J. Physiol. (1980), **299**, *pp.* 29–44
With 4 text-figures
Printed in Great Britain

THE EFFECT OF CARBONIC ANHYDRASE INHIBITORS ON THE ANIONIC COMPOSITION OF SHEEP'S PAROTID SALIVA

By J. R. BLAIR-WEST, R. T. FERNLEY, J. F. NELSON, E. MARELYN WINTOUR and R. D. WRIGHT

From the Howard Florey Institute of Experimental Physiology and Medicine, University of Melbourne, Parkville, 3052 Australia

(*Received 9 May 1978*)

With an Appendix on uncatalysed carbon dioxide–water kinetics by P. T. McTigue

From the Department of Physical Chemistry, University of Melbourne, Parkville, Australia

SUMMARY

1. The effects of the carbonic anhydrase inhibitors, acetazolamide, ethoxzolamide and benzolamide on the ionic composition of parotid saliva were studied in anaesthetized sheep with access to the parotid blood vessels.

2. The inhibitors were infused directly into the arterial blood supply to the gland to give blood concentrations in the range 10^{-5} to 10^{-2} M.

3. Mean anionic concentrations at basal flow rate before inhibitor infusion were, bicarbonate 98 m-mole/l., phosphate 15 m-mole/l. and chloride 26 m-mole/l. In the presence of inhibitors, bicarbonate concentration fell by 11 m-mole/ml. and phosphate and chloride concentrations rose. Secreto-motor nerve stimulation increased bicarbonate concentration by 13 m-mole/l. before infusion of inhibitors and the concentrations of the other anions fell. The bicarbonate rise was abolished by the inhibitors and the fall in phosphate concentration was balanced by a rise in chloride concentration.

4. These effects show that only a small component of the bicarbonate ion transfer system in the sheep parotid gland is sensitive to these inhibitors.

5. The relationship of these findings to a new enzyme with carbonic anhydrase action isolated from the sheep's parotid gland is discussed.

INTRODUCTION

Earlier reports (Coats, Denton, Goding & Wright, 1956; Denton, 1956; Coats & Wright, 1957; Coats, Denton & Wright, 1958; Blair-West, Coghlan, Denton, Nelson, Wright & Yamauchi, 1969; Kraintz, Blair-West, Coghlan, Denton & Wright, 1972), have examined the composition of sheep's parotid saliva and the changes of composition caused by stimulation of salivary secretion rate and by the action of aldosterone. The major cations are Na and K and the sum of their concentrations in end-duct saliva is usually 170–180 m-mole/l. At normal Na status, the concen-

0022-3751/80/6900-0451 $07.50

tration of Na is 165–175 m-mole/l. and K is 4–9 m-mole/l. but, in Na deficiency, the salivary Na concentration is reduced and the K concentration is elevated, the sum $[Na^+]+[K^+]$ staying approximately constant. In both of these conditions, stimulation of the secretomotor nerve (Moussu's nerve) or natural reflexes, e.g. rumination, increase salivary production over a range of 0·25–7 ml./min with $[Na^+]+[K^+]$ remaining almost constant in the outflow.

The anions demonstrated in sheep's parotid saliva are bicarbonate, phosphate and chloride and the sum of their concentrations is approximately equal to $[Na^+]+[K^+]$ (Coats & Wright, 1957; Coats *et al.* 1958). The predominant anion is bicarbonate, 90–120 m-mole/l. Electrical stimulation of the secretomotor nerve increases the bicarbonate concentration with corresponding changes in the phosphate and chloride concentration so that the total anion concentration remains almost constant (Coats & Wright, 1957). Maximal saliva flow under electrical stimulation was 5–7 ml./min Thus the parotid gland may secrete bicarbonate at 4–5 times plasma concentration at rates up to 50 m-mole/hr. Coats *et al.* (1958) calculated that the metabolic activity of a parotid gland under maximal nerve stimulation may produce bicarbonate at 75 μmole/min = 4·5 m-mole/hr, which is approximately 10% of the appearance rate in end-duct saliva at maximal flow. Therefore approximately 90% of the salivary HCO_3^- at maximal secretion must have been taken from the blood as CO_2 or HCO_3^- or both and appeared in the saliva as HCO_3^-. If it were a system involving transfer of CO_2 to the cells and then HCO_3^- to saliva, the rate of appearance at maximal flow would be faster than spontaneous hydration of CO_2 at the existing CO_2. It appeared worth testing whether carbonic anhydrase action was necessary for this secretion of HCO_3^-.

