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Study of Ducrey's Bacillus and Recognition of a Gram-Positive Smooth Phase.* (21068)

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In the current and generally accepted classification system, *H. ducreyi* appears to have a rather definite taxonomic position in the genus Hemophilus(1). A review of the published studies lends little support to this view, and reveals that the accumulated knowledge of this bacterium is exceedingly limited (2). The lack of basic information appears to be due primarily to the difficulties which have attended the cultivation of Ducrey's bacillus. *H. ducreyi* is the recognized cause of chancroid. Most investigators agreed that the causative microorganism is extremely difficult, if not impossible, to isolate from primary chancroidal lesions. Primary lesions are invariably contaminated with a variety of bacterial species and this together with the fastidious growth requirements of *H. ducreyi* appear to make identification of this organism impossible by present-day procedures(3). The well-known characteristics of Ducrey's bacillus seem to have resulted from studies of cultures which were obtained from bubo material. The successful isolation of *H. ducreyi* from aspirated bubo fluid has been reported on numerous occasions(4,5). In all investigations the importance of incorporating whole blood in culture media has been emphasized. It has been determined that a growth factor necessary for the Hemophilus group is also required for *H. ducreyi* cultivation(6). Previous bacteriologic procedures have not permitted detailed characterization of Ducrey's bacillus in respect to colonial morphology, or to its physiologic or serologic behavior. The bacteriologist, therefore, with limited means of identification, can offer little assistance in the diagnosis of chancroid as a disease entity. The diagnosis of chancroid, therefore, is dependent upon clinical impressions which are not yet clearly defined. The development of new methods and procedures for the cultivation and identification of Ducrey's bacillus could offer a partial solution to this diagnostic problem. The successful accomplishment of these objectives should eventually help to explain some of the unanswered questions pertaining to epidemiologic and immunologic aspects of the disease.

The present report deals with a description of our experimental methods and procedures applied to the study of Ducrey's bacillus.

Experimental. Stock *H. ducreyi* cultures were received from Dr. R. B. Dienst, Medical College of Georgia; Dr. J. D. Thayer, Venereal Disease Experimental Laboratory, Chapel Hill, N. C. and from the Lederle Laboratories.

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While it was not possible to obtain complete histories of all of the cultures it is assumed that they were originally isolated from bubo pus. The oldest culture of this group, strain "Hunt", was isolated by Dr. G. A. Hunt in 1935. The youngest culture, strain ATC, was listed as having been isolated in or about 1947. Most of the old cultures were received in the dried state and were reconstituted or sub-cultured to sterile defibrinated rabbit blood-infusion agar slants according to the method of Sanderson and Greenblatt(5). Cultures were incubated at 35°C and were examined microscopically at 24-hour intervals. All of the old stock cultures behaved in a manner which might be described as classical, that is when once growth had become established by any one strain, subsequent sub-cultures usually demonstrated good growth after a 24-hour incubation period. Microscopic examinations demonstrated typical gram-negative long-chained streptobacilli. Rabbit inoculation experiments with these cultures were invariably negative. In an effort to determine colonial characteristics the stock cultures were plated on Eugon Agar (BBL) plus 15% sterile rabbit blood. Both the candle and the Brewer anaerobic jar methods were used for petri plate incubation. Growth and colonial morphology was approximately the same by either method. The colony size of any one strain usually varied between 0.5-1.0 mm in diameter after 72 hours incubation. By reflected light, colonies showed a distinct roughness in surface texture and in some cases the margins were uneven. There was a decided tendency for most colonies to crumble when touched with the inoculating needle, some would slide along the surface of the agar when attempts were made to "pick" them. Many colonies were apparently made up of nonviable bacteria as evidenced by growth failure of numerous transplants. All our attempts to grow old stock cultures in media other than those rich in sterile blood were unsuccessful.

