sites of nephrotoxic antibodies with fluorescein-labeled antiglobulin.

In the present procedure used to detect *Anaplasma marginale*, the antigen-antibody reaction is a naturally occuring process within the blood-vascular system of infected animals. To demonstrate this reaction in vitro, thoroughly washed erythrocytes from infected cattle were exposed to the fluorescein-labeled bovine antiglobulin.

Normal bovine globulin was extracted from the serum of a normal calf, according to the method described by Dubert (*3*), which involves the precipitation of globulins with methanol. The antiglobulin was produced by injecting a rabbit three times a week for 3 weeks with 0.5 ml of normal bovine globulin in increasing concentrations (20, 40, and 80 mg). The globulin was precipitated from the antiserum and conjugated to fluorescein isothiocyanate, according to the method of Riggs *et al.* (*4*). To minimize nonspecific fluorescence, the conjugated material was absorbed with powdered rabbit liver prior to use. The technique of fixation and staining of blood films with labeled bovine antiglobulin was similar to that used by Ristic *et al.* (*5*) for detection of *A. marginale* by means of specific fluorescein-labeled antianaplasma antibody.

By the method described above it was possible to observe *A. marginale* growth forms, including marginal and initial bodies (*6*). It was also possible to detect a single initial body occurring within erythrocytes of carrier animals. As viewed with a microscope by ultraviolet light, the initial bodies appeared as punctiform, brilliant foci scattered throughout the erythrocytes of acutely infected animals (Fig. 1) or appeared singly within the erythrocytes of carrier cattle (Figs. 2, 3). The classical *A. marginale* occuring in acute infections appeared as brilliant yellow-green, sharply defined, round bodies (Fig. 1). In contrast, the erythrocytes were clearly seen as grayish-green background structures.

With this technique it was possible to demonstrate the presence of initial bodies not usually observed by conventional staining procedures. This is apparently due to the formation at the site of the organism of a specific, fluorescent-complex aggregate of sufficient size to be observable by microscope. While further evaluation of this technique with regard to its accuracy in detecting anaplasmosis carriers is needed, the principles upon which it is based offer a means of developing a serological test capable of revealing the organism rather than serum antibodies in the blood of anaplasmosis carriers.

It seems reasonable to believe that by the technique described above, other organisms capable of invading the blood-vascular system could be detected. In addition, this method may prove useful in demonstrating *Vibrio fetus, Trichomonas fetus, Leptospira* species, and other microorganisms which, after an acute stage of infection, may persist for long periods in extravascular localities of the body. In these areas complex cell systems apparently are capable of producing antibodies in local tissue that, when combined with specific antigens, offer the prerequisite for carrying out the technique described (*7*).

MIODRAG RISTIC*
F. H. WHITE
Department of Veterinary Science, University of Florida, Gainesville

References and Notes

1. T. H. Weller and A. H. Coons, *Proc. Soc. Exptl. Biol. Med.* **86**, 789 (1954).
2. R. C. Mellors, *J. Histochem. and Cytochem.* **3**, 284 (1955).
3. J. M. Dubert, P. Slizewicz, P. Rebeyrotte, M. Macheboeuf, *Ann. inst. Pasteur* **84**, 370 (1953).
4. J. L. Riggs, R. J. Seiwalf, J. H. Burckhulter, C. M. Downs, *Am. J. Pathol.* **34**, 1081 (1958).
5. M. Ristic, F. H. White, D. A. Sanders, *Am. J. Vet. Research* **18**, 924 (1957).
6. M. Ristic, *J. Am. Vet. Med. Assoc.*, in press.
7. This report is Florida Agricultural Experiment Stations Journal Series publication No. 987. This investigation was supported in part by a research grant (E-3315) from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health.
* Present address: Department of Pathology and Hygiene, College of Veterinary Medicine, University of Illinois, Urbana.