Previous reports on the effects of acetazolamide administration on bicarbonate concentration in saliva record variable and inconsistent effects, probably due to low dosage in some and trivial HCO_3^- secretion in other experiments, e.g. Chauncy & Weiss (1958); Niedermeier, Stone, Dreizen & Spies (1955); Yoshimura, Iwasaki, Nishikawa & Matsumoto (1952); Bruzilow & Diaz (1962); Young, Martin, Asz & Weber (1970); Somner, Kaiser & Drack (1975). Carbonic anhydrase inhibitors do influence the elaboration of acid and alkaline secretions in the alimentary tract (Maren, 1967) and the reabsorption of bicarbonate ion in the kidney (Pitts, 1963; Maren, 1967; Rector, 1973). In these studies however, the anionic balances between blood and secretion or blood and tubular fluid were not investigated. In view of our ability to isolate and sample the arterial blood supply and venous effluent of the parotid gland and thereby derive ionic balances (Coats & Wright, 1957; Coats *et al.* 1958), we have examined the effect of infusion of acetazolamide (Diamox) and other carbonic anhydrase inhibitors on the bicarbonate, phosphate and chloride relations in blood and parotid saliva at basal and stimulated flow rates.

METHODS

The experiments were performed on eleven cross-bred Merino sheep weighing 16·9–45·0 kg. The animals were usually given 10 g NaCl by intraruminal tube on the day before the experiment in order to avoid the possibility of sodium depletion. Anaesthesia was induced by I.V. Na thiopentone and was continued, after tracheal intubation, by closed-circuit halothane and O_2.

Breathing was regulated by forced ventilation at 20 c/min. Blood gases were monitored by the use of a Corning Model 165 Blood Gas Analyser, and the amplitude of ventilation was adjusted to control arterial blood pH, P_{CO_2} and P_{O_2}.

Dissection was performed with full asepsis. Cannulation of the parotid duct and facial artery, preparation of the secretomotor nerve (Moussu's nerve) for stimulation, and vascular isolation of the parotid gland have been described previously (Coats *et al.* 1956). The ipsilateral sympathetic trunk was divided in the neck, during the course of the preparation, to prevent uncontrolled effects of reflex stimulation.

All samples of blood and saliva were collected directly into syringes without obstruction or contact with air. The venous blood was withdrawn from the cannulated butt of the facial vein after clamping the jugular vein below it. Blood flow rate was assessed by stopwatch and plasma flow from the haematocrit. Saliva was collected by inserting a needle with a side-arm into the parotid duct cannula with the direct channel connected to a syringe and the side-arm connected to 45 cm of polythene tubing (1·5 mm i.d.). Saliva was collected by aspiration into the syringe keeping at least 10 cm of the side-arm tubing filled with saliva. This procedure was adopted after establishing: (i) that delivery of saliva from the parotid duct cannula to the sampling chamber of the gas analyser via long (transit time 2·5 min) or short (transit time 30 sec) polythene tubing made no difference to measurements of pH and P_{CO_2}; (ii) that measurements of pH and P_{CO_2} in saliva delivered through tubing to the chamber of the gas analyser were the same whether measurements were made during continuous or stopped flow; and (iii) that the bicarbonate concentration of saliva samples kept in syringes for 1 hr did not alter significantly.

Blood and saliva samples were assayed for pH, P_{CO_2} and P_{O_2} by the Corning Model 165 Blood Gas Analyser used for anaesthetic guidance. This instrument measures pH, P_{CO_2} and P_{O_2} independently of each other by means of three separate electrodes. Na, K, total and inorganic phosphate in plasma and saliva samples were estimated by Technicon Auto-analyser using standard methods. Bicarbonate was calculated from total CO_2 and pH.

Results are expressed throughout as mean $\pm$ S.E. of mean and statistical analysis of all results were performed by a paired t test, using each animal as its own comparison.

