

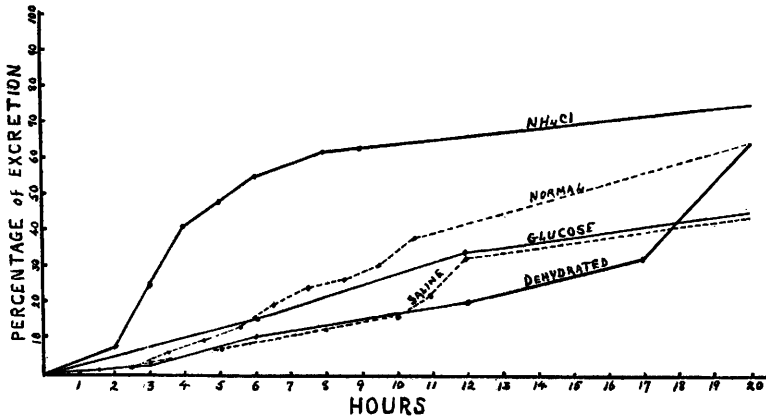


FIGURE 2.

Percentage of excretion of barbitol in urine of a dehydrated, normal, and 3 diuretic dogs (10% glucose, physiological salt solution, and ammonium chloride.)

As Table I shows, dehydration did not prolong the recovery time nor did the saline diuresis carried out according to the method of Gower and Van de Erve shorten the duration of the barbitol narcosis. In fact, no diuretic procedure seemed to have any significant effect on the duration of barbitol anesthesia. However, as the comparison of Graphs I and II shows, there is a very striking effect of NH_4Cl on the rate of elimination of barbitol.

In conclusion we might say that the supposed beneficial effects of glucose and physiologic saline in barbitol poisoning may be disregarded and that ammonium chloride diuresis even though it did not appreciably shorten the period of depression did definitely increase, unlike glucose or salt solution, the rate of elimination of barbitol.

7166

Failure to Enhance the Growth of *B. Leprae* by Wherry's Gaseous Method (CO_2 , O_2 Environment).

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One of us (Duval¹) reported the observation that the acid-fast bacillus of Hansen in removed pieces of leprous tissue continued slowly, over a period of months, to multiply and that the micro-

¹ Duval, C. W., *J. Exp. Med.*, 1910, **12**, 649.

scopic colony-like masses, known as "globi", attained many times their original size. Duval further observed that the proliferation of *B. leprae*, regardless of any special atmospheric environment, progressed *pari passu* with the autolysis taking place in the transplanted leprous tissue and that growth continued as long as the end-products of protein digestion were available. Continued growth in subsequent transplants occurred only in a medium containing certain protein-split products (amino-acids).

The recent claims of Wherry² and those of Soule and McKinley³ for the CO₂, O₂ method of culturing *B. leprae* led us to employ this method in our recent studies of the *in vitro* cultural requirements of the Hansen bacillus of human leprosy. Wherry notes that it is essential to furnish suitable nutritives as well as CO₂, O₂ environment for the proliferation *in vitro* of *B. leprae*. In contrast, Soule and McKinley emphasize the necessity of this atmospheric condition to such an extent that the nutritive requirement is lost and becomes a negligible factor.

The leprous tissue used in the cultivation experiments were obtained through the kindness of Dr. Denney of Carville, La. It consisted of the subcutaneous leprous nodules and other material known as the leprous "abscess" which were extremely rich in acid-fast bacilli. Some of this material was free from contamination as evidenced by microscopic and cultural examinations, while others were contaminated with some undetermined gram-positive micrococcus. All tissues were cut aseptically into small pieces or emulsed in a mortar. The contaminated material was further treated with a 3% NaOH for one hour and then washed several times in Ringer's solution.

A large series of cultures were prepared upon a variety of solid and liquid media, containing chick embryo tissue, placenta autolysate, banana extract and certain purified amino-acids in various proportions. The inoculated media were placed in Novy jars and maintained for weeks at 37°C. under a gaseous tension of 10% CO₂ and 40% O₂. Duplicate cultures were subjected to ordinary atmospheric environment, media and temperature.

Careful examination of the cultures after periods of 4 and 6 weeks failed to show that the special gaseous environment affected in any way the proliferation of *B. leprae*. The CO₂ and O₂ atmosphere neither inhibited nor enhanced the *in vitro* multiplication. Our negative results with the Wherry method are in entire accord with

² Wherry, *J. Infec. Dis.*, 1930, **46**, 263.

³ Soule and McKinley, *Am. J. Trop. Med.*, 1932, **12**, 1.

those of Oliver *et al.*⁴ and the British bacteriologists⁵ in India who likewise have found the method of no value in the cultivation of the Hansen bacillus.

