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# A comparison of some laboratory methods for the production of antibodies

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# NOTE TO USERS

# A COMPARISON OF SOME LABORATORY METHODS FOR THE PRODUCTION OF ANTIBODIES

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# INTRODUCTION

Further advances in immunochemistry require more definite knowledge regarding the mechanism of antibody formation and the chemical structure of antibodies. The experiments described in this thesis were attempts to obtain more information on the nature of antibody formation and attempts to develop a simple procedure for evoking precipitating antibodies. A method of producing antibodies more conveniently than is possible with the laboratory animals generally used for this purpose should facilitate studies on the nature of antibodies.

Many hypotheses of the mechanism of antibody production have been suggested. They may be divided into two general proposals. One concept is that antibody production is a synthetic function of cellular systems. Under the influence of an antigen, the formation of certain globulin molecules may be directed so that the resultant globulins have a surface structure complementary to the surface of the stimulating antigen. The second proposal is that antibodies may be produced by altering the stereochemical and/or electrochemical properties of non-antibody proteins. No synthetic mechanism is postulated. Instead it is suggested that under the influence of an antigen and various physical and chemical stimuli, non-antibody proteins may be converted into specific antibodies.

The first hypothesis is more widely accepted[1, 2]. Most immunologists also accept the evidence indicating that antibodies are synthesized by the cells of the reticulo-endothelial system of animals[2]. However, there are a number of reports in the literature of the demonstration of antibody formation in the absence of reticulo-endothelial cells[a]. Natural antibodies[b] have been observed in plants. Production of antibodies has been reported in plants, insects, and in acellular systems.

The specific purposes of the present study were to extend the recently reported observation of antibody formation by incubation of plasma and antigens <u>in vitro</u>[3] and to attempt to develop a rapid method for the production of the relatively large amounts of antibodies which are needed for chemical studies of antibody composition.

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1 Topley and Wilson, "Principles of Bacteriology and Immunity," 3rd. Ed., Williams and Wilkins Co., Baltimore, Md., (1946), p. 980, 1131.

2 Landsteiner, "The Specificity of Serological Reactions," Revised Ed., Harvard University Press, Cambridge, Mass., (1945), p. 146, 148.

3 Bacon, <u>Arch. Internal Med.</u> <u>72</u>, 581 (1943).

a Antibody formation may be demonstrated by any observable reaction between an antigen and its specific antibody or by the development of immunity to a specific antigen present in an organism.

b See Glossary, p. 3.

## GLOSSARY

Antigen - A substance which stimulates the production of an antibody and which, when mixed with that antibody, reacts with it in some observable way.

Antibody - A substance which is produced under the stimulus of an antigen and which, when mixed with that antigen, reacts with it in some observable way.

Natural Antibody - Substances found in animals and plants which have not been treated with an antigen, and which are similar in their effects to the antibodies developed by immunization.

Specific Antibody - An antibody which reacts in an observable way only with the antigen which stimulated its production and with closely related antigens.

Agglutinin - An antibody which reacts with a cellular antigen causing agglutination of the antigen.

Antitoxin - An antibody which reacts with a toxin; i.e., an antigen characterized by high toxicity, thereby neutralizing the physiological activity of the toxin.

Lysin - An antibody which reacts with a cellular antigen causing diruption or lysis of cells.

Immunity - The ability to resist the development of a disease caused by micro-organisms or their products because of the action of specific antibodies.

Active Immunity - Immunity resulting from the production of antibodies by an organism after stimulus with an antigen.

Passive Immunity - Immunity produced indirectly by introduction of antibodies formed by another organism.

Precipitin - An antibody which reacts with a soluble antigen to form a visible precipitate.

Resistance - The ability of an organism to withstand a disease or infection.

Tolerance - The natural or acquired capacity to withstand an antigenic substance.

## HISTORICAL

### Hypotheses on the Mechanism of Antibody Formation

Of the various hypotheses of the mechanism of antibody formation which have been proposed, the simplest is the assumption first advanced by Buchner[4] that the antigens enter into the composition of the antibodies. To this concept are opposed strong arguments. Berger and Erlenmeyer[5] after immunization of animals with a conjugated antigen containing arsenic, "horse serum diazo arsanilic acid", found no arsenic in the sera, and Heidelberger and Kendall[6], Hooker and Boyd[7] and Haurowitz, Vardar and Schwerin[8] have also reported results not in keeping with this hypothesis. Another objection to Buchner's suggestion is based upon the quantitative relationship between antigens and antibodies. According to a calculation made by Hooker and Boyd[9] a single antigen molecule may give rise to a quantity of antibody sufficient to react with several hundred antigen molecules.

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[4] Buchner, Zentr. Bakt. 5, 817 (1889).

[5] Berger and Erlenmeyer, Z. Hyg. Infektionskrank. 113, 79 (1931).

[6] Heidelberger and Kendall, Sci. 72, 252 (1930).

[7] Hooker and Boyd, J. Immunol. 23, 465 (1932).

[8] Haurowitz, Vardar and Schwerin, J. Immunol. 43, 327 (1942).

[9] Hooker and Boyd, J. Immunol. 21, 113 (1931).

A working hypothesis of the method of antibody formation which fits in well with our present knowledge of the structure of antigens and antibodies has been put forth by Breinl and Haurowitz[10] and Mudd[11]. These investigators proposed that in contact with the antigen, globulins are synthesized which correspond spatially and in chemical affinity to the antigen. This influence is exerted through a physical and/or chemical union between the peptides or amino acids and the antigen during globulin synthesis. Mudd suggests that this synthesis occurs at the interface between the antigen and the surrounding fluid. The globulin thus formed is assumed to be dissociated from the antigen after a certain stage has been reached and the antigen is thus able to leave its stereochemical imprint on successive molecules of antibody globulin. Breinl and Haurowitz suggest that this synthesis is located in the reticulo-endothelial cells.

Pauling[12] has reviewed the physicochemical basis of the hypothesis and considers that "all antibody molecules contain the same polypeptide chains as normal globulin and differ from normal globulin only in the configuration of the chain; that is, in the way that the chain is coiled in the molecule". Pauling suggests that when an antigenic substance with a well

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10 Breinl and Haurowitz, Z. physiol. Chem. 192, 45 (1930).

11 Mudd, J. Immunol. 23, 423 (1932).

12 Pauling, J. Am. Chem. Soc. 62, 2643 (1940).

defined configuration of active groups is present in the cell, it influences the configuration at the ends of the folded polypeptide chains of the normal globulins, thereby inducing the formation of antibody.

Burnet[13] has pointed out a number of features of antibody formation that are not explained by the above hypotheses. Burnet states that these hypotheses demand the presence of antigen in the tissues as long as active immunity lasts. Burnet prefers the assumption of an antibody forming capacity, independent of the continued presence of antigen, which is transmitted to descendant cells by some hereditary process. He suggests that antigens do not influence the antibody globulins directly during their formation, but modify the proteinases responsible for globulin synthesis. When the antigen is no longer present in the tissues, the proteinase is reproduced in the modified form, both in the original and in descendant cells.

Emerson[14] has suggested that the formation of antibodies as well as the formation of natural antigens and of enzymes may be genetically controlled.

There is very little knowledge of the mechanism of antibody formation based on direct experiment, and it is not possible to decide between these hypotheses.

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13 Burnet, Australian J. Sci. 1, 172 (1939).

14 Emerson, Ann. Mo. Botan. Gard. 32, 243 (1945).

## Antibody Production in Cellular Systems

### In Animals

Laboratories producing commercial antisera use the method of injection of animals to stimulate antibody production in animals. When an appreciable quantity of foreign protein has been injected into an animal in a series of small doses, the blood serum of the animal contains, in most cases, a large amount of antibody specific for the injected protein. Injections are usually made at three to four day intervals for a series of five to thirty treatments, and the amount of antigen administered is increased each time. This method, however inconvenient and time consuming, is at the present time the preferred method for the production of antisera for therapeutic use and for theoretical investigation.

### In Tissue Cultures

Antibody formation in cell cultures has been repeatedly reported. Carrel and Ingebrigsten[15] (1912) mixed goat erythrocytes with lymph node or bone marrow tissue of guinea pigs and after three to five days in culture obtained hemolysins to goat erythrocytes in extracts of the cultures. By a similar procedure Schilf[16] (1926) prepared bacteriolysins

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15 Carrel and Ingebrigsten, J. Exptl. Med. 15, 287 (1912).

16 Schilf, Zentr. Bakt. 97, 219 (1926).

to Vibrio cholerae. Schilf cultured cells from rabbit spleen in rabbit plasma and chicken embryo extract containing antigen at 37° for several days and was able to demonstrate the presence of specific vibriolysins in extracts of the cultures.

Fischer[17] (1922) reported that fibroblasts in vitro respond to the presence of an antigen in the culture medium by becoming immunized to its action. A large amount of foreign protein added to culture medium markedly decreased the rate of growth of fibroblasts previously cultured in homogeneous media, but had relatively little effect on those cells previously cultured in media containing the antigen. Fischer found a definite relation between the amount of antigen present in the media and the time of appearance of immunization and its duration.

Other workers have demonstrated formation of antibody for a variety of antigens when the tissue cultures were made from animals which had been inoculated previously with the antigen, but have obtained uniformly negative results by adding the antigen to the tissue culture in vitro. Lüdke[18] (1912) injected killed cultures of typhoid or dysentery bacilli into rabbits, removed spleen or bone-marrow cells after one to five days, and cultivated the cells in homologous plasma. After two to five days, he was able to detect lysins and agglutinins in the culture fluid. His results were generally

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[17] Fischer, J. Exptl. Med. 36, 535 (1922); 35, 661 (1922).

[18] Lüdke, Berlin. klin. Wochschr. 49, 1034 (1912).

negative when he added antigenic materials directly to the tissue cultures. Meyer and Loewenthal[19] (1928) reported that cultures of the spleen, lymph glands, and "milkspots" of rabbits form agglutinins for typhoid bacilli when taken from animals previously injected with the antigen. Agglutinin formation was noticed for two to five days. Cessation of the function after this period of time was assumed to be due to changes in the reticulo-endothelial system. Meyer and Loewenthal used the method of massive antigen injection followed by immediate death of the animal. They believe that this method is superior to the method of addition of antigen to tissue cultures in vitro only because it provides for greater diffusion of antigens into the cells. The time period between injection of the antigen and death of the animal was not long enough for activation of the antibody-synthesizing mechanism of the body. These investigators stress the importance of actively growing cells since they found that the amount of antibody production in vitro could be correlated with the amount of growth in the culture flasks. They were able to demonstrate antibody production only with tissues secured from the reticulo-endothelial system.

A type of anaphylactic reaction in tissue cultures was reported by Moen and Swift[20] (1936) who found that cells in spleen tissue cultures from rabbit previously injected with

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19 Meyer and Loewenthal, Z. Immunitäts. 54, 409 (1928).

20 Moen and Swift, J. Exptl. Med. 64, 339 (1936).

tubercle bacilli exhibit a high degree of sensitivity to tuberculin toxicity when grown in media containing tuberculin, as shown by the rate of cytolysis. Moen[21] (1936) also found that tuberculin-sensitive cells grown in vitro in normal media showed persistence of this cellular sensitivity through several transplantations, during which time many new generations of cells developed. Hwon[22] (1937) cultured rabbit spleen tissues in media containing diphtheria toxin and reported failure of the cultures to produce antitoxins. Antitoxin was produced, however, by tissue cultures from animals which had been inoculated with toxin. Spleen tissue from previously inoculated animals was cultured in media containing diphtheria toxin. After two to six days of culture in vitro, the presence of antitoxin in tissue extracts could be demonstrated. Salle and McOmie[23] (1937) also failed to produce precipitins, agglutinins, or hemolysins by in vitro culture of antigens with suspensions of chick embryo tissue or tissue cultures of rabbit or guinea pig splenic or pulmonary cells. Parker[24] (1937) reported another study of the formation of antibodies by tissue cultures in vitro. He was able to demonstrate antibody formation only when the tissue was taken from an animal which had been injected with an

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21 Moen, J. Exptl. Med. 64, 943 (1936).

22 Hwon, J. Immunol. 33, 471 (1937).

23 Salle and McOmie, J. Immunol. 32, 157 (1937).

24 Parker, Sci. 85, 292 (1937); Compt. rend. soc. biol. 125, 315 (1937).

antigen two to three days earlier. Negative results were obtained when the time of exposure of the animal to the antigen was shortened. Parker suggested that antibody production does not take place unless the tissues have been acted upon by some unknown mechanism within the body.

The reasons for failure of the *in vitro* immunization are not obvious. Beard and Rous[25] (1938), using a culture of Kupffer cells from the liver, noted that the cultivated cells had lost their normal power of ingesting antigenic particles. These investigators inoculated rabbits with vaccinia virus, removed the Kupffer cells (reticulo-endothelial cells lining the liver sinusoids) and mixed the cells with virus. The mixture was then reinoculated subcutaneously into rabbits and was found to be less pathogenic than was the virus alone. They suggest that the cells multiply in the cutaneous tissue and thus suppress virus activity. No antiviral effect was found if the cells failed to multiply, and no antiviral principle was elaborated by the cells under *in vitro* conditions. In fact, the virus concentration showed a great increase during six to eight days incubation in tissue culture.

The evidence indicating that the reticulo-endothelial system is not essential for antibody production is indirect. There is, for example, some evidence for the occurrence of the antibody mechanism in nature in lower forms. Antibody formation has been reported in lower vertebrates and in insects as well as in plants.

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[25] Beard and Rous, J. Exptl. Med. 67, 883 (1938).

In Insects

Immunization of insects by inoculation with attenuated culture, cultures killed by heating at 60°, and small doses of virulent cultures have been reported[26, 27, 28, 29]. Two typical reports are those of Chorine[30] and Metalnikov and Chorine[31]. Chorine (1928) demonstrated that caterpillars, Galleria Mellonella, could be passively immunized to diphtheria toxin by injecting the blood of immunized caterpillars into the body cavities. Subsequent injection of lethal doses of the toxin did not kill the insects. Chorine also showed that diphtheria toxin mixed with the blood of immunized caterpillars was neutralized. Metalnikov and Chorine (1930) reported immunization of caterpillars, Pyrousta nubilalis, to various virulent and avirulent strains of bacteria. Small doses of 24 hour cultures of bacteria such as Bacterium galleriae and Bacillus paratyphi were injected into the caterpillars. After one to several days, doses which were

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26 Metalnikov and co-workers, Ann. Inst. Pasteur 34, 888 (1920), 35, 363 (1929), 37, 528 (1923).

27 Chorine, Compt. rend. soc. biol. 96, 1289 (1937); Compt. rend. 186, 1859 (1928).

28 Paillot, Compt. rend. soc. biol. 83, 278 (1920), 84, 737 (1921); Compt. rend. 188, 1113 (1929).

29 Zernoff, Compt. rend. soc. biol. 97, 1697 (1927), 98, 1500 (1927), 99, 315 (1928).

30 Chorine, Compt, rend. 186, 657 (1928).

31 Metalnikov and Chorine, Ann. Inst. Pasteur 44, 273 (1930).

toxic to control insects did not kill the previously inoculated caterpillars. Thirty different bacteria were used as antigens. Five to ten insects were inoculated with each antigen. These workers were also able to demonstrate the presence of bacteriolysins in the blood of immunized insects.

In Marine Eggs

Lillie[32] (1913) noted the presence of sperm agglutinins in the egg water of sea urchins (Arbacia). In later studies (1919) on sea urchins of the genus Strongylocentrotus (S. francisonus and S. purpuratus), Lillie found that the reaction of the egg to the spermatozoon and the reaction of the spermatozoa, by agglutination, to egg secretions were both highly specific. He also obtained evidence indicating that there is a specific correlation between fertilization and the capacity for agglutination of the spermatozoa and concluded that the substance which agglutinates the spermatozoa is the same as that which activates fertilization of the egg. Tyler and Fox[33] (1940) obtained solutions of sperm agglutinins from sea-urchin eggs and keyhole limpet eggs by dissolving the jelly layer surrounding the egg with acidified (pH 3.5) sea water. Tyler[34] (1940) also found that cytolysis of the eggs

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[32] Lillie, J. Exptl. Zool. 14, 513 (1913), ibid. 16, 523 (1914); Biol. Bull. 40, 1 (1921).

[33] Tyler and Fox, Sci. 90, 516 (1939), Biol. Bull. 79, 153 (1940).

[34] Tyler, Proc. Nat. Acad. Sci. U.S. 26, 249 (1940).

released an antiagglutinin which inactivated the agglutinin. Production of sperm agglutinins by other marine eggs has been reported[a].

In Plants

Numerous investigators have reported the observation of normal agglutinins, lysins, precipitins and other antibodies in plants. Chester[35], in an excellent review of the literature points out the works of Marcusson-Begun[36], Wagner[37] and Wilkenko[38] as the most reliable investigations in this field.

