J. Physiol. (1981), **320**, *pp.* 139–148
With 2 plates and 2 text-figures
Printed in Great Britain

THE EFFECTS OF EXERCISE ON PROTEIN AND ELECTROLYTE SECRETION IN PAROTID SALIVA

BY C. DAWES
From the Department of Oral Biology, Faculty of Dentistry, University of Manitoba, Winnipeg, Canada R3E OW3

(*Received 18 April 1980*)

SUMMARY

1. Ten subjects collected ten 1 min samples of parotid saliva at a constant flow rate 1–2 hr before exercise, immediately after running 3–8 miles and 3 hr after exercise.

2. Exercise had no significant effects on the concentration of sodium, potassium, calcium, magnesium, chloride, bicarbonate or inorganic phosphate.

3. Exercise caused a marked elevation in total protein concentration and in seven of the subjects the initial samples after exercise were cloudy due to protein precipitation. The precipitated protein could be dissolved in sodium EDTA.

4. The saliva samples collected immediately after exercise from the seven subjects in which precipitation occurred contained higher concentrations of protein, calcium and phosphate than in those of the other three subjects.

5. Electrophoretic analysis revealed that all proteins appeared to show a proportional increase in the cloudy saliva, as compared with the clear saliva but differential precipitation of certain proteins occurred in the former which was largely restricted to proteins having a high affinity for calcium phosphate.

6. Electronmicroscopic examination of centrifuged cloudy saliva produced after exercise revealed the presence of rounded droplets of homogeneous structure in contrast to the fine granular deposits produced in clear parotid saliva by *in vitro* procedures causing precipitation of calcium phosphate crystals on which protein adsorbed.

INTRODUCTION

Following moderate exercise such as jogging it is a common subjective experience that the mouth feels dry and the saliva unusually viscous. Whether there is any physiological basis for the apparently increased viscosity does not appear to have received much study.

Salminen & Konttinen (1963) reported that following exercise the sodium concentration doubled and the protein concentration increased by about 50% but with no change in the potassium concentration in whole saliva. Shannon (1967) noted no effects of exercise on acid drop-stimulated parotid saliva flow rates or on the concentration of sodium, potassium, chloride or 17-hydroxycorticosteroids in the parotid saliva.

The present study was thus designed to test the effects of exercise on the electrolyte and total protein concentrations in parotid saliva collected under standardized conditions and to determine whether exercise caused any changes in the relative proportions of the different salivary proteins.

METHODS

Saliva collection

On three separate occasions, seven male and three female, physically fit young adults or adolescents collected ten successive 1 min samples of sour lemon drop-stimulated parotid saliva. The flow rates were maintained constant for each individual by the negative feed-back technique described by Dawes (1967) and all were between 0·6 and 1·0 ml./min. Briefly, after cannulation of the parotid duct (Dawes & Jenkins, 1964), saliva was collected into a graduated centrifuge tube positioned in front of a mirror. By observation the subject was able to calculate the flow rate with the aid of a stopwatch and regulate the degree of sucking on the acid drop to maintain a constant flow rate. The three saliva collections were made 1–2 hr before exercise, immediately after running at a moderately fast pace for 3–8 miles (depending on the physical fitness of the subject) and 3 hr after the exercise, which was carried out at about 1700 hr. The time of day of the experiment was chosen to minimize the influence of circadian variations in salivary composition (Dawes, 1974*a*). Lunch was consumed about 12 noon, 3 hr before the first saliva collection. A previous study (Dawes & Chebib, 1972) revealed that when similar saliva collections were separated from each other by two hours or more, there was no evidence of serial dependency.

One subject repeated the above procedure on several different days to cover a wide range of constant flow rates for parotid saliva, including collection of unstimulated parotid saliva. On one occasion, submandibular saliva was collected at a constant flow rate of 2 ml./min from both the right and left submandibular ducts using the collection device described by Truelove, Bixler & Merritt (1967).

Analytical techniques

The saliva was analysed for sodium, potassium, calcium and magnesium by atomic absorption spectroscopy (Dawes, 1967, 1969), chloride by a coulometric method (Cotlove, Trantham & Bowman, 1958), inorganic phosphate by the method of Chen, Toribara & Warner (1956) as modified for saliva (Dawes, 1969) and total protein by the method of Lowry, Rosebrough, Farr & Randall (1951). The bicarbonate concentration was computed as described previously (Dawes, 1974*b*). Anionic slab gel electrophoresis of salivary proteins (about 350 μg) in 7·5% polyacrylamide was carried out for 2·5 hr in a Multiphor (LKB Produkter AB. Bromma, Sweden) with a current of 45 mA and using the gel reagents described by Davis (1964). The gels were stained for protein with a mixture of 0·25% Coomassie Brilliant Blue 250 and 0·5% Wool Fast Blue BL in methyl alcohol, water, acetic acid (5:5:1) and destained in 7% acetic acid. A preliminary study showed that more bands were stained by the combination of dyes than by either dye alone.