Experimental protocol. Control saliva and arterial blood samples were collected and, in some experiments, saliva samples were then collected during stimulation of Moussu's nerve. Then the parotid gland was vascularly isolated and control saliva and facial arterial and venous blood samples were collected; in some experiments further samples were taken during stimulation of Moussu's nerve. Carbonic anhydrase inhibitor was infused into the carotid artery via the lingual artery stump and thus into the parotid arterial blood supply, except in the first experiment when a single dose was given I.V. Saliva was collected at basal salivary flow rate before inhibitor infusion, and 10 min after starting infusion at low and at high rates and during secretomotor nerve stimulation, beginning 5 min after starting stimulation.

Three carbonic anhydrase inhibitors were used: acetazolamide (Diamox, Lederle); ethoxzolamide (U4191, lot no. 1035A, Upjohn) and benzolamide (CL11,366, 5417B-168A, Lederle). All infusion solutions were prepared in 0·9% NaCl. Solution of ethoxzolamide and benzolamide was achieved by warming and by the dropwise addition of a minimum volume of 2 M-NaOH. The pH of the infusion solutions was 7–8. One inhibitor was tested in each experiment. Acetazolamide was given in six experiments, ethoxzolamide in three experiments and benzolamide in two experiments. Acetazolamide was given by single I.V. injection at 20 mg/kg in one experiment. In all other experiments, the inhibitor was given by continuous infusion at a volume flow (0·05–0·5 ml./min) and at concentrations that were adjusted to the prevailing parotid blood flow to give a steady blood concentration of inhibitor for each incident of infusion in the range 10^{-6} to 10^{-2} M. After infusion for 30–40 min at a low dose rate (calculated blood concentration of inhibitor 10^{-6} to 10^{-3} M), the rate was increased to a higher level for another 30–40 min (calculated blood concentration 10^{-3} to 10^{-2} M).

The blood concentrations of inhibitors, calculated from known infusion rates and venous blood flows, were checked by assay in one experiment with each inhibitor. Concentrations of acetazolamide and benzolamide in venous plasma and saliva samples were measured by an enzyme inhibitor assay similar to that described by Yakatan, Martin & Smith (1976). Purified ovine erythrocyte carbonic anhydrase was inhibited by known concentrations of acetazolamide or benzolamide and a standard curve (% inhibition *vs.* inhibitor concentration) was constructed. Inhibition caused by plasma and saliva samples gave the inhibitor concentration after correction for control plasma and saliva blanks. Ethoxzolamide in venous plasma and saliva in one experiment was estimated chemically (United States Pharmacopeia, 1975).

RESULTS

1. *Composition of end-duct parotid saliva without inhibitors*

Table 1 shows the mean composition of end-duct saliva and arterial and venous plasma after vascular isolation of the parotid gland. The pH of venous blood was consistently lower than the pH of arterial blood ($P < 0{\cdot}01$). The large arterio-venous phosphate difference ($1{\cdot}45 \pm 0{\cdot}09$ and $0{\cdot}90 \pm 0{\cdot}09$ m-mole/l. respectively) was highly significant ($P < 0{\cdot}001$). Falls in bicarbonate concentration, 27 ± 1 to 25 ± 2 m-mole/l., and rises in chloride concentration, 92 ± 1 to 95 ± 2 m-mole/l, from artery to vein were small but consistent and both changes were significant (for bicarbonate $P < 0{\cdot}05$, for chloride $P < 0{\cdot}001$). The rise in chloride is related to the relatively greater shift of water into the saliva.

Bicarbonate concentration in saliva varied from 93 to 113 m-mole/l. compared with variation from 17 to 32 m-mole/l. in arterial plasma and 16–30 m-mole/l. in venous plasma. That is, the salivary bicarbonate concentration remained relatively constant despite almost 100% variation in plasma concentrations. Phosphate concentration in saliva varied from 9 to 22 m-mole/l. compared with 1·1–1·9 m-mole/l. in arterial plasma and 0·4–1·4 m-mole/l. in venous plasma. Phosphate was concentrated nearly twentyfold in saliva compared with venous plasma. P_{CO_2} values in saliva and arterial and venous plasma, 45 ± 4, 43 ± 4 and 47 ± 5 mmHg respectively, were not significantly different.

