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.

We thank Miss S. McLaughlin and Miss M. Sutherland for technical assistance.

Owen, E. C. & Proudfoot, R. (1968). *Br. J. Nutr.* **22**, 331.
Owen, E. C. & West, D. W. (1970). *Br. J. Nutr.* **24**, 45.
Williams, P. C. (1964). *Analyst, Lond.*, **89**, 276.

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

By EDWIN C. OWEN and SHEILA A. R. DEMPSTER.
(Department of Biochemistry, The Hannah Dairy Research Institute, Ayr, U.K.)

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 *Eubacterium fissicatena* n.s. (M. M. Taylor, D. W. West & E. C. Owen, unpublished work) is one of these.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.

Hobson, P. N., Summers, R., Owen, E. C., Spencer, J. C. & West, D. W. (1969). *Proc. Nutr. Soc.* **28**, 53A.
McKillen, M. N. & Spencer, B. (1970). *Biochem. J.* **118**, 27P.
Owen, E. C. (1962). *Proc. 1st int. Congr. Food Sci. Technol.* vol. 3, p. 669.
Owen, E. C. & Pringle, J. (1970). *Biochem. J.* **119**, 23P.
Owen, E. C. & West, D. W. (1970). *Br. J. Nutr.* **24**, 45.
Owen, E. C., West, D. W. & Coates, M. E. (1970). *Br. J. Nutr.* **24**, 259.
West, D. W. & Owen, E. C. (1969). *Br. J. Nutr.* **23**, 889.

A Protected Intermediate for the Preparation of Semisynthetic Insulins

By F. BORRAS and R. E. OFFORD. (Molecular Biophysics Laboratory, Department of Zoology, Parks Road, Oxford, OX1 3PS U.K.)

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.

We now report the reversible blocking of the remaining amino groups of phenylthiocarbamoyl-phenylalanine (B-1) insulin with suitable protecting groups. These must survive the trifluoroacetic acid treatment employed to remove phenylalanine B-1 and expose the amino group of valine B-2. It is then possible to couple amino acids back into position B-1 with high efficiency and specificity of location, and we give examples of preliminary coupling experiments of this type. We intend to continue the degradation further to make a series of shortened insulins available for study and to recouple amino acids and peptides on to the shortened chain.

Several sets of conditions were used to produce the phenylthiocarbamoyl-insulin. Kinetic studies, monitored by isoelectric focusing in polyacrylamide gel (Wrigley, 1968), showed that the maximum yield of phenylthiocarbamoylphenylalanine B-1 insulin was obtained in pyridine-water (3:1, v/v) with a phenyl isothiocyanate/insulin ratio 1:2 (Brandenburg, 1969) after approx. 22h at 3°C. This derivative was separated from other phenylthiocarbamoyl-insulins by DEAE-cellulose chromatography at 3°C. The eluent was 0.01M-tris-1mM-EDTA-0.03M-NaCl-6.95M-urea, pH 7.7 (at room temperature). The phthaloyl and trifluoroacetyl groups were found to be resistant to trifluoroacetic acid. The former group was applied by using *N*-ethoxycarbonylphthalimide (Nefkens, Tesser & Nivand, 1960), but in our hands could not be removed easily. Phenyl trifluoroacetate (Weygand & Röpsch, 1960) was used for the second group (the more conventional thiol esters cause reduction of disulphide bridges). Conditions were: 0.04% solution of insulin in anhydrous dimethylformamide (obtained by rotary evaporation of an aqueous solution), 5% w/v MgO, 1.6% v/v reagent for 3h at 20°C. Removal was by *m*-NH₃ for 24h at 20°C. Trifluoroacetic acid treatment (1h at 20°C) of the trifluoroacetylated and phenylthiocarbamoylated derivative produced the required intermediate. Couplings with normal and radioactive trifluoroacetyl-amino acid active esters were carried out on the triethylamine salt of the derivative in anhydrous dimethylformamide.

We thank the Medical Research Council and the British Council for their support.

Brandenburg, D. (1969). *Hoppe-Seyler's Z. physiol. Chem.* **350**, 741.

Brandenburg, D. & Ooms, H. A. (1968). *Proc. int. Symp. Protein and Polypeptide Hormones, Liège*, p. 482.

Brown, H., Sanger, F. & Kitai, R. (1955). *Biochem. J.* **60**, 556.

Nefkens, G. H. L., Tesser, G. I. & Nivand, R. J. F. (1960). *Rec. Trav. chim. Pay-Bas Belg.* **79**, 688.

Offord, R. E. (1969). *Nature, Lond.*, **221**, 37.

Weygand, F. & Röpsch, A. (1960). *Chem. Ber.* **92**, 2095.

Wrigley, C. (1968). *Sci. Tools*, **15**, 17.

Chain Lengths of the Oligodeoxyribonucleotides Formed by the Nucleoside Triphosphate-Dependent Deoxyribonuclease of *Mycobacterium smegmatis*

By F. G. WINDER and M. LAVIN. (*Department of Biochemistry, Trinity College, Dublin 2, Irish Republic*)

The presence of a nucleoside triphosphate-dependent deoxyribonuclease in *Mycobacterium smegmatis* has been reported (Winder & Coughlan, 1967, 1969). The deoxyribonucleotides formed by this enzyme are largely short-chain oligonucleotides (Winder & Lavin, 1969). These have now been characterized further.

The enzyme was partially purified as described by Winder & Lavin (1969) and incubation mixtures were as described by these authors except that [³²P]DNA, prepared as described by Marmur (1967), was employed. Incubation periods ranging from 15 to 60min were used, the reaction was stopped with HClO₄, and the soluble fraction was neutralized with KOH.

This fraction was chromatographed on DEAE-Sephadex A-25 with 0.02M-tris-HCl buffer, pH 7.6, containing 7M-urea and a linear 0.1-0.27M-NaCl concentration gradient (Satoh & Inoue, 1969). This gave three major discrete peaks of ³²P radioactivity, corresponding to di-, tri- and tetra-nucleotides. Raising the NaCl concentration to 1M eluted some further material. The chain length of the oligonucleotides in each peak was determined by concentration (by using Dowex 1) followed by chromatography on Sephadex G-25 in 0.5M-NH₄HCO₃ containing 8M-urea (Stanley, 1967) and on DEAE-cellulose paper in one dimension (Furlong, 1969), and by determining the proportion of monoester phosphate (Furlong, 1969).

The results showed that the amounts of mononucleotide and P_i released from the DNA were small. The acid-soluble products consisted on average of 23% of dinucleotide, 40% of trinucleotide, 20% of tetranucleotide and 10% of oligonucleotides of greater chain length. Two-dimensional paper chromatography (Sulkowski & Laskowski, 1962) showed that at least eight dinucleotides were formed. Susceptibility to specific phosphatases showed that the oligonucleotides were 5'-phosphate-terminated.

These results, taken together with the equivalence of ATP used to deoxyribose solubilized, indicated that about 3 molecules of ATP were used/phosphodiester bond split. The findings were similar to