Salivary protein precipitation

Since protein precipitation was present in many of the saliva samples collected after exercise, various methods were used to induce salivary protein precipitation in clear saliva to allow comparison of the different precipitates both electrophoretically and electronmicroscopically. Precipitation was induced by addition of small volumes of either 1 M-calcium chloride or 1 M-potassium phosphate at pH 7·5 to clear parotid saliva which had been either untreated or mixed with sufficient disodium ethylenediaminetetracetate (sodium EDTA) to chelate all the calcium and then dialysed exhaustively against either 50 mM-sodium chloride, 50 mM-sodium chloride containing 4 mM-potassium phosphate at pH 7·5 or 50 mM-sodium chloride containing 2 mM-calcium chloride. The sodium EDTA was added initially to prevent the spontaneous precipitation which sometimes occurs when saliva is cooled, as during the dialysis at 4 °C. When precipitation of protein occurred, by adsorption on precipitated calcium phosphate, the saliva was centrifuged at 12,000 *g* and the protein precipitate redissolved in 3·75% sodium EDTA at pH 8·6 before further analysis by slab gel electrophoresis. The protein precipitate in saliva collected immediately after exercise was prepared for slab gel electrophoresis in a similar manner.

Protein precipitation was also induced in clear parotid saliva which had been mixed with sufficient sodium EDTA to chelate all the calcium, extensively dialysed against water, lyophilized and reconstituted in sufficient saliva or electrolyte solution to yield a protein concentration of 800–4000 mg/100 ml. The electrolyte solution had a composition similar to that of saliva and contained 15 mM-$NaHCO_3$, 5 mM-KH_2PO_4, 20 mM-KCl, 1 mM-$CaCl_2$ and 0·02 mM-$MgSO_4$, adjusted to pH 7·0.

Electron microscopy

With a technique similar to that described by Blomfield, Dascalu, van Lennep & Brown (1973), salivary protein precipitates were isolated by centrifugation, fixed with 5% glutaraldehyde in 0·1 M-phosphate buffer at pH 7·3, for 4 hr, washed in 5% sucrose buffer and post-fixed in 0·8% osmium tetroxide. Thin sections were stained with uranyl acetate and lead citrate and examined with a Hitachi HU-12 electron microscope. Addition of 5% glutaraldehyde in phosphate buffer to clear parotid saliva did not cause any detectable protein precipitation.

RESULTS

As tested by analysis of variance, the concentrations of sodium, potassium, calcium, magnesium, chloride, bicarbonate and phosphate were not significantly affected by the exercise or by the sex of the subjects and the combined results for the ten subjects are shown in Figs. 1 and 2. The initial increase in bicarbonate and decreases in potassium, magnesium, chloride, phosphate and protein with duration of stimulation have been reported previously (Dawes, 1967, 1969). The large standard errors for the sodium and chloride results (Fig. 1) are due to the very large inter- rather than intra-individual variation.

Preliminary studies in which saliva was collected into tubes under a stream of 5% carbon dioxide in nitrogen, to avoid changes in pH during collection, revealed that exercise had no specific effect on salivary pH which, because of the change in bicarbonate concentration (Fig. 1), increased progressively during the 10 min collection from about 6·9–7·5, as found previously (Dawes, 1969).

The total protein concentration was markedly elevated immediately after exercise (Fig. 2) and in seven of the subjects the initial samples were very cloudy due to protein precipitation. In the one subject studied in more detail, the cloudiness appeared after exercise at all flow rates, with both stimulated and unstimulated parotid saliva as well as in submandibular saliva. In all subjects the cloudy saliva regained its normal water-like appearance after a few minutes of continued stimulation but the unstimulated saliva remained cloudy and with elevated protein concentration for the duration of the 40 min collections. Both unstimulated and stimulated cloudy saliva became quite clear on addition of sufficient sodium EDTA to chelate all the calcium, whereas addition of a similar volume of a buffer with no calcium-complexing ability, such as Tris-glycine at the same pH, had no effect on the cloudiness.

