Our type of inactivation may be different from that of Block, Jones, and Gersdorff (*2*), since they found that heated and unheated casein yielded the same quantities of lysine after acid hydrolysis. Our conditions are certainly similar to those of Block, *et al.* (*1*), who obtained lysine inactivation in high protein cakes. Their recipe included 11% molasses as well as other browning reactants. Paper partition chromatography appears to provide evidence that we are dealing with actual amino acid destruction. In preliminary experiments we have found that an excess of glucose in a solution of the essential amino acids does not interfere with their chromatography unless the mixture has been heated. Upon chromatographing heated and unheated portions of the same glucose amino acid mixture, we have found that the amino acid spots from the heated sample have either declined in density or vanished completely. At the same time new, unidentified "spots" appear, with higher R_F values than the corresponding amino acids, which are visible by fluorescence under ultraviolet light *before heating the paper.*

Soy globulin was used instead of whole soybeans for the following reasons: There are difficulties in obtaining a true picture of the amino acid destruction in a heat-treated protein food or feed which do not seem to be universally realized. It is not possible to accomplish this with any degree of accuracy by hydrolysis in hot acid or alkali. We have found that, as hydrolysis proceeds, the carbonyl compounds present or produced from carbohydrates or other components react with the amino acids as they are released. This causes a non-specific, over-all destruction which tends to mask the true heat-processing losses. Enzymatic digestion cannot be used, because overheating is reported to decrease the amount of each of the essential amino acids liberated by enzymes (*6*). We believe this effect on enzymatic digestion is separate from the destructive effect of browning with which the present article is concerned.

We do not wish to emphasize the actual *percentages* of loss obtained; they probably apply only to the conditions of individual trials. Statistical analysis, as previously shown for casein (*5*), succeeds only in demonstrating that the precision of the microbiological assay is excellent. We do wish to point out, however, that there seems to be a pattern of destruction which is significant and would probably apply to other heat processes which cause nonenzymatic browning. Judging from experience with casein (*5*) and soy globulin, it appears that the amino acids which are attacked by browning an intact protein are chiefly those containing functional nitrogen groups unattached in peptide linkages. There seems to be little doubt that lysine is most susceptible to attack, followed by arginine, tryptophan, and histidine. Presumably the radicals involved are the epsilon amino, guanido, indole, and imidazole groups, respectively. There is no conclusive evidence that any of the other essential amino acids are significantly altered. Apparently the amino groups in peptide or other protein linkages are blocked and do not readily react until hydrolysis has liberated them. On the basis of this hypothesis, terminal amino groups should also react.

References

1. Block, R. J., Cannon, P. R., Wissler, R. W., Steffee, C. H., Straube, R. L., Frazier, L. E., and Woolridge, R. L. *Arch. Biochem.,* 1946, **10**, 295.
2. Block, R. J., Jones, D. B., and Gersdorff, C. E. S. *J. biol. Chem,* 1934, **105**, 667.
3. Hayward, J. W. Private communication.
4. Hill, E. G., and Patton, A. R. *Science,* 1947, **105**, 481.
5. Patton, A. R., Hill, E. G., and Foreman, E. M. *Science,* 1948, **107**, 623.
6. Riesen, W. H., Clandinin, D. R., Elvehjem, C. H., and Cravens, W. W. *J. biol. Chem.,* 1947, **167**, 143.
7. Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M. C. *J. biol. Chem.,* 1945, **160**, 35.

Dyes as Microchemical Indicators of a New Immunity Phenomenon Affecting a Protozoon Parasite (Toxoplasma)[1]

Albert B. Sabin and Harry A. Feldman[2]

The Children's Hospital Research Foundation
and *Department of Pediatrics,*
University of Cincinnati College of Medicine

The purpose of this preliminary communication is to describe a new immunity phenomenon in which dyes of certain chemical composition have been found capable of indicating the presence or absence of antibody activity. This phenomenon was discovered during the course of a search for some *in vitro* manifestation of the action of neutralizing antibody on toxoplasma, an obligate, intracellular protozoon parasite. After finding that toxoplasma in properly diluted mouse peritoneal exudate could be counted with great accuracy in a standard hemocytometer, we observed that in mixtures with immune serum the toxoplasma remained intact but lost the refractility they exhibited in mixtures with normal serum. When, after incubation at room temperature for several hours, large drops of such mixtures were allowed to dry slowly on slides overnight and then were stained with Wright's stain, large numbers of toxoplasma could be seen in the preparation from the normal serum mixture, whereas very few were found in that from the immune serum mixture. Small drops, spread thin and rapidly dried, revealed that, with few exceptions, the cytoplasm of the toxoplasma in the immune serum mixtures was distorted, poorly stained, or unstained as compared with the deep blue staining and granular structure of the cytoplasm of the toxoplasma in the mixtures with normal serum; the chromatin of the toxoplasma appeared the same in both types of mixtures. One of us (A. B. S.) had observed a number of years ago that, when alkaline methylene blue was added on a slide to a drop of peritoneal exudate containing toxoplasma, immediate deep purple staining of the parasites could be observed under the microscope. When this was done with