Of greater practical importance is the study of acquired disease resistance in plants. The studies in acquired immunity in plants were inaugurated by the reports of Ray[39] and Beauverie[40] in 1901 of successful vaccination of certain plants against fungus disease. Both of these workers vaccinated susceptible plants with attenuated strains of Botrytis cinerea, and found that on transfer of such immunized plants

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[a] See Tyler and Fox (33) and references.

[35] Chester, Quart. Rev. Biol. 8, 129, 275 (1933).

[36] Marcusson-Begun, Z. Immunitäts. 45, 59 (1926).

[37] Wagner, Zentr. Bakt. 42, 613 (1915), 44, 708 (1916).

[38] Wilkenko, Z. Immunitäts. 5, 91 (1910).

[39] Ray, Rev. gen. botan. 13, 145 (1901).

[40] Beauverie, Compt. rend. 133, 107 (1901).

to environments rich in virulent B. cinerea, the plants resisted the parasite while non-vaccinated controls were killed. Other investigators have confirmed these results using the same and other parasites.[a] Inoculation of plants with extracts of parasites has also been shown to result in increased resistance to subsequent parasitic infection. Jarach[41] (1932) grew sterile bean seedling in gradually increasing concentrations of Botrytis cinerea cultures. Subsequent infection showed that the vaccinated beans were more resistant to Botrytis infection than were the controls. Some of the resistant plants were killed by heating to 70°C., by ether vapor or by cold. Such killed plants were quickly disintegrated by the fungus, ruling out the possibility that autotoxic substances present in the cells were responsible for the immunity. It is interesting that the resistance of the plants seemed to be a function of the living tissues, as it seems to be in animals. Acquired resistance to bacterial infections, to animal parasites and to various virus infections have been reported[a, 42, 43]. Magrou[42] (1938) reported the observation of specific agglutinins and precipitins in juices of plants which had been infected with Phytomonas tumefaciens, the agent of plant tumors. Magrou removed

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[a] See Chester (31) and references.

41 Jarach, Phytopath. Z. 4, 315 (1932).

42 Magrou, Ann. Inst. Pasteur 60, 565 (1938).

43 Price, Quart. Rev. Biol. 15, 338 (1940).

tumors from plants such as Pelargonium zonate and Chrysanthemum frutescens which had been inoculated three to four months earlier with P. tumefaciens, ground the material in a mortar, and filtered the juices. When the tumor extracts were mixed with suspensions of the bacteria in physiological salt solution, the cells were agglutinated. The reaction differed from the reaction of serum agglutinins in that the agglutination products were flocculent and filiform. The tumor extracts were also observed to react with extracts of the bacterial cells in typical precipitin reactions.

There are reports of at least six different plant virus diseases in which recovery from the disease is followed by resistance from a second attack[43]. The characteristic features of the immunity are similar for all six diseases. Plants recover after an acute attack by production of shoots or leaves which appear healthy or show only mild symptoms of disease, which still harbor virus, and which are refractory to infection with the virus in question but not to unrelated viruses.

Several workers[44, 45] have contended that this type of acquired tolerance to virus infections is not immunological in nature. They suggest that cells become tolerant of the virus because of the absence of virus precursor materials,

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[44] Valleau, Ky. Agr. Expt. Sta. Res. Bull. 360, 181 (1935); Phytopath. 31, 522 (1941).

[45] Kunkel, Phytopath. 24, 437 (1934).

or because the conversion of such materials into virus is retarded. However, as Wallace[46] points out, this suggestion does not explain the specificity of reported reactions. Cells in which the supply of virus precursors is exhausted because of an infection should not be able to support the growth of other unrelated viruses. The fact that recovered plants still contain virus is not incompatible with accepted immunological phenomenon. Rivers[47] has reported the presence of virus in animals long after the symptoms of the disease had disappeared.

There have been several reports of the development of immunity by bacteria to foreign material in their substrates[a]. In most cases, there is considerable reason to believe that the phenomena observed were instances of increased tolerance rather than immunity or resistance in the serological sense. However, Rosenow[48] (1945) reported an interesting study on the production in vitro of substances resembling antibodies from streptococci, pneumococci, staphylococci, and Bacillus subtilis. Suspensions of organisms, containing two billion to one hundred billion bacteria per ml. in neutral saline solution were divided into three fractions. One fraction was made 0.5N in sodium hydroxide, and another made 0.5N in hydrochloric acid. Aliquots of the suspensions

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46 Wallace, J. Agr. Res. 69, 187 (1944).

47 Rivers, Sci. 95, 107 (1942).

48 Rosenow, J. Infectious Diseases 76, 163 (1945).

[a] See Chester (31) and references.

were heated at different temperatures ranging from 10 to 123°C. for periods of twenty minutes to twelve days. Agglutinin and precipitin tests were run on the neutralized supernatants of the suspensions. Altogether about 200 suspensions were studied. Maximum agglutination titers were obtained from supernatants of suspensions that had been autoclaved for 48 hours at 17 pounds pressure in acid solution. Tests were incubated at 50°C. and read after eighteen and forty hours. Specificity of agglutination was shown especially well in high dilutions. Rosenow interpreted the very high agglutinin titers as indicating that agglutinins actually were produced from the bacteria and not merely liberated as auto-antibodies. Titers as high as 1/31,250 were noted. Rosenow found that the agglutinins did not have any bacteriostatic or bactericidal action on homologous or heterologous organisms but had antibody like action in vivo. In these respects they resemble antisera. He concluded that as long as the toxic components within such bacteria are not completely destroyed, agglutinins are formed. These agglutinins have many of the characteristics of antibodies produced by animals under the stimulus of an injected antigen.

Antibody Production in Acellular Systems

In vitro formation of antibodies in the absence of cells has been claimed by many workers. Substances simulating specific antibodies formed outside the animal body were demonstrated by Ostromuislenskii[49] in 1915. By the incubation of a mixture of horse serum containing six percent sodium chloride and either diphtheria toxin or botulism toxin for 36 hours at 37°, a product was obtained with the therapeutic properties of an antitoxin. Inoculation of experimental animals with lethal doses of toxin plus an equivalent of the incubation product was harmless to the animals. Upon acidification of the incubation product, a solution was obtained which was lethal but which could be converted to a non-lethal solution by exact neutralization. Ostromuislenskii postulated that the natural globulins of animal serum adsorb the administered toxin first forming a non-poisonous salt readily decomposable by acid or alkali. The salt then undergoes a gradual profound change with the formation of antitoxin and the release of the toxin, which is then free to convert more globulin to antitoxin. This last change takes place only upon prolonged incubation, the time being dependent on the nature of the salt.

Variations of Ostromuislenskii's method have been used

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[49] Ostromuislenskii, J. Russ. Phys. Chem. Soc. 47, 263 (1915).

by other investigators with varying success. Kryshanowski[50] (1929) prepared two artificial diphtheria antitoxins of different therapeutic value by incubating diphtheria toxin with different protein fractions of normal horse serum, and found that the globulin fraction was much more effective than was the albumin fraction for this preparation. Baschkirzev[51] applied Ostromuislenskii's method to the production of agglutinins for gonococcus and noted that blood previously inoculated with gonococcus was bactericidal to the organism on subsequent reinoculation. Mez and Ziegenspeck[52] (1925) incubated normal plant juices with beef serum and reported the production of artificial precipitins specific for each of the juices. Eisler[53] (1927) was unable to confirm their results. He found that artificial antisera prepared by the method of Mez and Ziegenspeck reacted quite unspecifically both in precipitation and in agglutination reactions. Eisler concluded that the observed flocculation was a non-specific colloidal reaction between ingredients of the serum and the extract. However, Sasse[54] (1928) used the method of Mez with success. Sasse mixed plant juices with clear nonhemolytic

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50 Kryshanowski, Zentr. Bakt. 110, 1 (1929).

51 Baschkirzev, Z. Urol. 23, 92 (1929).

52 Mez and Ziegenspeck, Botan. Arch. 12, 163 (1925).

53 Eisler, Z. Immunitats. 53, 136 (1927).

54 Sasse, Beitr. Biol. Pflanz. 16, 351 (1928).

beef serum, incubated the mixture for eight days at 35°, and then diluted and centrifuged the mixture. The centrifugate then contained an antiserum with a titer as high as 1/1600 in some cases. Sasse concluded that although antisera could be produced by this method, the reaction has but little practical importance since the antibody titer is low and since non-specific reactions were obtained in certain cases. Nachmacher[55] (1929) also reported the observation of overlapping reactions and concluded that no specific antibodies were formed since no specificity of reaction was observed.

Sdravosmisslov and Kostromin[56] (1927) incubated diphtheria toxin with commercial trypsin for two and one-half hours at 37.5° and reported the successful synthesis in vitro of a toxin-trypsinate complex with the therapeutic properties of diphtheria antitoxin. These investigators had earlier (1923) subjected cholera virus and various bacteria to the action of trypsin[57] and had found specific agglutinins and lysins, as demonstrated by complement fixation tests. The first contact of antigen and enzyme resulted in production of some lysis but no agglutination. Addition of the bacterial antigen to the enzyme-antigen complex after digestion caused agglutination of the bacteria. However, Kimmelstiel[58] (1929),

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55 Nachmacher, Beitr. Biol. Pflanz. 17, 1 (1929).

56 Sdravosmisslov and Kostromin, Z. Immunitats. 54, 1 (1927)

57 Sdravosmisslov and Kostromin, Bull. Inst. Pasteur 21, 941 (1923).

58 Kimmelstiel, Z. Immunitats. 62, 245 (1929).

in an attempt to confirm their results, was unable to produce specific antibodies.

Bacon[3] (1943) reported production of antibodies by a procedure very similar to Ostromuislenskii's original method. Bacon lyophilized bovine plasma in the presence of two percent by volume of staphylococcus toxin and of diphtheria toxin and incubated the dried material at 37° for twelve hours. The plasma was then restored to its original volume with water, adjusted to pH 7.4 with 0.1 N acetic acid, and filtered. Precipitin tests on the filtrates gave strong tests, the precipitate filling from five to fifteen percent of the test tube. Control tubes gave light or negative tests. Bacon concluded that "the production of antibodies under laboratory conditions is within the bounds of possibility". He points out that fever, which accompanies infection in animals, may conceivably be due to a shortage of water available in the body for the adsorption and transportation of heat energy. He therefore suggests that dehydration of blood plasma, with its resultant alkalinization, may cause remodeling of normal globulins to antibody globulins *in vitro* as well as in infected animals. Unfortunately, Bacon did not test his antitoxin preparations for therapeutic value.

Pauling and Campbell[59] (1942) prepared antibodies *in vitro* by subjecting normal globulins or other proteins to

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[59] Pauling and Campbell, *Sci.* 95, 440 (1942), *J. Exptl. Med.* 76, 211 (1942).

the action of denaturing and renaturing reagents or conditions in the presence of an antigen. The procedure was based on the hypothesis that if globulins are treated with denaturing agents strong enough to cause the protein chain to uncoil, and if this agent is removed slowly while the antigen is present in solution in considerable concentration, the chain ends will coil up to assume configurations stable under these conditions and complementary to the antigen. The recoiled globulin will then have the properties of a specific homologous antibody. Various methods of denaturation were investigated. The most successful consisted of heating to 65°, slowly cooling, and incubating for several days at 57°. In a typical experiment, a 0.01 percent solution of 1,3-dihydroxy-2,4,6-tri-(p-azophenyl) arsonic acid in a 1 percent protein solution at pH 7.8 was held at 57° for about fourteen days. The mixture was then dialyzed against 1 percent solution of sodium chloride to remove excess antigen. Aliquots of the dialyzate, when mixed with antigen, gave specific tests. The tests were weak, however, and differed from those of immune sera in that more critical conditions were necessary for precipitation.

An unusual method of <u>in vitro</u> antibody production was reported by Laiseleur[60] (1938). Using an electrodialysis procedure, Laiseleur claims to have produced antibodies to

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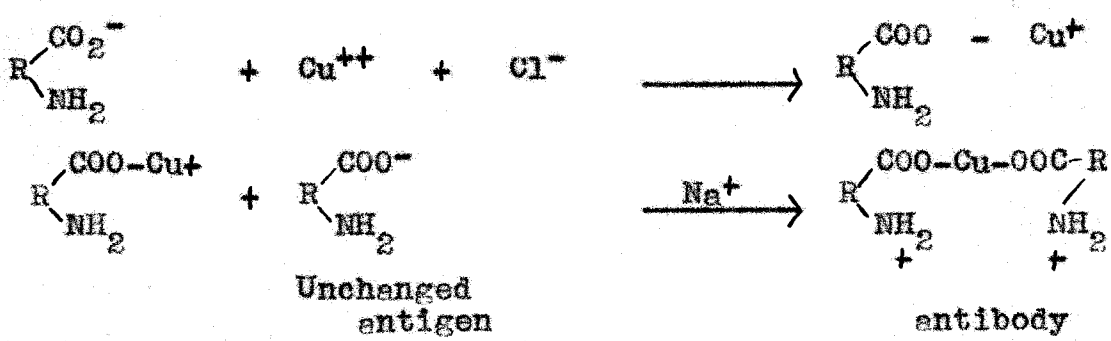
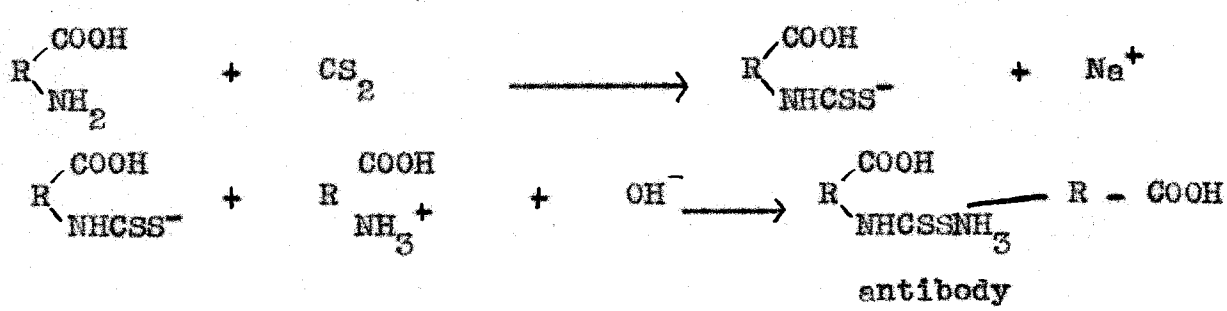
[60] Laiseleur, <u>Compt. rend. soc. biol. 129</u>, 172, 250, 358, (1938).

cobra and viper venoms, diphtheria and tetanus toxins and ovalbumin. Laiseleur assumes that antigens and antibodies are electrical images of each other, and that if the charge on each of the dissociated groups of an antigenic protein molecule is reversed with change in the protein structure, the theoretical conditions for antibody production will have been met. The procedure consists of dialysis of a solution of antigen in the presence of chloride and cupric ions for two hours (current, 110 volts at 20-50 milliamps.). At the end of two hours the solution was alkaline. Five mls. of $CS_2$ were added to the alkaline solution which was then allowed to react for another thirty minutes. After this period, the solution contained the material which Laiseleur considers to be a specific antibody. Laiseleur postulated the following series of reactions for the procedure.




In the presence of $CS_2$, the reactions are pictured as



Laiscleur claims to have obtained strong and specific agglutination reactions between antigens such as ovalbumin, casein and gelatin and antibodies prepared by this method, and to have produced antivenom solutions of such strength that 5 mg. of the antibody neutralized the toxic effect of 1 mg. of venom when mixtures were injected into rabbits. Little experimental evidence is given in his papers.

✓ * As far as could be determined, no methods other than the standard method of animal inoculation have been used for the preparation of antibodies for use in medical therapy. However, British Patent #239,302[61] covers the production of antitoxins with yeast cells. The patent states that yeast cells, grown in a culture of a toxin, or a culture of a toxin together with body fluids or organisms containing toxin within themselves, accumulate counter substances which may be used as medicaments. The yeast cells when dried, and formed into pills, are available for intravenous or oral administration. The therapeutic value of Deutschmann's preparation may be seriously questioned. No experimental confirmation of his claims was presented. He apparently did not employ any of the relatively precise tests in common use to check the antibody content of his preparations. Moreover, there are no other reports in the literature by Deutschmann or other investigators on production of antitoxins by yeast.

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[61] Deutschmann, Brit. Patent 239,302, June 11, 1924.

# EXPERIMENTAL

## Preparation of Compounds

### 2,6-Diiodo-4-nitrophenol

The method of Savitskiĭ[62] for the iodination of tyrosine was adapted for the preparation of this compound. Thirty-five g. (0.25 mole) of p-nitrophenol (Eastman Kodak Co.) were dissolved in 670 ml. of concentrated ammonium hydroxide and 755 ml. of water in a five liter flask. To this solution was added slowly, with constant stirring, 128 g. (0.50 mole) of iodine dissolved in 600 ml. of a 5 percent solution of potassium iodide. The solution was held at 6-8° during the reaction period of three hours. Three g. (0.024 mole) of sodium sulfite were added to the solution and allowed to react for five hours at room temperature. The solution was acidified with 700 ml. of concentrated hydrochloric acid, filtered and extracted with ether. Evaporation of the ether extracts yielded 23 g. (23%) of a yellow solid, which was soluble in alcohol, and which melted at 153-155° (uncor.).