In the first sample collected after exercise the mean and S.E. of mean values for the protein, calcium and inorganic phosphate concentrations for the seven subjects showing protein precipitation were 889 ± 181 mg/100 ml., $0{\cdot}85 \pm 0{\cdot}08$ mM and $5{\cdot}26 \pm 0{\cdot}69$ mM, respectively. For the three subjects whose saliva remained clear the corresponding values for protein, calcium and inorganic phosphate were 446 ± 46 mg/100 ml., $0{\cdot}58 \pm 0{\cdot}12$ mM and $3{\cdot}01 \pm 0{\cdot}53$ mM, respectively. These differences just failed to reach statistical significance possibly because of the small number of subjects in the second group. Of these three subjects, two showed no change in protein

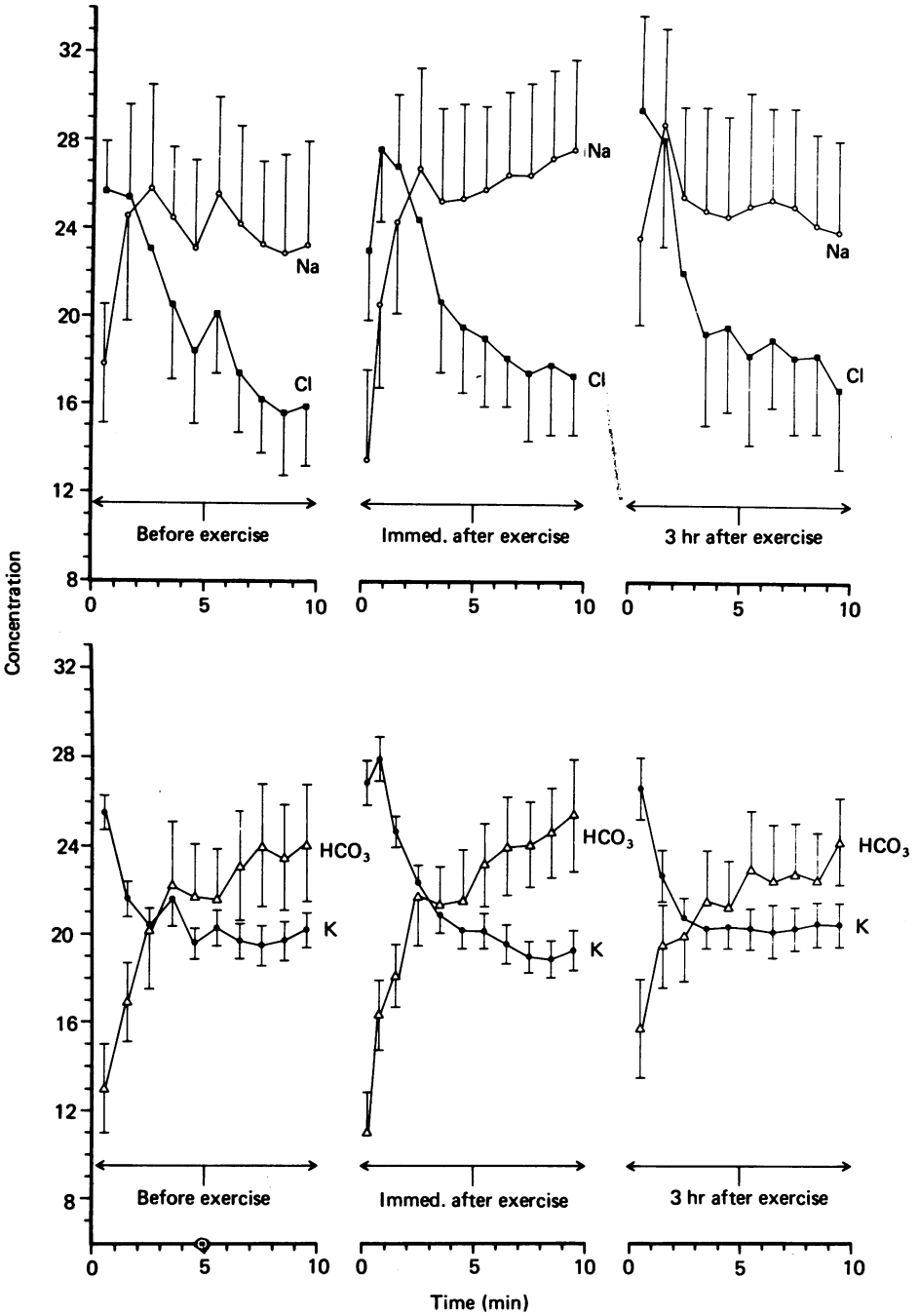


Fig. 1. Effects of exercise on the sodium, chloride, potassium and bicarbonate concentrations (mM) in parotid saliva from ten subjects. The bar lines indicate the standard error of the mean.

concentration in saliva collected following exercise, as compared with before or 3 hr after exercise whilst the other subject showed only a slight increase following exercise.

The gel electrophoresis revealed that in comparison with clear saliva produced before exercise or after several minutes of gustatory stimulation after exercise (Pl. 1*A*, slot 1), the cloudy saliva produced immediately after exercise showed no change in the relative proportions of the different proteins (Pl. 1*A*, slot 2).