The fact that *B. leprae* may continue to proliferate in leprous tissue that has been removed from the human host regardless of any specially supplied cultural conditions, possibly explains the so-called "positive culture" and "micro-colonies" claimed by Soule and McKinley. Furthermore, they state that *B. leprae* does not grow with greater ease throughout successive transplants, but with increasing difficulty. This indicates to us that proliferation became less and less and ultimately ceased, because the transferred culture inoculum no longer contained any of the original tissue products of protein digestion which Clegg,⁶ Duval,⁷ and Kedrowski⁸ have shown is necessary to sustain the primary *in vitro* growth of *B. leprae*. Soule and McKinley have failed apparently to appreciate the importance of the amino-acid nutritives in the initial recovery and maintenance of successive generations of *B. leprae* that are removed from the influence of the living host.

7167 C

Pathologic Changes in the Organs of Scorbatic Guinea Pigs.

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Recently Waugh and King¹ have shown that crystalline vitamin C isolated from lemon juice is identical with the hexuronic acid previously prepared from beef adrenals. With the more exact knowledge of the chemical nature of vitamin C it was deemed desirable to restudy some of the scorbutic lesions produced in organs. The most conspicuous gross lesions occur in the joints, bones and teeth and the pathologic appearance of these have been reported in

⁴ Oliver, *Philippine J. Sc.*, 1931, **46**, 611.

⁵ Leprosy in India, *Editorial Review*, Vol. 5, No. 4, October, 1933.

⁶ Clegg, *Philippine J. Sci.*, 1909, **4**, 403.

⁷ Duval, C. W., *J. Exp. Med.*, 1911, **13**, 365.

⁸ Kedrowski, *Z. F. Hyg. u. Infektionskrankheiten*, 1910, **66**, 1.

*Contribution No. 267 from the Department of Chemistry.

¹ Waugh, W. and King, C. G., *Science*, 1932, **75**, 357; *J. Biol. Chem.*, 1932, **97**, 325.

detail by Holst and Frölich,² Hess,³ Höjer and Westin,⁴ Meyer and McCormick,⁵ and others. Changes in the organs have received less attention.

Guinea pigs were fed on the basal vitamin C free diet of Sherman,⁶ supplemented by cod liver oil and yeast to supply adequate quantities of vitamins A, B, D, and G. After a preliminary survey of the histopathology of the organs and tissues, work was concentrated on adrenals, heart, testes and liver in which evidence of scorbutic effects was most marked. The pathologic bone changes studied by us did not differ from those reported by other investigators.

Microchemical preparations of formalin fixed and fresh tissue, both teased and cut with freezing microtome, were compared after treatment with Sudan iv, osmic acid, Nile blue sulphate and Nile pink⁷ for distribution of fat. Since practically identical pictures were obtained with all of these reagents, in later work Sudan iv and formalin fixed tissue was chosen. Hematoxylin and eosin stained sections were used for controls.

The distribution of cholesterol esters was indicated by the presence of doubly refractive materials in warmed formalin sections when examined with Nicol's prisms. The distribution and relative concentration of vitamin C in the tissues, particularly in the adrenals, was followed by the intensity of the reduction of 0.4% neutral silver nitrate.

The cortex of normal adrenals was invariably heavily laden with fat-staining material but this was not uniformly distributed. The zona fasciculata seldom showed any appreciable amount, the zona reticularis contained variable, but usually small amounts, and the zona fasciculata was loaded with lipids. The lipid was present only in the cytoplasm where it appeared to be located as a film on the periphery of fairly large granules. The nucleus was free from demonstrable fat. When considerable amounts of the fat had become free from the granules, it collected in droplets, which frequently fused to form long lines bordering the vascular channels. The unsaturated nature of these fatty materials was shown by the

² Holst, A., and Frölich, T., *Z. f. Hyg. u. Infektionsk.*, 1912, **72**, 1.

³ Hess, A. F., *Scurvy, Past and Present*, Lippincott, 1921, Phila.

⁴ Höjer, J., and Westin G., *Dental Cosmos*, 1925, **57**, 1.

⁵ Meyer, A. W., and McCormick, L. M., *Studies on Scurvy*, Stanford Univ. Press, 1928.

⁶ Sherman, H. C., La Mer, V. R., and Campbell, H. L., *J. Am. Chem. Soc.*, 1922, **44**, 165.

