayden, 1932; Ratner, 1957). Also, as evidenced by the effects of trypsin on proteins in vitro, digestive enzymes are more effective per unit time on partially denatured proteins than on native proteins (Wallen-Lawrence and Koch, 1930). This results in a marked loss of ability for the milk proteins to antigenically sensitize the individual by the oral route (Ratner and Gruehl, 1935).

Denatured proteins may act as simple or complex haptens, to block a reaction between antibody and native antigen without producing a visible reaction (Saperstein and Anderson, 1962). Some denatured proteins may act as antigens to induce production of antibodies to the denatured protein as well as to the unaltered protein. Habeeb and Borella (1966) found that the denaturation of bovine serum albumin in which the disulfide bonds were broken resulted in a loss of reactivity between antibody produced against the denatured bovine serum albumin and undenatured bovine serum albumin.

There is considerable dissension in the literature regarding the issue of whether a heated protein antigen has retained sufficient strength and specificity

to act as an allergen when orally administered. In the case of allergy to bovine milk, certain researchers feel that individuals hypersensitive to milk could tolerate a heat denatured product (Ratner, 1957; Crawford, 1960; Crawford and Grogan, 1961; Hinkle, Hong, and West, 1961). Other investigators, in contrast, believe that denatured milk is not a safe substitute for milk in the diet of hypersensitive individuals, and that instead such products as soy milk and meat base milks should be used (Saperstein and Anderson, 1962; Heiner, Sears, and Kniker, 1962; Lus and Todd, 1964). Saperstein and Anderson (1962) have suggested that the term heat denatured milk can be abandoned as a designation implying non-antigenicity or non-allergenicity of the proteins in these products.

Ratner and his associates believe that the majority of children hypersensitive to milk can safely consume heat treated milk, and that less than one fourth actually require a milk substitute (Ratner, Crawford, and Flynn, 1956; Ratner, 1957; Ratner et al., 1958a). According to Ratner, the lack of a favorable response to a heat treated milk in this latter 25% is due to a casein hypersensitivity. Saperstein (1960) believes that no evidence has been given for casein hypersensitivity, and that suspected cases were due to incorrect diagnoses made with casein that was

contaminated with lactoglobulin or lactalbumin. It cannot be disputed that incomplete purification of the milk protein fractions has resulted in many misleading conclusions, and early studies were carried out with casein fractions contaminated with lactalbumin and lactoglobulin, and lactalbumin fractions contaminated with lactoglobulin and serum albumin (Ratner et al., 1958a; Cole and Lees, 1963).

It would seem that the newly acquired specificity of the antigen should be considered as well as the original quantity of native protein remaining when trying to determine the value of a heat denatured milk product for use in the diet of hypersensitive individuals. Synthetic polypeptides of low molecular weight are antigenic in rabbits and guinea pigs, suggesting that severely denatured proteins could still be antigenic (Maurer, 1962, 1963; Maurer, Gerulat, and Pinchuck, 1963; Levine, 1965a,b). To be considered also is the fact that the ability of a substance to act as an antigen based on classical serological techniques of antibody detection is not necessarily indicative of the allergenic properties of that substance (Hanson and Mansson, 1961).

Furth (1925) noted that the heating of horse serum or crystalline egg albumin to 100 C created a new antigenic specificity. Antisera obtained by

injecting antigen heated to 100 C precipitated strongly the antigen used in its production, but caused only weak precipitation of unheated protein. Cutler (1929) found that heated whey proteins were more reactive in animals sensitized with heated milk than in animals sensitized with raw milk. Lewis and Haydon (1932) found that heating altered some antigens so that antisera produced against them were more or less specific for the heated antigens, the so-called cocto-antigens. Included in this group was bovine milk that had been heated to 116 C for 15 minutes.

Hirata and Sussdorf (1956) found that the partial denaturation of bovine serum albumin created a new antigenic specificity. When this partially denatured bovine serum albumin was injected into rabbits, it resulted in higher antibody titers and a larger number of reactors than did native bovine serum albumin when each antigen was tested with its homologous antiserum. Peters and Coetzl (1965) state that appreciable configurational changes can be induced in the bovine serum albumin molecule without markedly altering its antigenic properties, and that immunologic determinants unreactive in the native molecule may be uncovered by manipulations which do not break the peptide chain. Chromatography of a tryptic hydrolysate of beta lactoglobulin by Bing, Gusdon, and Stavitsky (1966) yielded

seven active peptide haptens. These inhibited the hemagglutination, precipitation, and passive cutaneous anaphylaxis reactions in a beta lactoglobulin anti-beta lactoglobulin system.

Although it is usually accepted that casein is the most heat stable protein in whole milk, considerable disagreement exists concerning the comparative heat stability of the more heat labile whey proteins. Larson and Rolleri (1955) concluded that alpha lactalbumin was the most heat stable of the whey proteins. Crawford and Grogan (1961), Collins-Williams (1962), and Lus and Todd (1964) maintain that alpha lactalbumin is the more heat labile, and that beta lactoglobulin is the most heat stable of the whey proteins. Hansson (1965) believes that serum albumin is the most heat stable of the serum proteins.

Wells (1908) stated that crystalline egg albumin and bovine milk do not lose their sensitizing power for anaphylaxis when heated to 100 C for 30 minutes. Attempts by Ratner et al. (1938c) to orally sensitize or challenge guinea pigs with heat denatured milk were invariably unsuccessful. Lus and Todd (1964) found that milk that had been heated to 245 F for 19 minutes showed only slight differences in antigenicity from unheated milk when tested by the Prausnitz-Kustner technique. Micro-Ouchterlony techniques show that

heating causes a loss of precipitability with the native protein, but in this case such a loss is not necessarily equivalent to a loss of antigenicity or allergenicity (Lus and Todd, 1964).

In 1929, Cutler failed to detect any significant differences in the degree of anaphylaxis elicited with caseins from pasteurized or commercially available evaporated milk that had been exposed to 220 F for 40 minutes or 240 F for 20 minutes. Rowland (1937) believed that denaturation of alpha lactalbumin and beta lactoglobulin was complete in five to ten minutes at 100 C. Brown, Aurand, and Roberts (1961) found that pasteurisation caused negligible milk protein denaturation, but autoclaving at 250 F for 15 minutes denatured all the whey proteins. According to Hanson (1965) casein can withstand 120 C for at least 15 minutes, and beta lactoglobulin 100 C for at least 15 minutes. Hanson and Hansson (1961) using immunoelectrophoresis could not demonstrate the presence of whey proteins from milk heated to 120 C for 15 minutes.

Ratner et al. (1958b) on the basis of systemic anaphylaxis tests, state that the allergenicity of alpha lactalbumin was eliminated in a heat denatured milk product, and that even casein evidenced a degree of heat lability. Cole and Dees (1963) claim that beta lactoglobulin in milk exposed to evaporation is

only partially denatured and still able to elicit anaphylaxis when guinea pigs are sensitized with unheated beta lactoglobulin.

Saperstein (1960) found that both alpha lactalbumin and beta lactoglobulin in commercial evaporated milk products were still antigenically intact. In a later study, Saperstein and Anderson (1962) concluded that heat treatment had some effect on alpha lactalbumin, but did not reduce the antigenicity of casein or beta lactoglobulin. With precipitation tests, milk products heated for ten minutes at 110 C or 30 minutes at 99 C all gave positive precipitates with beta lactoglobulin, alpha lactalbumin and casein. Three hour heating at 99 C did not inactivate the alpha lactalbumin, beta lactoglobulin or casein as tested by passive cutaneous anaphylaxis (Saperstein and Anderson, 1962).

According to Fries (1957, 1959) allergy to cow's milk is not as frequent as uncritical evaluation would make it seem, and many disturbances induced by milk should not be categorized as allergic in nature. The only satisfactory way to diagnose milk allergy is to show that symptoms are alleviated with removal of milk from the diet, and that they return when milk feedings are restored (Gold and Robbins, 1965).