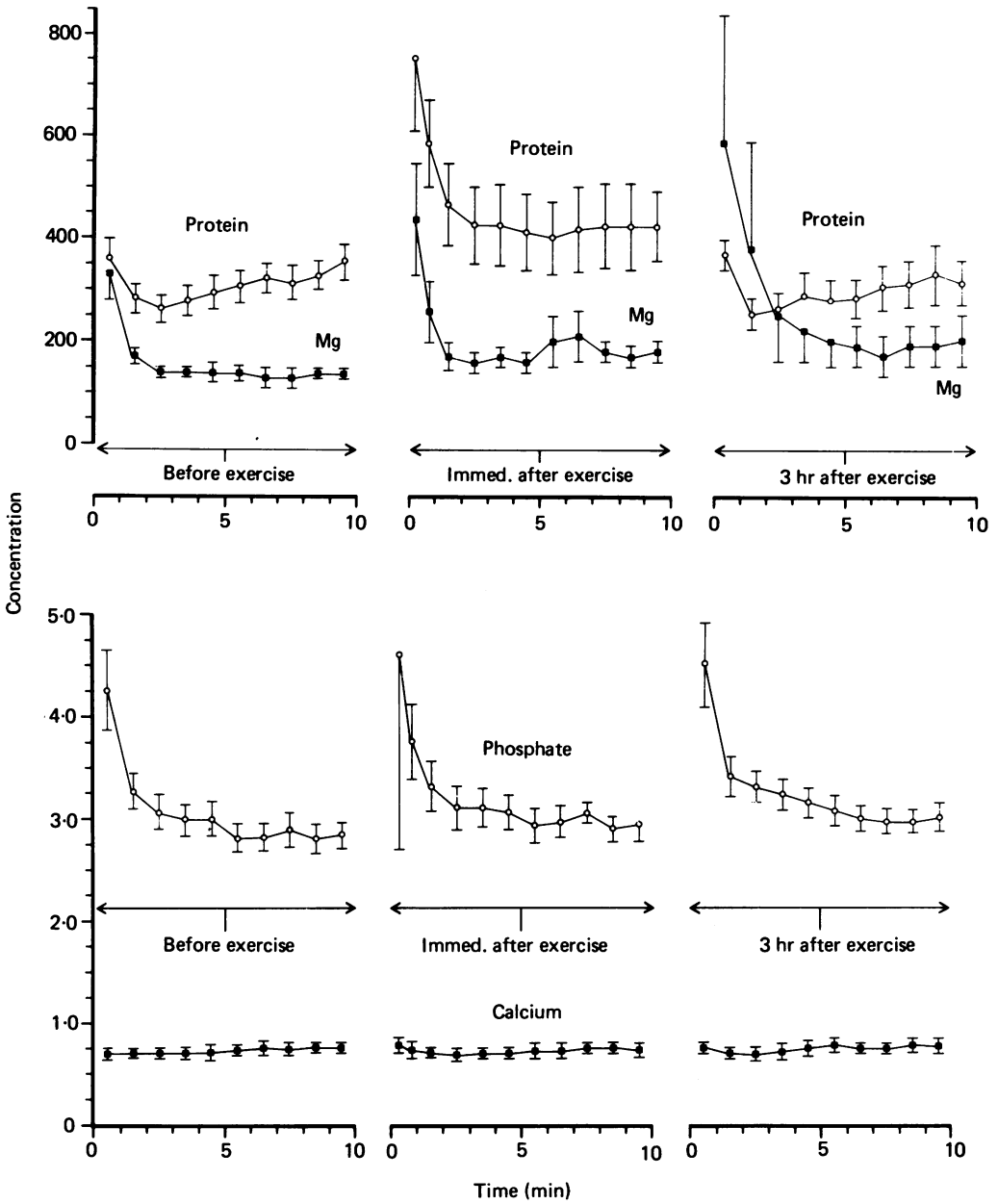


Fig. 2. Effects of exercise on the protein (mg/100 ml.), magnesium (μM × 10) calcium and inorganic phosphate concentrations (mM) in parotid saliva from ten subjects.

In the cloudy saliva, there was selective precipitation of particular proteins which was revealed by centrifuging 1 ml. cloudy saliva and redissolving the precipitate in sodium EDTA before slab gel electrophoresis (Pl. 1 *A*, slot 3). The identical proteins were precipitated (Pl. 1 *A*, slot 4) by first dialysing saliva to remove the electrolytes and then lyophilizing and reconstituting in an electrolyte solution to a higher concentration (about 2000 mg/100 ml.), as described in the Methods. Many of the same proteins, but not all, were precipitated from 10 ml. of clear parotid saliva (Pl. 1 *A*, slot 5) by the addition of as little as 10 μl. 1 M-calcium chloride, an amount sufficient to double the normal calcium concentration.

