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Physical and Chemical Properties of Rat Leprosy Bacilli.*

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It is known that percentages of serum Ca and P are not noticeably altered in human leprosy except in unusual cases or during the lepra reaction. In order to discover the changes, if any, in the lesions themselves a histospectrographic investigation was made of skin lesions obtained from Dr. O. E. Denney at the U. S. Marine Hospital, Carville, La. It was found[1] that the P/Ca ratios in the 5 cases studied were probably 3 times those in normal skins from the same age group. A fair correlation was obtained for the P/Ca ratios with known duration of the disease and volume of leprous cells in the tissues analyzed spectroscopically. The change from the normal may be conditioned by increase in P, decrease in Ca, but probably by both. Perhaps an increase in P may have been occasioned by the tremendous number of bacilli in the lesions. No notable deviations from normal were noted in the Na/Ca, Mg/CA and Fe/Ca ratios.

In the hope of relating these observations on mineral constituents more definitely to cells and groups of cells, the technic of microincineration[2] was applied to sections of human leprous nodules also secured from Carville. We found that the lepra cells showed a finely divided white ash, tending in some places to be slightly bluish and something like that observed in cancer cells.[3] However, we were not successful in differentiating the ash resulting from the bacilli on the one hand and from the cells containing them on the other.

Consequently we shifted the attack to sections of rat leprosy nodules with which it was obviously more easy to experiment in our laboratory. The strain of organisms was received from Dr. E. L. Walker, 12/11/34. The tissues were either fixed in 10% formalin in absolute alcohol or were frozen in liquid air and dehydrated while still frozen in a cryostat. Alternating sections were stained with Ziehl-Neelson-hematoxylin and were incinerated. Nevertheless, it

* Aided by a grant from the U. S. Public Health Service.

1 Cowdry, E. V., Heimburger, L. F., and Williams, P. S., *Am. J. Path.*, 1936, **12**, 13.

2 Scott, Gordon H., *McClung's Microscopical Technique*, 2d Edition, 1937, New York, Hoeber, pp. 643-666.

3 Scott, G. H., and Horning, E. S., *Am. J. Path.*, 1932, **8**, 329.

was often difficult to identify in the incinerated section the same cell, or group of cells, which had been stained for bacilli in the next section because there are so few landmarks in the nodules. What we did was to concentrate our attention on relatively large intracellular aggregates of bacilli known as rosettes.[4] In no instance could we locate any mass of ash corresponding to a rosette. We conclude, therefore, that the finely divided white ash residue in lepra cells is mainly an expression of the mineral constituents of the cytoplasmic ground substance and not of the intracytoplasmic bacilli.

Influenced by reports on differences in fluorescence of different tissues and of different kinds of bacilli (summarized by Radley and Grant[5]) we assembled a fluorescence-microscope with which to ascertain whether any significant alterations occur in leprous lesions or in the bacilli. An old Zeiss microscope was fitted with a quartz condenser. Light from a General Electric high-pressure mercury lamp (type H-4 with outer envelop removed) was directed into the condenser by a quartz 90° prism. A Wood's filter, 5 mm thick, placed between the arc and the prism, eliminated all visible light but the violet. The latter was removed by placing a Bausch and Lomb fluorescent filter over the ocular of the microscope. It is important to note that, when a Wood's filter 3 mm thick is employed, as by some investigators, much more violet and blue light will be admitted from the mercury arc so that some confusion will result in the proper description of the color of fluorescence. Since the filter over the ocular also depresses the blue, it enhances the other spectral colors. The conditions must be very carefully specified in order for the observations to be properly interpreted and compared with those made by other investigators.

Rat leprosy nodules were fixed in 10% formalin, dehydrated, cleared, and embedded in paraffin in the usual way. Serial sections were usually cut 4 microns thick. In special cases the thickness was 3 microns. They must be thin for otherwise the fluorescence is so intense as to hide structural details. For the sake of orientation in the lesion, sections alternating with those for fluorescent study, were stained and mounted in the usual way. After the others, intended for fluorescence, had been mounted on quartz or Corex-D slides, the paraffin dissolved with xylol and the xylol allowed to evaporate they were ready for examination.

[4] Cowdry, E. V., and Ravold, Amand, *Puerto Rico J. Pub. Health and Trop. Med.*, 1938, **2**.

[5] Radley, J. A., and Grant, G., *Fluorescence Analysis in Ultraviolet Light*, London, Chapman & Hall, Ltd., 1935, 326 pp.

The color of fluorescence of the nodule was found to be grayish white with a definite greenish cast. The light emitted was not as strong as that from the liver, myocardium, kidney, pancreas, and other normal tissues used for comparison, and consequently it was a little less white. These other tissues all gave a grayish-white fluorescent color with a greenish cast, but the green was not nearly as pronounced as in the case of the leprous nodule. Although the leprous tissue had many bacilli, none of them could be distinguished as such. It is likely, however, that the definite greenish color of the fluorescence was partly due to the great abundance of the bacilli. Despite this characteristic appearance under ultraviolet illumination, leprous tissues in our sections could not be analyzed by the fluorescent method with any accuracy.