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[62] Savitskiĭ, J. Gen. Chem. U.S.S.R. 9, 1342 (1939)/C.A. 34, 741 (1940).

Anal. Calc'd for $C_6H_3O_3N\,I_2$; I, 65.0%; N, 3.6%.

Found; I,[a] 64.8%; N, 3.9%.

2,6-Diiodo-4-aminophenol

The method given in Lassar-Cohn[63] for the preparation of m-amino-p-iodotoluene was adapted for this reduction. Thirty g. (0.20 mole) of ferrous sulfate was dissolved in 100 ml. of water and enough concentrated ammonium hydroxide to make the solution alkaline. Five g. (0.013 mole) of 2,6-diiodo-4-nitrophenol were dissolved in 100 ml. of ethanol. The solutions were mixed and refluxed on a boiling water bath for eight hours. The alcohol was then boiled

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63 Lassar-Cohn, "Arbeitsmethoden für Organisch-Chenische Laboratorien", 5th Ed. Voss, Leipzig (1923). p. 819.

a The method of Reimers, Z. Anal. Chem. 118, 399 (1940), for the estimation of iodine in thyroid tissue was adapted and found applicable for the determination of iodine in organic compounds of known constitution and in iodo proteins. The method has been used for analysis of the following compounds in addition to those reported in this thesis.

3,5-Diiodo-4-hydroxybenzoic acid
Anal. Calc'd for $C_7H_4O_3I_2$; I, 65.1%.
Found; I, 64.2%.

5-(3,5-Diiodo-4-hydroxybenzyl)-hydantoin
Anal. Calc'd. for $C_{10}H_8O_3N_2I_2$; I, 55.4%
Found; I, 54.7%, 54.2%.

α-Ureido-β-(3,5-Diiodo-4-hydroxyphenyl)-propionic acid
Anal. Calc'd. for $C_{10}H_{10}O_4N_2I_2$; I, 53.3%
Found; I, 52.6%.

The figures on the diiodotyrosine derivatives were made available through the kindness of Mr. F. N. Minard, who prepared and analyzed the compounds.

off; the aqueous solution decanted, and the residue extracted with 500 ml. of hot absolute alcohol. The alcohol was distilled off under reduced pressure, leaving a reddish brown residue which was dissolved in concentrated sodium hydroxide solution and extracted with ether. On evaporation of the ether in an air stream, a yield of 2.3 g. (49%) of yellow needlelike crystals was recovered. The material was recrystallized from ethanol. A sample melted at 214-215° (uncor.) under iodine vapor.

Anal. Calc'd for $C_6H_5ONI_2$; I, 70.4%; N, 3.9%.

Found; I, 70.2%; N, 4.0%.

Attempts to prepare 2,6-diiodo-4-aminophenol by reduction of 2,6 diiodo-4-nitrophenol with stannous chloride and hydrochloric acid[64] were unsuccessful.

3,5-Diiodo-4-hydroxybenzeneazo casein

The diazotization and coupling reaction of Landsteiner[65] and Van der Scheer for aniline and horse serum was adapted for the preparation of 3,5-diiodo-4-hydroxybenzeneazo casein. Two g. (0.005 mole) of 2,6-diiodo-4-aminophenol were dissolved in 100 ml. of water and 43 ml. of N hydrochloric acid, and diazotized at 0-5° by dropwise addition of a sodium

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64 Seifert, J. prak. Chem. 28, 437 (1883).

65 Landsteiner and Van der Scheer, J. Exptl. Med. 45, 1045 (1927).

nitrite solution until a starch-iodide end point was reached. The solution was then diluted to 1500 ml. with ice water, and added slowly to 18 g. of casein (Pfanstiehl, pure) dissolved in 180 ml. of a 0.2 N sodium carbonate solution. The mixture was allowed to react for three and one-half hours with constant stirring, and stored overnight in the ice box. The solution was next acidified with 10 percent hydrochloric acid. The precipitate which formed was centrifuged and washed with absolute alcohol until the alcohol washings were colorless. The protein was recentrifuged and dried over sulfuric acid in a vacuum desiccator for seven days. The protein and its solutions were light yellow in color. The protein was soluble in alkali and in neutral salt solutions.

Anal. Found; I, 2.7%.

Nitro casein

Nitro casein was prepared by the method of Mutsaars[66]. Twenty g. of casein (Pfanstiehl, pure) were dissolved in 200 ml. of a 5 percent solution of sodium carbonate and added dropwise to one liter of 6 N nitric acid. After five minutes at room temperature, the reaction mixture was poured into a three liter flask containing 1,000 ml. of ice water and was placed in an ice box for twelve hours. The precipitated protein was separated by centrifugation.

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[66] Mutsaars, Ann. Inst. Pasteur 62, 81 (1939).

The protein was purified by solution in 500 ml. of a 5 percent sodium carbonate solution and reprecipitation with 5 N hydrochloric acid, and by dialysis against distilled water. The protein was dried over sulfuric acid in a vacuum desiccator for seven days. The nitro protein and its solutions were yellow in color.

Anal. Found: Protein N (Kjeldahl), 15.8%; Total N (Dumas) 21.2%.

The Dumas method for nitrogen measures both amino and nitro nitrogen. The Kjeldahl method gives low results on nitro compounds.[67] The original Pfanstiehl casein contained 13.6% N as determined by the Kjeldahl method.

4-Sulfobenzeneazo casein[a]

A solution of 2.2 g. (0.013 mole) of sulfanilic acid (Baker and Adams) in 30 ml. of water and 15 ml. of 7 N hydrochloric acid was prepared. The solution was cooled to 0-5° in an ice bath and a solution of sodium nitrite was added dropwise until a positive reaction was obtained with starch-iodide paper. The solution was then diluted to 100 ml. with ice water and added to 21 g. of casein

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[67] Johns, "Laboratory Manual of Microchemistry," Burgess, Minneapolis, Minn., (1941), p. 12.

[a] All diazotization and coupling reactions were adapted from the general procedure of Landsteiner and Van der Scheer, J. Exptl. Med. 45, 1945 (1927).

(Pfanstiehl, pure) dissolved in 300 ml. of a 0.3 N sodium carbonate solution. The solution was kept alkaline throughout the reaction period by the addition of more sodium carbonate solution when necessary, and was held at 0-5° throughout the reaction period and for thirty minutes after completion of the reaction. The protein was then precipitated with 5 percent hydrochloric acid, dialyzed for 24 hours against distilled water and separated by centrifugation. The protein was orange-red in color. On desiccation the color darkened to a reddish brown. Solutions at pH 7.4-7.8 were red. The protein was dried over sulfuric acid in a vacuum desiccator for seven days.

Anal. Found: S, 2.5%; neut. equiv. 236.

Neut. equiv. of Pfanstiehl casein was found to be 800.

4-Carboxybenzeneazo casein.[a]

A solution of 1.8 g. (0.012 mole) of p-aminobenzoic acid in 30 ml. of distilled water and 15 ml. of 7 N hydrochloric acid was prepared. A 2 percent sodium nitrite solution was added dropwise until a positive test was obtained with starch-iodide paper. The solution was kept at 0-5° in an ice bath during diazotization. The solution was then diluted to 100 ml. with ice water and added to 21 g. of casein (Pfanstiehl, pure) dissolved in 300 ml. of a 0.3 N

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[a] All diazotization and coupling reactions were adapted from the general procedure of Landsteiner and Van der Scheer, J. Exptl. Med. 45, 1045 (1927).

sodium carbonate solution. The solution was held at 0-5° and was kept alkaline to phenolphthalein during the coupling reaction. The solution was then acidified with 5 N hydrochloric acid. The precipitated protein was dialyzed for 24 hours against distilled water and was dried over sulfuric acid in a vacuum desiccator for seven days. The protein was red in color. Its solutions were also red.

Anal. Found: neut. equiv., 101.

Arsanilic acid

The procedure of Cheetham and Schmidt[68] was followed with slight modifications. Forty-seven g. (0.33 mole) of arsenic acid (General Chem. Co.) and 152 ml. (1.63 moles) of aniline (General Chem. Co.) were placed in a Kjeldahl flask and heated on an oil bath at 150-160° for five hours, allowed to stand at room temperature overnight, and reheated to 150-160° for another five hours. The material was washed into a one liter beaker with 200 ml. of hot water, mixed with 60 ml. of a 6 N sodium hydroxide solution and cooled under a water tap. The aqueous layer was separated from excess aniline in a separatory funnel. Twenty gm. of Filter Cel were added to the solution, which was then filtered with suction. The filtrate was mixed with 50 ml. of 6 N hydrochloric acid and placed in the ice box. White needlelike crystals separated out in four hours. The crystals were

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[68] Cheetham and Schmidt, J. Am. Chem. Soc. 42, 828 (1920).

filtered and recrystallized from hot water. A sample melted at 230° (uncor.). The yield of arsanilic acid was 24 g. (34%).

Anal. Calc'd for $C_6H_8O_3N$ As; As, 54.5%; N, 6.50%

Found; As[a], 33.8%; N, 6.5%

4-Arsonobenzeneazo casein[b]

Two g. (0.009 mole) of arsanilic acid were dissolved in 10 ml. of 7 N hydrochloric acid, and diazotized at 0° with excess of a 2 percent solution of sodium nitrite. Starch-iodide paper was used as an indicator. The solution was diluted with 100 ml. of ice water and added slowly, with constant stirring, to 50 g. of casein (Pfanstiehl, pure) dissolved in 500 ml. of a 2 N sodium carbonate solution. The reaction mixture was held at 5-10° during the thirty minutes allowed for coupling, and was kept alkaline to phenolphthalein by addition of 2 N sodium hydroxide solution when necessary. The arsono protein was precipitated by acidifying the solution with 5 percent hydrochloric acid and was purified by reprecipitation from a 12 N sodium hydroxide solution by the addition of ten volumes of ethanol and enough hydrochloric acid to cause flocculation of the pro-

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[a] All arsenic analyses by the method of Ewins, J. Chem. Soc. 109, 1355 (1916).

[b] All diazotization and coupling procedures were adapted from the general procedure of Landsteiner and Van der Scheer, J. Exptl. Med. 45, 1045 (1927).

tein, and by dialysis against distilled water. The protein was dried over sulfuric acid in a vacuum desiccator for seven days. Both the dried protein and its solutions were a bright yellow in color.

Anal. Found; As, 1.0%.

A second preparation of 4-arsonobenzeneazo casein was made in order to obtain a casein with a higher arsenic content. Twenty-five g. of casein (Pfanstiehl, pure) were dissolved in 500 ml. of a 2 N sodium carbonate solution. Three g. of arsanilic acid were dissolved in 20 ml. of 7 N hydrochloric acid and diazotized with excess of 2 percent solution of sodium nitrite at 0-5°. Subsequent treatment was identical with that of the first preparation. The protein obtained was orange in color and formed orange-red solutions in dilute alkali. The protein was dried over sulfuric acid in a vacuum desiccator for seven days.

Anal. Found: As, 3.9%; N, 19.5%.

4-Arsonobenzeneazo egg albumen[a]

Four-tenths g. (0.002 mole) of arsanilic acid were dissolved in 5 ml. of 7 N hydrochloric acid and diazotized by the slow addition of a 2 percent sodium nitrite solution until the starch-iodide end point was reached. The solution

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[a] All diazotization and coupling procedures were adapted from the general procedure of Landsteiner and Van der Scheer, J. Exptl. Med. 45, 1045 (1927).

was diluted to 60 ml. with ice water and added slowly, with constant stirring, to 10 g. of egg albumen (Baker and Adams) in 500 ml. of a N sodium carbonate solution. The solution was held at 0-5° during diazotization and the thirty minute period allowed for coupling. The solution was acidified with 7 N hydrochloric acid and the protein which precipitated was separated by centrifugation. The protein was purified by three reprecipitations from a 12 N sodium hydroxide solution with ten volumes of ethanol and enough hydrochloric acid to cause flocculation of the protein, and by dialysis against distilled water. The protein was dried over sulfuric acid in a vacuum desiccator for seven days. The protein and its solutions were light orange in color.

Anal. Found; As, 2.4%; N, 22%.

4-Arsonobenzeneazo blood albumin[a]

Four-tenths g. (0.002 mole) of arsanilic acid were dissolved in 5 ml. of 7 N hydrochloric acid and diazotized at 0° by the addition of a 2 percent solution of sodium nitrite to a starch-iodide end point. The solution was diluted to 10 ml. with ice water, and coupled at 5-10° to 1 g. of blood albumin (of unknown history) dissolved in 45 ml. of a 1 N sodium carbonate solution. After 15 minutes, the mixture

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[a] All diazotization and coupling procedures were adapted from the general procedure of Landsteiner and Van der Scheer, J. Exptl. Med. 45, 1045 (1927).

was filtered to separate the small amount of protein which had not been in solution. The filtrate was acidified with 5 N hydrochloric acid. The protein which precipitated was purified by reprecipitation from 5 ml. of a 10 N sodium hydroxide solution by the addition of ten volumes of ethanol and enough hydrochloric acid to cause flocculation of the protein. The arsono protein was dried over sulfuric acid in a vacuum desiccator for seven days.

Anal. Found; As, 0.1%.

A second preparation of 4-arsonobenzeneazo blood albumin was made using two g. of bovine plasma albumin (Armour, Crystalline, lot 46). The albumin was dissolved in 200 ml. of a 1 N sodium carbonate solution and coupled with 0.1 g. (0.0045 mole) gm. of diazotized arsanilic acid by the procedure used for the first preparation. The protein was precipitated by acidification of the solution with hydrochloric acid, and was purified by two reprecipitations from sodium hydroxide solution with hydrochloric acid and ethanol, and by dialysis against distilled water. The protein was dried in a vacuum desiccator over sulfuric acid for seven days. Both the arsono protein and its solutions in neutral salts were orange in color.

Anal. Found: As, 3.9%; N, 20%.

Iodo casein

The method of Wormall[69] was followed. Twenty-five g.

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[69] Wormall, J. Exptl. Med. 51, 295 (1930).

of casein (Pfanstiehl, pure) were dissolved in 1,000 ml. of a 1 N sodium carbonate solution. Two g. of iodine in a 10 percent solution of potassium iodide were added to the protein solution in a two liter glass-stoppered flask. The mixture was allowed to react for 24 hours in the ice box. The solution was then acidified with 5 percent hydrochloric acid. The protein which precipitated was filtered with suction and was washed with water until the filtrate was chloride free and colorless. The iodo casein was dried over sulfuric acid in a vacuum desiccator for seven days. The protein was white in color and its solutions were colorless.

Anal. Found; I, 2.8%; N, 16.5%.

Iodo egg albumen.

The method of Shahrokh[70] was used. Ten g. of egg albumen (Baker and Adams) were dissolved in 1,400 ml. of solution containing 40 g. of dipotassium hydrogen phosphate and 40 g. of potassium dihydrogen phosphate. Five g. of iodine in 120 ml. of a 5 percent solution of potassium iodide were added and this solution was held at 5-10° for twenty hours. Excess iodine was then reduced by addition of a saturated solution of sodium thiosulfate. The iodo albumen was precipitated by the addition of an equal volume of a saturated ammonium sulfate solution and enough 1 percent acetic acid to make the solution distinctly turbid. The mixture was

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[70] Shahrokh, J. Biol. Chem. 151, 659 (1943).

placed in the ice box for six hours to allow the precipitate to settle out. The protein was then separated by centrifugation, purified by dialysis against distilled water, and dried over sulfuric acid in a vacuum desiccator for seven days.

Anal. Found; I, 2.6%; N, 14.5%.

Iodo plasma albumin

Iodo plasma albumin was also prepared by the method of Shahrokh. Two g. of bovine plasma albumin (Armour, crystalline, lot 46) were dissolved in 240 ml. of solution containing 8 g. of potassium dihydrogen phosphate and 8 g. of dipotassium hydrogen phosphate in a glass-stoppered flask. One-half g. of iodine in 120 ml. of a 10 percent potassium iodide solution were added to the clear protein solution and the flask was kept in the ice box for 24 hours. Ten ml. of a saturated sodium thiosulfate solution were added to reduce the excess iodine. The protein was precipitated by the addition of an equal volume of a saturated ammonium sulfate solution. The precipitate was separated by centrifugation, dialyzed against distilled water and dried over sulfuric acid in a vacuum desiccator for seven days.

Anal. Found: I, 0.4%; N, 22%.

S-Methylisothiourea sulfate

The procedure of Shildneck and Windus[71] was followed. Fifty g. (2/3 mole) of thiourea (Eastman Kodak 497) were mixed with 25 ml. of water in a liter round bottom flask. Thirty-six ml. (4 moles) of methyl sulfate (Eastman Kodak, practical) were added. The flask was heated with a moving flame until a spontaneous reaction started. After completion of the spontaneous reaction, the material was refluxed on an oil bath (bath temperature 130°-140°) for 1½ hours. The mixture was then cooled, and 200 ml. of absolute alcohol were added. There were formed immediately white crystals which, when filtered, washed with alcohol, and dried in air overnight, melted with decomposition at 228° (uncor.). The yield was 63 g. (68%).

