The Immunochemistry of *Shigella flexneri* O-Antigens: a New Serologically Active Trisaccharide from Variant Y

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The O-specific side chains of *Shigella flexneri* variant Y lipopolysaccharide are comprised of N-acetyl- α -D-glucosaminyl-(1 $\rightarrow$ 2)- α -L-rhamnosyl-(1 $\rightarrow$ 4)-L-rhamnose 'repeating units' linked together by 6-O-substitution of the N-acetylglucosamine residue in the adjacent unit (Simmons, 1969). Two trisaccharides, Y/3 and Y/4, previously isolated from this side chain by partial acid hydrolysis proved to be N-acetylglucosaminyl-(1 $\rightarrow$ 2)-rhamnosyl-(1 $\rightarrow$ 4)-rhamnose and rhamnosyl-(1 $\rightarrow$ 4)-rhamnosyl-(1 $\rightarrow$ 6)-N-acetylglucosamine respectively. The structure of a third, more recently isolated, trisaccharide (Y/3a) has now been elucidated by methods previously described (Simmons, 1969) as follows. On quantitative micro-analysis, a sample of compound Y/3a contained 218nmol of rhamnose and 96nmol of N-acetylglucosamine and on chromatography in butan-1-ol-acetic acid-water (4:1:5, by vol.) (Jermyn & Isherwood, 1949) it ran between trisaccharides Y/3 and Y/4 (R_{Glc} 0.56). These findings taken together imply that compound Y/3a is a trisaccharide containing rhamnose and N-acetylglucosamine in the molar proportions 2:1. Treatment with sodium borohydride reduced 48% of the rhamnose, which establishes this sugar as the reducing end group. After partial acid hydrolysis, α -L-rhamnosyl-(1 $\rightarrow$ 6)-N-acetylglucosamine was demonstrated among the products by comparative chromatography and high-voltage borate electrophoresis with an authentic sample of that disaccharide as a control. Trisaccharide Y/3a is therefore an α -L-rhamnosyl-(1 $\rightarrow$ 6)-N-acetylglucosaminylrhamnose. As the new saccharide gave no colour in the Morgan-Elson reaction, the reducing rhamnose is most probably 2-O-substituted. This view is consistent with the formation of 0.88mol of acetaldehyde/mol of trisaccharide after periodate oxidation. The above findings, and the structures of the oligosaccharides previously isolated from variant Y lipopolysaccharide, suggest that trisaccharide Y/3a is α -L-rhamnosyl-(1 $\rightarrow$ 6)-N-acetyl- α -D-glucosaminyl-(1 $\rightarrow$ 2)-L-rhamnose.

Complement-fixation inhibition studies in a homologous *Sh. flexneri* variant Y system showed that trisaccharides Y/3a and Y/4 were powerful inhibitors whereas trisaccharide Y/3 was virtually inactive. These findings imply that the terminal non-reducing rhamnose of trisaccharide Y/3a is the immunodominant residue and that the sequence

rhamnosyl-(1 $\rightarrow$ 6)-N-acetylglucosamine plays a major determinant role in the 3,4-group factors assigned to variant Y in the classification of Ewing & Carpenter (1966).

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Inhibition of Growth of *Lactobacillus casei* by Tungstate and its Reversal by Molybdate

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In studies of metabolites of riboflavin in animals we have used *Lactobacillus casei* A.T.C.C. 7469 for assaying riboflavin (Owen & West, 1970). Tungstate has been used to demonstrate a requirement for molybdenum by species ranging from bacteria to birds and mammals, in which either growth, or the production of enzymes that contain molybdenum as an essential constituent, can be inhibited by tungstate. In many such species, additional molybdenum overcomes the tungstate-induced inhibition (for references see Owen & Proudfoot, 1968). We now report a new example of competition between molybdenum and tungsten.