Evidently, in order to extend the observations on fluorescence as well as on mineral constituents, it was necessary for us to separate the bacilli from the lesions. This was done by employing a technic devised by Ravold according to which relatively large masses of bacilli-laden cells are dissected away from neighboring uninvolved tissue and from necrotic tissue when present in the centers of the nodules. They are placed in a Wueller press without addition of any fluid. On exertion of pressure many of the cells are ruptured and the tissue fluid, together with cytoplasm, nucleoplasm and some entire cells, passes through minute holes in the press and is collected, leaving most of the fibrous elements behind. Then a little saline solution is added and the material is ground up in sand and made up to a volume of about 50 cc. The sand is allowed to sediment out at the bottom of a centrifuge tube. The supernatant fluid is then centrifuged at low speed (300 r.p.m.). This throws all the rest of the debris to the bottom while the bacilli remain in suspension. The supernatant fluid, containing the bacilli, is again decanted and centrifuged at high speed (3500 r.p.m.) in an angle centrifuge for 1 hour. The supernatant fluid is discarded and the pasty material at the bottom of the tube, made up of bacilli, is diluted and washed by repeated centrifugation in some experiments with saline solution and in others with distilled water.

Beginning with a large nodule or with several small ones it is a simple matter to collect in 4 or 5 hours billions of bacilli. The pasty bacterial mass can be desiccated and weighed in grams. For our experiments we used only the wet bacilli, when viewed *en masse* they appear dense white with a faint shade of gray. They are not yellow or even yellowish. Examination of a thick smear, made after washing in saline, shows myriads of bacilli without any trace of cellular

material. The bacilli retain to a remarkable degree their characteristic morphology, as seen in sections and in smears of fresh tissue, and their acid-fast properties are not interfered with. After washing in distilled water until the supernatant fluid gave no precipitate when added to an aqueous solution of silver nitrate, the bacilli do not fuse together but still remain discrete bodies though their shape is different.

Despite the most stringent aseptic precautions, an organism other than *Mycobacterium lepræ muris* was detected both microscopically and culturally in some samples which were then discarded. Thus far we have not determined whether this organism occasionally occurs in the leprous nodules or whether it is a contaminant from the air.

Thick smears of the bacilli, washed in distilled water, gave little or no mineral residue after microincineration which lends support to the conclusion that the residue of sections of cells crowded with bacilli is mainly due to cellular components other than the bacilli. However, by the much more sensitive method of spectrographic analysis, some minerals were detected in the bacilli. We have not completed these analyses as yet, and do not know all that they will tell us, but in the spectrograms of washed bacilli, as compared with those of controls of fluid, obtained when normal tissues were treated in the same way as the lesions, it was found (1) that there is a significantly greater P/Ca ratio due to a considerable increase in P and to a lesser decrease in Ca. (2) That the bacilli contain traces of sodium and magnesium in about the same amounts as in the controls. (3) That traces of K are very slight in both the bacilli and in the controls, if anything less in the former.

In ultraviolet light the masses of distilled-water-washed bacilli on the special slides fluoresced faintly white definitely tinged with green. We believe, therefore, that the greenish fluorescence of cells charged with bacilli seen in sections is to be attributed chiefly to the bacilli. Unfortunately this observation, though interesting if other bacilli could be examined in precisely the same way, does not permit any deductions as to the chemical composition of the organisms. While every fluorescent substance has a characteristic color under ultraviolet light, the visual appearance of a great many compounds is so similar that accurate conclusions, regarding the constitution of the fluorescent material cannot be drawn. Spectrographic examination of the fluorescent emission is the only reliable method of fluorescent analysis and for this we are not equipped.

In conclusion, the available evidence indicates that the mineral ash of leprous nodules is mostly due to the cells of the host and that the

green shade of fluorescence is due mainly, but not entirely, to the contained bacilli. It is possible that the method devised for separating bacilli from the lesions and for collecting them in large numbers, which we have found useful in rats, may be helpful when extended to leprous lesions of humans.

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Influence of Anoxia on Glycogenolytic Action of Adrenalin.*

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In the course of a study of the interaction of hypoglycemia and anoxia in the rabbit[1] it was found that a short period of anoxia (7% oxygen for 15 minutes) enhances the return of the blood sugar to the control level, whereas the inhalation of 7% oxygen for 2 hours greatly aggravates the hypoglycemia. In spite of the fact that in the latter group the blood sugar averaged less than 30 mg % and was maintained at this level for 2 hours, no convulsions occurred, thus confirming McQuarrie and Ziegler's[2] experiments. The question was studied whether the differential reaction of the blood sugar to anoxia in the 2 groups of experiments is related to the effect of adrenalin on the liver. If this were the case a greater hyperglycemic effect of adrenalin would be expected after a short period of anoxia than is observed under control conditions. Furthermore, prolonged periods of anoxia should lead to a diminished glycogenolytic response of the liver.

Fifty-six experiments were carried out on rabbits which were *starved* for 18 hours and injected with adrenalin 1.9 γ/kilo 3 times in intervals of 10 minutes. The maximum rise of the blood sugar averaged 49% in this control group. The reaction was twice as great (average rise 97.9%) when this adrenalin experiment was repeated during the last half hour of an hour experiment in which the rabbits inhaled 7% oxygen. If, however, this period of anoxia was more prolonged (2 hours) and the same amount of adrenalin was

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1 Coffee, Ann, and Gellhorn, E., *Proc. Am. Physiol. Soc.*, Toronto, 1939, p. 51.

2 McQuarrie, I., and Ziegler, M. R., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 525.