Anal. Calc'd. for $C_4H_{14}O_4N_4S_3$; N, 20.3%

Found; N, 20.3%.

Guanidyl casein

The method of Schütte was used[72]. Twenty-five g. of casein (Pfanstiehl, pure) were dissolved in 500 ml. of concentrated ammonium hydroxide, and 8.5 g. of S-methylisothiourea sulfate were added to the protein solution in a ground glass stoppered flask. The reaction mixture was allowed to sit at

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[71] Blatt, "Organic Syntheses" Col. Vol. II, John Wiley & Sons, Inc., New York, N. Y. (1943), p. 411.

[72] Schütte, Z. physiol. Chem. 279, 59 (1943).

room temperature for three weeks. The protein was then dialyzed for three days against tap water and for three days against distilled water. The precipitated protein was then separated by centrifugation and dried over sulfuric acid in a vacuum desiccator for seven days. The proteins and their solutions were colorless.

Anal. Found: Protein N. (Kjel.), 23.2%.

A duplicate sample was allowed to react for only three days and was then dialyzed and dried in the same way.

Anal Found: Protein N (Kjel.), 13.6%.

The original Pfanstiehl casein contained 13.6% N.

Guanidyl egg albumen

The method of Schütte was followed. Twenty g. of egg albumen (Baker and Adams) were dissolved in 400 ml. of concentrated ammonium hydroxide, and 6.5 g. of S-methylisothiourea sulfate were added to the protein solution in a ground glass-stoppered flask. The mixture was allowed to react at room temperature for three weeks. The protein was then dialyzed for three days against tap water and for three days against distilled water. The precipitated protein was separated by centrifugation and dried over sulfuric acid in a vacuum desiccator for seven days. The protein and its solutions were colorless.

Anal. Found: Protein N (Kjel.), 23.1%.

A duplicate sample was allowed to react for only three days

and was then dialyzed and dried.

Anal. Found: Protein N (Kjel.), 26.1%.

The original egg albumen contained 14.5% N.

## Methods of Antibody Production

Three methods of antibody production were investigated; stimulation of antibody production by intravenous injection of antigens into rabbits, lyophilization and incubation of antigen and bovine serum mixtures as developed by Bacon[3] and culture of yeast in a media containing an antigenic protein. Yeast was tested because of its rapid synthesis of protein as demonstrated in the manufacture of nutritional concentrates.[73]

### Inoculation of Animals with Antigens

The method of rabbit inoculation used was substantially that given in Hawk and Bergeim[74]. Solutions of 4-arsonobenzeneazo casein (3.8 mg. N/ml.) and iodocasein (1.7 mg. N/ml.) were prepared and sterilized by passage through a Seitz filter. The antigen solutions were stored in the ice box at 5-10°. Rabbits were injected intravenously at three to four day intervals with one of the antigen solutions. The

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[73] Fink, Vorratspflege u. Lebensmittelforsch. 1, 52, 107 (1938).

[74] Hawk and Bergeim, "Physiological Chemistry", 11th Ed., The Blakiston Co., Philadelphia. Pa. p. 400.

amount of inoculum was increased from 0.5 to 1.0 ml. in a series of 4 to 12 injections. Blood was removed ten days after the last inoculation, preserved with 0.01 percent merthiolate, and stored in the ice box at 5-10°.

Incubation of Blood Plasma and Antigens

Bacon's method of antibody production was modified slightly. One hundred ml. of clear citrated or oxalated and centrifuged beef plasma were placed in a 1000 ml. suction flask. To the plasma was added an aliquot of an antigen solution containing 2 g. of the antigen. (Smaller amounts of antigen were used in the initial experiments. The better results were obtained when the relative concentrations indicated were used.) The solutions were thoroughly mixed and were lyophilized. The dried material was incubated at 37° for 12-14 hours, and was then reconstituted with distilled water and sufficient 0.1 N acetic acid to adjust the pH to approximately 7.4. (The majority of solutions prepared did not require addition of acid.) The reconstituted solution was filtered, and an aliquot of the filtrate tested for precipitin content by the following method. One to five ml. aliquots of the filtrate were mixed with 1 ml. of a 0.1 percent solution of the homologous antigen in 1 percent sodium chloride solution, and incubated for twelve hours at 37°. Readings were taken every hour. Incubation for 4-6 hours gave the most satisfactory readings. Intensity of reaction

increases with increased time of incubation, but non-specific precipitation interfered after about six hours.

Culturing Yeast in Media Containing Antigens

The yeast used was Saccharomyces cerevisiae, Strain No. 567 of the Northern Regional Research Laboratory[a]. The stock culture was carried on a 2.5 percent malt extract agar slant[b]. Cultures were transferred every three months. Loop transfers were made from the stock cultures to flasks containing 100 ml. of a 15-20 percent molasses solution and were then incubated for 24 hours at 30°. In order to make up media for any particular run, 80 ml. of molasses medium, 10 ml. of an approximately 1 percent solution of an antigen and a 10 ml. aliquot of a 24 hour yeast culture were combined in a 600 ml. flask. The number of yeast cells inoculated was approximately $6 \times 10^8$ in each case. The flasks were then incubated for 48 hours at 30°.

The molasses medium was prepared by dissolving the molasses (Brer Rabbit or Anheuser Busch) in hot water, boiling for 15-20 minutes, and filtering off the coagulated protein through a mat of Filter Cel. The medium was sterilized by autoclaving at fifteen pounds pressure for 20 minutes.

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[a] Kindly furnished by Dr. L. A. Underkofler and Dr. E. I. Fulmer.

[b] Twenty-five g. of wart agar, 25 g. of malt extract, and 20 g. of agar were dissolved in 1,000 ml. of water. The media was autoclaved for 15 minutes at 15 pounds pressure. Ten ml. aliquots of media were used to prepare agar slants.

One percent solutions of the antigens were prepared by dissolving 5 g. of the dried protein in 50 ml. of a 5 N sodium hydroxide solution, diluting with 400 ml. of distilled water, and neutralizing the solution with 50 ml. of 5 N hydrochloric acid. If the protein was insoluble in neutral solution, the pH was sometimes adjusted by dropwise addition of 10 N sodium hydroxide until the solution was clear. In other cases, antigen solutions containing suspended protein particles were used.

After incubation, the cells were separated by centrifugation, washed with distilled water, cytolyzed by fairly continuous grinding with sea sand and ether for 30 minutes, and extracted with 25 ml. of a 5 percent solution of sodium chloride. The sodium chloride extracts were decanted from the sand and centrifuged until clear. It was found that centrifugation did not remove all the suspended material, and in later work the centrifugates were treated with 0.1 g. of Filter Cel for 15-20 minutes and filtered. The extracts were then tested for precipitin content.

Extracts giving stronger precipitin tests were obtained from yeast cells which were transferred to fresh media containing the same concentration of antigen each 48 hours for a series of two to six transfers, and then were cytolyzed and extracted by the procedure given above.

## Methods of Testing for Antibodies

### Micro

Two methods of testing for precipitins were used, the micro method of Hanks[75] and the more commonly used macro precipitin test[76]. Hanks' method for determination of small amounts of precipitins was modified slightly. One ml. aliquots of gelatin base or antigen solutions in gelatin base were placed in 75 x 7 mm. test tubes. One ml. aliquots of the solutions to be tested for precipitin content were layered over the gelatin. The test tubes were incubated at 37° for 1-3 hours and then placed in an ice box for three days. Readings were taken after 1, 2, 3 and 72 hours of incubation.

Gelatin base was prepared by dissolving 25 g. of Difco gelatin in 400 ml. of distilled water, adding 10 g. of sodium chloride, 2.5 g. of phenol and 100 ml. of glycerol to the gelatin solution, and mixing thoroughly. The antigen solutions for testing were prepared by mixing 9 ml. of gelatin base and 1 ml. of a clear stock solution of the desired concentration of antigen in 1 percent sodium chloride solution. Stock solutions of antigens were prepared by the procedure given on p. 45.

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[75] Hanks, J. Immunol. 28, 95 (1935).

[76] Kolmer, "A Practical Textbook of Infection, Immunity, and Biologic Therapy", 3rd Ed., W. B. Saunders Co., Philadelphia, Pa., 1924. P. 1133.

Macro

The macro precipitin tests were set up by a modification of Kolmer's method[76]. One ml. of approximately 0.1 percent solutions of the antigens in 1 percent sodium chloride solution were placed in small test tubes (100 x 12.5 mm.) Antigen solutions were prepared by diluting 1 ml. of 1 percent solutions of protein prepared in the manner given on p. 45, with 9 ml. of a 1 percent sodium chloride solution. All antigen solutions used for testing were optically clear. One to five ml. aliquots of the solutions being tested for precipitin content were mixed thoroughly with the antigen solutions. The tests were incubated at 37° for 1-3 hours and then placed in an ice box overnight. Readings were taken after 1, 2 and 3 hours of incubation and after a night in the ice box.

# RESULTS

## Production of Antibodies by Experimental Animals

It seemed desirable to check the ability of some of the antigens to stimulate specific antibody production by the usual immunological procedure of animal inoculation. Four rabbits were employed. Two of the rabbits were injected intravenously with a sterile solution of 4-arsonobenzeneazo casein (3.8 mg. N/ml.). Two were treated in the same manner with a sterile solution of iodo casein (1.7 mg. N/ml.). The amount of inoculum was increased from 0.5 to 1.0 ml. in a series of four to twelve injections. Injections were made at 3-4 day intervals with a rest period of ten days after 6, 10, and 12 injections. Blood was removed for titer determinations at the end of each rest period.

In the tests, one ml. aliquots of serial dilutions of rabbit serum and 1 ml. of a 0.1 percent solution of the homologous antigen were mixed and incubated at 37° for two hours, and overnight in an ice box. Results of micro and macro tests are shown in Tables I and II.

To test the specificity of the antibodies present in the rabbit sera, cross tests were run using the serum dilution which gave the strongest precipitin reaction in the titer determinations. One ml. aliquots of serum were added

Table I

Titer Determined by Hank's Micro Method[a]

| No. of injections | Serum from rabbits injected with 4-Arsonobenzeneazo casein #1 | #2 | Iodocasein #1 | #2 |
|---|---|---|---|---|
| 6 | | 1/160 | 1/640 | 1/1280 |
| 10 | | 1/1280 | 1/1280 | 1/5120 |
| 12 | 1/40[b] | 1/2560 | 1/10,240 | 1/5120 |

[a] Titers recorded are the highest dilution which gave a precipitate.
[b] Rabbit sacrificed after four injections.

Table II

Titer Determined by Macro Method[a]

| No. of injections | Serum from rabbits injected with 4-Arsonobenzeneazo casein #1 | #2 | Iodocasein #1 | #2 |
|---|---|---|---|---|
| 6 | | 1/80 | 1/40 | 1/40 |
| 10 | | 1/80 | 1/640 | 1/320 |
| 12 | 1/8[b] | 1/2048 | 1/1024 | 1/512 |

[a] Titers recorded are the highest dilution which gave a precipitate.
[b] Rabbit sacrificed after four injections.

to 1 ml. portions of 0.1 percent solutions of a wide variety of antigens and were tested by the macro precipitin test (p. 47). Results are shown in Tables III and IV. Serum obtained from the animals prior to inoculation with the antigens did not form precipitates with any of the antigens. The results show considerable variation in the amount of precipitins and in the specificity of the precipitins produced by animals which had been inoculated with the same antigen.

## Production of Antibodies by *in vitro* Incubation of Antigens and Serum Proteins

### Production of Precipitins by a Modification of Bacon's Method

Since Bacon's procedure, if confirmed, would provide a method for rapid production of relatively large quantities of antibodies, it was applied to the preparation of precipitins against the chemically modified proteins being studied. The antigens tested were 3,5-diiodo-4-benzeneazo casein, nitro casein, 4-sulfobenzeneazo casein, 4-arsonobenzeneazo casein, 4-arsonobenzeneazo egg albumen, 4-arsonobenzeneazo blood albumin, iodo casein, and iodo egg albumen. A number of the antigens were used in two or more preparations. The preparations were tested for precipitins by the macro test (p. 47). See Table V.

Preparations which formed definitely heavier precipitates

Table III

Macro Tests for Cross Reactions of Antisera[a]

| | Control | One ml. aliquots of serum from rabbits injected with | | | |
|---|---|---|---|---|---|
| | | 4-Arsonobenzeneazo casein | | Iodo casein | |
| | 1% NaCl | Animal #1 | Animal #2 | Animal #1 | Animal #2 |
| Serum Dilution | | 1/8 | 1/256 | 1/128 | 1/512 |
| Test Antigens (1 ml. of 1% solutions in 1% NaCl solution.) | | | | | |
| 1% Sodium Chloride | — | —[b] | — | — | — |
| 4-Arsonobenzeneazo casein | Tr | + | Tr | — | — |
| Iodo casein | — | — | — | + | + |
| 4-Arsonobenzeneazo egg albumen | — | + | + | Tr | Tr |
| Iodo egg albumen | — | Tr | — | ++ | ++ |
| 4-Arsonobenzeneazo blood albumin | — | — | + | + | — |
| Iodo blood albumin | — | — | — | — | + |
| 3,5-Diiodo-4-hydroxybenzeneazo casein | — | — | — | — | — |
| Guanidyl casein (13.6% N) | O | + | — | + | — |
| Guanidyl casein (23.2% N) | + | ++ | ++ | ++ | Tr |

[a] Readings taken after incubation for two hours at 37°, and overnight in an ice box.

[b] Key: Tr, cloudy; O, opalescent; +, ++, precipitate.

Table IV

Micro Tests for Cross Reactions of Antisera[a]

| | One ml. aliquots of serum from rabbits inoculated with | | | |
|---|---|---|---|---|
| | 4-Arsonobenzeneazo casein | | Iodo casein | |
| | Animal #1 | Animal #2 | Animal #1 | Animal #2 |
| Serum Dilution | 1/40 | 1/640 | 1/320 | 1/2560 |
| Test Antigens (1 ml. of 0.1% solution in gelatin base) | | | | |
| Control, gelatin base | —[b] | — | — | — |
| 4-Arsonobenzeneazo casein | — | — | — | — |
| Iodo casein | — | — | — | + |
| 4-Arsonobenzeneazo egg albumen | — | + | — | — |
| Iodo egg albumen | — | — | — | — |
| 4-Arsonobenzeneazo blood albumin | — | — | — | — |
| Iodo blood albumin | — | — | — | — |
| 3,5-Diiodo-4-hydroxy-benzeneazo casein | — | — | — | — |
| Guanidyl casein (23.2% N) | — | ++ | — | — |
| Guanidyl egg albumen (23.1% N) | — | — | — | — |
| Nitro casein | — | — | — | — |

[a] Tests were incubated at 37° for three hours, and in an ice box for 72 hours.

[b] Key: +, # precipitates.

when tested with the homologous antigen than when tested with the control (1% sodium chloride) were reported as giving positive precipitin tests. Preparations which formed no

Table V

Precipitin Tests on Solutions Prepared by Bacon's Method

| Antigen Tested | No. of preparations tested | No. of preparations giving positive Precipitin tests | % of preparations giving positive precipitin tests |
|---|---|---|---|
| 3,5-Diiodo-4-hydroxy-benzeneazo casein | 4 | 4 | 100 |
| 4-Sulfobenzeneazo casein | 1 | 0 | 0 |
| Nitro casein | 2 | 1 | 50 |
| Iodo egg albumen | 2 | 2 | 100 |
| Iodo casein | 4 | 4 | 100 |
| 4-Arsonobenzeneazo casein | 5 | 5 | 100 |
| 4-Arsonobenzeneazo egg albumen | 2 | 2 | 100 |
| 4-Arsonobenzeneazo blood albumin | 1 | 1 | 100 |

precipitate when tested with the homologous antigen and preparations which formed as much precipitate with the control (sodium chloride) as with the homologous antigen were reported as giving negative precipitin tests. In the majority of the positive tests, a trace, at most, of precipitate was

formed in control tubes. The preparations gave positive precipitin tests in almost all cases. The plasma preparations did not, however, show the expected specificity of reaction. (See Table VI).

