The Identification of a Peptide in Human Parotid Saliva Particularly Active in Enhancing the Glycolytic Activity of the Salivary Micro-organisms

By IAN B. HOLBROOK and PETER C. MOLAN

Department of Dental Sciences, University of Liverpool, Pembroke Place, Liverpool L3 5PS, U.K.

(Received 12 May 1975)

A factor in saliva responsible for markedly activating the glycolytic activity of micro-organisms was isolated from parotid secretions and identified as a small basic peptide. Analysis of the peptide showed a high proportion of histidine, lysine and arginine. Its minimum molecular weight was calculated to be between 2500 and 3000.

The enhancement of glycolysis by oral micro-organisms by factors in saliva was first reported by Hartles & Wasdell (1955). It is considered to be of importance in dental health because of the implication of acid produced by microbial glycolysis in the initiation and progression of caries of teeth. Several workers have been concerned with the isolation and characterization of the factors responsible for the enhancement (Bramstedt *et al.*, 1957*a,b*; Wasdell, 1962; Hartles, 1963; Kawasaki, 1964; Hay & Hartles, 1965, 1966; Guggenheim, 1966; Mishiro *et al.*, 1966, 1969, 1970; Ishihara, 1967; Molan & Hartles, 1969, 1972; Kirimura *et al.*, 1970; Holbrook & Molan, 1973). However, only one of these factors, urea, has been positively identified (Molan & Hartles, 1969).

It has been observed (Holbrook & Molan, 1973) that enhancement of glycolysis by micro-organisms is brought about by many proteins present in saliva. The enhancement was correlated with the content of protein in the fractions obtained by gel-filtration chromatography of saliva. However, in one fraction the enhancement was disproportionately high with respect to the content of protein. It was decided to attempt to isolate and identify the factors responsible for this phenomenon.

Experimental

The initial fractionation of human parotid saliva was performed by gel-filtration chromatography on a column of Sephadex G-25 (3.8 cm × 75 cm, void volume 335 ml, eluted with 0.154 M-NaCl). The fractions exhibiting disproportionately high enhancing activity were eluted between 620 and 760 ml (see Holbrook & Molan, 1973). These fractions were pooled, dialysed against water and freeze-dried. A small portion was redissolved in water and an attempt was made to isolate the factors by chromatography on a column of Bio-Gel P-2. In view of the caution given by Bonilla (1969) that cationic salivary proteins are adsorbed on Bio-Gel P-2 when deionized distilled water is the eluent, the column was washed with 1.0 M-NaCl

after 1 bed volume of water had been eluted. A considerable proportion of the material applied to the Bio-Gel P-2 was eluted by the 1.0 M-NaCl and was thus indicated to be strongly cationic (Bonilla, 1969).

The enhancement of glycolysis effected by the fractions eluted from the column of Bio-Gel P-2 was assessed by the method of Hay & Hartles (1965), by using a Gilson differential respirometer. Bacteria obtained from the mouths of volunteers were incubated with Krebs–Ringer bicarbonate solution (Umbreit *et al.*, 1964), glucose solution and samples of the fractions to be tested. It was found that high glycolytic activity was associated with the material which had been adsorbed. Thus ion-exchange chromatography on a cation-exchanger was adopted in the subsequent isolation of the factor sought.

The remaining freeze-dried material was redissolved in 15 ml of 0.05 M-sodium phosphate buffer, pH 6.0, and loaded on a column (2.6 cm × 34 cm) of Sephadex CM-25 equilibrated with 0.05 M-sodium phosphate buffer, pH 6.0. The column was washed through with 100 ml of the same buffer and then with a linearly increasing salt gradient to 4.0 M-NaCl in 200 ml. The result of the ion-exchange chromatography is shown in Fig. 1. No further material was eluted by washing the column with 0.1 M-HCl.

A peak of material absorbing at 220 nm was eluted by the salt gradient. The fractions containing this peak (designated CM-2 in Fig. 1) were pooled and an attempt was made to remove salt by ultrafiltration by using an Amicon UM-10 membrane, but this membrane failed to retain any detectable quantity of material, which indicated that the components of fraction CM-2 were of molecular weight below approx. 10000. A further attempt was made, this time with an Amicon UM-05 membrane. The E_{220} values of the ultrafiltrate and the retained material were measured and found to be 0.37 and approx. 22 respectively (a portion of the retained material was diluted with water for measurement of the E_{220}). This indicated that the material designated fraction CM-2 contained components of molecular weight greater than 500. The material retained

Fig. 1. Ion-exchange chromatography of the disproportionately active factor from Sephadex G-25, concentrated to 15 ml, on Sephadex CM-25

The ion-exchange matrix was equilibrated with 0.05M-sodium phosphate buffer, pH6.0. The eluent was changed to a linearly increasing salt gradient after 100ml had been eluted. The E_{220} (●) and the molarity of NaCl (■) are shown. The fractions that were pooled to give fraction CM-2 are also shown.

on the Amicon UM-05 membrane was washed three times to complete the removal of salt and the residue was taken up in 20ml of water. The glycolysis-enhancing activity of fraction CM-2 was measured and samples of fractions of saliva with normal enhancing activity [eluted at 420ml and 460ml from the column of Sephadex G-25; see Fig. 3 in Holbrook & Molan (1973)] were taken as controls for comparison. All samples were diluted to similar E_{220} values. The samples were tested in triplicate and the results were as follows, expressed as percentage enhancement relative to blanks of water: both controls, 7.5 ± 0 ; fraction CM-2, 13.7 ± 1.3 . The excess of activity of fraction CM-2 was of the same order as that normally found in the disproportionately active fractions from chromatography on Sephadex G-25 (Holbrook & Molan, 1973).