Recorded incidences of clinical symptoms of cow's milk allergy in randomly selected children has

varied from 0.2% to 7.0% (Clein, 1954, 1958; Collins-Williams, 1956; Glaser, 1956; Bachman and Dees, 1957a). Based on clinical symptoms, reported incidences of sensitivity to cow's milk in allergic groups varied from 5.0% in asthmatics to 55% in allergic children under four years of age (Davidson, 1942; Fries, 1957). In allergic patients under two years, the incidence of hypersensitivity to cow's milk is high, being present in approximately one in three infants (Bachman and Dees, 1957b).

Ratner et al. (1953a) and Collins-Williams (1962) attribute the greatest allergenicity to milk proteins to the beta lactoglobulin fraction. Fries (1957) and Cole and Dees (1963) state that sensitization to milk is usually to the lactalbumin fraction. Goldman et al. (1963a) orally challenged children known to be allergic to milk with the milk protein fractions. The frequencies of reactions to the various fractions were: casein 57%, bovine serum albumin 51%, beta lactoglobulin 66%, and alpha lactalbumin 54%.

Detectable antibody titers to bovine milk in both healthy and allergic infants are considerably more common than clinical signs of milk allergy.

The precipitation test, as performed by the gel diffusion or capillary tube technique, consistently detects only the high titered sera. Twenty-five

percent of 170 normal and sick infants had precipitins to cow's milk by the gel diffusion technique (Peterson and Good, 1963). Precipitins are more frequently found in sera of children with chronic pulmonary disease or iron deficiency anemia than in normal controls. The incidence as determined by gel diffusion in iron deficient infants fed cow's milk prior to diagnosis was 75% to 80% (Wilson, Heiner, and Lahey, 1962).

Gunther et al. (1960) found that 96% of 286 serum samples taken from normal infants had hemagglutinin titers up to 1000 with a mode of 64. Similar samples from healthy adults and newborn infants before exposure to bovine milk showed titers of zero to four for the most part, but samples from infants seven to 97 weeks old had titers of two to 128 with a mean of 16 to 32. Saperstein et al. (1963) and Collins-Williams and Salama (1965) found similar patterns of hemagglutinins for normal children, children allergic to milk, and allergic children that were not allergic to milk. These findings disagree with those of Gunther et al. (1962) and Parish et al. (1964) that high hemagglutinin titers to milk proteins are correlated with susceptibility to anaphylaxis in infants. Peterson and Good (1963) found that 67% of 288 serum samples from both healthy and diseased children had a milk protein hemagglutinin titer of ten or more. The highest titers

of hemagglutinins were obtained from the sera of significantly disabled children. All sera with hemagglutination titers over 1,280 had precipitins also, but most sera showing hemagglutinating activity in low titers only did not have precipitins.

Skin testing techniques did not correlate with either hemagglutination or precipitin titers, giving negative tests with sera showing the highest positive in vitro titers by hemagglutination or precipitation, and sera with lower hemagglutinin or precipitin titers occasionally giving positive skin tests (Peterson and Good, 1963). Antibody specific for bovine serum albumin by the ammonium sulfate technique was in no way related to positive skin tests for milk or bovine serum albumin (Rothberg and Farr, 1962). Ratner, Crawford, and Flynn (1956) and Goldman et al. (1963b) report that strongly positive skin tests with purified milk proteins have considerable diagnostic value.

Gunther et al. (1960), using the hemagglutination technique, found antibody specific to casein, alpha lactalbumin, and bovine serum albumin in the serum of normal infants. No antibody was found specific to beta lactoglobulin. In contrast, Peterson and Good (1963) found precipitins to all milk protein fractions except casein in normal and allergic children. In these studies, the most common antibody to a milk protein fraction was

anti-beta lactoglobulin.

Using the ammonium sulfate test, Rothberg and Farr (1962) found circulating antibody to bovine serum albumin in serum samples from 72% of the children and 17% of the adults tested. Thirty-seven percent of the children and seven percent of the adults had antibody to alpha lactalbumin. Thus, although there is considerably more alpha lactalbumin than bovine serum albumin in bovine milk, the bovine serum albumin seemed to act as a more efficient antigen. However, it should also be considered that bovine serum albumin would be present in any beef consumed, whereas alpha lactalbumin would not (Farr, 1959).

Groups of healthy and diseased children showed no differences in incidence of antibodies to bovine serum albumin or alpha lactalbumin (Rothberg and Farr, 1963). It was concluded that when an immediate type of hypersensitivity was associated with the presence of circulating antibody, that antibody is necessary but not alone sufficient to cause symptoms on re-exposure to the antigen. Other factors such as variation of the type of antibody produced, end organ susceptibility and reactivity, permeability of the blood gut barrier, and dose of antigen or re-exposure all determine whether an individual will have an untoward reaction.

The possibility of a cross reaction between

antibody produced against bovine milk protein antigens, especially bovine serum albumin, and other heterologous antigens has been considered by Weigle (1962), Weigle and McConahey (1962), and Rothberg and Farr (1965). No shared antigenicity was detected between bovine serum albumin and other antigens to which the population might be frequently exposed, such as ovalbumin, polio vaccine, influenza vaccine, tetanus toxoid, diphtheria, and pertussis vaccine. In vivo reactions between rabbit anti-bovine serum albumin and ten cross reacting mammalian albumins were capable of producing systemic anaphylaxis, passive cutaneous anaphylaxis, and the Arthus phenomenon in sensitized guinea pigs. When equal amounts of precipitating rabbit anti-bovine serum albumin were used to passively sensitize the animals, some of the cross reacting albumins produced as severe symptoms as the homologous bovine serum albumin when a sufficient amount of antigen was used in the challenge dose (Weigle, 1962).

Saperstein et al. (1963) concluded that the passive cutaneous anaphylaxis test was more valid than tanned cell hemagglutination techniques, double gel diffusion, or skin testing techniques for determining titers of milk protein antibodies in the sera of allergic children.

Minden, Reid, and Farr (1966) studied the

relative sensitivity of several serological tests for their respective capacity to detect circulating antibody to bovine serum albumin in human sera. Those studied were double gel diffusion, hemagglutination, Praunitz-Kustner, passive cutaneous anaphylaxis, systemic anaphylaxis, radioimmunoelectrophoresis, spontaneous precipitation, and the ammonium sulfate test. The only consistently positive tests for detection of antibody were the ammonium sulfate and radioimmunoelectrophoresis techniques. Seventy-five percent of the sera were positive by hemagglutination, and 50% were positive by spontaneous precipitation. The remaining tests were considerably less sensitive in this study.

Antibody Production In The Rat

The rat has to be considered a poor antibody producer when exposed to commonly used antigens by conventional methods (Raffel, 1961; Kabat and Meyer, 1961; Benovitz and Tragani, 1963). Although this weak response becomes especially evident when trying to induce anaphylaxis in this species, rats are also relatively poor producers of precipitins and agglutinins (Lipton, Stone, and Freund, 1956; Wimebright and Fitch, 1962; Cody and Code, 1963). Anaphylaxis is also difficult to produce in the mouse, although this species is usually not as unreactive as the rat (Kind, 1957). This resistance to anaphylaxis is probably associated with the fact that both the rat and mouse are extremely tolerant to the effects of histamine, being unaffected by a dose at least five hundred times as large as that which will kill the guinea pig (Drill, 1958).