Bacon reported extremely heavy precipitin tests between his antibody preparations and the stimulating toxins. In certain cases, the precipitate was reported as filling as much as 10-15 percent of the test tube. In no case was so heavy a precipitate obtained in these experiments. The heaviest precipitate observed did not occupy more than 2-3 percent of the test tubes. An attempt was made, however, to compare the effectiveness of antigens in producing large amounts of precipitins. Calculations of percent incidence of precipitation, by the method of Rosenow[47] were made and are shown in Figure 1. Each reaction was scored according to the following scale.

| Reading | Interpretation | Score |
|---|---|---|
| — | No reaction | 0 |
| Tr, ±, o | Cloudy or opalescent solution | 1 |
| + | Slight precipitate | 2 |
| +± | Precipitates of increasing strength | 3 |
| ++ | " | 4 |
| +++ | " | 5 |

Table VI

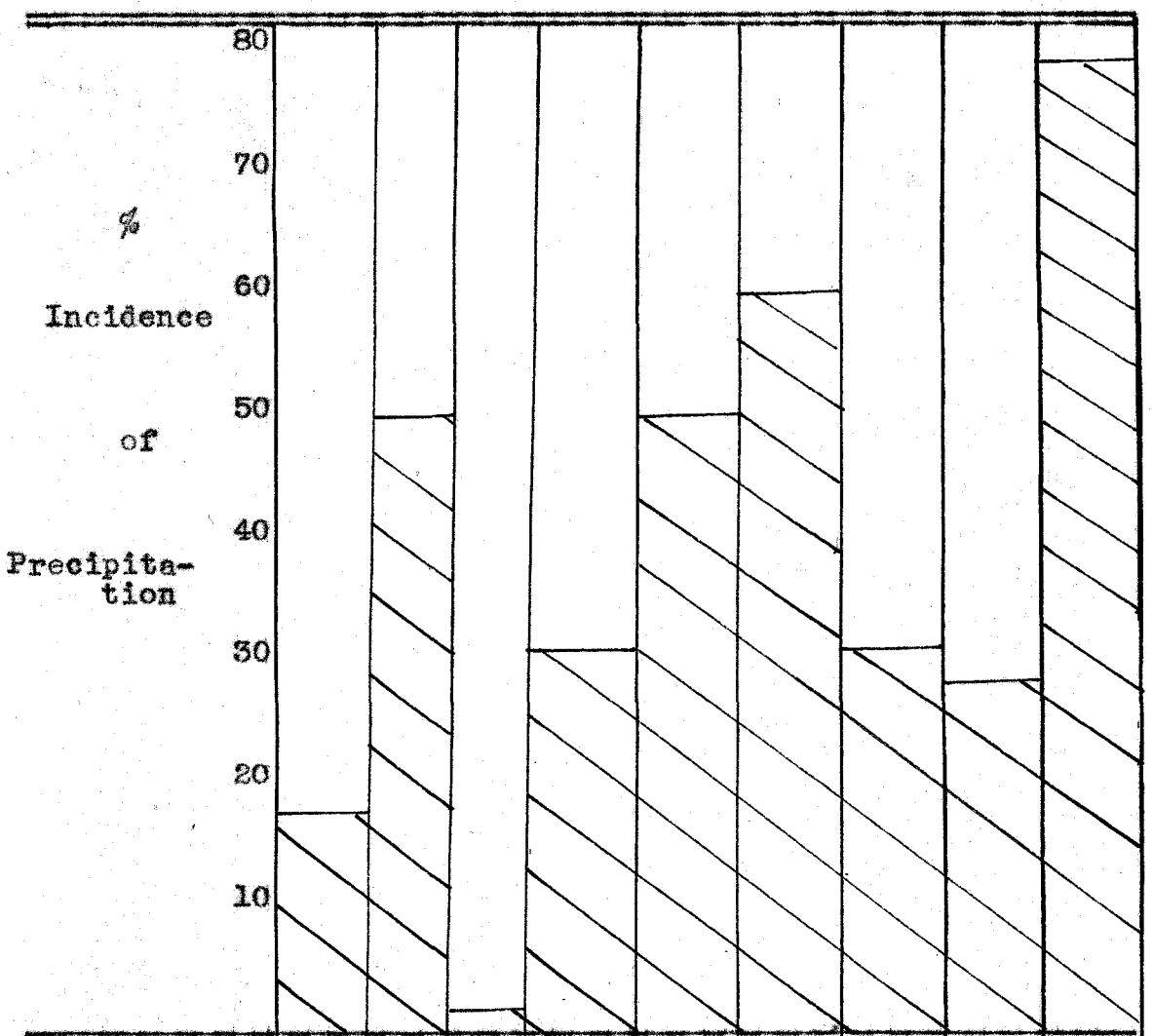
Specificity of Precipitin Preparations

| Antigen in Preparation | No. of preparations tested for specificity | No. of preparations showing specific precipitation[a] | No. of preparations showing precipitation of questionable specificity[b] | No. of preparations showing non-specific precipitation[c] |
|---|---|---|---|---|
| 3,5-Diiodo-4-hydroxy-benzeneazo casein | 2 | 1 | 1 | 0 |
| 4-Sulfo-benzeneazo casein | 0 | 0 | 0 | 0 |
| Nitro casein | 2 | 0 | 1 | 1 |
| Iodo egg albumen | 2 | 1 | 1 | 0 |
| Iodo casein | 4 | 1 | 1 | 2 |
| 4-Arsono-benzeneazo casein | 5 | 2 | 2 | 1 |
| 4-Arsono-benzeneazo egg albumin | 2 | 1 | 1 | 0 |
| 4-Arsono-benzeneazo blood albumin | 1 | 0 | 1 | 0 |

[a] In these cases, a given plasma preparation showed strongest precipitation with the homologous antigen, and either formed precipitates only with the homologous antigen or formed smaller amounts of precipitate with other antigens containing the same protein component or the same hapten.

[b] In these cases, a given plasma preparation showed strongest precipitation with antigens other than the specific antigen which contained either the same protein component or the same hapten.

[c] In these cases, an unexplainable cross reaction was noted.




[a] 1 - control
2 - 3,5-Diiodo-4-hydroxy-benzeneazo casein
3 - 4-Sulfobenzeneazo casein
4 - Nitro casein
5 - Iodo egg albumen
6 - Iodo casein
7 - 4-Arsonobenzeneazo casein
8 - 4-Arsonobenzeneazo egg albumen
9 - 4-Arsonobenzeneazo blood albumen

Figure 1

Strength of Precipitin Reactions

Percent incidence was found by dividing the total score from tests on plasma treated with a given antigen by the total possible score (5 x no. tests).

It should be noted that the number of preparations tested was small. Therefore, positive conclusions as to effectiveness of antigens cannot be drawn. However protein derivatives containing arsenic or iodine appeared to be the most effective of the antigens tested in stimulating production of materials giving relatively strong and specific precipitin tests.

Effect of Substitution of Skim Milk or Egg White Proteins for Serum Proteins

Since blood plasma is a relatively unavailable source of protein, it seemed desirable to see if another cheap, available protein could be used as a substitute. Blood plasma contains 1.8 percent of globulins. The two protein sources selected for substitutes, skim milk and egg white, contain 0.15 percent and 0.7 percent of globulins, respectively[74].

Sufficient sodium hydroxide was added to 200 ml. of skim milk to clarify the solution. The pH of the milk was then adjusted to approximately 7.4 with 10 percent acetic acid. Thirty ml. of the milk solution were mixed with a solution containing 0.3 g. of 4-arsonobenzeneazo egg albumen, and lyophilized. Thirty ml. of untreated milk were also lyophilized. The dried materials were incubated at 37° for twelve hours, and were then reconstituted. Macro tests

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[74] Hawk and Bergeim, op. cit. pp. 199, 407.

(Table VII) run on the preparations gave only negative results (See p. 53). No precipitation occurred in any of the tubes, although all solutions became cloudy after four hours of incubation.

Table VII

Skim Milk as a Blood Plasma Substitute

| | Preparation tested[a] | |
|---|---|---|
| | Milk (Control) | Milk and 4-Arsonobenzeneazo egg albumen |
| Test antigens (1 ml. of a 1% solution in 1% NaCl) | | |
| 1% NaCl (Control) | $T_R$[b] | $T_R$ |
| 4-Arsonobenzeneazo egg albumen | $T_R$ | $T_R$ |
| 4-Arsonobenzeneazo casein | $T_R$ | $T_R$ |
| Iodo casein | $T_R$ | $T_R$ |

[a] Results after incubation for four hours at 37°. No change in the tests was noted during six additional hours of incubation.

[b] $T_R$, trace.

Lyophilized samples of egg white and 4-arsonobenzeneazo casein, egg white and iodo casein, and egg white containing no antigen were incubated, reconstituted and tested for precipitin content. Only non-specific precipitation was observed when macro tests (Table VIII) were run on aliquots of the preparations.

Neither egg white nor milk was a substitute for blood plasma for production of antibodies under the conditions of these experiments.

Table VIII

Egg White as a Blood Plasma Substitute[a]

| | Preparation tested | | |
|---|---|---|---|
| | Egg white Control | Egg white plus Iodo casein | Egg white plus 4-Arsonobenzeneazo casein |
| Test antigens (1 ml. of 1% solution of antigen in 1% NaCl). | | | |
| 1% NaCl (Control) | $T_R$ [b] | + | $T_R$ |
| 4-Arsonobenzeneazo casein | $T_R$ | + | $T_R$ |
| Iodo casein | $T_R$ | $T_R$ | + |
| Iodo egg albumen | $T_R$ | ++ | $T_R$ |
| 4-Arsonobenzeneazo egg albumen | $T_R$ | + | $T_R$ |

[a] Results after incubation for eight hours at 37°.
[b] $T_R$, trace; +, ++, precipitates.

Comparison of Precipitin Production by Bacon's and Ostromuislenskii's Methods

As has been mentioned, Bacon's procedure for preparations of antibodies resembles the method developed by

Ostromuislenskii in 1915. It seemed of interest to compare the strength and specificity of the precipitin reactions of preparations obtained by the two methods.

One g. of iodo casein was dissolved in 5 ml. of 10 N sodium hydroxide and 10 ml. of water. One g. of 4-arsono-benzeneazo casein was treated in the same way. The solutions were then neutralized with 5 ml. of 10 N hydrochloric acid. A 10 ml. aliquot of each of the protein solutions was mixed with 1 g. of sodium chloride and 10 ml. of blood plasma, and incubated at 37° for 36 hours. After incubation, the solutions were filtered and tested for precipitin content. A 10 ml. aliquot of each of the protein solutions was also used for the preparation of antibodies by Bacon's procedure. These preparations were also tested for precipitin content.

The test results, shown in Table IX, indicate that precipitins of comparable concentration and specificity may be prepared by these two methods.

## Production of Antibodies by Yeast Cultured in Media Containing Antigenic Proteins

Since yeast growth might be retarded by too high a concentration of foreign protein in the medium, it seemed advisable to determine the effect on growth of various concentrations of antigen. Cell counts were made on cultures containing 3,5-diiodo-4-hydroxybenzeneazo casein in concentrations of $10^{-1}$ to $10^{-5}$ percent after incubation for 48 hours at 30°.

Growth was found to be fairly uniform (15-30 fold) over this range of antigen concentrations, and the observed variations in growth could not be correlated with antigen concentration. Twenty to thirty fold growth was noted in antigen-free cultures.

Table IX

Precipitin Tests after Incubation at 37° for six hours[a]

| Method of preparation | Preparations tested: Ostromuislenskii's | | | Bacon's | | |
|---|---|---|---|---|---|---|
| Antigen in preparation | Blood Plasma plus | | | Blood Plasma plus | | |
| | None | Iodo casein | 4-Arsono-benzeneazo casein | None | Iodo casein | 4-Arsono-benzeneazo casein |
| Test Antigen (1 ml. of a 0.1% solution of antigen in 1% NaCl) | | | | | | |
| None (1% NaCl) | — | Tr[b] | Tr | — | — | — |
| Iodo casein | — | + | Tr | — | Tr | — |
| 4-Arsono-benzeneazo casein | — | Tr | + | — | + | Tr |

[a] The tests were incubated for twelve hours at 37°. Readings taken after 7-12 hours of incubation showed less specificity than those taken after 4-6 hours.

[b] Tr, trace; +, precipitate.

In the initial experiments on the production of precipitins by S. cerevisiae, yeast was cultured for 48 hours at 30° in a

15-20 percent solution (g./ml.) of molasses containing antigen. Antigen concentrations varied from $10^{-1}$ to $10^{-5}$ percent. The following proteins were used as antigens: casein (Pfanstiehl, pure), egg albumen (Baker and Adams), gluten (Waxy maize, Iowa 939), tyrocidin (Merck), papain (Merck), iodo casein, nitro casein, 4-arsonobenzeneazo casein, 4-sulfobenzeneazo casein, 4-carboxybenzeneazo casein, 3,5-diiodo-4-hydroxybenzeneazo casein, iodo egg albumen, 4-arsonobenzeneazo egg albumen, guanidyl casein and guanidyl egg albumen.

After incubation, the cells were separated by centrifugation and were washed twice with distilled water. The cells were transferred to a mortar and were cytolyzed by fairly continuous grinding with sand and ether for thirty minutes. The cells were then extracted with 25 ml. of a 5 percent sodium chloride solution. The extracts were decanted from the sand and were cleared by centrifugation. One ml. aliquots of the extracts were tested for precipitin content by the micro precipitin test.

Results of some typical tests are shown in Tables X and XI. Precipitin formation was observed in each case but the tests showed some non-specific precipitation. There was, also, considerable variation between the reactions of the several extracts of cells grown in the presence of the same antigen (Table XI). No correlation could be made between the number of cells extracted or the fold growth of cells in the culture and the specificity and/or strength of the precipitin tests.

In tests of 95 of such extracts, 60 (63 percent) of the extracts gave positive precipitin tests (See Table XII). Extracts which formed distinctly more precipitate or cloud-

Table X

Precipitin Formation by Yeast Grown in Media Containing Some Casein Derivatives

| Antigen in Media | One ml. aliquots of sodium chloride extracts of yeast grown in media containing 0.01% concentraion of the indicated antigen[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | None | | Casein | Iodo Casein | | 3,5 Diiodo-4-hydroxybenzene-azo casein | |
| | #1 | #2 | #1 | #1 | #2 | #1 | #2 |
| Test antigen (1 ml. of 0.1% solution in gelatin base) | | | | | | | |
| None | — | $T_R$[b] | $T_R$ | $T_R$ | $T_R$ | — | — |
| Casein | — | — | + | — | + | — | — |
| Iodo casein | — | — | ++ | + | ++ | $T_R$ | ++ |
| 3,5-Diiodo-4-hydroxybenzene-azo casein | — | — | ++ | + | ++ | + | ++ |

[a] Reading taken after incubation for three hours at 37° and 72 hours in an ice box.
[b] $T_R$, trace; +, ++, increasing amounts of precipitates.

iness when tested with a 0.1 percent solution of the homologous antigen in gelatin base than when tested with the control (gelatin base) were reported as giving positive precipitin

Table XI

Precipitin Formation by Yeast Grown in Media Containing Some Casein Derivatives

| Antigen in Media | One ml. Aliquots of Sodium Chloride extracts of Cells Grown in Media Containing 0.1% Concentration of the Indicated Antigen[a] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | None | | | | 3,5-Diiodo-4-hydroxybenzeneazo casein | | | | 4-Sulfobenzeneazo casein | | | |
| | #1 | #2 | #3 | #4 | #1 | #2 | #3 | #4 | #1 | #2 | #3 | #4 |
| Test Antigen (1 ml. of a 0.1% solution in gelatin base) | | | | | | | | | | | | |
| None | − | − | − | +[b] | − | − | − | + | − | − | − | − |
| 3,5-Diiodo-4-hydroxybenzeneazo casein | − | − | − | − | + | + | − | + | − | $T_R$ | − | − |
| 4-Sulfobenzeneazo casein | − | − | − | − | − | − | − | + | + | $T_R$ | − | − |
| 4-Carboxybenzeneazo casein | − | − | − | − | − | − | − | + | − | − | − | − |
| Iodo casein | − | − | − | − | − | − | − | + | − | − | + | − |
| Nitro casein | − | − | − | − | + | − | − | + | − | $T_R$ | + | − |

[a] Readings taken after incubation for three hours at 37° and overnight in an ice box.

[b] $T_R$, trace; +, ++, increasing amounts of precipitate.

Table XI (Continued)

| Antigen in Media | 4-Carboxy-benzeneazo casein #1 | #2 | #3 | #4 | Iodo casein #1 | #2 | #3 | #4 | Nitro casein #1 | #2 | #3 | #4 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Test Antigen (1 ml. of a 0.1% solution in gelatin base) | | | | | | | | | | | | |
| None | + | − | − | − | − | − | − | − | − | − | − | − |
| 3,5-Diiodo-4-hydroxy-benzeneazo casein | ++ | $T_R$ | + | $T_R$ | − | − | + | − | $T_R$ | + | − | − |
| 4-Sulfo-benzeneazo casein | + | − | − | − | − | − | − | − | $T_R$ | − | − | − |
| 4-Carboxy-benzeneazo casein | + | + | + | $T_R$ | − | − | − | − | $T_R$ | − | − | − |
| Iodo casein | + | − | + | − | − | − | $T_R$ | − | $T_R$ | − | − | − |
| Nitro casein | + | + | − | − | − | − | $T_R$ | − | $T_R$ | + | − | − |

tests. No reaction was noted in control tests on 44 (73 percent) of the 60 extracts which showed positive precipitin reactions. In tests of the other 16 (27 percent) extracts which gave positive precipitin tests, control tubes showed some cloudiness or a trace of precipitate.

Extracts which formed no precipitate when tested with a solution of the homologous antigen were reported as giving negative precipitin tests. Extracts which formed precipitates with solutions of the homologous antigen but which showed equally strong reactions with the gelatin base control were also reported as giving negative tests.