The basal flow rate of parotid saliva was $23{\cdot}8 \pm 1{\cdot}0$ μl./g.gland.min^{-1} and neurally stimulated flow rate was 392 ± 44 μl./g.gland.min^{-1} frcm a large series of observations. Stimulation of Moussu's nerve (six of eleven experiments) increased mean salivary flow approximately twelvefold. Na and K concentrations in saliva, being close to 165 and 10 m-mole/l. respectively, were not significantly changed (cf. Coats *et al.* 1957; Blair-West *et al.* 1969). Bicarbonate concentration rose significantly ($P < 0{\cdot}01$) and the mean rise of 12·7 m-mole/l. was approximately compensated by the combined mean fall of 2·0 m-mole/l. in phosphate concentration and the mean fall of 7·0 m-mole/l. in chloride concentration although these changes separately did not reach statistical significance. Approximately 100% of phosphate occurs as HPO_4^{2-} at parotid salivary pH and approximately 80% occurs as the divalent ion at blood pH.

2. *Effect of carbonic anhydrase inhibitors on the composition of end-duct saliva (Table 1)*

Relations between calculated blood concentration and measured concentrations in venous blood plasma and saliva were as follows. Measured concentrations of acetazolamide in venous plasma in one experiment were $2{\cdot}5 \times 10^{-5}$ M for the lower infusion rate (calculated blood value 10^{-5} M) and $1{\cdot}5 \times 10^{-3}$ M at the higher infusion rate (calculated blood value 10^{-3} M). The same assay gave acetazolamide concentrations of $1{\cdot}1 \times 10^{-6}$ M and $0{\cdot}8 \times 10^{-3}$ M respectively in the matching saliva samples. In another experiment, estimates of benzolamide in venous plasma were 4×10^{-6} and $1{\cdot}4 \times 10^{-3}$ M compared with calculated blood values of 10^{-5} and 10^{-3} M respectively. Saliva samples collected at the same time contained benzolamide at $1{\cdot}2 \times 10^{-8}$ and

TABLE 1. Composition of end-duct parotid saliva and arterial and venous plasma (mean ± S.E. of mean)

	n	Flow rate (ml./min)	pH	P_{CO_2} (mmHg)	Na (m-mole/l.)	K (m-mole/l.)	Phosphate (m-mole/l.)	HCO_3 (m-mole/l.)	Cl (m-mole/l.)
Plasma									
Arterial	11	—	7·46 ± 0·04*	43 ± 4*	146 ± 1	3·7 ± 0·2	1·45 ± 0·08	27 ± 1	92 ± 1
Venous	11	7·3 ± 1·6*	7·38 ± 0·04*	47 ± 5*	146 ± 1	3·5 ± 0·2	0·90 ± 0·09	25 ± 2	95 ± 2
Saliva									
Control									
Basal 1	11	0·25 ± 0·02	7·97 ± 0·05	45 ± 4	159 ± 3	12·9 ± 2·3	15 ± 2	99 ± 3	29 ± 2
Stimulated	6	4·2 ± 0·6	8·06 ± 0·04	43 ± 7	165 ± 2	7·5 ± 1·7	11 ± 1	118 ± 3	20 ± 4
Basal 2	11	0·30 ± 0·04	7·97 ± 0·05	43 ± 4	160 ± 3	12·1 ± 2·5	15·1 ± 1·5	98 ± 2	26 ± 2
Acetazolamide (all infusion rates)									
Basal	11	0·27 ± 0·05	7·92 ± 0·03	46 ± 3	164 ± 4	10·3 ± 2·7	19·8 ± 1·3	91 ± 3	28 ± 1
Stimulated	6	2·9 ± 0·8	7·93 ± 0·07	42 ± 10	168 ± 2	8·1 ± 1·1	15·3 ± 1·2	93 ± 4	36 ± 4
All inhibitors (high infusion rates)									
Basal	11	0·24 ± 0·04	7·88 ± 0·03	46 ± 5	169 ± 4	13·5 ± 3·7	22·1 ± 1·6	87 ± 3	29 ± 2
Stimulated	11	2·54 ± 0·47	7·93 ± 0·10	42 ± 6	169 ± 3	10·5 ± 4·1	17·0 ± 1·1	91 ± 3	34 ± 3

* Whole blood measurements.

$1{\cdot}4 \times 10^{-5}$ M respectively. Ethoxzolamide, chemical assay in one experiment, gave values of $4{\cdot}8 \times 10^{-5}$ M in venous plasma and $1{\cdot}7 \times 10^{-5}$ M in saliva when the calculated blood concentration was $1{\cdot}4 \times 10^{-4}$ M.