Despite the difficulties involved, early workers Parker and Parker (1924), Dellaway (1930), and Hochwald and Rackemann (1946) reported success in attempts to produce anaphylaxis in rats. More recently, a wide variety of techniques have been employed for increasing the rat's ability to respond to sensitization and subsequent challenge with foreign proteins. These include hypophysectomy, fasting, insulin induced hypoglycemia, the administration of ascorbic acid, thyroid

hormones, zymosan, Brucella abortus vaccine, Freund's adjuvant, Bordetella pertussis cells, and Bordetella pertussis vaccine (Hochwald and Rackemann, 1946; Parfentjev and Goodline, 1948; Malkiel and Margis, 1952a,b; Dews and Code, 1953; Kind, 1957; Sanyal and West, 1958; Spencer and West, 1962; Munoz, 1963; Adamkiewicz, Sacra, and Ventura, 1964; Binaghi and Benacerraf, 1964b).

A partial list of the protein antigens used in the successful antigenic sensitization of rats includes human serum, horse serum, egg albumin, bovine serum albumin, human serum albumin, human gamma globulin, bovine gamma globulin, fibrinogen, the flagellar antigen from Salmonella typhosa, and the cell extract from Nippostrongylus brasiliensis (Hochwald and Rackemann, 1946; Sanyal and West, 1958; Winebright and Fitch, 1962; Binaghi and Benacerraf, 1964a; Wilson, Bienenstock, and Bloch, 1966). Mota (1964a) found that egg albumin was a more effective antigen than human serum albumin, rat gamma globulin, horse gamma globulin, human gamma globulin, or bovine gamma globulin for the production of antibody in the rat as tested by the passive cutaneous anaphylaxis and hemagglutination techniques.

Various routes of antigen injection have been employed in the rat, including intraperitoneal, subcutaneous, intravenous, and intramuscular routes

(Mota, 1964a). Using ten microgram doses of antigen, Winebright and Fitch (1962) found negligible differences between the effectiveness of intraperitoneal, intravenous, and subcutaneous routes of injection for the primary production of agglutinins to the particulate flagellar antigen of Salmonella typhosa in normal and splenectomized rats. With a smaller dose of antigen, 0.001 micrograms, the intravenous or intraperitoneal routes were more effective than the subcutaneous route. Mota (1958) reported that sensitization by the intraperitoneal route is more effective than the subcutaneous route for producing mast cell disruption and the appearance of detectable plasma histamine. Binaghi and Benacerraf (1964b), Mota (1964a), and Binaghi, Oettgen, and Benacerraf (1966) report that subcutaneous injection of antigen into the footpad, along with Freund's adjuvant or Bordetella pertussis organisms is the most effective procedure for the production of rat anaphylactic antibody.

The two most common methods of sensitizing rats for the production of anaphylactic antibody includes the injection of the antigen; a) with Bordetella pertussis organisms or vaccine, or b) with complete Freund's adjuvant.

Rats immunized with bovine serum albumin and complete Freund's adjuvant produce high precipitin

titers, which Lipton, Stone, and Freund (1956) believe to be correlated with susceptibility to systemic anaphylactic death. Mota (1964a) found that the rat produced a high titer of hemagglutinating antibody when injected with ten milligrams of egg albumin with complete Freund's adjuvant intramuscularly every week for two months plus two injections of alum precipitated egg albumin intramuscularly at a two week interval. This same procedure resulted in no detectable passive cutaneous anaphylaxis antibody titer. Munoz (1963) reported that susceptibility to fatal anaphylaxis in mice was equally enhanced whether complete Freund's adjuvant or Bordetella pertussis cells were used with a large dose of antigen; but with a small dose of antigen, Freund's adjuvant was more effective. Binaghi and Benacerraf (1964a) concluded that for the production of antibody demonstrating activity by the passive cutaneous anaphylaxis test, the use of pertussis vaccine as an adjuvant resulted in an earlier primary response than did the use of Freund's adjuvant. By the eleventh day, serum titers of antibody, as measured by the passive cutaneous anaphylaxis test, were essentially equal, whether pertussis vaccine or Freund's adjuvant had been employed.

Probably the most widely accepted technique for inducing anaphylaxis, or the production of anaphylactic

type of antibody in the rat or mouse, is the administration of Bordetella pertussis cells or vaccine with the antigen. Mice injected with pertussis vaccine are more susceptible to histamine, serotonin, passive and active anaphylaxis, the gram negative bacterial vaccines, and infection with living bacteria; although in these cases the mouse is not in a debilitated condition (Kind, 1958).

Because histamine and serotonin are the usual mediators of anaphylaxis in many species, this has led to the invalid assumption that increased sensitivity to these compounds in the rat and mouse is responsible for increased susceptibility to anaphylaxis. According to Sanyal and West (1958) the potentiating action of Bordetella pertussis vaccine in the enhancement of anaphylaxis could be due to: 1) increased antibody formation, 2) increased production and release of the amines, or 3) increased tissue sensitivity to the amines. Kind (1958) has based the sensitizing properties of B. pertussis on two assumptions: 1) that sensitivity to histamine, serotonin, anaphylaxis, and endotoxins is due to a defect in steroid metabolism, and 2) that increased susceptibility to the lethal effects of endotoxins, anaphylaxis, peptones, and other agents is due to the release of histamine and serotonin. The action of B. pertussis on steroid metabolism may be enhancement

of destruction of these compounds or the ability to increase tissue requirements for the steroids. B. pertussis also either elicits a hypoglycemic response by the animal, or prevents the development of compensatory hypoglycemia (Pieroni and Levine, 1967).

Munoz and Bergman (1966) and Pieroni, Broderik, and Levine (1965) have successfully characterized the histamine sensitizing factor of B. pertussis. As a result, an entirely new theory has emerged regarding the ability of B. pertussis to enhance anaphylaxis in rodents (Lawler et al., 1965; Keller, 1966). Numerous investigators have demonstrated that B. pertussis does play a significant role in blocking adrenergic receptors, thus interfering with the action of epinephrine (Gozy and Kato, 1962a,b; Bergman and Munoz, 1965; Munoz and Bergamn, 1965; Keller, 1966). If this theory is accepted, the enhancement of anaphylactic shock by adrenalectomy could be explained on the basis of loss of the adrenal medulla, rather than the loss of the adrenal cortex.

Sanyal and West (1958) noted that four days after treatment with B. pertussis vaccine, rats were five times more sensitive to histamine, and by the tenth to twelfth day were three times more sensitive to serotonin. This information led these investigators to support the theory that the potentiating action of pertussis vaccine is due to the induction of increased

tissue sensitivity to these agents. Munoz (1963) and Weta (1964b), however, disagree, and maintain that following sensitization, increased sensitivity to anaphylaxis is still present at a time when hypersensitivity to histamine and serotonin has disappeared. In addition, the administration of antihistamines and anti-serotonin compounds could not fully protect rats from anaphylactic death.

Recent work supports the theory that the most important action of B. pertussis in sensitizing the rat to anaphylaxis is the enhancement of anaphylactic antibody production. Although the mechanism by which pertussis organisms enhance the production of anaphylactic antibody is still theorized, the fact that passive transfer of the induced hypersensitivity is possible with serum or purified antibody rules out the possibility that pertussis organisms were only modifying the physiological response of the animals to the mediators involved in anaphylaxis (Dinaghi and Benacerraf, 1964a).

Munoz (1963) feels that the main mechanism by which B. pertussis increases susceptibility of mice to actively induced anaphylaxis is by stimulating the antibody response to the sensitizing antigen. That pertussis vaccine enhances the production of agglutinins in mice has been demonstrated by Kind (1957). However, unlike other phenomena induced with pertussis vaccine, the

agglutinin response occurs only when pertussis vaccine is administered at the same time and by the same route as the antigen. Nota (1963a) believes that B. pertussis vaccine in the rat enhances the production of an antibody type different from the precipitin, having an affinity for the mast cell. Solliday, Rowley, and Fitch (1967) state that B. pertussis vaccine enhances the antibody response in the rat by inducing proliferation of the antibody forming cells, rather than by affecting the antigen or the initial antibody response by the immunocompetant cells already present.

