dogs this could be shown as early as 5 months after the spleen had been removed; in others the polycythemia appeared later. In none of the animals did it reach the same height as observed before the operation. The highest value of adrenalin polycythemia could be observed in the splenectomized puppy (P_2).

Conclusions. Repeated examinations of the hematocrit value of the peripheric blood following the intravenous administration of adrenalin in 4 dogs before and for a period of 18 months following splenectomy could demonstrate that (1) the acute polycythemia following adrenalin which is regularly observed in the normal dog disappears after splenectomy; (2) after that period the polycythemia returns but does not reach the same degree as in the normal dog. These results suggest a slow substitution of the erythrocyte-storing function of the spleen by another capillary system.

7505 C

Conditions of the Halo Formation Around Subtilis Colonies and Growth of Halo Transplants.

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An interesting phenomenon was described[1] which certain *B. subtilis* strains show when grown on nutrient agar containing a small amount of saccharose. The amorphous, stringy material which is present in abundance in the cultures as an intercellular substance spreads out from the colonies and forms a wide halo around them. In the halo bacteria are not visible and transplants of it do not grow on the usual media. Transplanted on agar plates containing saccharose the halo substance grows in tiny transparent colonies. The observations described in the following note[2] furnish convincing evidence that these growth phenomena are caused by the growth of living elements entirely different in morphology from the usual forms of bacteria.

The observations here described concern the conditions under which the halo develops and which determine the abundance of growth in the transplants.

[1] Dienes, L., PROC. SOC. EXP. BIOL. AND MED., 1932, **29**, 1205.

[2] Dienes, L., PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 1211.

The formation of the halo depends on the addition of saccharose to the nutrient agar. Usually as small an amount as 0.02% is sufficient and about 0.1% gives the best results. It is probable that the saccharose itself is effective and not an impurity in the saccharose preparation; because, by fractionation of saccharose with alcohol, no fractions were obtained which had increased or decreased potency. Other sugars have no effect on the halo formation. The formation of the halo depends largely upon the condition of the surface of the plate. The production of the halo can be greatly improved by rubbing the surface of the agar plate lightly with a glass rod. The different batches of agar vary very slightly and the addition of different animal proteins, fresh plant tissue, cystein, etc., produced no effect. When the pH is varied between 6 and 8.5 there are no marked changes.

For the closer study of the conditions upon which the growth of the halo substance depends we started with an agar medium containing no nutrient substances besides saccharose. The growth of our strains, as was already indicated in another place, is very characteristic on this medium. No halo is formed but the bacterium colonies are surrounded by a haziness which under the microscope consists of tiny colonies. These tiny colonies vary in size from a few hundredths to a few tenths of a millimeter and are situated mostly deep in the agar. In the tiny colonies no bacteria are visible. If a small piece of agar containing these colonies is cut out and transferred to another agar plate a similar growth is produced without bacterial growth. Transplants from the halo formed on nutrient agar plates also grow similarly on non-nutrient agar plates which shows that the formation of haziness is an analogous phenomenon to the halo formation. Haziness in the non-nutrient agar plates is produced more often and more constantly by bacteria than a typical halo.

Various substances were added to the non-nutrient agar and the effects on the formation of the tiny colonies were studied. In all cases where the medium contained vegetable or animal substances although the growth of bacteria was markedly increased the formation of the tiny colonies was inhibited. If the substances were added in very small concentrations the growth of tiny colonies reappeared, but their growth was never improved by these additions. The addition of various inorganic salts was either detrimental or without effect. Only increasing the concentration of the agar from 2% to 4 or 6% materially improved the growth of the tiny colonies. Halo was never produced in these experiments. CO_2 and absence of O_2 did not affect the development of the tiny colonies.

It was evident from these observations that the agar itself contains the substances which make the development of the tiny colonies possible. The agar used in this experiment was prepared by autoclaving commercial agar in tap water and pouring off the clear part of the agar after sedimentation. The medium always contained impurities including dead bacteria. The next step in the study was to free the agar from the impurities by filtration. A weak solution (0.5%) of agar was first passed through paper pulp, then through a Berkefeld filter and concentrated to 4% on the water bath. A perfectly clear agar was obtained. After the addition of saccharose to this agar the growth of the bacteria was very poor—in contrast to the abundant growth on the non-filtered plates—and the tiny colonies did not develop. By adding different kinds of nutrient substances the growth of the bacteria was greatly increased but neither the tiny colonies nor the halo was obtained. Filtering the agar simply through paper also considerably decreases the growth of tiny colonies.

Since the tiny colonies in the non-nutrient agar tend to grow around the impurities present in the agar we tried to add various kinds of non-soluble material to the filtered agar. Tiny bits of meat thoroughly extracted by water, starch, $CaCO_3$, iron oxide, carbon obtained by heating saccharose, all were without effect. Impressive effect was obtained by the addition of autoclaved earth and infusorial earth suspensions. The whole medium was full of tiny colonies growing around the particles. Filtered or thoroughly centrifuged earth extract did not have this effect. The ignition of earth or the extraction of infusorial earth with HCl followed by ignition did not decrease their effectiveness. The addition of ground glass was also effective. It is probable, however, that the filtration of the agar affects the growth of tiny colonies not only by the elimination of silicon dust but also by the elimination of other substances.

With the help of infusorial earth the surface growth of the halo colonies can be made more abundant. The surface of a non-nutrient agar plate is sprinkled lightly with sterile infusorial earth and the transplant from the halo is made on this area. Without infusorial earth the growth is poor and only the haziness developing in the agar is visible without a hand glass. The soft transparent growth obtained with the help of infusorial earth may be as abundant as, for instance, a confluent growth of pneumococcus. The second transplant made in this way is also usually abundant. The third less so, and in the fourth only a few colonies develop.

The negative or depressing effect of all kinds of nutrient material and the stimulating effect of earth on the expansion of the halo on non-nutrient agar plates are observations which probably will be of interest for the further study of the halo organisms and their relationship to the bacteria. Our strains were obtained usually from contaminated blood cultures and originated probably from dust. Numerous colonies showing halo formation grew from dust collected under the roof of the laboratory building. Adding earth to the agar probably makes the medium more similar to the natural habitat of our strains and the addition of nutrient material less similar. It seems rather probable from this observation that the peculiar process which we observed is a regular process in the natural life of our strains, which is usually not apparent in artificial growth but under the influence of saccharose and other appropriate conditions will be noticeable. The halo formation on nutrient agar is a peculiar case of growth and expansion of the halo substance, the conditions of which are entirely different from the conditions which regulate the extension of the halo substance into the medium of non-nutrient plates.

7506 P

Morphologic Elements in the Halo of Subtilis Colonies.*

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The most interesting question in connection with the phenomena which are the subject of the previous note[1] is whether they are life phenomena produced by the growth of living organisms or whether they represent only a peculiar combination of diffusion phenomena and enzymatic action. The number of successive transfers in which the halo substance so far was grown is not sufficiently high to exclude the supposition that the mucoid substances are synthesized in the medium by an enzyme excreted from the bacteria. The observations which will be described in this note show that the halo consists of organized elements and is not an amorphous substance.

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1 Peirson, O., and Dienes, L., PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 1208.