⁷ Rettie, T., *J. Path. and Bact.*, 1931, **34**, 595.

rapidity with which they reacted with osmic acid. The doubly refractive cholesterol esters were uniformly distributed in the zona fasciculata, where they were at a maximum in the normal adrenal. These substances were not apparent in the remainder of the cortex. A high content of fat is maintained in the adrenal until a fairly severe scurvy develops. In guinea pigs deprived of vitamin C, little or no apparent reduction appeared in the fat content until several days before death. At this time the fat in the middle third of the zona fasciculata stained less intensely due to the fatty covering of the granules having been transferred into the intervening thinner cytoplasm, whence it passed in droplets to the vessels, which transported it towards the medulla. Occasionally capillaries loaded with this freed fat and stained with osmic acid, appeared as thin black lines lying at right angles to the capsule of the gland. This depletion of fat spread towards the periphery of the fasciculata, and, if the animal showed a prolonged survival, the fat entirely disappeared from the outer two-thirds of this zone. The inner third retained an apparently normal fat content. In a few animals a thin rim of fat remained in that part of the fasciculata contiguous to the zona glomerulosa. Immediately beneath this was a fat free band comprising about one-fourth of the fasciculata with the remaining inner half of the fasciculata showing a fairly normal amount of fat. The zona glomerulosa at this stage stained more deeply. The zona reticularis likewise showed a deeper color than normal. Coterminous with the decrease of demonstrable fat in the fasciculata there occurred a comparable diminution in doubly refractive material. These observations agree in detail with those reported by Iwabuchi.⁸ In guinea pigs, where the scurvy developed rapidly, patchy and irregular fat depletion separated by intervening fat stained areas was observed. Such a distribution of fat always occurred in association with hemorrhagic foci. In such animals the transport of the freed fat towards and in the vessels was more or less limited to those hemorrhagic foci and at death the picture of a uniformly depleted band comprising the outer half or two-thirds of the fasciculata was lacking. When deviation from the first fat picture described occurred, undoubtedly contributory factors, *e. g.*, infection, were involved in the rapid withdrawal of vitamin C from the adrenals. The medulla of adrenals, with fat depleted cortices, showed little or no chromaffin pigment which is consistent with the decrease of adrenalin found by McCarrison⁹ and Ohata.¹⁰

⁸ Iwabuchi, T., *Beitrage z. path. Anat. u. z. Allgem. Path.*, 1922, **19**, 440.

⁹ McCarrison, R., *Brit. Med. J.*, 1919, **2**, 200.

¹⁰ Ohata, S., *J. Biochem. (Tokyo)*, 1930, **12**, 419

Parallel to the disappearance of cortical fat staining material the doubly refractive cholesterol also disappeared. This bears out the chemical findings reported by Nagayama and Tagaya¹¹ on the decrease in cholesterol in the adrenal cortex in scurvy. The amount of doubly refractive material was directly proportional to the amount of demonstrable fat in the fasciculata. These various lipid pictures we have found to appear consistently in several dozen guinea pigs. Inanition *per se* did not produce a comparable change in lipid distribution. Comparison of hematoxylin sections with the microchemical preparations showed swelling, pyknosis and lysis of cells. In animals dying acutely, cortical hemorrhage was common. The intimate relationship of vitamin C to fat content of adrenal was shown by a series of studies with 0.4% neutral silver nitrate. Indeed, this technic offers a rough assay of the vitamin C content of tissue in agreement with the findings in these laboratories.

Marked differences were observed in the staining of the cut surface of the adrenals in normal and scorbutic pigs similar to that reported by Moore and Ray,¹² Miller, Siehrs and Brazda¹³ and Hamilton.¹⁴ Immersion of the cut surface of the adrenal of a normal guinea pig, one fed on a liberal diet of spinach, or injected with the pure vitamin C, in 0.4% of neutral silver nitrate, produced a rapid and intense blackening. This blackening developed in a few minutes in the light but required about 30 minutes in the dark. In animals deprived of vitamin C the intensity of this silver deposit decreased gradually until the sixth day, when it appeared to reach a minimum which was maintained until the death of the animal from scurvy (16-48 days). The adrenals from pigs which had received no vitamin C for 10 days and were then fed liberal quantities of spinach showed intense staining 3 hours subsequent to the feeding. A similar effect was noticeable in a shorter time after an intramuscular injection of pure vitamin C. The adrenals from pigs fed on a vitamin C level, such that at the end of a 60 day period showed distinct scorbutic lesions, stained like those glands from pigs on a 5-day vitamin C free diet.

The black silver deposit begins to appear first in the zona fasciculata and then the reduction gradually spreads to include the zona reticularis. The change in the cells during freezing and cutting and

¹¹ Nagayama, T., and Tagaya, T., *J. Biochem.* (Tokyo), 1929, **11**, 225.