27 November 1959

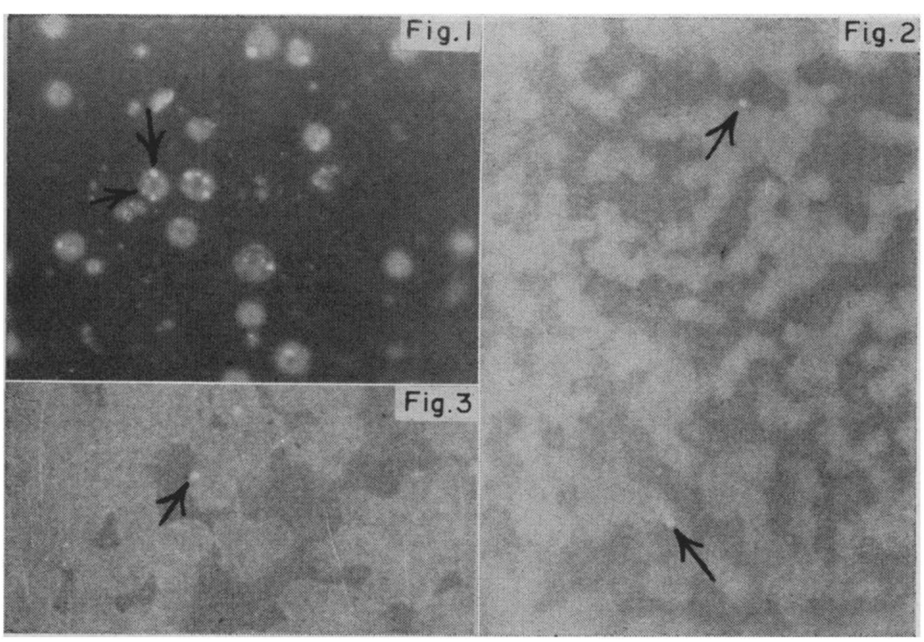

Fig. 1. Appearance of classical *Anaplasma marginale* (larger form) and initial anaplasma body (smaller form) in the blood film from an acutely infected cow after staining with fluorescein-isothiocyanate-labeled bovine antiglobulin (about × 600). Figs. 2 and 3. Erythrocytes of two anaplasmosis carriers, stained with fluorescein-isothiocyanate-labeled bovine antiglobulin. Note the presence of three initial anaplasma bodies (about × 900).

Significance of the Presence of Exchangeable Magnesium Ions in Acidified Clays

Abstract. Magnesium ions (Mg^{++}) were shown to constitute a substantial percentage of the total exchangeable cations in acidified clay samples from various sources. This finding helps in solving several problems of long standing in the fields of soil chemistry, soil formation, and geochemical weathering.

Many investigators have concluded that the acidity of naturally occurring soils and artificially acidified soils and clays is due mainly to the presence of exchangeable Al ions rather than to exchangeable H^+ (*1*). It is postulated that after the exchange of the basic cations with H^+ ions, the H^+ ions disappear from the exchange positions and are replaced by Al ions which are either a part of the interior of the crystal lattice (tetrahedral or octahedral positions) or part of free $Al(OH)_3$ and Al_2O_3 which are present in the soil or clay as impurities. Several investigators, however, were aware that the H^+ ions are replaced not only by Al ions but also by Mg^{++} ions (*2*), but the factors affecting this replacement and its significance were only studied recently. The present report is a result of this study.

Briefly, the relative amounts of Mg^{++} and Al^{+++} ions were found to depend on the total MgO and Al_2O_3 contents and the crystal structure of the acidified minerals and on the nature of the acidifying solution and the technique of acidification.

Table 1 is an example of the kind of results obtained for clay minerals and soil clays of varying MgO content acidified by very dilute (0.05 to 0.1*N*) HCl at room temperature. It is clearly seen that in many of the samples exchangeable Mg^{++} ions are even more abundant than exchangeable Al ions. It is also important to note that the Al ions have undergone varying degrees of hydroxylation.

The finding that Mg^{++} is an important exchangeable ion in acidified clay minerals and soils is of great significance to students of soil chemistry and pedology, for it sheds light on several important problems of long standing in these fields.

1) It explains the occurrence of the high percentage of Mg^{++} saturation commonly found in solonetz soils—soils which are formed by leaching sodium saline soils (*3*). Due to the high *p*H of such soils the exchangeable H^+ gained by the soil through hydrolysis tends to be replaced mainly by Mg^{++} rather than Al^{+++}. Crystal lattice Al^{+++} which may be replaced by H^+ would tend to precipitate as $Al(OH)_3$ rather than remain as an exchangeable ion.

2) It explains the occurrence of the high percentage of Mg^{++} saturation and nearly neutral *p*H of soils developed from serpentine rocks in areas where other soils developed from other types of rocks are quite acid (*4*). Furthermore, the finding suggests that the relative composition of the exchangeable ions in soils is strongly influenced by the total composition of the clay minerals and possibly of others. Among California soils formed under similar soil forming factors except parent material, the ratio of exchangeable Mg^{++} to Ca^{++}, the major exchangeable bases, tends to be in the following order: serpentine soils > basaltic soils > granitic soils—the order of their content of MgO (*4*).