In order that virulent and non-virulent strains might be compared, isolation of new cultures was attempted. Three cultural methods were used, Eugon Agar (BBL) plus 15% sterile defibrinated rabbit blood plates were streaked with freshly aspirated bubo pus and incubated at 35°C in both candle and Brewer anaerobic jars; infusion agar slants overlayed with sterile defibrinated rabbit blood were inoculated according to the method of Sanderson and Greenblatt(5). Plates were examined both macro and microscopically after 72 hours. Blood tube cultures were examined microscopically at 24-hour intervals. In the 3 cases reported here the cultural results were identical; growth occurred only on plates which had been incubated under anaerobic conditions. Colonies from each of the three cases were similar but in no way resembled the colonies previously described for old stock cultures. Colonies obtained from the bubo material measured from 1-3 mm in diameter, were smooth, white, and dew-drop in appearance, and almost opaque by reflected light. Most of the colonies were surrounded by a distinct zone of hemolysis. The staining and morphologic appearance of the bacteria composing these newly isolated colonies was entirely dissimilar to those described as occurring in the old stock cultures. All of the new cultures contained gram-positive rods of various sizes, the predominant type being long and slender, the dimensional proportion being 8-10 times as long as their width. In some cases more deeply staining granules were present within the rods. There was little if any evidence of chain formation; a few diplobacilli could be seen in the average microscopic field. The majority of the organisms were seen as scattered individual rods or were assembled in irregular groups.

When our new cultures were transplanted and maintained in rabbit blood a rather rapid variation in colony type, bacterial morphology and staining characteristics was observed. Five to eight weekly transfers were usually sufficient to complete these changes. After this period of time the new cultures were similar in every respect to the old stock cultures previously described. We have been able to maintain the new strains without change by frequently subculturing under anaerobic conditions. Carrying media are being investigated, but at present all of the stock cultures, new and old, have been preserved by the freeze-dry process.

Several investigators have reported the pro-

duction of chancroidal lesions in rabbits(7,8). From their descriptions it is apparent that success was dependent upon the use of freshly-isolated bubo cultures.

Rabbit virulence studies were conducted using our new strains. Best results were obtained when 0.2-0.3 ml of a 72-hour rabbit blood culture was inoculated intradermally. Most of the inoculations have been made into the shaved abdominal skin. Other inoculation sites appear to be quite as satisfactory. Virulent cultures usually demonstrated evidence of infection within 24 hours.

The lesion commonly had the appearance of an erythematous nodule occurring superficially in the epidermis at the site of injection. There was slight induration palpable beneath the lesion. The area of surrounding erythema extended approximately 2 centimeters beyond the nodule. Lesions appeared somewhat edematous and were larger than the original bleb produced by the inoculum. There was at times slight scaling of the surface of the lesion. With the passage of 4-5 days nodular lesions gradually increased in size to about 2 cm and take on the characteristics of a subcutaneous abscess, together with a decreasing surrounding erythema. There was a palpable softening of the contents of the abscess and after 5 days was frequently ulcerated and exuded a gummy purulent exudate. The ulcer appearing at the apex of the abscess did not generally enlarge beyond the limits of the nodule delineated prior to its ulceration. After rupture the abscess gradually discharges its contents, the ulceration produced heals slowly but spontaneously over a period of about 2 weeks leaving a slight scar.

Viable Ducrey's bacilli were always demonstrable in these lesions before ulceration had occurred. On the day following rupture of the abscess we have usually been unsuccessful in our attempts to recover the causative bacterium.

