ISOLATION AND CHARACTERIZATION OF MURAMIC ACID FROM TWO SPIROCHÆTES: *BORRELIA DUTTONI* AND *LEPTOSPIRA BIFLEXA*

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THERE is considerable confusion about the nomenclature and taxonomic position of helical, motile micro-organisms. Some are definitely classified with the bacteria (Spirillaceae)[1], others (Spirochaetales), with either the bacteria[1] or the Protozoa[2], and it has been suggested that certain members of the Spirochaetales should be classified with the Cyanophyceae[3].

One biochemical character which has proved useful in bacterial classification is the presence of cell wall mucopeptides, which on hydrolysis give a small range of sugars and amino-acids together with the amino-sugars glucosamine, galactosamine and muramic acid[4]. Apart from the true bacteria, muramic acid has been found only in the Actinomycetales[5] Cyanophyceae[6] and Rickettsiae[7]. The purpose of this article is to report that muramic acid has been isolated and characterized from two members of the Spirochaetales; *Borrelia duttoni*, the spirochæte of relapsing fever, and *Leptospira biflexa*, a free-living spirochæte found in natural waters.

B. duttoni is an old laboratory strain, maintained in young rats by syringe passage. Parasites were collected by bleeding the rats at the height of the first parasitæmia and were then separated from the blood by differential centrifugation. *L. biflexa* was cultured in Korthof's medium with added hæmoglobin, yeast extract and rabbit serum, and concentrated by centrifugation. The organisms were thoroughly washed and freeze-dried, and after extraction of lipids were treated by the method of Cummins and Harris[8] for isolation of mucopeptide from cell walls. Ribonuclease digestion was replaced by extraction of ribonucleic acid with 0·3 N potassium hydroxide for 5 h at 37° C, and deoxyribonucleic acid was extracted with 5 per cent trichloroacetic acid for 30 min at 70° C. The dried residue was hydrolysed in a sealed ampoule with 4 N hydrochloric acid for 4 h at 105° C, and dried *in vacuo* over potassium hydroxide. Muramic acid was identified in this hydrolysate by two-dimensional paper chromatography using *n*-butanol/pyridine/water (6:4:3) and *n*-butanol/acetic acid/water (63:10:27) as solvents, and spraying with ninhydrin reagent. Muramic acid was isolated from the remainder of the hydrolysate by concentration of amino-sugars on a 'Zeokarb 225 (H^+)' column, followed by further separation of muramic acid from glucosamine on a mixed 'Norit'/'Celite' column[9].

Part of the material obtained in this way was chromatographed on paper in the *n*-butanol/acetic acid/water system, together with standard preparations of glucosamine, galactosamine and synthetic muramic acid. The spirochæte material showed a spot which had the same mobility as muramic acid, and which gave positive reactions with ninhydrin and the Elson–Morgan[10] reagents. The remainder of the column eluate was finally purified by preparative paper chromatography in the same solvent system, which removed other faint ninhydrin-positive spots having greater mobility than muramic acid. The purified material eluted from the paper gave a chromogen in the Rondle and Morgan reaction[11] which had all the properties of the muramic acid chromogen[12], that is, the absorption maximum, if read immediately, was at 510 mμ and after 24 h at 505 mμ, with an approximate two-fold increase in intensity (Fig. 1). The substituted pentose obtained by treatment of the sample with ninhydrin[13] was identical in both colour and mobility with that obtained from synthetic muramic acid, when subjected to paper chromatography in *n*-butanol/pyridine/water, and sprayed with anisidine hydrochloride reagent[14].

This demonstration of muramic acid in the Spirochaetales suggests that they have a cell wall similar in structure to that of the bacteria, and provides support for their classification in this group, and not in the Protozoa. Other characters, which also point to their bacterial nature, are their general morphology, with a distinct cell wall and capsule[15], resistance to nuclear staining[16] and sensitivity to antibiotics[17] and to lysozyme[18]; these last two characters are presumed to be a consequence of the muramic acid content[4,19].

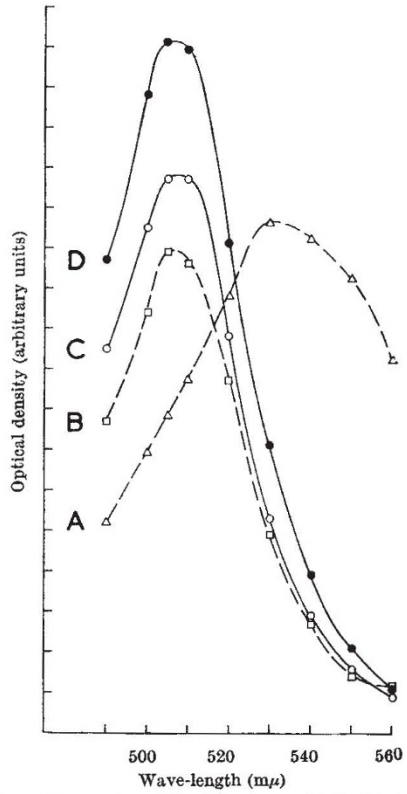

Fig. 1. Absorption spectra of the chromogens obtained in the reactions for amino-sugars described by Rondle and Morgan[11]. *A*, 20 μg of glucosamine, readings taken immediately; *B*, 20 μg of synthetic muramic acid, readings taken after 24 h; *C*, material from *B. duttoni*, readings taken after 24 h; *D*, material from *L. biflexa*, readings taken after 24 h

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