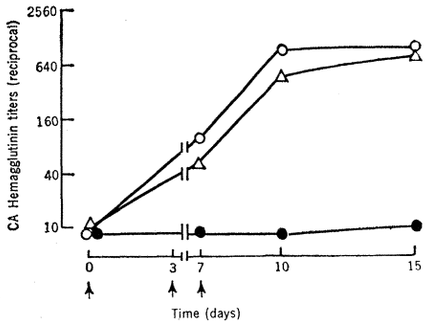

Fig. 1. The effect of normal rabbit serum on hemagglutinin response to common enterobacterial antigen. Groups of rabbits were injected intravenously on days 0, 3, and 7 with 1 ml of ethanol-soluble CA from *Salmonella typhimurium* in aqueous solution (1 part) mixed with either rabbit serum (1:10) or buffer (9 parts). Group 1 (●——●) received the mixture of antigen and serum incubated at 37°C for 30 minutes before injection; group 2 (△——△) received the same mixture without prior incubation; group 3 (○——○), serving as control, received antigen alone.

back mechanism, may be responsible for inhibition of the immune response. The following observations suggest that this is not the case. Calf serum obtained before and after colostrum feeding (*8*) were equally effective as immunosuppressants. Calf serum devoid of γ-globulin (*7*) inhibited the antibody response as well as whole calf serum did. Removal of the bacterial antibodies by absorption of normal rabbit serum with erythrocytes modified by antigen did not abolish its immunosuppressive effect. Finally, antiserum against CA obtained from rabbits, after dilution to antibody concentrations present in normal serum, did not inhibit the antibody response under identical conditions. Thus, serum of several animal species has immunosuppressive properties provided that the common enterobacterial antigen and serum interact first in vitro prior to immunization of rabbits.

Immunosuppression by normal serum was supported by experiments with another antigen shared by gram-positive bacteria and first described by Rantz *et al.* (*9*). The methods of antigen preparation and hemagglutinin titration have been described (*10*). The results of a representative experiment with this antigen (in a dilution of 1 : 10) obtained from *Staphylococcus pyogenes* and normal rabbit serum, incubated before immunization for 30 minutes at 37°C, are summarized in Table 1. Undiluted serum almost completely prevented the antibody response, and dilution of 1 : 10 was moderately effective. We conclude, then, that normal serum associated with either of two bacterial antigens inhibits their immunogenicity.

Studies directed toward elucidation of the mode of action of normal serum as immunosuppressant indicate that the serum does not hinder, bind, or destroy the antigenic determinant. This conclusion is based on the observation that antigen, either in the presence or absence of normal serum used in identical proportions as employed for immunization, neutralizes antibodies equally well. In these experiments, the materials were mixed with the appropriate antiserum, and the mixtures were tested for antibody by means of the passive hemolysis test (*5*). Nor do antibodies account for the immunosuppressive effects of normal serum. The possibility may be considered that serum alters enzymatically the antigen carrier. This is unlikely in view of the fact that other substances, such as lipopolysaccharide, lipid A, and cardiolipin (*2*, *10*, *11*) inhibit the immune response under identical conditions. Rather, it is postulated that antigen and inhibitor interact and form a complex and that the antigen-associated immunosuppressant affects early events of the immune response, such as antigen uptake or processing. Experiments are needed to determine the identity of the immunosuppressant component of normal serum. Immunosuppression by serum, and particularly by alpha proteins, has been reported (*12*), although some of the results could not be fully confirmed (*13*). At any rate, in these studies immunosuppression did not depend upon the injection of antigen-inhibitor mixtures. Our experiments then, extend previous observations of immunosuppression by antigen-associated inhibitors, to include normal serum, and suggest the possibility of other substances being immunosuppressants that are ineffective or less effective when given independently of the antigen.

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Sclerospora graminicola
Axenic Culture

Abstract. Sclerospora graminicola *(Sacc.) Schroet., the obligate pathogen causing downy mildew (green-ear) of pearl millet (*Pennisetum typhoides *Stapf. and Hubb.) has been successfully cultured for the first time on a known semisynthetic medium with no evident loss of fructifications.* Sclerospora graminicola *was first grown on host callus tissue and subsequently on a modification of White's basal medium that contained casein hydrolyzate (Oxoid), 2,4-dichlorophenoxyacetic acid, and kinetin.*

Plant pathologists have attempted without much success to culture viruses, downy mildews, powdery mildews, and rusts on nutrient media (*1*). Morel cultivated *Plasmopara viticola* (Sew.) Burr. on grape stem callus tissues in culture (*2*). Several other obligate parasites have been grown recently on tissue cultures of their respective hosts (*3*). We have grown *Sclerospora graminicola* (Sacc.) Schroet. on the callus tissues of pearl millet (*Pennisetum typhoides* Stapf. and Hubb.) and have maintained it for 4 years (*4*).