When larger amounts of calcium (250 μl. 1 M-calcium chloride) were added to 10 ml. parotid saliva, a much heavier precipitate was produced which only partially redissolved in 60 μl. sodium EDTA. After centrifugation in a Beckman Airfuge at 160,000 *g*, gel electrophoresis of the supernatant gave the results shown in Pl. 1 *A*, slot 6. The undissolved precipitate was suspended in 1 ml. sodium EDTA and extensively dialysed against water before lyophilization and addition of 60 μl. sodium EDTA. The lyophilized material dissolved almost completely and gel electrophoresis revealed the protein pattern shown in Pl. 1 *A*, slot 7.

Addition of 1 M-calcium chloride to the saliva dialysed against 50 mM-sodium chloride did not cause precipitation of protein but such precipitation occurred when as little as 10 μl. 1 M-calcium chloride was added to 3 ml. saliva dialysed against 50 mM-sodium chloride containing 4 mM-phosphate. Likewise, addition of 1 M-potassium phosphate at pH 7·5 to saliva dialysed against 50 mM-sodium chloride did not cause protein precipitation but precipitation occurred when the phosphate was added either to normal, clear parotid saliva or to the saliva dialysed against 50 mM-sodium chloride containing 2 mM-calcium chloride. The precipitated proteins, after centrifugation and solubilization in sodium EDTA gave electrophoretic patterns similar to those shown in Pl. 1 *A*, slot 5.

In one study on submandibular saliva, exercise also had no obvious effects on electrolyte secretion but caused a very significant increase in protein concentration to values about 150% of those in the collections preceding exercise and 3 hr afterwards.

Electronmicroscopy of the material obtained by centrifugation of the cloudy saliva obtained immediately after exercise from three of the subjects gave the results shown in Pl. 1 *B*. The precipitate appears to consist mainly of rounded droplets of a homogeneous consistency.

Plate 2 illustrates results obtained on the precipitate formed after addition of calcium chloride to clear parotid saliva. This precipitate appears to be of a fine, granular nature. A similar picture was obtained after electronmicroscopic examination of the insoluble protein remaining after lyophilization of clear parotid saliva and reconstitution in either water or electrolyte solution.

DISCUSSION

The present study confirms the findings of Shannon (1967) that moderate exercise has no significant effects on the sodium, potassium and chloride levels in parotid saliva. In addition, the results show no effect of exercise on the calcium, magnesium,

bicarbonate or phosphate concentrations. Shannon (1967) also found no significant effects of exercise on the flow rates elicited by extra-strong acid drops and this study (where flow rates were maintained constant but at relatively high rates) confirms his findings to some extent in that immediately after exercise none of the subjects had any difficulty in maintaining the required flow rate.

In contrast, Salminen & Konttinen (1963) reported higher sodium and protein concentrations in unstimulated whole saliva following exercise. Unfortunately they did not record flow rates, although both sodium and protein concentrations are known to be flow-rate dependent and, in addition, the relative contributions to whole saliva from the different glandular sources may well have been altered by exercise.

Gilman, Thornton, Miller & Biersner (1979) reported, in abstract form, that potassium and α-amylase concentrations were significantly elevated in parotid saliva after exercise. Unfortunately no data were given, flow rates were not mentioned and it was not stated whether unstimulated or stimulated saliva was collected.

A striking finding of the present study was the very high protein concentration in the saliva collected from most of the subjects immediately after exercise (Fig. 2). The elevated protein concentration after exercise may account for the subjective feeling of increased salivary viscosity which is typically experienced.

Exercise is known to increase sympathetic activity (Ganong, 1979) and the high protein concentration following exercise may be due to increased β-sympathetic activity to the salivary glands as protein secretion from human parotid glands is increased by sympathetic activity created by sudden body cooling or inhalation of isoprenaline (Speirs, Herring, Cooper, Hardy & Hind, 1974).

Denniss, Schneyer, Sucanthapree & Young (1978) have shown that sympathomimetic agents can influence electrolyte transport by the isolated ducts of the rat and rabbit submandibular glands. However, the present study could not demonstrate any changes in salivary electrolyte composition during the period immediately after exercise when sympathetic activity was presumably at its highest, as judged by the markedly elevated protein secretion.

In the majority of subjects, exercise caused differential precipitation of certain salivary proteins (Pl. 1 *A*, slot 3) which bear a strong resemblance, as judged by their electrophoretic mobility, to the parotid salivary proteins found by Hay (1973) to adsorb to hydroxyapatite and which may be involved in deposition of the acquired pellicle on exposed surfaces of enamel.