It seemed possible that many of the extracts which formed equally heavy precipitates with the control and with the homologous antigen, and which, consequently, were recorded as giving negative tests, actually contained precipitins. In order to obtain extracts which would give consistently negative control tests, methods of removing non-specific precipitating materials from the yeast extracts were investigated. It was subsequently found that treatment of the centrifuged extracts for 15-20 minutes with 0.1 g. of Filter Cel would remove much of the non-specific precipitating material. Micro precipitin tests on 25 of such treated extracts were positive in 18 (72 percent) cases. (See Table XII). Extracts treated with Filter Cel also showed fewer aberrant cross reactions. (See Tables XIII and XV).

Table XII

Micro Precipitin Tests on Yeast Extracts

| Antigen Tested | No. of extracts tested | No. of extracts giving positive precipitin tests | Percent of extracts giving positive precipitin tests |
|---|---|---|---|
| | Extracts not treated with Filter Cel | | |
| 3,5-Diiodo-4-hydroxybenzeneazo casein | 29 | 22 | 76 |
| Casein | 11 | 4 | 36 |
| Iodo casein | 15 | 6 | 40 |
| 4-Arsonobenzene-azo casein | 2 | 0 | 0 |
| 4-Carboxybenzene-azo casein | 11 | 9 | 82 |
| 4-Sulfobenzene-azo casein | 16 | 14 | 88 |
| Nitro casein | 6 | 3 | 50 |
| Egg albumen | 2 | 1 | 50 |
| Gluten | 1 | 0 | 0 |
| Papain | 1 | 0 | 0 |
| Tyrocidin | 1 | 1 | 100 |

Table XII (Continued)

| Antigen Tested | No. of extracts tested | No. of extracts giving positive | Percent of extracts giving positive precipitin tests |
|---|---|---|---|
| | Extracts treated with Filter Cel | | |
| 3,5-Diiodo-4-hydroxybenzeneazo casein | 1 | 1 | 100 |
| Iodo casein | 8 | 3 | 38 |
| Iodo egg albumen | 2 | 2 | 100 |
| 4-Arsonobenzeneazo casein | 8 | 6 | 75 |
| 4-Arsonobenzene-azo egg albumen | 2 | 2 | 100 |
| Guanidyl casein | 2 | 2 | 100 |
| Guanidyl egg albumen | 2 | 2 | 100 |

Table XIII

Effect of Treatment with Filter Cel on Specificity of Precipitin Reactions

| Antigen in Media | None | Iodo casein | Iodo egg albumen | 4-Arsono-benzeneazo casein | 4-Arsono-benzeneazo egg albumen |
|---|---|---|---|---|---|
| | One ml. aliquots of sodium chloride extracts of cells grown in media containing 0.1% concentration of the indicated antigen[a] | | | | |
| | Untreated extracts | | | | |
| Test Antigen (1 ml. of 0.1% solution in gelatin base) | | | | | |
| None | [b]+ | — | + | TR | TR |
| Iodo casein | + | TR | ++ | TR | TR |
| Iodo egg albumen | + | TR | + | TR | — |
| 4-Arsono-benzeneazo casein | ++ | TR | c | + | TR |
| 4-Arsono-benzeneazo egg albumen | + | — | + | TR | — |

Table XIII (Continued)

| Antigen in Media | None | Iodo casein | Iodo egg albumen | 4-Arsono-benzeneazo casein | 4-Arsono-benzeneazo egg albumen |
|---|---|---|---|---|---|
| | | Extracts treated with 0.1 g. Filter Cel for 15 min. | | | |
| Test Antigen (1 ml. of 0.1% solution in gelatin base) | | | | | |
| None | — | — | Tr | — | — |
| Iodo casein | — | Tr | + | — | — |
| Iodo egg albumen | — | Tr | ++ | — | — |
| 4-Arsono-benzeneazo casein | + | Tr | ++ | Tr | — |
| 4-Arsono-benzeneazo egg albumen | — | — | Tr | — | Tr |

[a] Readings taken after incubation for three hours at 37° and 72 hours in an ice box.
[b] Tr, trace; +, ++, +++, precipitate.
[c] Test missing.

The use of Filter Cel on the extracts shown in Table XIII eliminated some of the non-specific precipitation in cross tests and control tests. The treated extract of the control culture of yeast showed three fewer non-specific reactions than did the untreated extract. Treated extracts of cells cultured in media containing iodo casein, iodo egg albumen, 4-arsonobenzeneazo casein, or 4-arsonobenzeneazo egg albumen showed zero, zero, three, and three fewer cross reactions, respectively, than did the corresponding untreated extracts. In general, the use of Filter Cel eliminated some of the non-specific precipitation in cross tests and control tests which confused the results of tests of untreated extracts. (See Tables XII and XIV).

It is probable that some of the specific precipitins are also removed during the treatment, resulting in smaller amounts of precipitate in homologous tests. Table XV shows the results of precipitin tests on aliquots of yeast extracts treated with Filter Cel for 10, 20, and 30 minutes. After treatment for ten minutes with Filter Cel, extracts of cells grown in medium containing iodo casein failed to give positive tests. Extracts of cells cultured in iodo egg albumen showed the same result after thirty minutes of treatment. In general, in tests of extracts of cells cultured in media containing the various proteins, the extracts which had been exposed to the action of Filter Cel for twenty minutes gave reactions which were most satisfactory, considering both strength and specificity of reaction.

Table XIV

Specificity of Precipitin Reactions

| Antigens Tested | Extracts not treated with Filter Cel | | |
|---|---|---|---|
| | No. of extracts tested for specificity[a] | No. of extracts showing specific precipitation[b] | No. of extracts showing precipitation of questionable specificity[c] |
| 3,5-Diiodo-4-hydroxybenzene-azo casein | 14 | 6 | 3 |
| Casein | 5 | 1 | 1 |
| Iodo casein | 4 | 1 | 3 |
| Egg albumen | 1 | 0 | 0 |
| 4-Sulfobenzeneazo casein | 4 | 0 | 0 |
| 4-Carboxybenzeneazo casein | 4 | 0 | 0 |
| Nitro casein | 2 | 1 | 0 |
| 4-Arsonobenzeneazo casein | 2 | 0 | 2 |
| Gluten | 1 | 0 | 0 |
| Tyrocidin | 1 | 0 | 0 |
| Papain | 1 | 0 | 0 |
| Guanidyl casein | 2 | 0 | 2 |
| Guanidyl egg albumen | 1 | 0 | 1 |

Table XIV (Continued)

| Antigens Tested | Extracts treated with Filter Cel | | |
|---|---|---|---|
| | No. of extracts tested for specificity[a] | No. of extracts showing specific precipitation[b] | No. of extracts showing precipitation of questionable specificity[c] |
| 3,5-Diiodo-4-hydroxybenzeneazo casein | 1 | 1 | 0 |
| Iodo casein | 4 | 1 | 2 |
| Iodo egg albumen | 3 | 2 | 0 |
| 4-Arsonobenzeneazo casein | 6 | 5 | 0 |
| 4-Arsonobenzeneazo egg albumen | 3 | 2 | 1 |

[a] Only extracts giving positive precipitin tests (p. 63) were considered in tests of specificity.

[b] In these cases, a given extract gave a positive reaction with the homologous antigen, and either gave no reaction with other antigens or gave weaker reactions with antigens containing the same protein or hapten component.

[c] In these cases, a given extract gave a positive reaction with the homologous antigen, but gave stronger positive reactions with other antigens containing the same protein or hapten component.

The strength and specificity of the observed precipitin reactions varied widely in experimentation during twelve months. (See Table XIV and Fig. 2). The data presented in Figure 2 must be interpreted cautiously. It should be noted

Table XV

Effect of Time of Treatment with Filter Cel on Strength of Precipitin Reactions

| | One ml. aliquots of sodium chloride extracts of cells grown in media containing 0.1% concentration of the indicated antigen.[a] | | | | |
|---|---|---|---|---|---|
| Test Antigens (1 ml. of 0.1% solution in gelatin base): | None | 4-Arsono-benzeneazo egg albumen | Iodo egg albumen | 4-Arsono-benzeneazo casein | Iodo casein |
| | Untreated extracts | | | | |
| None | TR [b] | + | + | ++ | — |
| 4-Arsono-benzeneazo casein | TR | + | TR | ++ | — |
| 4-Arsono-benzeneazo egg albumen | TR | TR | TR | ++ | TR |
| Iodo egg albumen | TR | TR | TR | ++ | TR |
| Iodo casein | TR | TR | + | + | TR |

[a] Readings taken after incubation for three hours at 37° and overnight in an ice box.

[b] TR, trace; +, ++, increasing amounts of precipitate.

that the number of tests on extracts of yeast cultures grown in a given antigen varies widely. Several extracts which formed heavy precipitates with the homologous antigen, also showed non-specific precipitation with heterologous antigens. In general, arsenic and iodine containing proteins were found

to be the most satisfactory antigens of those tested in producing specific precipitins by yeast cultures as well as by the incubation procedure of Bacon.

There are indications that at least some of the variability may be biological. Experiments in which three or four

Table XV (Continued)

| Test Antigens (1 ml. of 0.1% solution in gelatin base) | None | 4-Arsono-benzeneazo egg albumen | Iodo egg albumen | 4-Arsono-benzeneazo casein | Iodo casein |
|---|---|---|---|---|---|
| | Extracts treated with 0.1 g. Filter Cel for 10 minutes | | | | |
| None | — | — | — | — | — |
| 4-Arsono-benzeneazo casein | — | Tr | Tr | Tr | — |
| 4-Arsono-benzeneazo egg albumen | — | Tr | Tr | + | — |
| Iodo egg albumen | — | — | Tr | — | — |
| Iodo casein | — | — | — | — | — |

cultures were carried out simultaneously showed differences which are difficult to explain on any other basis. Table XVI shows results which are typical of the reactions obtained in experiments designed to determine if cultures carried out simultaneously would produce comparable amounts of specific precipitins. Extracts from the three yeast cultures grown in the presence of 4-arsonobenzeneazo casein all appear to

contain precipitins, but differences in strength and specificity of reaction are shown by the tests. The extracts from yeast cultures grown in the presence of iodo casein show even greater variations. One extract gave fairly specific reactions. One gave weak and non-specific reactions. One

Table XV (Continued)

| Test Antigens (1 ml. of 0.1% solution in gelatin base) | None | 4-Arsono-benzeneazo egg albumen | Iodo egg albumen | 4-Arsono-benzeneazo casein | Iodo casein |
|---|---|---|---|---|---|
| | Extracts treated with 0.1 g. Filter Cel for 20 minutes | | | | |
| None | — | TR | — | TR | — |
| 4-Arsono-benzeneazo casein | — | + | — | ++ | — |
| 4-Arsono-benzeneazo egg albumen | — | ++ | — | ++ | — |
| Iodo egg albumen | — | TR | TR | TR | — |
| Iodo casein | — | — | — | — | — |

extract showed no precipitin concentration. The results show that sodium chloride extracts of similar cultures, treated under the same cultural conditions, varied in strength and/or specificity of precipitin reactions.

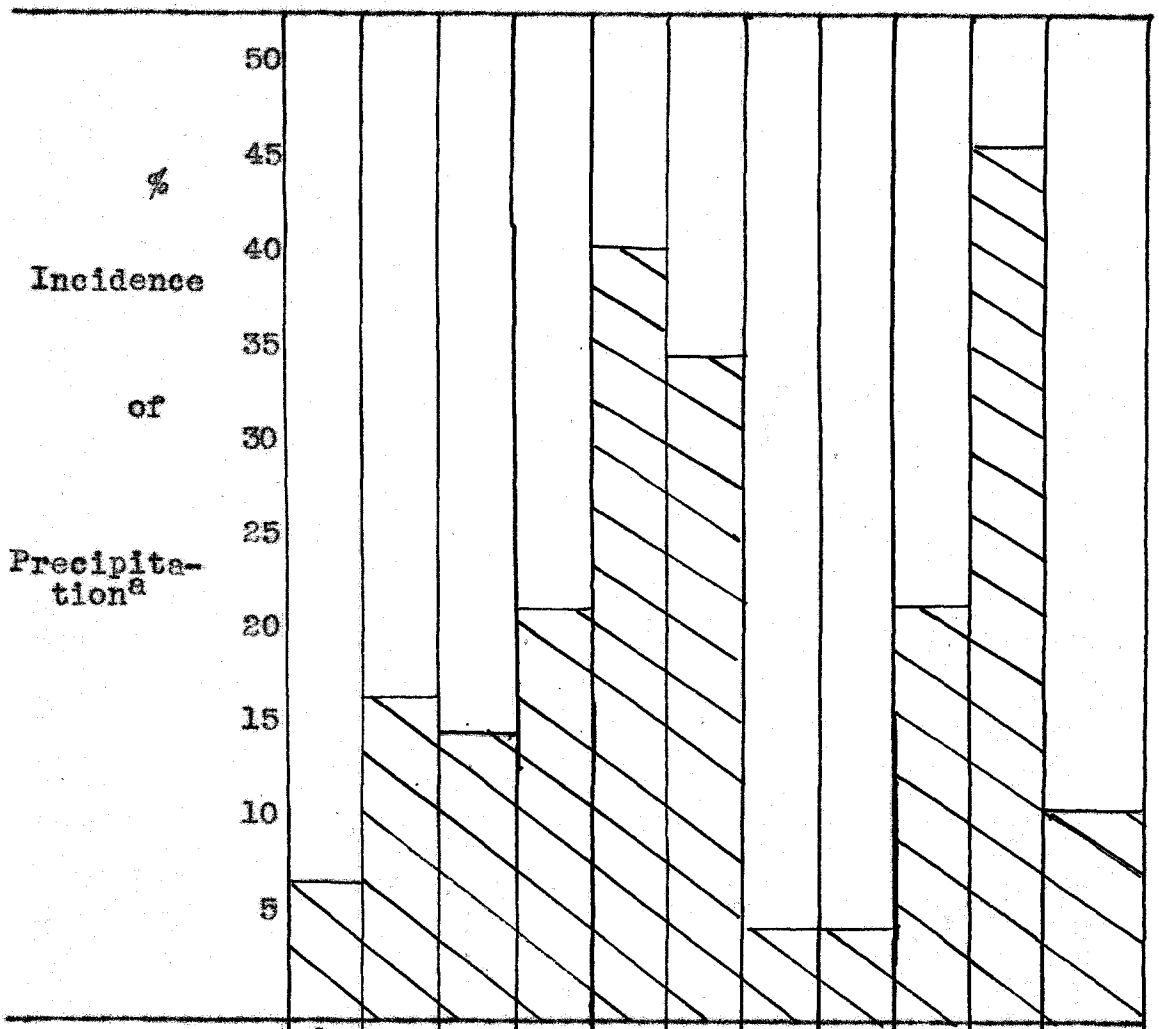
The molasses medium from which the cells had been separated was also tested for precipitins by Henk's micro test. No precipitins could be demonstrated in the medium. Further-

more, diffusion of the colored constituents of the medium into the gelatin layer results in formation of a dark colored ring which would hide the formation of a colorless precipitate at the gelatin-molasses interface. Dialysis of the molasses for seven days against distilled water failed to remove the coloring matter.

Table XV (Continued)

| Test Antigens (1 ml. of 0.1% solution in gelatin base) | None | 4-Arsono-benzeneazo egg albumen | Iodo egg albumen | 4-Arsono-benzeneazo casein | Iodo casein |
|---|---|---|---|---|---|
| | Extracts treated with 0.1 g. Filter Cel for 30 minutes | | | | |
| None | — | — | — | Tr | — |
| 4-Arsono-benzeneazo casein | — | Tr | — | — | — |
| 4-Arsono-benzeneazo egg albumen | — | + | — | ++ | — |
| Iodo egg albumen | — | — | — | — | — |
| Iodo casein | — | — | — | — | — |

In the course of experimentation, it was noted (Table XVIII) is typical) that the amount of precipitate formed when sodium chloride extracts of yeast were tested for precipitin content frequently decreased when the extracts were stored in the ice box (5-10°C.) for more than a few days.




| Antigen in Media | 1[b] | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| No. of Tests | 15 | 29 | 9 | 18 | 1 | 2 | 7 | 6 | 6 | 10 | 2 |

[a] See Fig. 1 and discussion.

[b] 1 - Control
2 - 3,5 Diiodo-4-hydroxybenzeneazo casein
3 - Casein
4 - Iodo casein
5 - Egg Albumen
6 - Iodo egg albumen
7 - 4-Sulfo-benzeneazo casein
8 - 4-Carboxybenzeneazo casein
9 -Nitro casein
10 - 4-Arsonobenzeneazo casein
11 - 4-Arsonobenzeneazo egg albumen

Figure 2

Strength of Precipitin Reactions of Yeast Extracts with Homologous Antigens

It is conceivable that proteolytic enzymes freed during cytolysis of the yeast cells may completely or partially hydrolyze the proteins responsible for the observed precipitation. As the quantity of precipitins present was reduced by hydrolysis, smaller amounts of precipitate would be expected.