(a) *At basal flow rates of saliva*

(i) *Acetazolamide.* Analysis by 'paired t test' of the changes that occurred during acetazolamide infusion at all rates in the six experiments showed that changes in salivary flow rate, pH, P_{CO_2} and K concentration were not significant. Salivary bicarbonate concentration fell in five of the six experiments. The fall increased with increased blood level of acetazolamide. Control bicarbonate concentration was 101 ± 3 m-mole/l. and fell to 91 ± 3 m-mole/l. during acetazolamide infusion. The mean difference was highly significant ($P < 0{\cdot}01$). This fall was associated with significant rises in salivary phosphate concentration, from $13{\cdot}7 \pm 1{\cdot}0$ to $19{\cdot}8 \pm 1{\cdot}3$ m-mole/l. ($P < 0{\cdot}001$), in chloride concentration, from $23{\cdot}5 \pm 1{\cdot}2$ to $28{\cdot}3 \pm 1{\cdot}3$ m-mole/l. ($P < 0{\cdot}05$), and in Na concentration, from 156 ± 4 to 164 ± 4 m-mole/l. ($P < 0{\cdot}01$).

The sum of the anionic concentrations, bicarbonate + phosphate + chloride, tended to increase during acetazolamide infusion. The mean rise was $8{\cdot}7 \pm 1{\cdot}8$ m-equiv/l. ($P < 0{\cdot}01$) at the higher dose of acetazolamide. This trend was associated with an increase in the sum of the Na and K concentrations, mean rise $11{\cdot}5 \pm 3{\cdot}2$ m-equiv/l. ($P < 0{\cdot}05$), which was mainly due to the rise in Na concentration.

(ii) *Ethoxzolamide and benzolamide.* Changes in the anionic composition of parotid saliva during three experiments with ethoxzolamide were similar to the changes observed with acetazolamide. Salivary bicarbonate concentration fell by approximately 14 m-mole/l., i.e. 13%, and phosphate concentration rose during ethoxzolamide infusion. Changes in Na and chloride concentrations were small. Benzolamide had no consistent effect on salivary composition, in two experiments.

(iii) *All inhibitors at high dosage.* The salivary data at the higher level of inhibitor in the eleven experiments were taken as a group and analysed by 'paired t test' against the appropriate control data. Results were: a significant fall in salivary flow rate from $0{\cdot}30 \pm 0{\cdot}04$ to $0{\cdot}24 \pm 0{\cdot}04$ ml./min (significance of mean difference, $P < 0{\cdot}05$), a significant rise in Na concentration from 160 ± 3 to 169 ± 4 m-mole/l. ($P < 0{\cdot}05$), a very highly significant rise in phosphate concentration from $15{\cdot}1 \pm 1{\cdot}5$ to $22{\cdot}1 \pm 1{\cdot}6$ m-mole/l. ($P < 0{\cdot}001$), and a highly significant fall in bicarbonate concentration from 98 ± 2 to 87 ± 3 m-mole/l. ($P < 0{\cdot}01$). The change of chloride concentration from 26 ± 2 to 29 ± 2 m-mole/l. was not significant. Changes in salivary pH, P_{CO_2} and K concentration were also not significant.

Infusion of carbonic anhydrase inhibitors had no regular effect on parotid blood flow or venous blood composition. Control blood flow was $7{\cdot}3 \pm 1{\cdot}6$ ml./min and the value after infusion of inhibitors at the higher dosage was $8{\cdot}3 \pm 1{\cdot}1$ ml./min (mean difference n.s. by paired t test).

Paired comparison of saliva with venous blood P_{CO_2} levels during the control period and during infusion of carbonic anhydrase inhibitors showed that differences were not significant. Mean control values (mmHg) were $43 \pm 3{\cdot}6$ in saliva and $45 \pm 5{\cdot}2$ in venous blood; mean values during low dosage of inhibitors were $40 \pm 3{\cdot}3$ and $47 \pm 4{\cdot}3$, and at high dosage $46 \pm 4{\cdot}8$ and $44 \pm 4{\cdot}7$, for saliva and venous blood respectively. Differences between saliva and simultaneous arterial blood P_{CO_2} levels in control and

both inhibitor infusion periods were not significant. Arterio-venous differences in P_{CO_2} during these periods were also not significant. Blood samples were collected during stimulation of Moussu's nerve in the presence of carbonic anhydrase inhibitor in five experiments. There were no significant differences between saliva and arterial and venous blood P_{CO_2} values at stimulated flow rates.