A portion of the desalted CM-2 fraction was subjected to cationic polyacrylamide-gel disc electrophoresis on gels prepared by the method of Neidle & Waelsch (1964). The reservoir buffer was 0.35M- β -alanine-0.13M-acetic acid, pH4.6. The gels were subjected to a current of 2mA/tube for 2h and then stained with Amido Black, as described by Davis (1964), or Coomassie Brilliant Blue R250, as described by Meyer & Lamberts (1965).

A single fast-moving band was detected, corresponding to the fastest-moving band in parotid saliva. This band in parotid saliva was increased in intensity when fraction CM-2 was added.

Table 1. Amino acid composition of the desalted CM-2 fraction

For details, see the text. n.d., Not determined; —, not detected.

Amino acid	Amino acid composition (residues/1000 residues)
Aspartic acid	63
Threonine	—
Serine	75
Glutamic acid	45
Proline	—
Glycine	79
Alanine	31
Cystine	—
Valine	13
Methionine	—
Isoleucine	—
Leucine	—
Tyrosine	80
Phenylalanine	46
Lysine	177
Histidine	263
Arginine	128
Tryptophan	n.d.

Thus it was concluded that fraction CM-2 was in fact identical with the fastest-moving basic protein in parotid saliva.

A portion of the desalted CM-2 fraction was freeze-dried and samples of the dry material were weighed out. Each was hydrolysed with 6M-HCl *in vacuo* at 110°C. The HCl was removed by evaporation under reduced pressure and the amino acids were analysed on a Bio-Cal BC100L machine. The average results of the analyses are given in Table 1.

Discussion

The factor isolated from parotid saliva has been found to be a basic peptide, and may well be of low molecular weight, since it is eluted from Sephadex G-25 just before urea. However, some proteins are known to be retarded on Sephadex and Bio-Gel (Bonilla, 1969; Jacobsen & Sønju, 1971), and consequently larger molecules may be eluted at similar volumes to the smaller molecules.

The passage of the basic peptide through an Amicon UM-10 membrane indicates its molecular weight to be less than approx. 10000. Its retention by an Amicon UM-05 membrane indicates that it has a molecular weight greater than approx. 500. Although the cut-off values for Amicon membranes are not absolutely precise, they do indicate that the peptide is of low molecular weight.

From the results of the amino acid analyses, an estimate of the minimum molecular weight of the

peptide was made by taking aspartate, glutamate, alanine, tyrosine and phenylalanine as unity. Depending on the particular amino acids used in the estimation, the molecular weight was calculated to be between 2500 and 3000.

The retention of the factor on Bio-Gel P-2 indicates that the pI is 10.8 or higher (Bonilla, 1969). This observation is supported by the behaviour of the factor on electrophoresis in polyacrylamide gels at acid pH. The high mobility of the factor towards the cathode under these conditions suggests a small basic molecule. The high percentage of basic amino acids (50%) also suggests a high pI.

Other workers have also reported the discovery of basic peptides and proteins in saliva (Bonilla, 1969; Azen, 1972; Balekjian & Longton, 1973). The two most basic histones of the three reported by Balekjian & Longton (1973) have amino acid compositions very similar to that of the basic factor reported in the present paper.

Our observations on the nature of the factor may help to explain its mode of action in enhancing glycolysis. The hypothesis of Hay & Hartles (1965) was that the glycolysis-enhancing properties of saliva are, in the main, due to a mixture of free amino acids, peptides and proteins. They pointed out that the concentration of free amino acids alone is too low to provide more than sub-optimal stimulation, but that the peptides and proteins act as a potential source of amino acids for the stimulation of the anabolic processes of oral flora. They suggested that the nitrogenous compounds induce growth in the oral micro-organisms and thus an increased requirement for ATP results in increased glycolysis.

The sediment from centrifuged saliva, used as the source of salivary micro-organisms, is rich in proteolytic enzymes (Weinmann, 1934; Chauncey, 1961; Mäkinen, 1966a,b,c,d; Söder & Frostell, 1966; Critchley, 1967; Frostell & Söder, 1970; Söder, 1972). It is conceivable that they could break down proteins and peptides sufficiently rapidly to produce amino acids that could be utilized by the micro-organisms, and thus produce the almost immediate enhancement of glycolysis on the addition of supernatant that was observed by Molan & Hartles (1972).