3) The observation (*5*) that in some soils, even though they are acid in reaction, the cation-exchange capacity by the NH_4Ac method is lower than the total bases replaced by the NH_4Ac may be explained as follows: Due to the weak acid character of clay acidity, clay minerals adsorb H^+ ions during the leaching process even though the solutions are neutral. These adsorbed H^+ ions enter the crystal lattice and displace Mg^{++} and Al^{+++} ions to the surface. In NH_4Ac the Al^{+++} ions become either hydroxylized to some form of $Al(OH)_x{}^{(3-x)+}$ or precipitate as $Al(OH)_3$, but the Mg^{++} ions are again replaced by NH_4^+, and thus they appear as replaceable ions which were not on the surface in the first place. This conclusion raises doubts as to whether the method of determining the naturally occurring exchangeable ions by replacement techniques yields results which truly represent their distribution, particularly for soils high in total MgO.

4) The presence of exchangeable Mg^{++} in acidified clays raises doubts as to whether it is possible to prepare 100-percent homoionic saturated clays, other than Mg^{++}, by the common method which involves saturating the clays first with "acid" and then adding in proper amounts the desired cation in its basic form.

5) Due to the presence of exchangeable Mg^{++} in acidified clays it is not surprising to find that in many published titration curves of clay minerals the full cation-exchange capacity is observed at a *p*H between 10.3 and 10.6, since at this *p*H Mg^{++} precipitates as $Mg(OH)_2$.

6) Most significant of all, the finding that adsorbed H^+ ions on clay surfaces or other minerals readily enter the interior of the crystal lattice to displace Al^{+++}, Mg^{++}, and possibly Fe^{++} or Fe^{+++} whenever these are present, suggests a mechanism by which the chemical alteration and breakdown of the silicate mineral proceeds beyond the first stage of hydrolysis. If the H^+ is replaced by Mg^{++} ions, then the Mg^{++} ions can again be replaced by other H^+ ions, and this process can be repeated until all of the Mg^{++} is exhausted. If the H^+ ions are replaced by Al^{+++} ions and the *p*H of the system is buffered, as by CO_2 or organic substances, the Al^{+++} will either precipitate as $Al(OH)_3$ or become hydroxylized to various degrees as $Al(OH)_x{}^{(3-x)+}$. These ions remain adsorbed on the clay, but they leave several exchange spots free to adsorb more H^+, which again can enter the crystal lattice and replace more crystal-lattice ions. If Fe^{++} iron is present in the crystal lattice it too would be replaced, but as it reaches the surface and comes in contact with dissolved oxygen it becomes oxidized to Fe^{+++}. The Fe^{+++} iron, due to its chemical behavior will become hydroxylized even more readily than Al^{+++}. In the *p*H range of most soils the Fe^{+++} will precipitate as $Fe(OH)_3$. The replacement of Fe^{++}, therefore, would be expected to proceed even faster than that of Al^{+++}, and indeed minerals which contain large amounts of Fe^{++} do weather more rapidly than those which do not.

From a consideration of the geometry of the crystal structure of the clay mineral, it is necessary to conclude that the H^+ ion enters the interior of the crystal lattice as a bare proton rather than as a hydronium ion (H_3O^+).

I. BARSHAD
Department of Soils and Plant Nutrition, University of California, Berkeley

Table 1. Exchangeable ions in various clay samples after their sodium-saturated forms were leached with a dilute acid*.

Sample	Clay minerals in sample†	Total octahedral MgO (gm / 100 gm)	Total exchangeable cations (meq / 100 gm)	Saturation (%)				X‡ OH per Al^{+++}
				Ca^{++}	Mg^{++}	$Al(OH)_x{}^{(3-x)}$†	H^+	
Kerrite	V	28.1	169	0.0	52.0	48.0	0.0	1.20
Hectorite	S	25.9	55.0	0.0	100.0	0.0	0.0	
Bentonite No. 2	M	7.9	111.0	0.0	17.0	83.0	0.0	0.75
Bentonite No. 7	M	4.5	103.0	0.0	12.0	64.0	24.0	0.0
Bentonite No. 5	M	2.9	86.0	0.0	7.0	74.0	19.0	0.0
Soil clays								
Dubakella	Ch, V	35.5	12.0	0.0	90.0	10.0	0.0	2.00
Maxwell	Ch, M	10.3	61.6	2.2	90.0	7.8	0.0	2.00
Sweeney	V, M	12.0	59.4	2.5	53.5	44.0	0.0	1.12
Rosamond	V, M, Mic	6.7	33.2	33.1	55.8	11.1	0.0	2.00
Yolo	V, M, K	4.3	43.7	2.4	31.8	65.8	0.0	1.23
Holtville	M, K	3.9	43.4	0.0	30.3	69.7	0.0	1.27
Fresno	V, K, Mic	3.5	18.2	3.4	30.9	65.7	0.0	1.75
Cayucos	M	3.0	63.1	1.2	9.5	89.3	0.0	0.87
Huerohuero (solonetz colloid)	M, V		49.3	0.0	17.5	82.5	0.0	1.00