Electronmicroscopically (Pl. 1 *B*), the protein precipitate had the appearance of droplets similar to those described by Blomfield, Allars, Rush, van Lennep & Brown (1974) as being present in parotid saliva from children with cystic fibrosis which had become turbid after cooling. Blomfield *et al.* (1974) argued that the droplets were not identical with the zymogen granule spherules present in some samples of turbid submandibular saliva from normal subjects (Caldwell & Shackleford, 1967) or from subjects with cystic fibrosis (Blomfield *et al.* 1973) but that they were colloidal suspensions of aggregated protein. If the precipitate in the cloudy saliva produced after exercise had been composed of zymogen granule spherules, which would presumably contain most of the salivary proteins, depending on the contribution from granular ducts, one would have expected the electrophoretic pattern (Pl. 1 *A*, slot 3) to be largely the same as that for whole parotid saliva (Pl. 1 *A*, slot 1). However, these

patterns are clearly different from each other. This suggests that the cloudiness is due to selective precipitation of salivary proteins in colloidal aggregates and that these proteins are electrophoretically identical to those which do not redissolve after reconstitution of lyophilized saliva in water or electrolyte solution (Pl. 1*A*, slot 4). However, these two types of precipitates were not identical at the ultrastructural level since the latter proteins had a similar appearance to the fine granular deposits produced in clear parotid saliva by procedures causing precipitation of calcium phosphate with subsequent protein adsorption on the calcium phosphate crystals (Pl. 2).

The results suggest that calcium was necessary for the protein precipitation after exercise since the cloudy saliva could be clarified by addition of sodium EDTA, but not by other buffers lacking the ability to chelate calcium. In addition, the results of the experiments in which protein could not be precipitated by addition of calcium or phosphate ions to saliva dialysed against saline alone but only when the saliva had been dialysed against saline containing phosphate or calcium, respectively, indicate that calcium phosphate rather than calcium alone, is the key factor. This conclusion is also supported by the finding that when lyophilized salivary protein, which had previously been dialysed against sodium EDTA and then water, was reconstituted in electrolyte solution to a high protein concentration, precipitation only occurred when the solution contained both calcium and phosphate ions. Leach (1977), in studies on calcium precipitation of submandibular salivary proteins, also concluded that the presence of phosphate was critical and that the proteins which precipitated were actually adsorbed to calcium phosphate crystals. Thus it seems significant that the saliva samples collected immediately after exercise in the three subjects who did not show protein precipitation contained the lowest concentrations of protein, calcium and phosphate.

The proteins which could be precipitated by addition of small amounts of calcium chloride to clear parotid saliva (Pl. 1*A*, slot 5) were similar to, but not identical with the proteins precipitated after exercise. The addition to clear saliva of larger amounts of calcium chloride caused a much heavier precipitation of calcium phosphate and the deposition of other proteins which normally adsorb less strongly to calcium phosphate. Hence these redissolved more readily in amounts of sodium EDTA insufficient to dissolve completely all the calcium phosphate precipitated (Pl. 1*A*, slot 6). Complete solution of the precipitate in an excess of sodium EDTA and subsequent removal of the latter and the calcium phosphate by dialysis left the proteins which have a strong affinity for calcium phosphate (Pl. 1*A*, slot 7).

The precipitation of protein after exercise occurred without a significant change in the salivary calcium and phosphate concentrations or in the pH and is presumably due simply to the increased protein concentration. However, the degree of cloudiness in the saliva produced after exercise appeared to be greater than that in dialysed saliva which had been lyophilized and reconstituted in either electrolyte solution or in clear parotid saliva to an equivalent protein concentration. This difference is presumably caused by differences in the actual mechanism of protein precipitation because although the same types of proteins were present in the two types of precipitate, their ultrastructural appearances were entirely different.

Saliva is normally supersaturated with respect to hydroxyapatite (McCann, 1968)

and a very important function of certain salivary proteins is that they inhibit calcium phosphate precipitation in saliva by binding onto the surfaces of early crystal nuclei (Gron & Hay, 1976). One might thus have anticipated that an increased salivary protein concentration would, if anything, have inhibited precipitation in association with calcium phosphate. Further studies are needed of the factors influencing protein precipitation in saliva.

I thank the subjects for their cooperation, Mrs T. Beaton for excellent technical assistance, Dr G. R. Holland and Mr D. Craw for the electron microscopy, Dr F. S. Chebib for statistical advice, and the Medical Research Council of Canada for financial support.

REFERENCES

BLOMFIELD, J., ALLARS, H. M., RUSH, A. R., VAN LENNEP, E. W. & BROWN, J. M. (1974). Parotid serous hypersecretion in cystic fibrosis. *Aust. paediat. J.* **10**, 75–81.