## Effect of Repeated Introduction of Antigen to Yeast Cultures

Since the antibody titer of the plasma of animals is higher when a given amount of antigen is injected in a series of small doses than when a single massive dose is given, it seemed desirable to determine the effect of repeated addition of small amounts of antigen to yeast culture medium. In experiments, using different antigens, extracts of cells removed after several additions of antigen failed to give significantly stronger precipitin tests than extracts of cells removed from the cultures after one addition of antigen. Both micro and macro methods of testing were used. In a typical experiment, flasks were set up containing 400 ml. of a 15 percent (g./ml) molasses solution, approximately $5 \times 10^9$ yeast cells and 40 ml. of a 0.1 percent solution of either 4-arsonobenzeneazo casein or iodo egg albumen. The cultures were incubated at $30^\circ$ for 48 hours. At the end of the first 48 hour period, a 100 ml. aliquot of each culture medium was withdrawn and 30 ml. of antigen were added to the remainder of the culture medium, which was then incubated at $30^\circ$ for another 48 hour

period. Antigen was added after each 48 hour incubation period for a series of five additions. Aliquots were removed after 48, 144, and 240 hours. Cells were separated by centrifugation, cytolyzed with sand and ether and extracted with 25 ml. of a

Table XVI

Variations in Precipitin Production in Triplicate Cultures

| Test Antigens (1 ml. of 0.1% solutions in gelatin base) | One ml. aliquots of sodium chloride extracts of cells grown in media containing 0.1% concentration of the indicated antigen[a]. | | | | | | |
|---|---|---|---|---|---|---|---|
| | None | Iodo Casein | | | 4-Arsonobenzeneazo casein | | |
| | | (1) | (2) | (3) | (1) | (2) | (3) |
| None | —[b] | — | — | — | — | — | Tr |
| 4-Arsonobenzeneazo casein | — | — | — | — | + | + | ++ |
| Iodo casein | — | — | — | + | Tr | Tr | — |
| 4-Arsonobenzeneazo egg albumen | — | — | — | — | Tr | + | + |
| Iodo egg albumen | — | — | — | + | Tr | Tr | Tr |
| 4-Arsonobenzeneazo blood albumin | — | — | — | — | Tr | + | + |
| Iodo blood albumin | — | — | + | Tr | Tr | Tr | — |

[a] Readings taken after incubation for three hours at 37° and 72 hours in an ice box.

[b] Tr, trace; +, ++, increasing amounts of precipitates.

5 percent solution of sodium chloride. The extracts were clarified by centrifugation and treatment with Filter Cel. Aliquots of the clarified extracts were tested for precipitin content by the macro method. Results are shown in Table XVIII. In this experiment, yeast cultured in medium containing iodo egg

Table XVII

Decrease in Amount of Precipitins in Stored Extracts of Yeast Cells

| Antigen in Media | One ml. aliquots of sodium chloride extracts of cells grown in media containing 0.1% concentration of the indicated antigen[a]. | | | | | |
|---|---|---|---|---|---|---|
| | None | 4-Arsono-benzene-azo egg albumen | 4-Arsono-benzene-azo casein | None | 4-Arsono-benzene-azo egg albumen | 4-Arsono-benzene-azo casein |
| | Fresh Preparations | | | After storage at 5-10° for two weeks | | |
| Test Antigens (1 ml. of 0.1% solutions in gelatin base) | | | | | | |
| None | — | Tr[b] | Tr | — | — | — |
| 4-Arsono-benzeneazo egg albumen | — | + | ++ | — | — | — |
| 4-Arsono-benzeneazo casein | — | +++ | ++ | + | — | ++ |
| Iodo egg albumen | — | Tr | Tr | — | — | — |
| Iodo casein | — | — | — | — | — | — |

[a] Readings taken after incubation for three hours at 37° and 72 hours in an ice box.

[b] Tr, trace; +, ++, +++, increasing amounts of precipitates.

albumen failed to produce specific precipitins. Extracts of cells grown in medium containing 4-arsonobenzeneazo casein did, however, give specific precipitin tests. The largest amount of precipitate was obtained from a test on

Table XVIII

Effect of Repeated Additions of Antigen on the Production of Precipitins by Cultures of Yeast

| | One ml. aliquots of sodium chloride extracts of cells grown in media containing 0.1% concentration of the indicated antigen[a]. | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| No. of Additions of Antigen | Zero | | | | Two | | | | |
| Antigen in Media | None | 4-Arsono-benzeneazo casein | | Iodo egg albumen | None | 4-Arsono-benzeneazo casein | | Iodo egg albumen | |
| | | (1) | (2) | (1) | | (1) | (2) | (1) | (2) |
| Test Antigen (0.2 mls. of a 0.1% solution in 1% NaCl) | | | | | | | | | |
| None (1% NaCl) | — | —[b] | $T_R$ | $T_R$ | — | $T_R$ | $T_R$ | $T_R$ | — |
| 4-Arsono-benzeneazo casein | — | ++ | $T_R$ | $T_R$ | — | — | + | $T_R$ | $T_R$ |
| Iodo egg albumen | — | — | $T_R$ | $T_R$ | — | — | — | $T_R$ | $T_R$ |

[a] Readings taken after incubation for three hours at 37°, and overnight in an ice box.
[b] $T_R$ , trace; +, ++ , increasing amounts of precipitates.

an extract of cells which received one addition of antigen.

## Effect of Repeated Transfers of Yeast Cells to Fresh Media Containing Antigen

In the course of experiments on the effect of repeated

introduction of antigen into yeast culture medium it was noted that the yeast multiplied very little after the first 48 hours of incubation. It appeared likely that the amount of precipitin production by yeast would be closely correlated with the total amount of protein synthesis taking

Table XVIII (Continued)

| No. of Additions of Antigen | Four | | |
|---|---|---|---|
| Antigen in media | None | 4-Arsono-benzeneazo casein (1) | Iodo egg albumen (1) (2) |
| Test Antigen (0.2 mls. of a 0.1% solution in 1% NaCl) | | | |
| None (1% NaCl) | — | TR | — — |
| 4-Arsono-benzeneazo casein | — | TR | — — |
| Iodo egg albumen | ++ | TR | TR TR |

place in the culture. Therefore, it seemed advisable to administer repeated doses of antigen to cultures of yeast under conditions which sustain a fair amount of yeast growth.

Accordingly, yeast were cultured at 30° for 48 hours in medium containing 100 ml. of a 15-20 percent solution (g./ml) of molasses and 0.1 percent concentration of antigen. At the end of this incubation period, the cells were separated

by centrifugation and were transferred to fresh medium containing the same antigen and again incubated at 30° for 48 hours. The cells were transferred each 48 hours and the flasks were set up at intervals which provided, at the end

Table XIX

Macro Tests on Extracts of Yeast Cultured by the Transfer Technique

| | Five ml. aliquots of sodium chloride extracts of cells grown in media containing 0.1% concentration of the indicated antigen[a]. | | | | | |
|---|---|---|---|---|---|---|
| Antigen in Media | None | 4-Arsono-benzene-azo casein | Iodo casein | None | 4-Arsono-benzene-azo casein | Iodo casein |
| No. of transfers | 0 | | | 2 | | |
| Test Antigens (1 ml. of 0.1% solution in 1% NaCl) | | | | | | |
| None (1% NaCl) | TR[b] | TR | TR | TR | TR | — |
| 4-Arsono-benzeneazo casein | TR | TR | TR | TR | ++ | — |
| Iodo casein | TR | TR | TR | + | TR | TR |

[a] Readings taken after incubation for two hours at 37° and overnight in an ice box.
[b] TR, trace; +, ++, increasing amounts of precipitates.

of the culture period, cells which had been transferred zero, two, four, and six times. It was possible by this procedure to obtain precipitin extracts which gave positive macro tests. Table XIX shows the results of the first experiment using this method of transfers.

In this case, the extracts of yeast cultures which had been transferred six times gave exceedingly strong precipitin reactions. In the above tests, adequate specificity of reaction was also demonstrated. Since it had usually been

Table XIX (Continued)

| Antigen in Media | None | 4-Arsono-benzene-azo casein | Iodo casein | None | 4-Arsono-benzene-azo casein | Iodo casein |
|---|---|---|---|---|---|---|
| No. of transfers | 4 | | | 6 | | |
| Test Antigens (1 ml. of 0.1% solution in 1% NaCl) | | | | | | |
| None (1% NaCl) | TR | c | TR | — | TR | — |
| 4-Arsono-benzeneazo casein | TR | | — | TR | +++ d | TR |
| Iodo casein | TR | | + | TR | TR | +++ e |

c Solution missing
d 46 mg.
e 73 mg.
Only the precipitates d and e were weighed. The test solutions were slightly contaminated by mold. The precipitates were, however, separated, washed, dried and weighed after removal of the mold.

impossible to obtain positive macro tests with extracts of cells cultured by the original method, it seemed from this and subsequent tests (Tables XX-XXV) that the method of transfers increased the production of precipitins.

In the above tests, the largest amounts of precipitate were formed by extracts of cells which had been transferred

the most (six) times. Therefore, it seemed desirable to extend the number of transfers in order to determine if there were a transfer level giving maximum precipitin production. Yeast cultures were set up and transferred, according to the

Table XX

Precipitin Tests on Extracts of Yeast Cultured by the Transfer Procedure

| T | Five ml. aliquots of sodium chloride extracts of cells grown in media containing 0.1% concentration of the indicated antigen[a]. | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Antigen in Media | None | | 4-Arsono-benzene-azo casein | | Iodo casein | | None | | 4-Arsono-benzene-azo casein | | Iodo casein | |
| | #1 | #2 | #1 | #2 | #1 | #2 | #1 | #2 | #1 | #2 | #1 | #2 |
| No. of transfers | No transfers | | | | | | Two transfers | | | | | |
| Test Antigens (1 ml. of 0.1% solution in 1% NaCl) | | | | | | | | | | | | |
| None (1% NaCl) | – | – | +[b] | + | – | – | – | – | – | – | – | TR |
| 4-Arsono-benzeneazo casein | + | + | ++ | ++ | – | – | – | – | TR | ++ | – | TR |
| Iodo casein | + | + | +± | +± | + | TR | TR | – | – | ++ | TR | + |

[a] Readings taken after incubation for three hours at 37° and overnight in an ice box.
[b] TR, trace; +, ±, ++, increasing amounts of precipitate.

procedure given on p. 83, each 48 hours. The flasks were set up at intervals which provided, at the end of the culture period, cells which had been transferred zero, 2, 4, 6, 8 and 10 times. Table XX shows the results of macro precipitin tests on extracts of the cells.

In this experiment, more precipitate was formed in tests of extracts of cells which had been transferred zero, two or four times than was formed in tests of extracts of cells which were transferred a greater number of times. Extracts of cells

Table XX (Continued)

| Antigen in Media | None | | 4-Arsono-benzene-azo casein | | Iodo casein | | None | | 4-Arsono-benzene-azo casein | | Iodo casein | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | #1 | #2 | #1 | #2 | #1 | #2 | #1 | #2 | #1 | #2 | #1 | #2 |
| No. of transfers | Four transfers | | | | | | Six transfers | | | | | |
| Test Antigens (1 ml. of 0.1% solution in 1% NaCl) | | | | | | | | | | | | |
| None (1% NaCl) | — | — | TR | — | + | — | — | — | — | TR | — | — |
| 4-Arsono-benzeneazo casein | TR | — | TR | ++ | + | TR | — | — | + | TR | — | — |
| Iodo casein | TR | — | TR | ±+ | + | TR | — | — | — | TR | — | TR |

grown in medium containing iodo casein and removed after zero or two transfers showed the expected specificity of reaction. Extracts of cells grown in medium containing 4-arsonobenzeneazo casein and removed after zero and four transfers showed only slight specificity. More specific reactions were noted in tests of extracts of cells transferred six times to medium containing 4-arsonobenzeneazo casein.

However, in the procedure described above, cultures transferred different numbers of times bore no relationship to each other, since each culture was started from a separate

inoculum. In order to establish a correlation between number of transfers and the amount of precipitate formed in macro precipitin tests it seemed desirable to remove and test a fraction of the same yeast culture at each transfer level.

Table XX (Continued)

| Antigen in Media | None | | 4-Arsono-benzene-azo casein | | Iodo casein | | None | | 4-Arsono-benzene-azo casein | | Iodo casein | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | #1 | #2 | #1 | #2 | #1 | #2 | #1 | #2 | #1 | #2 | #1 | #2 |
| No. of transfers | Eight transfers | | | | | | Ten Transfers | | | | | |
| Test Antigens (1 ml. of 0.1% solution in 1% NaCl) | | | | | | | | | | | | |
| None (1% NaCl) | — | — | — | — | TR | TR | — | — | — | — | — | — |
| 4-Arsono-benzeneazo casein | — | — | — | TR | TR | TR | — | — | TR | TR | — | — |
| Iodo casein | — | — | — | — | TR | TR | — | — | — | TR | — | — |

Therefore, yeast were cultured at 30° for 48 hours in 400 ml. of medium containing 15-20 percent (g./ml) concentration of molasses and 0.1 percent concentration of antigen, and were transferred each 48 hours to fresh medium containing the same antigen and again incubated at 30° for 48 hours. One hundred ml. aliquots were removed after zero, 2, 4, and 6 transfers. The cells were separated by centrifugation and were cytolyzed and extracted in the usual manner. Results of macro tests on extracts of a series of cultures treated in this way are presented in Table XXI.

In this series the strongest precipitin reactions were shown by extracts of cells removed from the culture after two or no transfers. Extracts of cells removed after further transfers failed to show increasing strength of reaction.

Table XXI

Precipitin Tests on Aliquots of Yeast Cultures Taken at Different Transfer Levels

| | Five ml. aliquots of sodium chloride extracts of yeast cells grown in media containing 0.1% concentration of the indicated antigen[a]. | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Antigen in Media | None | 4-Arsono-benzeneazo casein | | Iodo egg albumen | | None | 4-arsono-benzeneazo casein | | Iodo egg albumen | |
| No. of Transfers | Zero | | | | | Two | | | | |
| | #1 | #2 | | #1 | #2 | #1 | #2 | | #1 | #2 |
| Test Antigen (1 ml. of 0.1% solution in 1% NaCl) | | | | | | | | | | |
| None (1% NaCl) | —[b] | TR | TR | TR | — | — | TR | TR | TR | TR | TR | TR |
| 4-Arsono-benzeneazo casein | TR | TR | TR | TR | — | TR | TR | ++ | ++ | TR | TR |
| Iodo egg albumen | TR | TR | TR | + | + | — | — | TR | TR | + | + |

[a] Readings taken after incubation at 37° for three hours and overnight in an ice box.
[b] TR , trace; +, ++ , precipitates.

In transfer experiments, maximum precipitation was shown by extracts of cells removed after zero, two, four, and six transfers, varying in different runs and with different antigens. (See Tables XX through XXIV). In tests on extracts of aliquots of a total of seventeen different cultures, treated by the second transfer procedure, specific macro tests were

obtained, at some transfer level, in fourteen cases. At other transfer levels, weaker or less specific precipitation was noted. In the other three cases, only non-specific precipitation was observed. (Table XXII)

Table XXI (Continued)

| Antigen in Media | None | | 4-Arsono-benzeneazo casein | | Iodo egg albumen | | None | | 4-Arsono-benzeneazo casein | | Iodo egg albumen | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. of Transfers | Four | | | | | | Six | | | | | |
| | #1 | #2 | #1 | #2 | #1 | #2 | #1 | #2 | #1 | #2 | #1 | #2 |
| Test Antigen (1 ml. of 0.1% solution in 1% NaCl) | | | | | | | | | | | | |
| None (1% NaCl) | $T_R$ | $T_R$ | − | $T_R$ | $T_R$ | $T_R$ | $T_R$ | $T_R$ | + | + | $T_R$ | $T_R$ |
| 4-Arsono-benzeneazo casein | $T_R$ | $T_R$ | $T_R$ | $T_R$ | $T_R$ | $T_R$ | $T_R$ | $T_R$ | + | + | $T_R$ | $T_R$ |
| Iodo egg albumen | $T_R$ | $T_R$ | − | − | $T_R$ | + | $T_R$ | $T_R$ | + | + | $T_R$ | $T_R$ |

In the course of experimentation, it was found that the maximum amount of precipitate formed varied considerably from one experiment to another. The precipitates from certain of the tests were centrifuged, washed with water until the washings were free of chloride ion, dried at 60°C., and weighed. Table XXIII presents data on the amount of precipitates noted in some of the tests reported in Table XXI.

The previously noted type of cultural variation was

observed in tests of precipitin content of extracts of cells treated by the transfer procedure under the same experimental conditions. Table XIV shows results of macro tests on extracts of triplicate cultures of yeast grown in medium containing 4-arsonobenzeneazo casein.