Axenic culture of *Gymnosporangium juniperi-virginianae* Schw. on a relatively simple medium containing mineral salts, a carbon source, ascorbic acid, and yeast extract represented a major

breakthrough (*5*). Limited saprophytic growth of a *Puccinia* species (*6*), and *Peronospora brassiceae* Gaum. with conidiophores (*7*) have been reported. More recently Williams *et al.* grew *Puccinia graminis tritici* Eriks. and Henn. in culture medium containing Czapek's minerals with additional supplements (*8*). Lately we have obtained axenic cultures of *Sclerospora graminicola* which contained all sexual and asexual stages.

The medium used (CHDK) was a modified medium consisting of White's basal mineral salts (*9*) and containing the following (per liter): glucose, 20 g; agar, 5 g; casein hydrolyzate (Oxoid), 3 g; calcium pantothenate, 3.5 mg; inositol, 300 mg; cyanocobalamin, 0.05 μg; glycine, 3.0 mg; folic acid, 0.1 μg; nicotinic acid, 1.5 μg; thiamine hydrochloride, 0.1 mg; ferric tartrate, 40 mg; 2,4-dichlorophenoxyacetic acid, 6.0 mg; ascorbic acid, 25 mg; 6-furfurylaminopurine (kinetin), 0.1 mg; and alpha-naphthaleneacetic acid (NAA), 1.0 mg. The medium was adjusted to *p*H 5.9, and NAA was added before autoclaving it at 15 pounds (1 atm) for 20 minutes. Healthy tissue cultures were obtained from the hypocotyl region of a germinated seed of *Pennisetum typhoides* on the above medium. Infected proliferated flowers were teased out and transferred aseptically on to CHDK medium, which allowed growth of downy mildew on the proliferated floral pieces. Such proliferated flowers with sporangia, antheridia, and oogonia were transferred to healthy callus which became subsequently infected. The flasks were incubated in a weakly lighted (216 lumen/m²) room at 26°C (65 percent relative humidity). After 20 days incubation, luxuriant fungus growth was obtained covering the entire surface of the callus. Aerial mycelium bore sporangia, antheridia, and oogonia. The fungus grew over the surface of the medium; after 20 days it thinly covered the entire surface of the medium in 100 ml flasks. The fungus maintained this saprophytic growth independent of that of the host callus during two subsequent subcultures made at intervals of 20 days on the same fresh medium. Further subcultures produced extremely thin growth.