Nota (1964b) concluded that anaphylaxis in rats is a result of a mixed reaction in which both immediate and delayed hypersensitivity are present, and that actively sensitized rats are fully susceptible only when both immediate and delayed hypersensitivity are fully established. Anaphylactic shock in the rat can usually only be produced between the tenth and twenty-first days after sensitization. Sanyal and West (1958) found the twelfth day to be the most successful for producing severe anaphylaxis. When the reaction is fatal, death usually occurs several hours after antigen injection (Nota, 1964b).

Systemic anaphylaxis in the rat is a progressive circulatory collapse with the small intestine as the target organ (Sanyal and West, 1958). With severe

anaphylaxis, the first symptoms seen in the rat are ruffled hair, loss of reaction to painful stimuli, falling of blood pressure, and lowering of body temperature. These are followed by weakness, cyanosis, severe prostration, exophthalmos, convulsions, and often death. This same general syndrome is seen in rats following the injection of anaphylatoxin, a toxic principle produced by incubation of certain antigen-antibody complexes in normal rat serum (Mota, 1959).

Once sensitization has occurred for the development of systemic anaphylaxis in the rat, the severity of shock can be depressed with such procedures as splenectomy, thymectomy, or the administration of antihistamines, anti-5-hydroxytryptamines, and glucocorticoids (Spencer and West, 1962; Cody and Code, 1963). Thompson (1966) reported that anaphylaxis and histamine shock are depressed during the fall season. Although the reason for this is unknown, this phenomenon is probably associated with imbalances in sulfur metabolism (Perera and Mongar, 1965).

Active cutaneous anaphylaxis has also been produced in the rat, by injecting bovine serum albumin and complete Freund's adjuvant. For producing the cutaneous type of anaphylaxis, shocking doses of diluted antigen are reportedly more effective than highly concentrated doses of antigen. The

histopathological changes in the sites of intense skin reactions were similar to those seen in the Arthus reaction of the rabbit (Lipton, Stone, and Freund, 1956).

Both passive cutaneous and passive systemic anaphylaxis have also been successfully produced in the rat (Nota, 1962). Reports on the value of B. pertussis for use in passive anaphylaxis have been contradictory and confusing.

Attempts to produce passive systemic anaphylaxis in rats, using serum from rats sensitized with antigen and B. pertussis organisms have been largely unsuccessful (Nota, 1961; Binaghi and Bonaccorraf, 1964a). Coulson and Stein (1965), however, successfully produced passive systemic anaphylaxis in rats using rat antisera produced by inoculation of egg albumin emulsified in Freund's adjuvant into the footpad with an additional intraperitoneal injection of B. pertussis organisms. Sanyal and West (1958) report that passive transfer of rabbit or rat antibody into rats that had received an injection of B. pertussis vaccine twelve days before resulted in susceptibility to anaphylaxis. Nota (1962) disagrees, stating that pretreatment of the serum recipient with B. pertussis does not influence either passive anaphylaxis, or the anaphylactoid reaction induced by the injection of soluble antigen-antibody complexes.

Passive cutaneous anaphylaxis has been produced in rats using rat, rabbit, and human antibody (Ovary, 1952; Nota, 1963a; Sonnetag and Marcus, 1963; Lovett, Movat, and Wardlaw, 1966). While Ovary (1952) reports that a positive passive cutaneous anaphylaxis reaction can be obtained for only a few hours after injection of antibody in the skin, Binaghi and Benacerraf (1963a) report that sensitization was maximal at sixteen hours, and positive reactions persisted for many days without significant change.

The rat serum titers of passive cutaneous anaphylaxis antibody are rarely very high, although Wilson, Bienenstock, and Bloch (1966) obtained titers up to one to one thousand using Nippostrongylus brasiliensis as the antigen. Connell and Sherman (1965) report that the titer of hemagglutinating antibody in rat serum is directly correlated with the ability to induce passive cutaneous anaphylaxis, but that the titer of skin sensitizing antibody bears no relation to the capacity of a serum sample to induce passive cutaneous anaphylaxis.

Ottgon, Binaghi, and Benacerraf (1966) reported that treatment of rats with cortisone or diethylstilbesterol resulted in increased serum concentration of anaphylactic antibody.

Recent findings by Nota (1958, 1961, 1962) and Keller (1957) have stressed the importance of the

mast cell in producing anaphylaxis in the rat. Tissue mast cells show qualitatively similar alterations in all types of shock, suggesting that these cells and damage or destruction of them is associated with histamine liberation (Riley, 1953).

Histamine and serotonin, two compounds thought to be important in the shock syndrome, are found in highest concentration in the mast cells, and released into the extracellular fluid following the action of a chymotrypsin-like enzyme upon the mast cells (Mota, 1953; Keller and Boeger, 1953; Austen et al., 1965). Though antihistamines protect rats against anaphylactic shock, they do not prevent mast cell disruption and the release of histamine. Plasma histamine normally reaches a maximum five minutes after injection of antigen (Mota, 1957).

Binaghi and Benacerraf (1964a) found that when tissues from actively sensitized rats were incubated with labeled specific antigen, the uptake of antigen was much higher for tissues from animals in which mast cell damage could be induced than for tissues from sensitized animals not showing mast cell damage or from normal untreated animals. When mesentery from normal rats was sensitized in vitro with anaphylactic antibody, subsequent contact with the antigen produced extensive mast cell damage with extrusion of the granules.

Both passive cutaneous anaphylaxis and sensitization of mesentery gave similar results in their effect on mast cells.

Although anaphylaxis in the mouse seems similar in many respects to anaphylaxis in the rat, Zinbinder (1964) feels that the mast cell plays no essential role in mouse anaphylaxis. Further, tissues containing the most mast cells are not necessarily the tissues showing the greatest histopathological effects following anaphylactic shock.

According to Mota (1963a) sensitization to anaphylaxis in the rat involves the production of two distinctly different types of antibody. The first type is the classical precipitin; and the second type is an antibody with an affinity for mast cells which is termed mast cell lytic antibody, mast cell sensitizing antibody, reaginic antibody, or rat anaphylactic antibody. In this thesis, the term rat reagin will be used.

The rat precipitating and reaginic types of antibodies have many important contrasting characteristics (Benacerraf and Kabat, 1949; Mota, 1961; Mota, 1963a,c; Binaghi et al., 1964; Ovary, Bloch, and Benacerraf, 1964; Stanworth and Kulms, 1965). The most important of these are listed in Table 2.

Table 2. Properties of rat antibodies.

1) Rat Precipitin

a) circulating type of antibody
b) sedimentation constant usually 7s
c) unlike human skin sensitizing antibodies
d) readily detectable with in vitro techniques
e) stable at 56 C for 30 minutes
f) passive cutaneous anaphylaxis reactions induced with this antibody last for only a few hours
g) antihistamines have no effect on ability to produce passive cutaneous anaphylaxis
h) titer increased after multiple antigen injections
i) Freund's adjuvant most effective for production
j) produce no apparent mast cell damage and histamine release
k) usually a gamma-2-globulin

2) Rat Reagin

a) a cytophilic type of antibody
b) sedimentation constant greater than 7s
c) similar to human skin sensitizing antibody
d) detectable only by in vivo techniques
e) destroyed by 56 C for 30 minutes
f) passive cutaneous anaphylaxis reactions induced with this antibody last for several days
g) antihistamines abolish ability to produce passive cutaneous anaphylaxis
h) titer decreased after multiple antigen injections
i) adjuvant Bordetella pertussis most effective for production
j) produce mast cell damage and histamine release
k) probably a gamma-1-globulin or a beta-2-globulin

Longcope (1922) was the first to suspect the possibility of an antibody other than the precipitin as being responsible for anaphylaxis in the rat. Kellaway (1930) disagreed, maintaining that the interaction of precipitating antibody fixed to smooth muscle cells and specific anaphylactic antigen was responsible for anaphylaxis in the rat uterus.