Standard solutions of riboflavin, such as we routinely use for riboflavin assays, supplemented by $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ or $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ or both, were added to Difco assay medium before its inoculation with *L. casei*. With every test a normal assay with riboflavin alone was used as a control. Though growth of *L. casei* was completely inhibited by 0.2% W (calculated as the element) 0.1% Mo had no inhibitory effect at all. With 0.1% W either 0.002 or 0.01% Mo permitted only negligible growth equal to 0.15 times the standard. In contrast, 0.15% W with either 0.1% or 0.4% Mo was not inhibitory at all. In all these experiments growth was measured turbidimetrically at 600nm. With 150 ng of riboflavin/tube of 11 ml of medium $E_{1\text{cm}}^{1\%}$ 600 nm was 0.671. As a check on the method, bacteria were harvested by centrifugation after incubation and their nitrogen content was determined by using Nessler's reagent (Williams, 1964). The results showed that there was no growth in the absence of riboflavin irrespective of additions of molybdate or tungsten. With 100 ng of riboflavin/tube 138, 123, 105 and 128 μg of N/tube corresponded to no ad-

dition, 0.1% Mo, 0.15% W + 0.1% Mo and 0.15% W + 0.4% Mo respectively, whereas 0.15% W gave only 28 µg of N. With 50 ng of riboflavin/tube the tubes containing 0.15% W showed only 5 µg of N and the nitrogen in the other tubes was halved.

We believe that, considered in relation to our own earlier work and to that of others to whom we made reference (Owen & Proudfoot, 1968), the present results indicate that *L. casei* requires molybdenum. The implications of this finding on the microbiological assay of riboflavin will be discussed elsewhere.

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Growth of the Riboflavin-Splitting Rumen Organism *Eubacterium fissicatena* in the Presence of Tungstate and Molybdate

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Owen and his colleagues showed that ruminants produce metabolites of riboflavin and that these originate from bacteria in the gut (Owen & West, 1970). Owen, West & Coates (1970) found the chief of these metabolites, which Owen (1962) had identified as 10-(2'-hydroxyethyl)isoalloxazine (I), in conventional but not in germ-free rabbits, and West & Owen (1969) found the metabolites in the urine of man. Known species of rumen bacteria could not produce riboflavin metabolites (Hobson, Summers, Owen, Spencer & West, 1969), but t.l.c. of extracts from riboflavin-enriched cultures revealed new species of anaerobes that could produce compound (I). *Eubacterium fissicatena* n.s. (M. M. Taylor, D. W. West & E. C. Owen, unpublished work) is one of these.

We now report a study of this bacterium in the presence of molybdate and tungstate in two different media, A and B. Each medium contained Oxoid yeast extract, Oxoid proteose peptone, NaCl and riboflavin, but medium A also contained Oxoid liver digest, whereas medium B also contained rumen fluid, a salt mixture providing Mg^{2+} , Zn^{2+} , Mn^{2+} , Fe^{2+} , Cu^{2+} , Co^{2+} and MoO_4^{2-} , EDTA and phosphate buffer.

Turbidimetry of 20 ml cultures in Universal bottles and of uninoculated incubated blanks was used to assess growth at 37°C. The extinctions at 600 nm of seven triplicated cultures with neither molybdate nor tungstate averaged 0.478 (± 0.043 s.d.) for medium A and 0.791 (± 0.090 s.d.) for

medium B. Two series of cultures were grown in the presence of 0.2%, 0.4%, 0.6% and 0.8% Mo or W (calculated as the element), a third series had 0.2% Mo added at each concentration of tungsten and a fourth series had 0.2% W added at each concentration of molybdenum. Thus the Mo/W concentration ratio varied over the third and fourth series from 4:1 to 4:16.

Differences between the effects on growth of *E. fissicatena* of tungstate and molybdate in either medium were small, but molybdate or tungstate or their mixtures stimulated growth in medium A and diminished it in medium B, for the average extinctions of all the cultures, excluding those to which neither molybdate nor tungstate had been added, were 0.762 (± 0.061 s.d.) for medium A and 0.619 (± 0.046 s.d.) for medium B. Thus unlike *Lactobacillus casei* (see Owen & Pringle, 1970) *E. fissicatena* shows no antagonism between molybdenum and tungsten. It is of interest that McKillen & Spencer (1970) reported that *Salmonella typhimurium* is inhibited by molybdate.

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A Protected Intermediate for the Preparation of Semisynthetic Insulins

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Porcine insulin has only three amino groups, the α -amino groups of glycine A-1 and phenylalanine B-1 and the ϵ -group of lysine B-29 (Brown, Sanger & Kitai, 1955). Our experiments form part of an attempt to develop methods that make it unnecessary to synthesize a whole protein when a structure is required that departs only slightly from a naturally occurring one (Offord, 1969). In its application to insulin it resembles that of Brandenburg & Ooms (1968), who used the phenyl isothiocyanate procedure to attack, and remove, the N-terminal residues of both chains. They subsequently coupled an amino acid back to replace them. During this procedure lysine B-29 was (irreversibly) protected by acetylation.