[1] Aided by a grant from the National Foundation for Infantile Paralysis.

[2] Senior Fellow, National Research Council.

toxoplasma in mixtures with normal or immune serum (incubated at room temperature for several hours), it was found that with few exceptions the toxoplasma in the "normal" mixtures (Fig. 1, 1) stained deeply, whereas

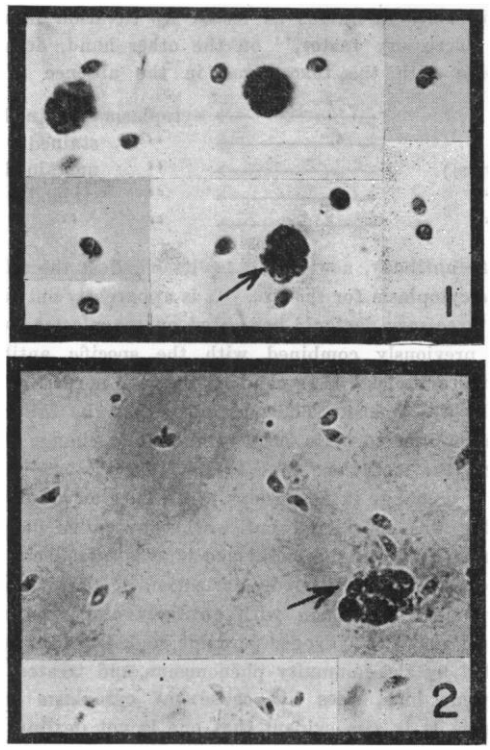

FIG. 1. (1) Toxoplasma incubated with *fresh normal serum* and then mixed with methylene blue. Note that the organisms are rounded or oval, the cytoplasm stains dark, and the lighter-staining chromatin body is surrounded by a light zone. Arrow points to group of intracellular toxoplasma. Different fields are presented because of difficulty of getting organisms into focus in wet preparation. (×650) (2) Toxoplasma incubated with *fresh immune serum* and then mixed with methylene blue. Note that the organisms are mostly crescentic, the cytoplasm is unstained except in the intracellular group (arrow), and the chromatin remains stained. Wet preparation. (×650)

in the "immune" mixtures (Fig. 1, 2) the cytoplasm of the *extracellular* toxoplasma was completely unstained. The toxoplasma which were still in their intracellular habitat within the large monocytes all stained deeply in the "immune" mixtures and were apparently protected from the antibody which affected the extracellular parasites. It has long been postulated on the basis of good circumstantial evidence that intracellularly situated viruses, bacteria, and larger parasites are protected from the effects of antibody, and here was visual evidence to confirm it. When these basic observations were once made, a systematic study was undertaken of the characteristics and mechanism of this immunity phenomenon, of the relationship of the antibody involved to the known neutralizing antibody, and of the capacity of dyes of varying composition to act as indicators. The results of these studies are summarized below.