While many investigators have reported that rat antiserum is ineffective for passive sensitization of rat tissues, others have successfully produced this reaction. Much of the discrepancy is due to the predominant type of antibody present in the sera used for passive sensitization, which in turn depends on the type of adjuvant and method of antigen injection used for production of the antiserum. Thus, rats receiving multiple injections of the antigen emulsified in complete Freund's adjuvant would be expected to have a high titer of precipitins, but a lower titer of reagins. Conversely, rats receiving a single injection of antigen plus B. pertussis vaccine or organisms would be expected to have a high titer of reagins, and a low titer of precipitins. For some unknown reason, multiple injections of antigen results in the appearance of a factor in serum that is able to reverse an already established sensitization for the production of reaginic antibody (Mota, 1963b). Also, mast cell

damage and histamine release can be expected to occur only when rats have been sensitized with a single injection of antigen plus B. pertussis (Mota, 1961).

Mota (1964a) uses three criteria to estimate the content of rat reagin in a given serum sample: 1) histamine index = the percentage of histamine released in a given system of rat mesentery and antigen following addition of the antiserum to be tested, 2) mast cell index = the percentage of mast cells damaged after the addition of antigen following incubation of rat mesentery with the unknown antiserum, and 3) passive cutaneous anaphylaxis titer = the highest dilution of antiserum able to induce passive cutaneous anaphylaxis in rats seventy-two hours after antiserum injection. Since precipitating antibody does not induce passive cutaneous anaphylaxis after seventy-two hours, this reaction is considered specific for reaginic antibody.

Binaghi and Benacerraf (1964a,b) concluded that only a minor fraction of the circulating antibody of the rat possesses anaphylactic properties, but that the very low serum titer of the reagin can't be taken as an indication of the level of its synthesis if one considers that this antibody probably has a high affinity for certain rat tissues. Serum titers of significance may only be found after these tissues

are saturated.

Sensitization to rat reaginic antibody can be detected one minute after incubation of the tissue with antiserum. Reagins can persist in skin for at least one month, the time of persistence being related directly to the amount of antibody injected. Nonspecific gamma globulins have an inhibitory effect on passive sensitization induced with rat reagin, but considerably more gamma globulin is necessary to produce this effect than is needed for passive cutaneous anaphylaxis in the guinea pig with rabbit antibody (Biozzi, Halpern, and Binaghi, 1959). Rat reaginic antibody is unstable in serum, because exposure at room temperature for a few hours, or at 4 C for several days resulted in a complete loss of anaphylactic activity. As with most types of antibody, certain individual rats are considerably more prone to producing reagins than others.

Reaginic antibodies in rats were shown to be intermediate in molecular size, sedimenting between the 7s and 19s globulins in sera from both singly and multiply injected animals (Jones and Ogilvie, 1967). It is possible that the rat reagin may exist as a polymer of 7s components (Binaghi et al., 1964). Electrophoretically, rat reagin migrates with the fast immunoglobulins, but does not seem to be related to either the IgG or the IgA rat immunoglobulins (Jones

and Ogilvie, 1967).

Mota (1966) noted that although splenectomized rats responded with very much diminished production of other types of antibody, their production of reaginic antibody was similar to that of non-splenectomized animals. This suggested that the spleen plays little or no role in the production of rat reaginic antibody.

The rat reagin appears to have properties similar to the human reagin and to the skin sensitizing antibody produced by the dog (Oettgen, Binaghi, and Benacerraf, 1966; Schwartzmann and Rockoy, 1966). All are present in serum in only trace amounts as compared with the concentration of gamma-2-antibody, all appear to remain attached to tissues for extended periods without loss of sensitizing properties, and all are heat labile. These reaginic antibodies also produce their reactions in their own species only. It has not been possible to passively sensitize the skin of rats with human reagins (Binaghi et al., 1964; Ovary, Bloch, and Benacerraf, 1964). The rat and human reagins both have sedimentation constants greater than 7s.

In contrast with the rat reaginic antibody, the guinea pig anaphylactic antibody exists in high concentrations in serum, is heat stable, and has a sedimentation constant of 7s. The guinea pig antibody does not attach to tissues for as long a period of

time as rat or human reaginic antibody (Binaghi et al., 1964).

Rat antiserum has been ineffective in passively sensitizing guinea pig tissues for general anaphylaxis, passive cutaneous anaphylaxis, the Schultz-Dale reaction, and mast cell degranulation in vitro (Humphrey and Mota, 1959; Binaghi and Benacerraf, 1964a). Antigen labeled with I^{131} showed that antibody adsorbed on guinea pig mesentery could combine equally well with antigen, whether the antibody came from rabbit or rat, although the anaphylactic response failed to result (Humphrey and Mota, 1959). Mota (1964a) has reported successful passive transmission of rat reaginic antibody to the mouse.

The Germfree Rat

The historical aspects of gnotobiology have been adequately reviewed by Reyniers (1959) and Luckey (1961). The germfree animal may be defined as an animal reared in the absence of demonstrable living bacterial, mycologic, protozoan, and macroparasitic associates according to the test procedures routinely employed. Sufficient studies have not been conducted with pleuropneumonia-like-organisms, rickettsia, and viruses to decide whether they should be included in the list of organisms known to be absent from the environment of the germfree animal (Westmann and Gordon, 1960). Because of the many varied and cumbersome techniques needed for isolation and identification of the many known groups of viruses, and the possibility of the presence of as yet unidentified viruses, germfree animals are not likely to be proven virus-free for a long period of time.

Although some animals, such as chickens, grow just as well under germfree conditions as under conventional conditions, this is not so with the rat. Normal growth is slightly retarded in this species when reared under germfree conditions (Gordon, 1959).

Animals raised under germfree conditions can be considered to have much less contact with antigenic materials than animals raised in a normal environment,

although a complete lack of contact with antigenic materials such as food constituents, dead bacteria, dust constituents, and viruses cannot be excluded (Thorbecke, 1959).

Lagercrantz et al. (1965) found that antigenic material of dietary origin in the gastrointestinal tract of man and conventional rats was capable of inhibiting the passive hemagglutination reaction between the antigenic intestinal material in germfree rats and the homologous antiserum to this material. In contrast, the intestinal antigenic material from germfree rats was not capable of inhibiting the reaction between dietary antigen from conventional rats or man and homologous antisera from these same conventional rats or man, thus indicating that although antigenic material is present in the gut of germfree rats, there is still additional antigenic material present in the intestinal contents of conventional rats or man.

Lodinova, Jouja, and Lane (1967) report that newborn human infants receive maternally produced antibodies against Escherichia coli and other intestinal bacteria across the placenta. If this is the case with lower animals, those cesarean derived germfree animals would be expected to have higher levels of antibody against the intestinal bacteria than would those germfree animals born to germfree parents.

Excluding the cecum, the intestinal tissue in germfree animals weighs less than a comparable section in conventional animals (Gordon, 1959; Smith, 1966). This is largely due to a decrease of lymphoid tissue and moisture content of the intestinal tissue in germfree animals. In some instances the liver weighs less in germfree animals than in conventional animals, but the adrenal glands are often heavier in germfree rats than in conventional rats. The organs normally free from bacteria in normal life are similar in size in germfree or conventional animals, whereas the organs normally in contact with bacteria are reduced in size in germfree animals, with the exception of the cecum which is enlarged in germfree rodents, although not in germfree chickens (Gordon, 1959).