¹² Moore, T., and Ray, S. N., *Nature*, 1932, **130**, 997.

¹³ Miller, C. O., Siehrs, A. E., Brazda, F. G., *Proc. Soc. Exp. Biol. and Med.*, 1933, **30**, 686, 696.

¹⁴ Hamilton, R. H., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1933, **30**, 1355.

the subsequent film deposit of silver on the cut surface presents difficulty in determining the exact location at which the reduction appears. The most intensely stained areas are in the small channels emptying into the blood vessels of the zona fasciculata. The reducing property of the adrenal cortex is undoubtedly intimately associated with the presence of both vitamin C and lipids. An attempt to use the silver nitrate solutions suggested by Macallum to demonstrate the distribution of chlorides and phosphates was unsuccessful, because of the rapid reduction of silver nitrate by the vitamin. The demonstration of potassium by the Macallum reagent was likewise unsuccessful in the adrenal. Although quantities of potassium were present in the normal as well as the scorbutic adrenal so much was freed during the freezing and manipulation of the tissue that a thin film formed on the surface of the section and obscured all cellular detail.

Coincident with the extreme lipid alteration in the adrenal extensive fatty degeneration occurred in foci irregularly distributed in the myocardium. This began by a shifting of the fat from the dark or anisotropic band into the light band where it progressively increased until droplets were formed. These fused with loss of morphology of the myocardial cell. This apparently constituted an irreversible stage and formed the beginning of the typical patches of fatty degeneration similar to those seen in diphtheria. Fatty changes in skeletal muscle were similar in pathogenesis to those formed in the myocardium.

Fatty infiltration of the liver was found in many of the animals with varying degrees of scurvy and was so marked in the terminal stages that the cells of the entire lobule were almost entirely composed of fat. This infiltration was associated with fatty degeneration which probably formed the primary lesion. Marked congestion of the liver was frequently encountered and in a few animals irregularly distributed hemorrhages were present.

In severe scurvy, extreme degenerative changes, with death of germinal epithelium and spermatozoa, were noted in the testis. The heads of the spermatozoa sometimes persisted as homogeneous dark staining masses in deeply eosin stained cytoplasm. Persistence of fairly well stained cells of Sertoli, among degenerating epithelium, as reported by Medes¹⁵ was found only in the early and milder stages of scurvy. Another occasional observation was the swollen edematous appearance of the connective tissue in animals dying with extreme manifestations of scurvy. This was well exemplified in the

¹⁵ Medes, G., and Lindsay, B., *Am. J. Anat.*, 1926, **87**, 213.

periportal connective tissue, splenic septa, as well as in the media of small arterioles of the lungs. In the lung such vessels occasionally presented the appearance of obliterative endarteritis. While it is impossible to exclude such contributory factors as may be supplied by spontaneous infections in the development of these vascular changes we feel such changes are to be interpreted primarily as alterations in the colloidal state of the collagen due to lack of vitamin C as postulated by Wolbach and Howe.¹⁶ The other lesions noted were less constant. Patches of degenerating intestinal mucosa formed the basis of the ulcerated areas seen in the gross, mostly in the small gut. In the spleen no characteristic scorbutic changes were observed unless one considers the brown hemolyzed intra and extra cellular pigment as such. The presence of occasional hydropic degeneration of the islets of Langerhans was probably only an indication of spontaneous intestinal infection.¹⁷

Summary. The most characteristic lesion found in the organs of scorbutic guinea pigs was the depletion of fat and cholesterol from the cortex of the adrenal. Fatty degeneration of the myocardium, marked degeneration of testicular germinal epithelium and spermatozoa and swelling of connective tissue were also observed in extreme scurvy.

7168 P

Chemical Determination of Pregnancy.

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We have found that a fair degree of accuracy can be obtained in testing for pregnancy by oxidizing 1 cc. of urine with 1 drop of 0.5% hydrogen peroxide, after which 5 drops of a 1% aqueous solution of phenylhydrazine hydrochloride are added and 5 drops of a 5% aqueous solution of methyl cyanide, followed by 5 drops of concentrated hydrochloric acid. It is then put into a boiling water bath and allowed to react for 25 minutes. After this time the reaction is said to be positive if a russet color develops and a flocculent precipitate appears. A negative reaction is shown by the absence of

¹⁶ Wolbach, S. B., and Howe, P. R., *Arch. Path.*, 1926, 1, 1.

¹⁷ Thomas, B. G. H., *J. Infect. Dis.*, 1924, 35, 407.