Table XXII

Precipitin Reactions of Extracts of Yeast Cells

| | 5 ml. aliquots of sodium chloride of extracts of cells cultured in media containing 0.1% concentration of the indicated antigen[a]. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Antigen in Media | None | | 4-Arsono-benzeneazo casein | | | Iodo egg albumen | | | None | 4-Arsono-benzeneazo casein | | Iodo egg albumen | |
| | #1 | #2 | #1 | #2 | #3 | #1 | #2 | #3 | | #1 | #2 | #1 | #2 |
| No. of Transfers | No transfers | | | | | | | | | Two transfers | | | |
| Test Antigen (1 ml. of 0.1% solution in 1% NaCl) | | | | | | | | | | [c] | | | |
| None (1% NaCl) | — | [b] — | — | — | [d] Tr | Tr | — | Tr | — | — | | — | — |
| 4-Arsono-benzeneazo casein | Tr | [d] — | — | + | [d] Tr | Tr | — | — | Tr | Tr | | Tr | Tr |
| Iodo egg albumen | Tr | — | — | — | [d] Tr | Tr | — | — | — | Tr | | Tr | Tr |

[a] Readings taken after incubation for three hours at 37° and overnight in an ice box.
[b] Tr, trace; +, ++, increasing amounts of precipitate.
[c] Solution missing
[d] See Fig. 3.

Considerable variation was observed between tests on extracts of the several cultures. Cultures #1 and #3 showed maximum precipitation after no transfers. Culture #2 showed maximum precipitation after six transfers. The quantity

Table XXIII

Amount of Precipitate Formed in Precipitin Tests of Extracts of Yeast[a]

| | 5 ml. aliquots of sodium chloride extracts of cells cultured in media containing 0.1% concentrations of the indicated antigen[b]. | | |
|---|---|---|---|
| Antigen in Media | None | Iodo Egg albumen | 4-Arsonobenzeneazo casein |
| Test Antigen (1 ml. of 0.1% solution in 1% NaCl) | | | |
| None (1% NaCl) | 0.3 mg. | 0.4 mg. | 0.4 mg. |
| Iodo egg albumen | 0.0 | 11.6 | 0.4 |
| 4-Arsonobenzeneazo casein | 0.0 | 0.2 | 9.7 |

[a] See Table XXI.
[b] Two transfers in this set. Tests were incubated three hours at 37°, stood overnight in ice box, centrifuged eight hours later. All figures are averages of duplicates.

Table XXIV

Variation in Tests on Triplicate Cultures

| | 5 ml. aliquots of NaCl extracts of cells cultured in media containing the indicated antigen[a]. | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Antigen in Media | None | 4-Arsonobenzeneazo casein | | | None | 4-Arsonobenzeneazo casein | | |
| | | (1) | (2) | (3) | | (1) | (2) | (3) |
| No. of Transfers | None | | | | Two | | | |
| Test Antigen (1 ml. of 0.1% solution in 1% NaCl) | | | | | | | | |
| None (1% NaCl) | $T_R$[b] | $T_R$ | – | $T_R$ | + | + | + | + |
| 4-Arsonobenzeneazo casein | – | + | – | ++ | ++ | ++ | + | ++ |

[a] Readings taken after incubation for three hours at 37° and overnight in an ice box.

[b] $T_R$, trace; +, ++, +++, increasing amounts of precipitates.

of precipitate formed also varied considerably. Extracts of culture #3 gave the strongest tests in this experiment.

Figure #3 shows reactions (See Table XXII) which are illustrative of results of macro precipitin tests. The

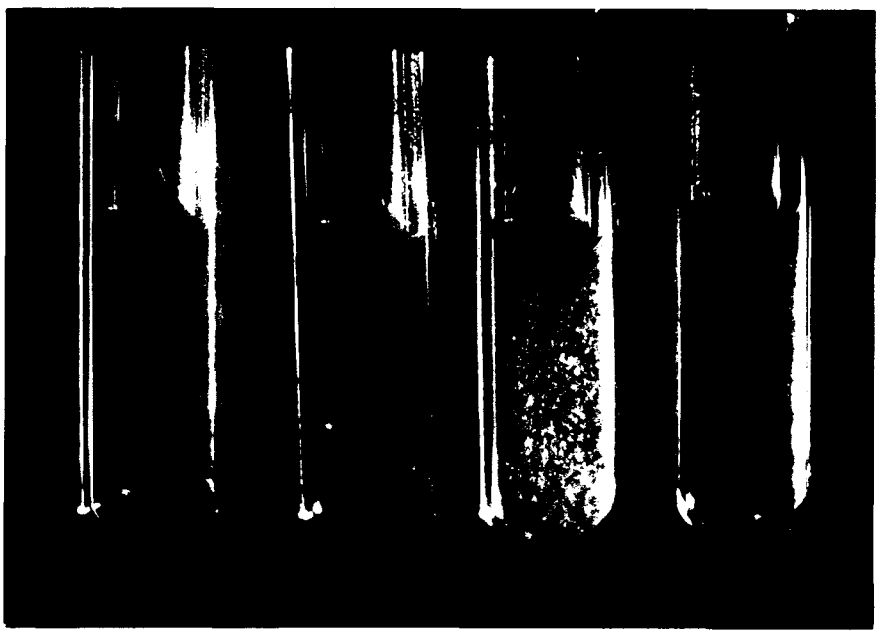
Table XXIV (Continued)

| Antigen in Media | None | 4-Arsono benzeneazo casein (1) | (2) | (3) | None | 4-Arsono-benzeneazo casein (1) | (2) | (3) |
|---|---|---|---|---|---|---|---|---|
| No. of Transfers | Four | | | | Six | | | |
| Test Antigen (1 ml. of 0.1% solution in 1% NaCl) | | | | | | | | |
| None (1% NaCl) | Tr | Tr | Tr | Tr | — | — | Tr | — |
| 4-Arsonobenzeneazo casein | + | + | Tr | ++ | — | Tr | + | Tr |

contents of the tubes are as follows:

| Tube # | Test antigens (0.2 ml. of a 0.1% solution in 1% NaCl) | Extract 1 ml. of an extract of cells cultured in media containing |
|---|---|---|
| 1 | None (1% NaCl) | 4-Arsonobenzeneazo casein |
| 2 | Iodo egg albumen | " |
| 3 | 4-Arsonobenzeneazo casein | " |
| 4 | 4-Arsonobenzeneazo casein | No antigen |

Tube #3 contained, as expected, much more precipitate than did the other tubes.



#1 #2 #3 #4

Figure 3

Macro Precipitin Tests on Extracts of Yeast

# DISCUSSION AND CONCLUSIONS

## Comparison of the Reproducibility of Precipitin Formation

Topley and Wilson[77], in an introduction to the study of immunology, call attention to the fact that various workers have reported violently conflicting reports on the efficacy of various methods of immunization. He states that such divergence is to be expected since workers in this field cannot avoid an element of randomness in observations, due to interplay of factors about which little is known, except that they are very numerous and may be very important. Topley suggests that whenever possible it is advisable to apply statistical methods to analyses of the results obtained.

In immunological studies using animals to produce antibodies, it is common practice to select for testing the serum of those animals which indicate the highest antibody concentrations[74,78]. Animals which fail to produce antibodies or whose sera contain low concentrations of antibodies are discarded. There are, unfortunately, few reports in the literature indicating the amount of this type of selection involved in serological experiments. It is known, however, that there is considerable variation in the response of individuals to antigen stimulation[77].

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[77] Topley and Wilson, op. cit. p. 980.

[78] Mayer and Heidelberger, J. Am. Chem. Soc. 68, 18 (1946).

Tables V and XII show that precipitin reactions were positive for 90 percent of the antibody solutions prepared by the lyophilization and incubation procedure, and for 65 percent of the preparations obtained by extraction of yeast cells cultured in media containing antigenic proteins.

The effect of variation in individual responses to antigenic stimuli was not determined for the solutions prepared by Bacon's method. The blood plasma used was obtained at various times from the Veterinary Clinic, Iowa State College. The animal source was unknown.

In the yeast culture methods the total number of cells extracted was approximately $2 \times 10^9$ to $7 \times 10^9$ in the original experiments. In spite of the large numbers of cells involved, experiments indicate that certain biological and/or cultural factors influence the precipitin formation in duplicate or triplicate cultures. See Tables XVI and XXV.

It seems probable that the indicated failure (Table XII) to obtain precipitin formation in 35 percent of the preparations from yeast after a 48 hour incubation period may have been due, in part, to failure to remove non-specific precipitating materials from the extracts and, in part, to formation of weak antibody solutions rather than to failure of the cultures to produce precipitins. It was seldom possible to demonstrate the presence of precipitins in these extracts by macro tests, and in many cases the micro tests showed only weak precipitin reactions.

When using the transfer procedures to increase the antibody content of extracts it was possible in 80 percent of the cultures studied to obtain positive macro precipitin tests at selected optimal transfer levels. (Tables XX-XIV). However, due to this selection of optimal transfer levels, the reproducibility of tests on extracts of cells cultured by the transfer procedures cannot be compared with the reproducibility of micro precipitin tests on extracts of 48 hour yeast cultures. No selection, of this type, was possible in tests on extracts of 48 hour yeast cultures.

By use of the transfer technique the number of cells in a 100 ml. aliquot was increased to a range of $4 \times 10^9$ to $1 \times 10^{10}$. No definite correlation can be shown, however, from the data available at this time, between the number of cells extracted and the strength or reproducibility of the precipitin tests.

## Comparison of the Strength of Precipitin Reactions

The antibody solutions prepared by Bacon's method or by the yeast incubation methods were, in general, much lower in antibody concentration than the sera of the rabbits injected with the corresponding antigens. Titer determinations on a few of these preparations gave maximum titers of 1/20 for a plasma preparation of anti-3,5-diiodo-4-hydroxybenzeneazo casein and 1/8 for an extract of yeast cells cultured in media containing 4-sulfobenzeneazo casein. The titers of the rabbit

sera were considerably higher as shown in Tables I and II.

Extracts of yeast cultured by the transfer method gave precipitin reactions which were, in many cases, heavier than any of the reactions shown by solutions prepared by Bacon's incubation procedure. The precipitates, however, differed in physical form from the precipitates obtained in tests of rabbit sera or of the antibody solutions prepared by *in vitro* incubation of plasma and antigen. The precipitates formed by antigen and yeast extracts were flocculent while precipitates formed by antigen and animal serum or plasma preparations were generally granular.

## Comparison of the Specificity of Precipitin Reactions

The term "specific" (See Glossary) as applied to serological reactions should not be interpreted too rigorously. Landsteiner[79] points out that immune sera vary in the range and strength of cross reactions when produced in individuals of the same species, or obtained from the same animal at different times. Referring in particular to various azo proteins he states that it is not always easy and may require a number of animals to obtain an immune serum specific for an azo component. He indicates that often, depending upon the response of the individual animal, sera are produced which precipitate strongly the immunizing azo protein

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[79] Landsteiner, *op. cit.*, Revised Ed. pp. 19, 159, 267.

and other antigens containing kindred proteins, and says, "Of course such sera are not suitable for the studies on serological specificity in relation to chemical constitution, a point which has sometimes been overlooked."

He also indicates that the presence of characteristic reactive groupings on an antigenic molecule does not necessitate reaction only with antibodies specific for that grouping. According to Landsteiner the fact that no strict correlation exists between antigens and antibodies is shown clearly by the occurrence of non-reciprocal reactions. Thus when immune sera for 2-carboxybenzeneazo proteins react strongly with 2-sulfobenzeneazo proteins, while sera for 2-sulfobenzeneazo proteins do not precipitate 2-carboxybenzeneazo proteins, this must be due to the difference in specificity between the two sera. He concludes that this phenomenon proves that even a small determinant structure can combine with quite different antibodies.

In a test on the specificity of an unadsorbed anti-3-sulfobenzeneazo horse serum solution, Landsteiner[80] reports the results shown in Table XXV. The reaction of the antiserum with the homologous antigen is stronger than any of the cross reactions but considerable precipitate was noted with antigens containing markedly different determinant groups.

The antigens which proved most satisfactory in stimulating the production of antibodies by yeast were iodo

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80 Ibid. p. 270.

proteins and 4-arsonobenzeneazo proteins. Landsteiner[79, 81] makes the following generalizations regarding the specificity of antisera for such antigens.

> The effect of iodination of a protein is loss of the original specificity, partial or complete, depending upon the intensity of the treatment, and appearance of a new specificity for iodinated proteins. However, immune sera for iodo proteins may contain distinct antibodies reacting with the homologous antigen alone, with all iodo proteins, and with the original protein, their relative proportion (and occurrence) depending on the degree of iodination.

Table XXV

Cross Reaction of Serum

| Test Antigens | Serum from animals injected with 3-sulfobenzeneazo horse serum. |
|---|---|
| 2-Sulfobenzeneazo chicken serum | +++ |
| 3-Arsonobenzeneazo chicken serum | ++ |
| 3-Carboxybenzeneazo chicken serum | ++± |
| 3-Sulfobenzeneazo chicken serum | +++ |

> Immune sera against azo proteins precipitate not only the homologous azo antigen but also mostly the original protein as well as various azo derivatives thereof, and there is evidence to show that the reactions are not to be explained solely by some uncoupled protein admixed with the immunizing antigen but to antibodies stimulated by the antigen.

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81 Landsteiner, ibid. p. 50.

> Further, on testing azo antigens prepared with the same azo component but with different proteins, the reactions, as a rule, vary in intensity according to the relationship of the proteins to that contained in the immunizing antigen.

Some of the cross reactions noted in studies of the specificity of the antibody solutions prepared by the acellular and yeast culture methods (Tables VI and XII) are explainable on the basis of the above correlations of Landsteiner.

Table VI indicates that in tests of nineteen antibody solutions prepared by Bacon's procedure, fourteen showed cross reactions only with antigens containing similar hapten or protein constituents.

Specificity tests on yeast extracts show a somewhat higher percentage of aberrant cross reactions. Table XV shows that in tests of extracts of yeast cultures grown for 48 hours at 30° 35 (59 percent) of 59 extracts clarified only by centrifugation showed the expected specificity of reaction. However, 82 percent of the 17 extracts which had been centrifuged and then treated with Filter Cel to remove non-specific precipitating materials gave positive precipitin reactions only with the homologous antigen and antigens containing the same hapten or protein.

All extracts of yeast grown by the transfer methods were treated with Filter Cel. Tests on extracts of cells removed from seventeen of such cultures after zero, two, four or six transfers indicated the presence of specific precipitins in

14 (80 percent) of the cultures at one or another transfer level. However, tests on extracts of cells removed from a given culture at other than the optimal transfer level frequently showed considerable non-specific precipitation.

Too little data are available at present on the production of antibodies by yeast or by incubation of plasma and antigens in vitro for a full statistical analysis of the observations. Further investigation of both methods of antibody formation is desirable.

With further study, it should be possible to find improved conditions for yeast culture and for the extraction of precipitins from yeast, and thus to obtain solutions of higher precipitin concentration. Development of other methods of adsorption of non-specific precipitins from the yeast extracts may conceivably lead to separation of specific antibodies, suitable for chemical analysis.

## SUMMARY

1. Using a slight modification of Bacon's procedure[55] precipitins have been prepared *in vitro* for such chemically modified proteins as 3,5-diiodo-4-hydroxybenzeneazo casein, iodo casein, 4-arsonobenzeneazo casein, iodo egg albumen, and 4-arsonobenzeneazo egg albumen. Antigens were mixed with bovine blood plasma and lyophilized. The dried materials were incubated at 37° for twelve hours, reconstituted, and tested for precipitin content. Precipitins could be demonstrated in ninety percent of the twenty-one preparations studied. Cross tests indicated specificity of reaction in approximately 2/3 of the preparations.

2. Precipitins have been demonstrated in extracts of a yeast, *Saccharomyses cerevisiae*, cultured in media containing antigenic proteins. The antigens tested were 4-sulfobenzeneazo casein, 4-carboxybenzeneazo casein, nitro casein, 3,5-diiodo-4-hydroxybenzeneazo casein, guanidyl casein, guanidyl egg albumen, and the 4-arsonobenzeneazo and iodo derivatives of casein, egg albumen and crystalline plasma albumen.

In the method employing *S. cerevisiae*, inoculae of Strain No. 567 of the Northern Regional Research Laboratory were incubated in 100 cc. of 15-20 percent molasses solution for 24 hours at 30°, aliquots containing approximately $6 \times 10^8$ cells were transferred to fresh medium (100 cc.) containing

the antigen in 0.1 percent concentration and reincubated for 48 hours. The cells were centrifuged, washed with water, and cytolyzed with sand and ether by vigorous grinding. The cytolyzates were each extracted with 25 cc. of a 5 percent sodium chloride solution. The extracts were cleared by centrifugation, and by stirring for 20 minutes with 100 mg. of Filter Cel. In 72 percent of eighteen of such preparations tested by micro-precipitin tests, homologous precipitin reactions were positive.

Stronger precipitin extracts, demonstrable with macro tests, were obtained from cells which were transferred each 48 hours to fresh medium containing the same antigen. In 80 percent of seventeen of such cultures, macro tests indicated the presence of specific precipitins at selected transfer levels.

Considerable variations in strength and specificity of precipitin reactions were noted in tests of yeast extracts prepared during sixteen months of experimentation.

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