Adult germfree rats have a five to seven fold increase in cecal weight over conventional rats (Westmann and Bruckner-Kardoss, 1959). Cecal enlargement in germfree rats starts during the second week of life, and reaches the degree of distension seen in adult germfree rats by the twenty-fifth day. Upon exposure of the germfree rat to a normal microflora, the cecum shrinks to normal size within a few weeks (Gordon, 1959). Westmann and Bruckner-Kardoss (1959) suspect the enlargement of the cecum to be due to the absence of stimulation by the normal intestinal organisms

rather than due to a nutritional imbalance. In other species, especially the guinea pig and rabbit, it is suspected that the enlarged cecum has an adverse effect on reproduction due to ovarian degeneration and physical obstruction.

In all species investigated, an underdeveloped immunological defense system is present under germfree conditions (Miyakawa, 1959; Wostmann and Gordon, 1960). The germfree state is characterised by a low number of reticuloendothelial cells, and a decrease in lymphoid tissue, especially in such organs that in conventional animals harbor or are in close association with an abundant bacterial flora, such as the intestinal tract (Wostmann and Gordon, 1960).

In conventional animals, lymphoid tissue actively concerned with defense against invading bacteria or other antigens gives rise to secondary nodules. The cells in the secondary nodules probably do not produce any antibody, but give rise to reactive nodules, which are areas of basophilic blast cells or plasmacytic cells. The basophilic blast cells or plasmacytic cells adjacent to the lymphoid nodules probably produce the antibody (Thorbecke et al., 1957; Thorbecke, 1959; Gordon and Wostmann, 1960; Soll and Fahey, 1964). Although rarely seen in germfree animals, reactive nodules have been observed in the lymphatic tissue of

germfree rats (Smith, 1966).

Plasma cells are found in the spleen, mesenteric lymph nodes, and the mucosal lining of the intestine of germfree rats, but usually only in small numbers as compared to conventional or ex-germfree rats (Thorbecke, 1959). Conventional mice reportedly have three times as many blast cells and potential antibody forming cells as germfree mice (Olson and Wostmann, 1965, 1966a). Yet after stimulation with the antigens human gamma globulin and Salmonella typhosa vaccine, germfree mice show a proportionally greater increase in the production of plasmacytic cells, blast cells, and large lymphocytes than do conventional mice, indicating that these cells are probably less committed in germfree animals due to a lack of previous antigenic stimulation (Olson and Wostmann, 1966a).

Although immunologic activity may be retarded in germfree animals as compared to conventional animals, the production of specific antibody following antigenic stimulation is still evident (Olson and Wostmann, 1965). Bosma, Makinodan, and Walburg (1966) claim that growth of the spleen and the primary antibody forming potential of spleen cells is the same in conventional and germfree animals. They concluded that exposure to naturally occurring antigens, excluding those such as food that germfree animals would have contact with,

is not a prerequisite for normal development of the immune potential. Lerner (1964) reported that following injection of germfree and conventional guinea pigs with ovalbumin, the germfree animals produced high levels of circulating antibody, and showed considerable hypertrophy of lymphoid tissue.

Although the serum of germfree rats contains the same major antigenic components as the serum of conventional rats, certain constituents, especially those of low mobility, are present in such small amounts that detection of them is difficult, even with the most sensitive precipitation methods. Germfree rat gamma globulins show a relatively high mobility, and injection of germfree rat serum into rabbits causes production of the entire spectrum of anti-gamma antibody otherwise found in rabbit anti-conventional rat serum (Grabar, Courcon, and Wostmann, 1962).

In germfree animals, especially the rat, the serum fractions alpha-2-globulin, beta globulin, and gamma globulin are lower than in the conventional animal. Therefore, all three of these fractions could be regarded as possibly harboring antibacterial antibodies. When the germfree rat is exposed to a conventional flora, an antibody titer to many of the intestinal organisms occurs in about two weeks, and this is roughly paralleled by a rise in beta globulin.

The lower content of immuno globulins in the serum of germfree animals is compensated for with a higher albumin content, resulting in a similar total serum protein content for germfree and conventional animals (Wostmann and Gordon, 1960; Asofsky, 1965; Asofsky, Ikari, and Hylton, 1966).

Wostmann (1959) reported that higher levels of serum gamma globulins are present in germfree rats fed milk than in germfree rats fed other diets. Smith (1965) has noted that germfree lambs and goats immunologically respond to orally administered sterile bovine milk.

Coates and O'Donoghue (1957) found that infant germfree rabbits given bovine milk in the diet often manifested an anaphylactic type of allergic reaction shortly after each feeding. The diet was sterilized by ultra high temperature heat treatment for short periods, a process which resulted in little loss of nutrient value, and probably also a negligible loss of the milk's ability to function as an antigen. This allergic condition seemed similar to the "cot-death" syndrome described in human infants.

MATERIALS AND METHODS

Antigens

The antigens employed were whole pasteurized homogenized milk, beta lactoglobulin[1] (BLG), and alpha lactalbumin[2] (ALA). All the antigens were of bovine origin. The whole milk was used as an antigen without heating beyond the original pasteurization process (UHWM), and after autoclaving at 121 C for 15 minutes (WM15) and 30 minutes (WM30) at 15 pounds pressure in glass two liter Square Pak flasks[3].

Incomplete Freund's adjuvant[4] and Bordetella pertussis vaccine[5] were used to enhance the effectiveness of the whole milk as an antigen.

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1. Nutritional Biochemicals Corp., Cleveland, Ohio
2. Nutritional Biochemicals Corp., Cleveland, Ohio
3. American Sterilizer Co., Erie, Pennsylvania
4. Difco Laboratories, Detroit, Michigan
5. Michigan Department of Health, Lansing, Michigan

Animals

Adult convantional New Zealand white rabbits, and adult conventional and adult germfree rats of the Fischer strain were used in this study.

The rabbits were alloted into six groups of two each, groups RI-RVI, and the rabbits were treated at three to four day intervals in the following manner:

Group RI- Ten intramuscular injections of 3.0 ml UHSM

Group RII- Ten intramuscular injections of 3.0 ml WM15

Group RIII- Ten intramuscular injections of 3.0 ml WM30

Group RIV- Eight intramuscular injections of 150 mg DLG suspended in 3.0 ml sterile physiological saline solution

Group RV- Seven intramuscular injections of 150 mg ALA suspended in 3.0 ml sterile physiological saline solution

Group RVI- Controls- no treatment

The germfree rats were purchased as weanlings[6], and reared in plastic isolators until a weight of 200 to 300 gm was reached, at which time they were used for the experiment. The germfree rats were fed Purina 50-100[7], a special diet designed to contain additional heat labile ingredients, so that after sterilization

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6. The Charles River Breeding Laboratories, Brookline, Massachusetts
7. Purina Laboratories, St. Louis, Missouri

the resulting diet will be nutritionally the same as Purina Laboratory Chow. The principal protein used to supply the amino acids is soybean meal.

The germfree rats were alloted into groups MI-MIV according to the treatment received:

Group MI- Seven germfree rats fed WM30 as the only source of fluid for a period of 62 days, at which time the experiment was terminated

Group MII- Seven germfree rats given subcutaneous injections of 1.0 ml WM30 at four to six day intervals, with each receiving a total of eight to ten injections

Group MIII- Six germfree control rats receiving no treatment

Group MIV- Four germfree rats; each given a single intraperitoneal injection of 1.0 ml WM30, and at the same time a single intraperitoneal injection of 1.0 ml Bordetella pertussis vaccine

The conventional rats used for the experiment were born and raised in a conventional environment, but were second and third generation descendants of ex-germfree parents originally obtained from the same source as the weanling germfree rats. These rats were fed Purina Laboratory Chow[8]. The conventional rats were alloted into six groups MV-MX:

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8. Purina Laboratories, St. Louis, Missouri

Group MV- Six conventional rats subjected to the same treatment as the germfree rats in group MI

Group MVI- Seven conventional rats subjected to the same treatment as the germfree rats in group MII

Group MVII- Fourteen conventional control rats receiving no treatment

Group MVIII- Five conventional rats receiving the same treatment as the germfree rats in group MIV

Group MIX- Five conventional rats receiving a single intramuscular injection of 1.0 ml WM30, and a single intraperitoneal injection of 1.0 ml Bordetella pertussis vaccine

Group MX- Five conventional rats receiving a single intraperitoneal injection of 1.0 ml WM30 emulsified in 1.0 ml incomplete Freund's adjuvant

In every instance, the only milk preparation used for all studies, with both conventional and germfree rats, was WM30.