* The dilute acid was 0.05 to 0.1*N* HCl (350 ml per 1.0 gm of sample). The free acid was removed with H_2O before the samples were extracted with neutral 1*N* KCl to determine the exchangeable ions. † Ch, chrysotile; K, kaolinite; M, montmorillonite; Mic, mica; S, saponite; V, vermiculite. ‡ Determined from the total titratable acidity and total Al^{+++} in the KCl extract.

References

1. H. Paver and C. E. Marshall, *Chem. & Ind. (London)* **12**, 750 (1934); R. K. Schofield, *Soils and Fertilizers, Commonwealth Bur. Soil Sci.* **9**, 265 (1946); V. A. Chernov (Press of Acad. Sci., U.S.S.R., Moscow, 1947); N. T. Coleman and M. E. Harword, *J. Am. Chem. Soc.* **75**, 6045 (1953); P. F. Low, *Soil Sci. Am. Proc.* **19**, 135 (1953); H. Laudelout and J. P. Eekmann, *Vortr. zweiten vierten Komm. IBG* **II**, 193 (1958).
2. I. Barshad, *Soil Sci.* **78**, 64 (1954); G. F. Walker, *Clay Minerals Bull.* **3**, 309 (1958); A. C. Mathers, S. B. Weed, N. T. Coleman, "Clay and Clay Minerals," *Natl. Acad. Sci.–Natl. Research Council Publ. No. 395* (1955), p. 403; G. T. Kerr, R. H. Zimmerman, H. A. Fox, Jr., F. H. Wells, "Clay and Clay Min-

erals," *Natl. Acad. Sci.–Natl. Research Council Publ. No. 456* (1956), p. 322.
3. W. P. Kelley, *Am. Soil Survey Assoc. Bull.* **15**, 45 (1935); L. D. Whittig, *Soil Sci. Soc. Am. Proc.* **23**, 469 (1959).
4. Henneke and Josephene, unpublished; P. J. Zinke, thesis, University of California (1956).
5. I. Barshad, *Soil Sci.* **61**, 432 (1946).

18 November 1959

Epinephrine, Norepinephrine, and Acetylcholine as Conditioned Stimuli for Avoidance Behavior

Abstract. Conditioned leg-flexion responses in dogs were developed with electric shock as an unconditioned stimulus and intestinal stimulation or the effects of injections of various drugs as conditioned stimuli. It is concluded that physiological effects can play a role in the development and maintenance of conditioned avoidance behavior.

Many investigators have suggested the importance of physiological correlates of behavior (*1*). However, few workers have considered the possible role of physiological changes as stimuli (*2*). The studies reported here were conducted to determine whether physiological changes produced by *l*-epinephrine, *l*-norepinephrine, acetylcholine, or stimulation of a Thiry-Vella jejunal loop can become conditioned stimuli in avoidance conditioning.

Beagles, surgically prepared with Thiry-Vella jejunal loops, were restrained in a harness in a soundproof chamber. A balloon inserted into the Thiry-Vella loop could be inflated remotely with 10 cm-Hg pressure. The conditioned stimulus was a balloon inflation lasting 2 seconds, terminated by the unconditioned stimulus, a brief electric shock (of intensity sufficient to cause leg flexion) delivered to the left hind leg. After an appropriate number of trials, balloon pressure *alone* consistently produced leg flexion, the conditioned avoidance response.

Other experiments were conducted to determine whether physiological changes produced by pharmacological agents could also act as conditioned stimuli in avoidance conditioning. A polygraph simultaneously recorded respiration, electrocardiogram, and intestinal activity of Thiry-Vella jejunal loops. The electrocardiographic recordings were made with surface electrodes fixed over the heart apex and the right paravertebral line (modified CR_{6L} lead) in order to minimize artifacts from gross body movements (*3*). Two fine polyethylene catheters were inserted into the external saphenous vein of the right hind leg and attached to syringes outside the soundproof chamber. These catheters permitted the injection of *l*-epinephrine, *l*-norepinephrine, or acetylcholine under remote control.