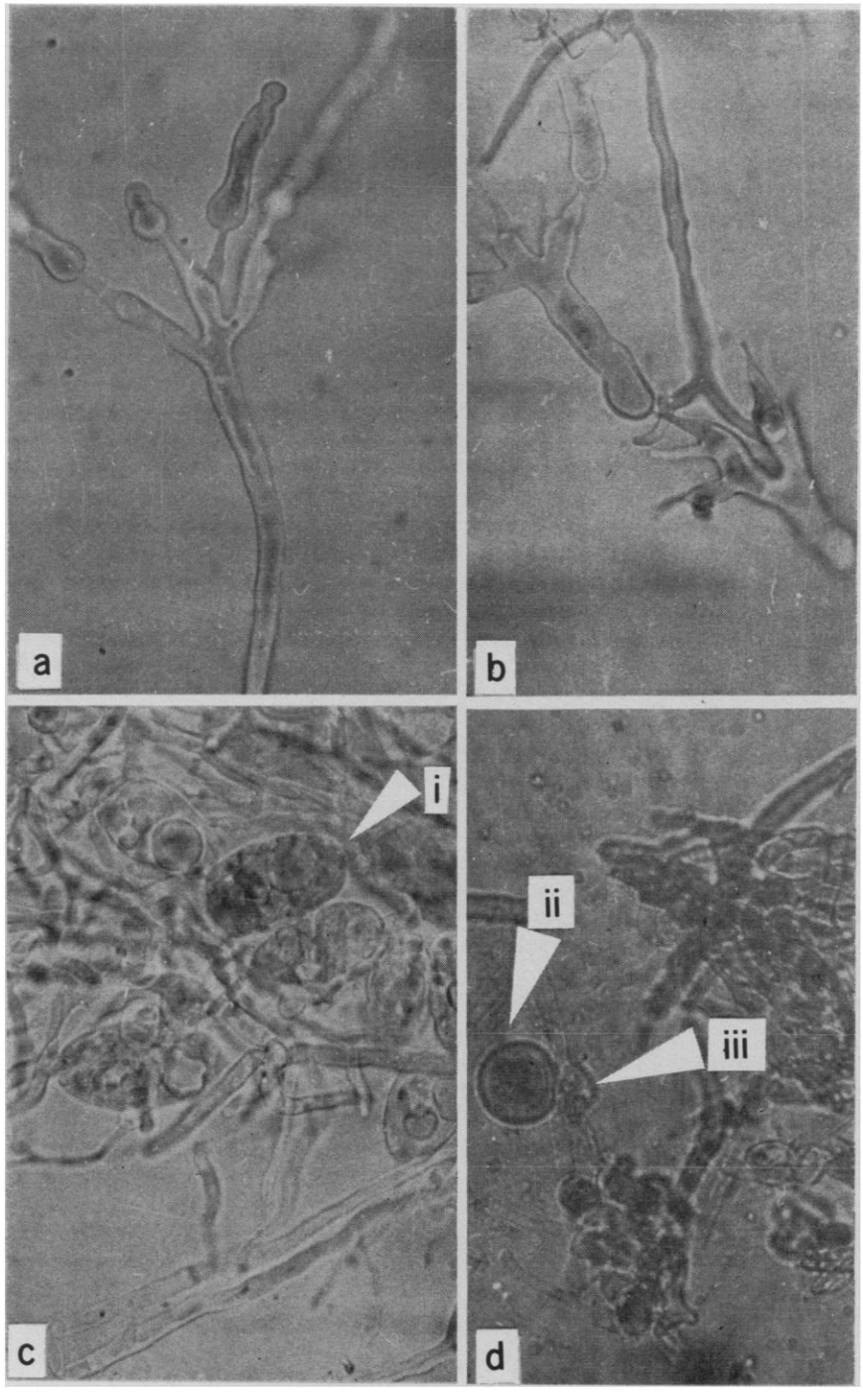

Fig. 1. (a) Initiation of abnormal production of sporangia (× 1500). (b) Sporangiophore gives rise to secondary and tertiary sporangiophores and a sporangium finally produced on the sterigma of tertiary sporangiophore (× 1500). (c) Sporangia with germinating zoospores inside as indicated by *i* (× 3000). (d) *ii*, Oogonium in axenic culture; *iii*, antheridium in axenic culture (× 1500).

Microscopic observations of the fungus from the subcultures revealed typical aseptate coenocytic mycelium with sporangia borne at the ends of sterigmata. Sporangia were produced in abundance, and abnormal structures were often formed (Fig.1, a–c). Under conditions of natural infection, mycelium gave rise to sporangiophores which exhibited di- or trichotomy in bearing sporangia on sterigmata. In this case sometimes primary sporangiophores gave rise to secondary or tertiary sporangiophores borne on typical sterigmata. Ultimately a sporangium was produced. The behavior of such a sporangium was also abnormal. Zoospores germinated while still inside the sporangium. Antheridium was placed lateral to oogonium which was of normal structure (Fig. 1d). The mycelium was 6.0 μ thick, whereas in vivo it measured 5.0 μ.

Other reproductive structures in tissue in vivo, in infected callus, and in axenic culture showed similar variations in size. Size of sporangia increased as the fungus changed its habitat from in vivo to in vitro, to axenic culture (15.5 by 21.0 μ, to 19.0 by 26.5 μ, to 20.0 by 29.0 μ) respectively. However, the size of oogonia was reduced in this sequence, the diameters being: 41.5 to 37.5 to 30.5 respectively. The differences in measurements were statistically significant. No oospores were noticed in the axenic culture.

The sudden emergence of saprophytic growth of *S. graminicola* from infected host callus tissues on artificial media, and, subsequently, the growth of two subcultures on the same medium still cannot be explained. The stepwise appearance from infected callus tissues of a saprophytic mycelium in the case of *Gymnosporangium juniperi-virginianae* Schw. by Cutter was explained as perhaps being due to mutation (*5*). Another possible explanation for the growth of *S. graminicola* is that the fungus adapted itself through gradual but persistent infection of host callus tissues to grow on the nutritive medium which supported good growth of the host tissues. The fungus growth obtained on the nutritive medium was limited in character, and proper nutritional requirements of the fungus need to be worked out to obtain continuous copious growth.