Germfree Procedures

The general procedures as described by Allen (1963) and Bardes (1964) were used for establishment and maintenance of the isolators. Plastic film isolators, similar to those described by Trexler and Reynolds (1957), measuring 24 in X 24 in X 36 in were used. Each isolator contained two or three plastic cages with two to four rats per cage. Water, diet, bedding, and other supplies were sterilized by autoclaving in stainless steel drums at 121 C and 15 pounds pressure before being transferred into the isolators. An aqueous solution of 2.0% peracetic acid[9] and 0.1% Hacconol[10], an alkyl aryl sulfonate, was used as the disinfectant for sterilization of the plastic transfer chambers used to connect the stainless steel supplying drums with the germfree isolators. Drinking water was sterilized in two liter volumes by autoclaving at 121 C and 15 pounds pressure for 30 minutes.

The general procedure used for determination of germfree status was that of Wagner (1959). Animals were checked for bacterial contamination each time the isolator system was supplied, which was every two to four weeks, and again just prior to termination of

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9. Becco Chemical Co., Buffalo, New York
10. National Aniline Division of Allied Chemical Corp., New York, New York

the experiment. Tubes of liquid thioglycollate medium B256[11] were inoculated with fecal samples and then incubated at 25 C, 37 C, and 55 C for three weeks. Glass slides were smeared with the fecal specimens and stained by Gram's technique. These slides were then thoroughly examined for the presence of bacteria using the oil immersion lens of a light microscope.

Bleeding Techniques And Collection Of Serum

The rabbits were bled by incising the dorsomedial artery of the ear no less than three days after the last previous injection of antigen. Each rabbit received seven to ten antigen injections at intervals of several days. From each rabbit, 25-50 ml of blood were collected a minimum of six times, at approximately four day intervals. The blood was allowed to clot, and the resulting serum was clarified by low speed centrifugation. The serum was then stored at -14 C.

All rats were bled from the venous plexus located caudal to the eye by puncturing the medial canthus of the orbit with a Pasteur pipette. From the original technique described by Riley (1960) for bleeding mice, several modifications were used to facilitate the bleeding of germfree rats in isolators and conventional

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11. Difco Laboratories, Detroit, Michigan

rats outside of isolators.

Several minutes before the bleeding procedure, the rats were given a tranquilizing dose of 23 mg/kg sodium pentobarbital intraperitoneally[12]. During the bleeding process the rat was held in the left hand with the head of the rat toward the floor, and with the thumb and index finger of the left hand being positioned to pinch off the jugular vein. The rats were held in this position to increase venous pressure in the area of the orbit. Samples of four to six ml of blood could be taken from adult rats without undue risk for the life of the animal. Using this technique, up to seven serial blood specimens were taken from each rat.

The rats receiving antigen were bled at least five days after the previous antigen injection. Table 3 lists the days when additional injections of antigen were given and when blood was collected for each group.

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12. Halatal- Jenson-Salsbery Laboratories, Kansas City, Missouri

Table 3. Days for feeding or injecting antigen and for collection of serum in germfree and conventional rats. First antigen was given on day 1.

Group	Antigen Injection (day)	Serum Collection (day)
MI	oral antigen only-continuous	18, 23, 30, 35, 46, 58, 64
MII	1, 4, 7, 10, 17, 25, 31	21, 24, 29, 38, 50, 60, 62
MIII	control	1, 7, 13, 20, 31, 43
MIV	1	7, 12
NV	oral antigen only-continuous	18, 23, 28, 33, 45, 57, 59
NVI	1, 4, 7, 10, 24, 35, 51	18, 23, 28, 33, 45, 57, 59
NVII	control	1, 6, 11, 16, 28, 39, 45
NVIII	1	7, 12
MIX	1	7, 12
MX	1	7, 12

Interfacial Test

At least one serum sample from each animal was screened for precipitins with the interfacial test. The procedure according to Campbell et al. (1964) was followed. The antigens employed were UHWM, WM15, WM30, unheated HLG, and unheated ALA. For use in the interfacial test the antigens UHWM, WM15, and WM30 were adjusted to contain 0.033 mg/ml protein antigen and

BLG and ALA were adjusted to contain 0.0033 mg/ml protein antigen.

Undiluted and tenfold dilutions of rabbit antisera from at least one of the last three bleedings were tested with the interfacial test. Undiluted samples of the last sera prepared from the last bleedings of rats in groups MI-MIII and groups MV-MVII were also tested. For rats in groups MIV, MVIII, MIX, and MX, samples taken on days seven and twelve were tested.

Passive Cutaneous Anaphylaxis

For the passive cutaneous anaphylaxis (PCA) tests, the procedures described by Ovary (1958), and Ovary and Bier (1953) were followed with minor modifications.

PCA in the Guinea Pig Four hundred to five hundred gram albino guinea pigs were used for testing at least one serum specimen from every rabbit and rat used in the experiment. One day before the PCA test, the hair on the entire back of the guinea pig was clipped, and a depilatory agent[13] was applied. A total volume of

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13. Surgex Crookes-Barnes Co., Wayne, New Jersey

0.05 ml of undiluted antiserum was injected intracutaneously in each of six sites on the back of the animal with a 27 gauge needle. For challenge, 5.0 mg Evans blue dye[14] and 33.3 mg of protein antigen in a single 2.0 ml volume were given intravenously in the saphenous vein. The reactions were read 15 minutes after challenge.

At least one of the last three sera collected from each of the rabbits in groups RI-RVI was tested with five undiluted antigens (UHWM, WM15, WM30, BLG, and ALA). For the guinea pig PCA tests using rabbit sera, a three hour incubation period was employed.

The last serum sample taken from each of the rats in groups MI-MIII and MV-MVII, and samples taken on days seven and twelve from groups MIV and MVIII-MX was tested for PCA activity in guinea pigs. All sera were used undiluted, and only the antigens UHWM and WM30 were used for challenge. Incubation periods of three hours and 16 hours were used.

PCA in the Rat Conventional rats of the Fischer strain weighing 200 gm were also used for the PCA test. The procedure for PCA as described for the guinea pig was followed with these exceptions: 1) the rat was subjected

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14. Matheson Scientific Inc., Detroit, Michigan

to light pentobarbital anesthesia, a dose of 23 mg/kg, while the intracutaneous injections of antisera were given, 2) for challenge, a dose of 3.75 mg Evans blue dye and 16.5 mg milk protein antigen were given intravenously in the tail vein, 3) only a three hour incubation period was used, and 4) only UHWM and WM30 were used as challenge antigens.

Rabbit sera from each rabbit in groups RI and RIII (those rabbits which received UHWM and WM30) were tested by the PCA test in the rat. Only the rabbit antisera that had already given positive reactions on the interfacial test and the PCA test in the guinea pigs were used.

Sera from terminal bleedings of at least two rats in each of the groups MI-MX were also tested for PCA activity in the rat.

RESULTS

Interfacial Test

The results of the interfacial tests with rabbit antisera are shown in Table 4.

Table 4. Interfacial tests with rabbit antisera produced against antigens UHWM, WM15, WM30, BLG, and ALA.