BLOMFIELD, J., DASCALU, J., VAN LENNEP, E. W. & BROWN, J. M. (1973). Hypersecretion of zymogen granules in the pathogenesis of cystic fibrosis. *Gut* **14**, 558–565.

CALDWELL, R. C. & SHACKLEFORD, J. M. (1967). A chemical, immunological and electron-microscopic study of centrifuged human submaxillary saliva. *Archs oral Biol.* **12**, 333–340.

CHEN, P. S., TORIBARA, T. Y. & WARNER, H. (1956). Microdetermination of phosphorus. *Analyt. Chem.* **28**, 1756–1758.

COTLOVE, E., TRANTHAM, H. V. & BOWMAN, R. L. (1958). An instrument and method for automatic, rapid, accurate, and sensitive titration of chloride in biological samples. *J. Lab. clin. Med.* **51**, 461–468.

DAVIS, B. J. (1964). Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121**, 404–427.

DAWES, C. (1967). The secretion of magnesium and calcium in human parotid saliva. *Caries Res.* **1**, 333–342.

DAWES, C. (1969). The effects of flow rate and duration of stimulation on the concentrations of protein and the main electrolytes in human parotid saliva. *Archs oral Biol.* **14**, 277–294.

DAWES, C. (1974*a*). Rhythms in salivary flow rate and composition. *Int. J. Chronobiol.* **2**, 253–279.

DAWES, C. (1974*b*). The effects of flow rate and duration of stimulation on the concentrations of protein and the main electrolytes in human submandibular saliva. *Archs oral Biol.* **19**, 887–895.

DAWES, C. & CHEBIB, F. S. (1972). The influence of previous stimulation and the day of the week on the concentrations of protein and the main electrolytes in human parotid saliva. *Archs oral Biol.* **17**, 1289–1301.

DAWES, C. & JENKINS, G. N. (1964). The effects of different stimuli on the composition of saliva in man. *J. Physiol.* **170**, 86–100.

DENNISS, A. R., SCHNEYER, L. H., SUCANTHAPREE, C. & YOUNG, J. A. (1978). Actions of adrenergic agonists on isolated excretory ducts of submandibular glands. *Am. J. Physiol*, **235**, F548–F556.

GANONG, W. F. (1979). *Review of Medical Physiology*. 9th edn., p. 441. Los Altos: Lange.

GILMAN, S., THORNTON, R., MILLER, D. & BIERSNER, R. (1979). Effects of exercise stress on parotid gland secretion. *Horm. Metab. Res.* **11**, 454.

GRON, P. & HAY, D. I. (1976). Inhibition of calcium phosphate precipitation by human salivary secretions. *Archs oral Biol.* **21**, 201–205.

HAY, D. I. (1973). The interaction of human parotid salivary proteins with hydroxyapatite. *Archs oral Biol.* **18**, 1517–1529.

LEACH, S. A. (1977). Mode of action of chlorhexidine in the mouth. In *The Borderland between Caries and Periodontal Disease*, ed. LEHNER, T., pp. 105–128. London: Academic.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265–275.

MCCANN, H. G. (1968). Inorganic components of salivary secretions. In *Art and Science of Dental Caries Research*, ed. HARRIS, R. S., pp. 55–73. New York: Academic.

SALMINEN, S. & KONTTINEN, A. (1963). Effect of exercise on Na and K concentrations in human saliva and serum. *J. appl. Physiol.* **18**, 812–814.

SHANNON, I. L. (1967). Effect of exercise on parotid fluid corticosteroids and electrolytes. *J. dent. Res.* **46**, 608–610.

SPEIRS, R. L., HERRING, J., COOPER, W. D., HARDY, C. C. & HIND, C. R. K. (1974). The influence of sympathetic activity and isoprenaline on the secretion of amylase from the human parotid gland. *Archs oral Biol.* **19**, 747–752.

TRUELOVE, E. L., BIXLER, D. & MERRITT, A. D. (1967). Simplified method for collection of pure submandibular saliva in large volumes. *J. dent. Res.* **46**, 1400–1403.