In each conditioning trial, electric shock was delivered to the left hind limb 30 seconds after the start of the injection. Injections were spaced 5 to 10 minutes apart, to allow time for physiological responses to return to normal levels. No stimuli, other than the physiological effects produced by the injected agents, preceded the shock. Preliminary experiments demonstrated that the monitored physiological responses were generally maximal 30 seconds after injection. Leg flexions occurring within the 30-second interval automatically prevented the shock. Prior to conditioning, none of the agents studied produced leg flexion. The effects of *l*-epinephrine (10 μg/kg), *l*-norepinephrine (10 μg/kg), or acetylcholine (20 μg/kg) came to serve as conditioned stimuli for the avoidance response after an appropriate number of training trials.

Figure 1 shows representative curves for the development of avoidance behavior with the various types of conditioned stimuli. For comparative purposes, other dogs were conditioned to an auditory stimulus (tone) as the conditioned stimulus. Differences in rates of acquisition are apparent; however, direct comparison of these rates is limited because the patterns of stimuli vary, due to differences in dose and in the intensity of effects. Figure 2 is a polygraphic recording of a representative trial, showing physiological effects and conditioned leg-flexion avoidance response. Jejunal activity, respiration, and electrocardiograph were monitored for indications of the occurrence of physiological changes and the temporal relationships of these changes to conditioned leg flexion. It was observed that physiological changes consistently preceded the occurrence of the avoidance response.

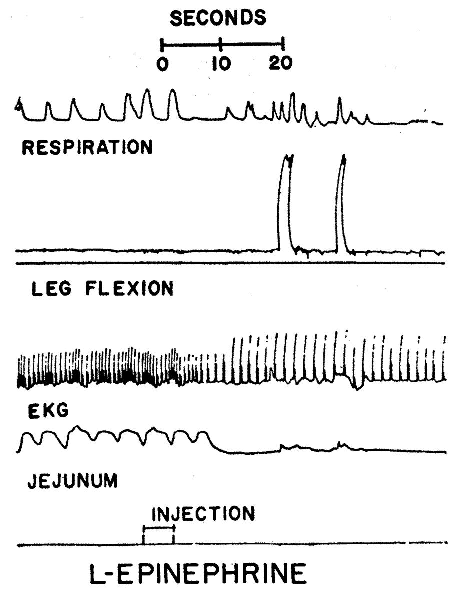

Fig. 2. Polygraphic record showing a trial in which the dog avoided shock. The leg flexion (avoidance response) followed the compensatory bradycardia and decreased intestinal motility produced by the injection of epinephrine.

After dogs had been conditioned to the effects of 10 μg of *l*-epinephrine per kilogram, doses of 1 or 2.5 μg of *l*-epinephrine per kilogram could produce avoidance responses. In a similar manner, dogs conditioned to 20 μg of acetylcholine per kilogram manifested avoidance responses after the injection of 10 μg of acetylcholine per kilogram. Saline, in comparable volumes, was injected on a random basis with all drug injections through the second catheter; saline injections never produced an avoidance response. These control injections eliminate the possibility that local sensation at the site of injection or volume changes acted as the conditioned stimulus. Injections of glucose

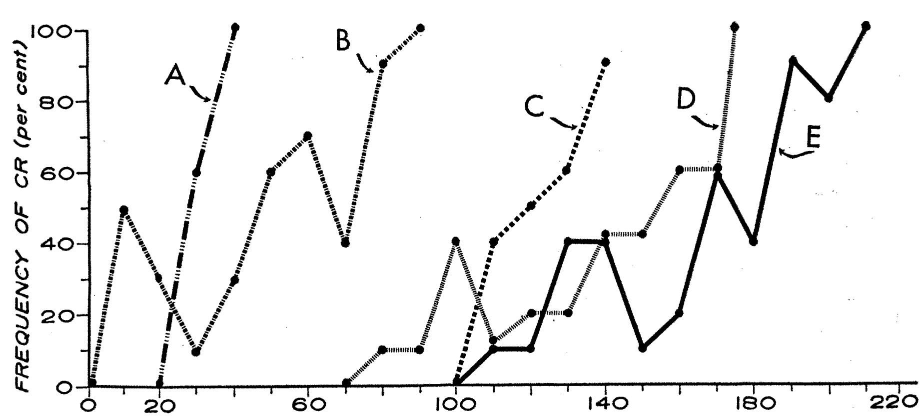

Fig. 1. Representative avoidance acquisition curves for each type of conditioned stimulus; each curve represents the results obtained with a single animal. Conditioned stimuli: *A*, tone; *B*, acetylcholine; *C*, *l*-norepinephrine; *D*, jejunal pressure; *E*, *l*-epinephrine. Different dogs were used in each experiment. Each point represents the percentage of conditioned leg flexions occurring in blocks of ten successive trials.