Antisera Group	Dilution	Antigens UHWM	WM15	WM30	BLG	ALA
RI (anti-UHWM)	UN*	+	+	+	+	+
	10	+	+	+	+	+
RII (anti-WM15)	UN	+	+	+	+	+
	10	+	+	+	-	-
RIII (anti-WM30)	UN	+	+	+	+	-
	10	+	+	+	-	-
RIV (anti-BLG)	UN	+	+	+	+	-
	10	+	+	+	+	-
RV (anti-ALA)	UN	+	+	+	-	+
	10	+	-	-	-	+

*Undiluted

In a few instances only one of the two rabbits within the group reacted positively when the serum was diluted tenfold, although both reacted positively

when the serum was undiluted. In these cases a positive reaction for that group is recorded in Table 4 for the tenfold dilution.

It should be noted in Table 4 that WM30 retained sufficient antigenicity to sensitize rabbits for the production of precipitins. Further, these precipitins reacted with either WM30, WM15, or UHWM antigen in vitro. Antisera produced by injecting WM30 had no detectable antibody to ALA antigen in the interfacial test. Tenfold dilutions of WM30 antisera did not react with BLG antigen, but undiluted WM30 antisera did.

The rat antisera were invariably negative for precipitins with the interfacial test.

Passive Cutaneous Anaphylaxis in the Guinea Pig

The results of the PCA test in guinea pigs using rabbit antisera are shown in Table 5. Only those reactions showing a well differentiated deposition of the blue dye with a diameter of at least 3/16 in were considered positive.

Table 5. PCA activity in guinea pigs using rabbit antisera.

Antisera Group	Antigens UHWM	WM15	WM30	BLG	ALA
RI (anti-UHWM)	+	+	+	+	+
RII (anti-WM15)	+	+	+	+	-
RIII (anti-WM30)	+	+	+	+	-
RIV (anti-BLG)	+	+	+	+	-
RV (anti-ALA)	+	+	-	-	+

Table 5 shows that sufficient activity remained in WM15 and WM30 antigens to sensitize rabbits for antibody production. These results coincide with the results obtained with the interfacial test. The alpha lactalbumin contained in the WM30 antigen seems to be severely altered by the thirty minute heat treatment as evidenced by failure of the WM30 antigen to produce detectable antibody to this component when tested with the ALA antigen.

As with the interfacial test, the rat antisera were invariably negative for PCA activity in guinea pigs.

Passive Cutaneous Anaphylaxis in the Rat

Only the rabbit antisera from group RI, the anti-UHWN sera, showed any possibility of reacting positively, and this reaction was quite diffuse and indistinct when compared with the clearly interpretable PCA reactions obtained when using the same sera in guinea pigs.

All the rat sera tested were again negative.

DISCUSSION

The results of this study clearly show that whole milk subjected to severe heat treatment remains antigenic. This was demonstrated in rabbits using whole milk that had been autoclaved for 30 minutes at a temperature of 121 C. These results are in agreement with those of Lua and Todd (1964) and Saperstein and Anderson (1962), but do not support the results of Ratner et al. (1958b,c) and Crawford (1960). It is difficult to compare those publications with the results presented here, because most of the work involving the antigenicity of heated milk is based on systemic anaphylaxis in guinea pigs or clinical skin tests in hypersensitive human patients.

The results presented here offer no support to the hypothesis originated by Furth (1925) that the heating of a milk protein creates a new antigenic specificity which in turn will produce antibody specific for the heated antigen. My results indicate that anti-WM30 sera reacted at least as well with the UMWN antigen as with the WM30 antigen.

It is realized that although the two liter volumes of milk were heated in an autoclave for

15 or 30 minutes at 121 C and 15 pounds pressure, the actual temperature in the center of the flask probably did not reach 121 C for the entire 15 or 30 minute time period. This is expected because sterility checks showed the two liter volumes of milk were not sterile until heated for 25 - 30 minutes at this temperature and pressure. Since smaller volumes of milk could be sterilized by autoclaving for only eight to ten minutes at 121 C and 15 pounds pressure, it is expected that the actual effective heat treatment on the milk protein antigens was considerably less than 121 C for 15 or 30 minutes.

It must also be considered that if it took longer for the two liter volume of milk to reach and maintain a temperature of 121 C, then logically it must also take longer for this volume to cool down to room temperature after removal from the autoclave. Additional denaturation of the protein antigen would then take place during the cooling period.

It is expected that the antigenicity of at least two of the milk proteins, beta lactoglobulin and alpha lactalbumin was considerably reduced as indicated by the interfacial test. The anti-WM30 serum from only one rabbit reacted positively with BLG, and neither of the WM30 antisera reacted with ALA.

Both the interfacial test and the PCA test indicate that the anti-WM30 sera possessed more activity for the BLG antigen than for ALA. These results agree with those of Crawford and Grogan (1961), Collins-Williams (1962), and Lux and Todd (1964) in that BLG is more heat stable than ALA. It is expected that the more heat stable casein would play a significant part in the total amount of undenatured protein remaining in the WM30 antigen.

Failure to detect antibody in the conventional and germfree rats exposed to WM30 could be explained on the basis of one or several possibilities; 1) that the WM30 did not retain sufficient antigenicity for the rats to elicit the production of antibody; 2) that precipitating antibody was produced but was present in such low quantities that the antibody could not be detected with the relatively insensitive interfacial and PCA techniques, whereas if a more sensitive technique such as radioimmunoelectrophoresis or tanned cell hemagglutination had been employed, antibody would have been detected; and 3) that a reaginic type of antibody was produced, but because of its cytophilic nature was not detectable with the in vitro or passive recipient tests. If the first mentioned possibility was responsible for the absence of detectable antibody in rats, it must be assumed that

the rat would require a larger dose of effective antigen for antibody stimulation than did the rabbit, since the rabbit readily produced precipitins to the WM30.

The fact that severely heated milk (WM30) is still antigenic suggests that heat denatured milk products for use in the diet of milk hypersensitive infants is highly questionable. Despite the probability that considerable denaturation of the milk proteins may take place upon heating, it is evident that a slight degree of antigenicity remains. This antigenicity could be crucial in circumstances where the shocking dose of antigen is provided to a milk hypersensitive human infant potentially susceptible to the "cot-death" syndrome.

Germfree animals offer many advantages over conventional animals for certain types of immunological investigation, and in some instances the germfree animal is almost a necessity for obtaining valid results. Unfortunately, the rat and mouse, which are relatively easily reared under germfree conditions, are poor subjects from the standpoint of humoral antibody production. In contrast, the rabbit is a good producer of precipitins, and the guinea pig an excellent choice for anaphylaxis testing, but both of these animals are very difficult to rear under germfree conditions.

Thus, the question concerning the intestinal absorption of antigen, and antigenicity of milk in germfree animals remains largely unanswered.

SUMMARY

Five groups of conventional rabbits were immunized with five antigens: 1) unheated whole milk (UHWM), 2) whole milk heated for 15 minutes at 121 C (WM15), 3) whole milk heated for 30 minutes at 121 C (WM30), 4) beta lactoglobulin (BLG), and 5) alpha lactalbumin (ALA). Each group received only one of the five antigens.

After several antigen injections, all groups had specific serum antibodies to the antigen injected, when tested with the interfacial and passive cutaneous anaphylaxis tests. In addition, the WM15 and WM30 antigens cross-precipitated the UHWM antisera, and the UHWM antigen also cross-precipitated the WM15 and WM30 antisera.

The BLG protein was less severely denatured by heat than the ALA antigen, because the WM30 antisera reacted positively with BLG, but negatively with ALA for both the interfacial and passive cutaneous anaphylaxis tests.

Passive sensitization of the conventional rat with rabbit anti-WM30 sera and rabbit anti-UHWM sera was unsuccessful. Active sensitization of the germfree or conventional rat using WM30 was also unsuccessful.

Several methods were employed in attempting to sensitize the rats. These included varying the route of antigen injection as well as the use of Freund's incomplete adjuvant and Bordetella pertussis vaccine.

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