McIlwain (1946) proposed that the utilization of glutamine in synthetic processes by streptococci (the major type of micro-organism in saliva) would require energy and material made available by glycolysis.

The particularly high enhancement found with the basic peptide may be due to a number of reasons. The peptide, being a relatively small molecule, may be more easily hydrolysed and incorporated by the micro-organisms. The nitrogen content of the basic peptide is 20.5% [Dixon & Webb (1964) quote 12–19% for average proteins],

and this could require more energy for its utilization and thus stimulate glycolysis to a greater degree. Finally, oral micro-organisms are known to be fastidious in their nutritional requirements (Critchley, 1970), but many can synthesize purines and pyrimidines 'provided that a suitable nitrogen and energy source is available and that folic acid is provided or can be synthesized'. The high content of histidine in the basic peptide may be utilized in the synthesis of folic acid, stimulating the metabolic activity to a greater degree than other components of saliva.

We thank Professor R. L. Hartles for his helpful interest and encouragement and Miss J. Taylor and Miss J. Sherriff for help in the preparation of this manuscript. I. B. H. is in receipt of a Medical Research Council scholarship.

- Azen, E. A. (1972) *Science* **176**, 673–674
 Balekjian, A. Y. & Longton, R. W. (1973) *Biochem. Biophys. Res. Commun.* **50**, 676–682
 Bonilla, C. A. (1969) *Anal. Biochem.* **32**, 522–529
 Bramstedt, F., Kröncke, A. & Naujoks, R. (1957a) *Odontol. Revy* **8**, 201–206
 Bramstedt, F., Kröncke, A. & Naujoks, R. (1957b) *Naturwissenschaften* **44**, 331
 Chauncey, H. H. (1961) *J. Am. Dent. Ass.* **63**, 360–368
 Critchley, P. (1967) *J. Dent. Res.* **46**, 1295, Abstr. no. 104
 Critchley, P. (1970) *J. Dent. Res.* **49**, 1283–1292
 Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
 Dixon, M. & Webb, E. C. (1964) *Enzymes*, 2nd edn., p. 30, Longmans, Green and Co., London
 Frostell, G. & Söder, P.-Ö. (1970) *Int. Dent. J.* **20**, 436–450
 Guggenheim, B. (1966) *Helv. Odontol. Acta* **10**, Suppl. 3, 59–93
 Hartles, R. L. (1963) *J. Dent. Res.* **42**, 553–558
 Hartles, R. L. & Wasdell, M. R. (1955) *Br. Dent. J.* **99**, 334–337
 Hay, D. I. & Hartles, R. L. (1965) *Arch. Oral Biol.* **10**, 485–498
 Hay, D. I. & Hartles, R. L. (1966) *Arch. Oral Biol.* **11**, 337–347
 Holbrook, I. B. & Molan, P. C. (1973) *Arch. Oral Biol.* **18**, 1275–1282
 Ishihara, H. (1967) *Odontology* **54**, 320–334
 Jacobsen, N. & Sønju, T. (1971) *Scand. J. Dent. Res.* **79**, 193–201
 Kawasaki, T. (1964) *Bull. Tokyo Dent. Coll.* **5**, 25–32
 Kirimura, K., Morita, M. & Mishiro, Y. (1970) *J. Dent. Res.* **49**, 890
 Mäkinen, K. K. (1966a) *Acta Odontol. Scand.* **24**, 579–604
 Mäkinen, K. K. (1966b) *Acta Odontol. Scand.* **24**, 605–617
 Mäkinen, K. K. (1966c) *Acta Odontol. Scand.* **24**, 619–632
 Mäkinen, K. K. (1966d) *Acta Odontol. Scand.* **24**, 709–721
 McIlwain, H. (1946) *Biochem. J.* **40**, 460–464
 Meyer, T. S. & Lamberts, B. L. (1965) *Biochim. Biophys. Acta* **107**, 144–145
 Mishiro, Y., Kirimura, K. & Ishihara, H. (1966) *J. Dent. Res.* **45**, 1824
 Mishiro, Y., Kirimura, K. & Morita, M. (1969) *Jpn. J. Oral Biol.* **11**, 24–29
 Mishiro, Y., Morita, M. & Kirimura, K. (1970) *Arch. Oral Biol.* **15**, 465–467

- Molan, P. C. & Hartles, R. L. (1969) *Arch. Oral Biol.* **14**, 421–426
- Molan, P. C. & Hartles, R. L. (1972) *Arch. Oral Biol.* **17**, 1671–1678
- Neidle, A. & Waelsch, H. (1964) *Science* **145**, 1059–1061
- Söder, P.-Ö. (1972) *J. Dent. Res.* **51**, 389–393
- Söder, P.-Ö. & Frostell, G. (1966) *Acta Odontol. Scand.* **24**, 501–515
- Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1964) *Manometric Techniques*, 4th edn., p. 132, Burgess Publishing Co., Minneapolis
- Waddell, M. R. (1962) *Arch. Oral Biol.* **7**, 25–37
- Weinmann, J. (1934) *J. Dent. Res.* **14**, 205, Abstr. no. 60