We used the "R.H." strain of toxoplasma, which has had over 400 serial passages in mice since its original isolation from a human case of encephalitis (*2*). The peritoneal exudate obtained from mice, 4 days after intra-abdominal injection of a large dose (about 0.1 cc of fresh exudate or 0.5 cc of a 10% suspension of freshly passaged mouse brain), was optimum for use because (a) it had the largest number of extracellular toxoplasma (3,000,000–30,000,000/cc) and (b) the toxoplasma were not yet affected by the immune response of the host. In most experiments the exudate was diluted 1: 5 either in physiological salt solution containing heparin 1: 5,000 or serum containing heparin 1: 5,000 (to prevent coagulation of the fibrinogen in the exudate). For quantitative experiments in which the number of stained and unstained toxoplasma were counted to determine the 50% end point of activity, *it was important to use the exudate not later than 1 hr after its removal from the peritoneal cavity of the mouse.* Toxoplasma immune sera from human beings, monkeys, rats, guinea pigs, etc., were stored in an insulated box containing dry ice, because the neutralizing antibody had previously been found to be thermolabile (*4*). The mixtures of toxoplasma suspension and sera were prepared in tubes, incubated for 1 hr in a water bath at 37° C (or otherwise, depending on the experiment), and then stored in the refrigerator while the microscopic examinations of the contents were being made, as follows: 0.02 cc of the mixture was put on a slide, one loopful (5 mm in diameter delivering about 0.01 cc) of dye mixed with it, covered with a cover slip (22 mm square), and examined at a magnification of 475 for counting of the stained and unstained parasites, or with the oil immersion lens for studying details of staining. After some trial and error the following dye was selected as standard and *prepared fresh* every 3–4 days: 3 cc of saturated alcoholic solution of methylene blue + 10 cc of alkaline soda-borax buffer solution of pH 11 (9.73 cc of 0.53% Na_2CO_3 + 0.27 cc of 1.91% $Na_2B_4O_7 \cdot 10H_2O$). The alkaline pH is important for *immediate* staining. The alcohol is not necessary except to keep the methylene blue in readily available stock solution. A freshly prepared 0.25% methylene blue solution in the pH 11 buffer also works rapidly and well.

When fresh immune serum (or freshly thawed from the frozen state) is added to a suspension of toxoplasma and the mixture is immediately examined microscopically with the aid of methylene blue, the toxoplasma appear stained (as in a mixture with normal serum), and continued observation for several hours indicates that none becomes decolorized as long as the preparation remains moist. When one makes a fresh microscopic preparation from such a mixture after it has stood for 20 min at room temperature (28° C), one may find that the cytoplasm of 40% of the extracellular toxoplasma is unstained (the chromatin stains blue, red, or not at all, depending on the age of the dye used); at 1 hr this increases to 62%; at 2 hrs to 86%; and at 4½ hrs, to 96% (a varying percentage usually remains unaffected). If the same mixture is incubated at 37° C, the whole process is usually complete in 1 hr. If the immune serum is heated at 56° C for 30 min or left at room temperature for

more than 3 days, it loses its effect completely. It has been found, however, that the specific antibody is not destroyed by this procedure but rather a heat-labile "accessory factor," present in fresh, normal human serum in low concentration (even less in guinea pig serum and not at all in mouse serum), which is necessary for the completion of the immunity phenomenon, as shown in the following summary:

Fresh immune serum	+ toxoplasma	(exudate 1:5 in *saline*)	⟶	cytoplasm	unstained
Heated "	" + "	" " " "	⟶	"	stained
" "	" + "	(exudate 1:5 in *fresh normal serum*)	⟶	"	unstained
" "	" + "	" " *heated* " "	⟶	"	stained
Fresh normal "	+ "	" " *fresh* " "	⟶	"	"

The amount of this "accessory factor" in human serum is so small that reduction of its final concentration in a mixture to less than 40–50% is sufficient to prevent the immunity phenomenon from affecting any more than 20–30% of the toxoplasma. In searching for a source of "accessory factor," it was found that fresh, undiluted sera of the sheep, cow, horse, dog, monkey, rabbit, rat, and guinea pig could, to a varying extent, by themselves deprive the toxoplasma cytoplasm of its affinity for the dye and therefore did not lend themselves for use as "accessory factor." This substance in "normal" animal sera was proved to be different from specific toxoplasma antibody by the fact that, unlike the antibody in the immune sera from some of these species, it could not be reactivated after heating by the addition of human "accessory factor." This "normal, heat-labile, anti-toxoplasma factor" has not been encountered in human or mouse sera and is present in only low concentration in some of the animals tested.

When a fresh, human immune serum is diluted in saline and tested against toxoplasma in saline, the effect on the cytoplasmic affinity for the dye is rapidly lost, less than 50% of organisms showing the phenomenon at a 1:2 dilution of the serum. It was found, however, that the limiting factor in this is not the specific antibody but rather the "accessory factor," since immune sera diluted in saline have been found effective in dilutions ranging from 1:16 to 1:1,000 and more when they were mixed with toxoplasma suspended in fresh, undiluted human serum containing the "accessory factor." While the "accessory factor" behaves like complement in many respects, it differs from that operating in the lysis of red cells or in bactericidal and bacteriolytic systems, in that larger amounts of it (or one of its component parts) are necessary for the consummation of the immunity phenomenon affecting toxoplasma. Furthermore the addition of concentrated preparations of the heat-labile C_1' and C_2' fractions of human complement (kindly supplied by L. Pillemer) to heated normal human serum failed to restore its toxoplasma "accessory factor" activity but did restore its capacity to hemolyze sensitized sheep erythrocytes. A purified, concentrated preparation of "accelerator globulin" (*5*) from bovine plasma (the heat-labile AcG factor which accelerates the conversion of prothrombin into thrombin), kindly supplied by W. H. Seegers of Detroit, was inactive as "accessory factor" for the toxoplasma immunity phenomenon.