Discussion. Our comparative study of old and freshly-isolated cultures of Ducrey's bacillus indicates that past descriptions of this species have dealt for the most part with a rough non-pathogenic variant. Our observations correspond to the well-known smooth (S)—rough (R) variations which have been observed in other bacterial groups. Our observations appear unique in respect to the radical nature of the variation. The difference is so great in the case of Ducrey's bacillus that the parent or "S" form would necessarily be classified in an entirely different family than the variant "R" form. It seems likely that the previous well-known and described characteristics of *H. ducreyi* strains are those of a "loss variant". The loss in this case appears to be associated with both antigenic and physiologic behavior. In our investigations which are in progress, we have discovered only slight antigenic relationships between the "S" parents and "R" variants. If growth factors found in whole blood are indeed necessary for the cultivation of either the "S" or the "R" forms, their effects are much more pronounced in connection with the "R" forms. We believe that the principal part played by whole blood in past experiments has been its ability to produce a favorable anaerobic environment. In concurrent experiments excellent growth of various anaerobic species in whole blood tubes without the use of special seals or other anaerobic equipment has been observed. The results of our observations require a reclassification of Ducrey's bacillus, the establishment of its exact taxonomic position however awaits more extensive investigation of its physiologic and antigenic behavior. With the information now known progress may be expected pertaining to immunologic and epidemiologic aspects of the disease chancroid.

Summary. 1. Stock strains of Ducrey's bacillus obtained from various sources have been found to conform to classical and current descriptions of *H. ducreyi.* 2. Comparison of stock "R" strains with freshly isolated anaerobic cultures, indicates that the "S" form of this species has not been previously recognized. 3. Our experiments indicated that blood is not required for growth of the "S" form and that its use leads to rather rapid production of the classical "R" variant currently classified as *H. ducreyi.*

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Weight, Ascorbic Acid and Cholesterol Changes in Adrenals of Pyridoxine-Deficient Adult Male Rat. (21069)

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Impaired adrenal functions in the pyridoxine-deficient rat have been reported by several workers employing histochemical methods(1), water diuresis tests(2), survival rates during cold exposure(3), and lymphopenic responses (4).

In this study an endeavor was made to measure the functional capacities of the adrenal of the pyridoxine-deficient rat by the responses to two stimuli, a) the administration of adrenocorticotropic hormone (ACTH) in hypophysectomized animals, and b) the exposure of intact animals to hypoxia. Depletion of the ascorbic acid and cholesterol content of the adrenal of the normal rat follows the administration of ACTH(5) or subjection to hypoxia(6,7). This depletion of adrenal ascorbic acid and cholesterol has been widely employed for the assay of ACTH preparations as well as for an index of the functional capacities of the adrenal gland.

Procedure. Long-Evans strain male rats were employed for all experiments. At 103 days of age the animals were placed on a diet of 24% casein, 10% hydrogenated vegetable oil, 4% salt mix(8) and 62% sucrose. A vitamin mixture† was fed 3 times weekly and pyridoxine was omitted from the mixture given the deficient group. Desoxypyridoxine, 30 mg/kg diet, was added directly to the ration of the deficient group. Three groups were studied, deficient, pair weighed and *ad libitum*. During the 8th week on the diet, 60 animals were subjected to 24 hours of fasting. For the last 20 of these hours the rats were placed in individual glass chambers, 30 of which were maintained at a pressure of 349 mm of Hg equivalent to 20,000 ft altitude and the remainder at sea level pressure. All animals received water *ad libitum* through a tube entering each glass jar. *Hypophysectomy* operations were performed on another group of 60 animals at the end of the 7th week on the regimen. For 2 days after the operation they received daily injections of 2 cc of 20% glucose intraperitoneally and their drinking water contained 10 mg % aureomycin and 5% glucose. The glucose intake was then discontinued and all animals were fasted for a total period of 18 hours. Half of these animals were injected during the fasting period with ACTH (Armours, ACTHAR, lot L5072) dissolved in 0.9% NaCl. The remaining animals received equal volumes of 0.9% NaCl.

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† Water soluble vitamins were dissolved in 20% aqueous ethanol and fed 3 times weekly in individual cups. This supplied each rat daily the following amounts in mg; thiamine hydrochloride, riboflavin, pyridoxine and folic acid, .02 each, calcium pantothenate and paraminobenzoic acid, .10 each, niacin amide, .07, inositol, 2.5, biotin, .002, and choline, 5.0. Vit. A and D and mixed tocopherols were dissolved in vegetable oil and were supplied separately in amounts of 1000 I.U., and 100 I.U., and 3 mg/rat/week.