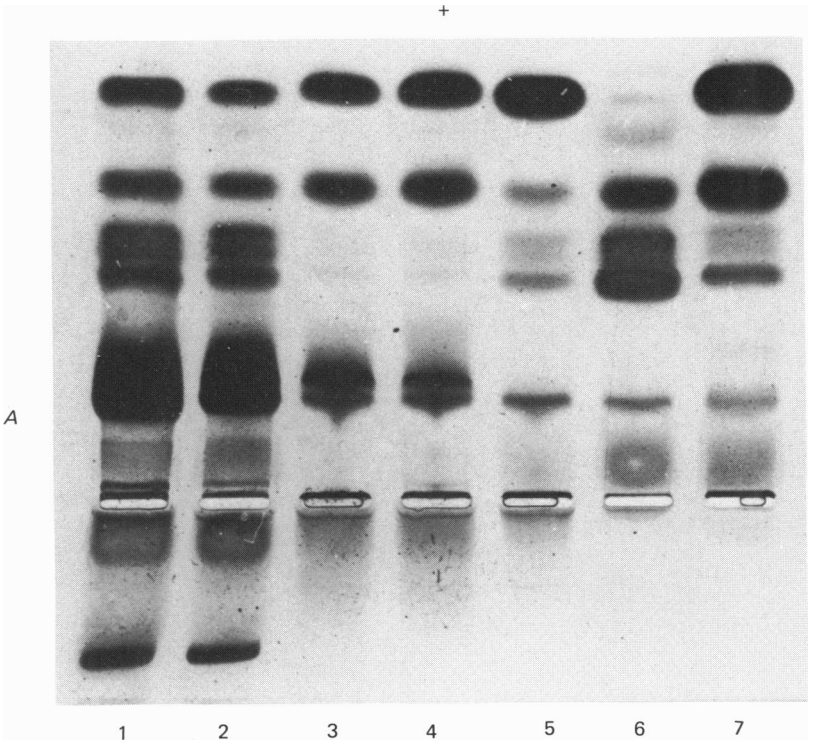
EXPLANATION OF PLATES

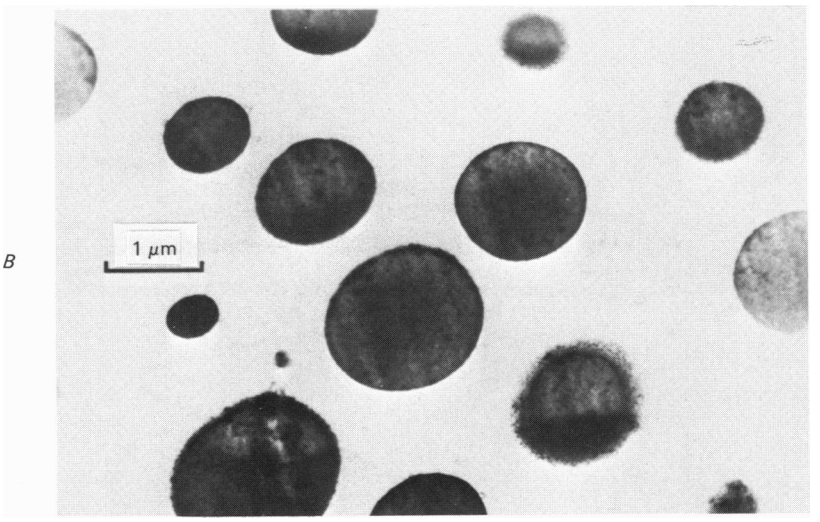
PLATE 1

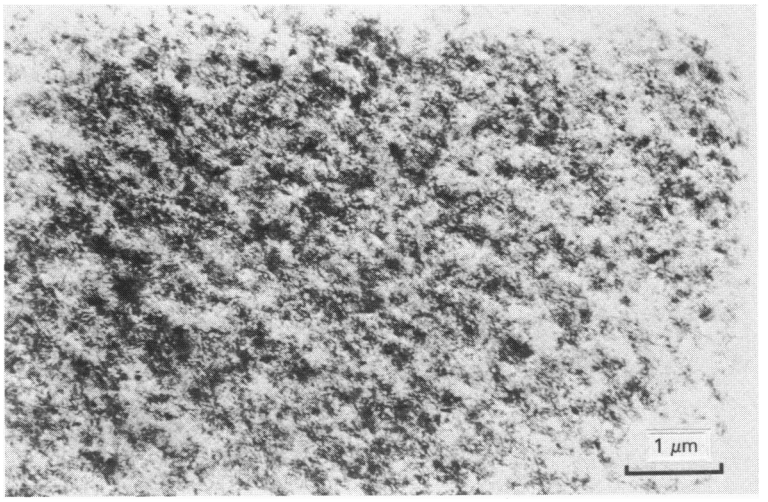
A, Slab gel anionic polyacrylamide gel electrophoresis of parotid salivary proteins. See text for details of source of proteins in the seven slots. The plus sign indicates the position of the anode. *B*, electron micrograph of the centrifuged deposit of cloudy parotid saliva obtained immediately after exercise.

PLATE 2

Electron micrograph of the centrifuged precipitate formed after addition of calcium chloride to clear parotid saliva.


+
A
1
2
3
4
5
6
7


B
1 μm



C. DAWES