It was possible to demonstrate by suitable experiments (to be described in detail elsewhere) that the specific antibody can combine with the toxoplasma in the absence of "accessory factor," and that such toxoplasma are not deprived of their affinity for the dye and, furthermore, remain fully infectious as tested by titration in mice. The "accessory factor," on the other hand, does not combine with the toxoplasma in the absence of the specific antibody, nor does it by itself affect the affinity of the cytoplasm for the dye. It is apparently only after the "accessory factor" has acted on toxoplasma, which have previously combined with the specific antibody, that the cytoplasm is so changed that the normal affinity for the dye is lost. The possibility that the failure of the cytoplasm to stain may be due to a change which reduces the methylene blue to its "leuco" base rather than to a change in the groups which have an affinity for the dye was considered but was regarded as unlikely, because (1) when the methylene blue is introduced into the toxoplasma first, no decolorization of the cytoplasm occurs upon incubation with antibody and "accessory factor," and (2) hydrogen peroxide, added to toxoplasma affected by the immunity phenomenon and treated with methylene blue, does not cause the cytoplasm to be stained. It is also evident that one is not dealing here with a change in the permeability of the cell wall of the organisms affected by the immunity process because the dye diffuses into them and stains the chromatin without affecting the cytoplasm. Although hypertonic salt solution (8.5%) and formalin (0.5%) do not interfere with the staining of toxoplasma, the immunity phenomenon just described does not occur in their presence.

The dyes of the thiazin (thionin, toluidine blue, methylene blue), oxazin (brilliant cresyl blue), and amino-azin (neutral red) groups behave alike in that they stain (in 0.5% concentration) the cytoplasm of normal toxoplasma but not of organisms modified by the effect of specific antibody and "accessory factor." In aqueous solution, without adjustment of the pH to the alkaline side (pH 11), all these dyes act slowly on the cytoplasm and may require from 30 min to 2 hrs for optimum staining. The "acid" character of the normal cytoplasm is indicated by its red staining with neutral red, and the absence of the red color in the cytoplasm affected by the immunity phenomenon—the chromatin stains a deep red—suggests that these "acid groups" have been changed. It is of interest, therefore, that phloxine (xanthene group of dyes) in 2.5% concentration (either aqueous or in buffer of pH 11) fails to stain the normal toxoplasma cytoplasm but stains the cytoplasm modified by the immunity phenomenon. Commercial basic fuchsin (triamino, triphenyl methane group) stains the "im-

mune'' cytoplasm more readily than the normal cytoplasm (even at pH 11), whereas in the presence of carbolic acid (as in the Ziehl-Neelsen stain) both types of cytoplasm are stained rapidly and well. On the other hand, another triamino, triphenyl methane dye, crystal violet (hexamethyl pararosanilin) stains the normal more deeply than the immune serum treated cytoplasm. Acid fuchsin (the sulfonated derivative of basic fuchsin) as well as Congo red, brilliant vital red, trypan red (dis-azo group), and sodium 2,6-dichlorobenzenone-indophenol fail to stain the cytoplasm of both normal and immune serum treated toxoplasma.

Toxoplasma killed by freezing and thawing, by heating at 56° C for 30 min, by storage *in vitro* or even after certain intervals in dead animals, exhibit the same lack of affinity for methylene blue as those treated with antibody and ''accessory factor,'' *i.e.* the cytoplasm is unstained and the chromatin is stained. There is a difference, however, in the behavior of the intracellularly situated toxoplasma, *i.e.* the physical methods of killing toxoplasma affect intracellular as well as extracellular organisms, while in the immune process the intracellular ones are spared. On the other hand, toxoplasma killed by heating at 50° C for 15–30 min or by 0.2–0.5% formalin retain the cytoplasmic affinity for methylene blue. Thus, while affinity of the toxoplasma cytoplasm for methylene blue is not an index of life, loss of this affinity is, nevertheless, an index of death.