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Sweetclover-Weevil Feeding Deterrent B: Isolation and Identification

Abstract. *Deterrent B, a compound apparently involved in the resistance of* Melilotus infesta *to the sweetclover weevil* Sitona cylindricollis, *has been isolated from leaves by a combination of preparative paper chromatography, sublimation, and crystallization. The compound has been identified as ammonium nitrate. Isolated deterrent B and ammonium nitrate have identical feeding deterrent activities. Although the deterrent principle was isolated as the ammonium salt, nitrate ion is probably responsible for the deterrent activity in vivo.*

The sweetclover weevil *Sitona cylindricollis* (Fåhraeous) is the major insect pest of sweetclover (*Melilotus* spp.) in the northern Great Plains of the United States. The adult insects feed on sweetclover leaves, leaving characteristic crescent-shaped notches. If present in large numbers, the weevils can completely defoliate young plants. One sweetclover species, *M. infesta* Guss., is resistant to weevil feeding (*1*).

A bioassay has been developed for the detection of factors which influence feeding by the sweetclover weevil (*2*). In the bioassay, washed disks of sweetclover root tissue are impregnated with the extracts to be tested and offered to adult weevils for feeding. The extent of feeding is measured by determining the percentage of disk area consumed during a given feeding period. In a bioassay of various extracts of leaves from the weevil-resistant *M. infesta* and a susceptible species, *M. officinalis* (L.) Lam., feeding was strongly stimulated by chloroform-soluble substances from both species. Also, water-methanol or water extracts of *M. officinalis* leaves stimulated extensive feeding in the bioassay system, but little feeding was observed on bioassay disks treated with corresponding extracts of *M. infesta* leaves (*2*, *3*).

Bioassay of leaf extracts separated by chromatography has shown that *M. infesta* leaves contain at least three water-soluble substances which influence weevil feeding (*4*). Two of these substances, stimulant A and deterrent A, apparently occur in both *M. officinalis* and *M. infesta*. The third, deterrent B, was detected only in *M. infesta* extracts. Available evidence indicates that deterrent B is involved significantly in resistance of *M. infesta* to weevil feeding. Deterrent B has now been isolated and identified as ammonium nitrate.

Young *M. infesta* leaves were autoclaved in water as described (*3*, *4*) to provide an extract of which 5 ml corresponded to 1 g of dry tissue. A 100-ml sample of crude extract was first subjected to preparative paper chromatography on Whatman No. 3 MM filter paper with ascending solvents. Solvents, in order of use, were: (I) isopropyl alcohol–concentrated ammonium hydroxide–water (8 : 1 : 2); (II) isopropyl alcohol–glacial acetic acid–water (8 : 1 : 3); and (III) methanol. Water was used to elute the deterrent B band (location determined by bioassay) after each chromatographic step. After chromatography with methanol, the eluate containing deterrent B was lyophylized to dryness.

The powder obtained was next sublimed at 0.2 mm-Hg and 100°C. The sublimate was washed from the cold finger with a small quantity of methanol, and the resultant solution was transferred to a graduated centrifuge tube. Deterrent B was precipitated from the solution by the addition of five volumes of ethyl ether. After centrifugation (2000*g*, 5 minutes), the supernatant was decanted and the precipitate was taken up in a small volume of methanol. To

Table 1. Feeding deterrent activity of various levels of isolated deterrent B and ammonium nitrate. Quantities shown were mixed with a quantity of stimulant A extracted from 8 mg (dry weight) of young *M. officinalis* leaves in a total of 0.15 ml of H_2O. The resultant solution was applied to five bioassay disks. A single experiment consisted of five disks of each treatment offered to a population of two adult weevils per disk in a 145-mm petri dish as previously described (*2*). The percentages are means (± S.E.) based on five such experiments.

Treatment		Disk area consumed (%)
Quantity (mg)	Substance	
	Stimulant A (control)	56.1 ± 3.1
0.05	Deterrent B	36.6 ± 2.8
0.05	NH_4NO_3	35.9 ± 2.4
0.10	Deterrent B	17.0 ± 2.5
0.10	NH_4NO_3	16.8 ± 1.5
0.20	Deterrent B	7.6 ± 1.4
0.20	NH_4NO_3	6.6 ± 1.1
0.40	Deterrent B	1.4 ± 0.5
0.40	NH_4NO_3	2.1 ± 1.0
0.80	Deterrent B	0.0 ± 0.0
0.80	NH_4NO_3	0.0 ± 0.0