Adult albino rats develop a nonfatal infection after intra-abdominal injection of toxoplasma. The heat-stable, cytoplasm-modifying antibody appeared on the 5th day after inoculation in a titer of 1:16 to 1:64 and on the 7th day reached a peak titer of 1:256 to 1:1,024 which was maintained for at least 4 weeks. The titer end point is the highest dilution of serum (previously heated at 56° C) which, upon addition to toxoplasma in fresh human serum and incubation for 1 hr at 37° C, effects a loss of cytoplasmic affinity for methylene blue in 50% or more of extracellular toxoplasma. In the rhesus monkey this antibody was not demonstrable in significant amount 3 days after inoculation, was present in a dilution of 1:4 on the 5th day, 1:16 on the 7th, 1:256 on the 10th, 1:1,024 on the 14th, and 1:4,096 on the 21st. Titers of 1:4,096 were also found in sera of immune guinea pigs and rabbits. Many of the sera containing the antibody in high titer exhibited a *prozone phenomenon,* in that the undiluted serum and sometimes also the 1:4 and 1:16 dilutions were either ineffective we determined whether some of the sera may have ''anti- or affected less than 50% of the toxoplasma. The mechanisms of the prozone has not yet been elucidated, nor have accessory factor'' properties just as some sera are known to anticomplementary.

Many comparative tests have shown that sera which have titers of 1:16 or more of the cytoplasm-modifying antibody also exhibit neutralizing properties in the rabbit skin test (*3*). It has also been shown that the loss of neutralizing activity resulting from heating or dilution (*4*) can be restored by the addition of a suitable amount of fresh, normal human serum containing the ''accessory factor.'' It would appear, however, that the complement-fixing antibody (*6, 7*) is different from both of these, not only because it may appear later and disappear earlier, but also because sera containing a high titer of the cytoplasm-modifying antibody can be devoid of complement-fixing antibody.

Over 100 sera from human beings with and without histories of toxoplasma infection have now been tested. In mothers without signs of illness who had given birth to children with toxoplasmosis and in children with clinical signs suggestive of the disease, titers of 1:256 to 1:16,384 have been found as long as 2–5 years after probable onset of infection. Titers of 1:16 to 1:64 were found in those with a history suggesting infection 6–7 years ago or longer. Individuals without history of infection or events suggesting infection, whose sera contain neutralizing antibodies, have yielded titers of 1:16 to 1:64. This test has been found more useful than the neutralization test because of its simplicity and because the quantitative data permit differentiation between very old and more recent infection, which is of importance in deciding whether or not certain clinical manifestations in infancy are due to toxoplasmosis, especially when the mother may have neutralizing antibodies as a result of an infection long ago.

This test can also be used to survey the occurrence of the disease in animals. The high titers exhibited by infected rabbits, guinea pigs, rats, and monkeys have already been mentioned. In a recent study (*1*) on the occurrence of toxoplasma among pigeons in Cincinnati, we found that the one pigeon whose tissues yielded toxoplasma had a serum with a titer of 1:256, while most other pigeons had no demonstrable antibody.

The possibility that cytoplasm-modifying antibodies demonstrable *in vitro* by a suitable dye-test may occur against such protozoa as *Leishmania,* trypanosomes, plasmodia, *Endamoeba histolytica,* etc. needs to be investigated. It is also probable that bactericidal antibodies which have hitherto been demonstrated by cultural methods might become demonstrable *in vitro* by the use of suitable dyes. Some of the neutralizing antibodies against viruses are heat stable (e.g. the antibody against poliomyelitis virus), whereas others exhibit varying degrees of lability (e.g. dengue neutralizing antibodies become ineffective after heating at 56° C), suggesting the possible operation of a heat-labile ''accessory factor.'' It may be possible to apply the dye technique to the study of some of the viruses by investigating the staining properties of collodion particles with adsorbed virus before and after exposure to action of immune sera.

References

1. Feldman, H. A., and Sabin, A. B. Unpublished observations.
2. Sabin, A. B. *J. A. M. A.,* 1941, **116**, 801–807.
3. Sabin, A. B. *Proc. Soc. exp. Biol. Med.,* 1942, **51**, 6–10.
4. Sabin, A. B., and Ruchman, I. *Proc. Soc. exp. Biol. Med.,* 1942, **51**, 1–6.
5. Ware, A. G., and Seegers, W. H. *J. biol. Chem.,* 1948, **172**, 699–705.
6. Warren, J., and Russ, S. B. *Proc. Soc. exp. Biol. Med.,* 1948, **67**, 85–89.
7. Warren, J., and Sabin, A. B. *Proc. Soc. exp. Biol. Med.,* 1942, **51**, 11–14.