Salivary and concurrent blood P_{CO_2} values (Fig. 1) gave correlation coefficients of 0·94 ($P < 0·001$) between control values, 0·72 ($P < 0·001$) between values during

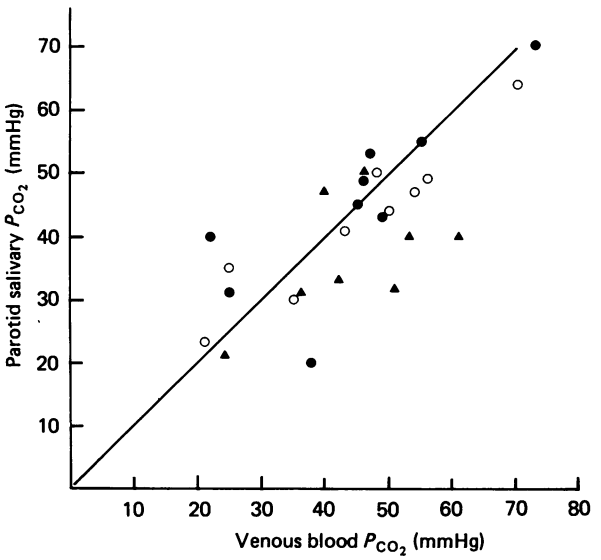

Fig. 1. Relation between parotid venous blood and salivary P_{CO_2}. ○, control; ▲, low-dose carbonic anhydrase inhibitors; ●, high dose carbonic anhydrase inhibitors. The line shows a 1:1 relationship.

inhibitor infusion, and 0·79 ($P < 0·001$) between all values taken together. Regression coefficients for these three sets of values were each not significantly different from 1·00.

Correlation coefficients were calculated to assess the significance of relations between salivary bicarbonate ion concentration and blood P_{CO_2} and bicarbonate levels (Figs. 2 and 3). In the control period, there were no significant correlations between salivary bicarbonate concentration and arterial or venous P_{CO_2} and bicarbonate levels (all values of $r < 0·27$; $n = 11$). Correlations between these variables during infusion of carbonic anhydrase inhibitors were also not significant (all values of $r < 0·31$, $n = 11$).

(*b*) *At high flow rates of saliva*

Stimulation of Moussu's nerve during infusion of carbonic anhydrase inhibitors increased parotid blood flow from $8·3 \pm 1·1$ to $27·5 \pm 5·5$ ml./min, thus reducing the blood concentration of inhibitor to about 30% of the value at basal flow, i.e. from 10^{-3} to 10^{-2} M down to 3×10^{-4} to 3×10^{-3} M. The normal time delay of 2–3 sec for onset of increase in saliva flow following secretomotor nerve stimulation was not

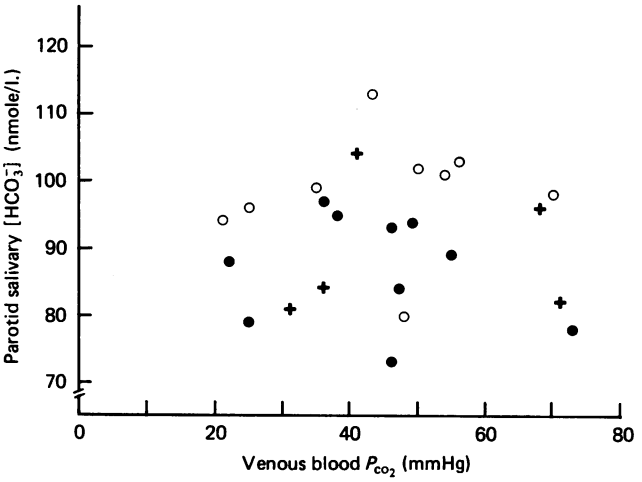

Fig. 2. Relation between parotid venous blood P_{CO_2} and salivary [HCO_3^-]. ○, control (high dose); ●, carbonic anhydrase inhibitors; +, carbonic anhydrase inhibitors (high dose) + stimulation.

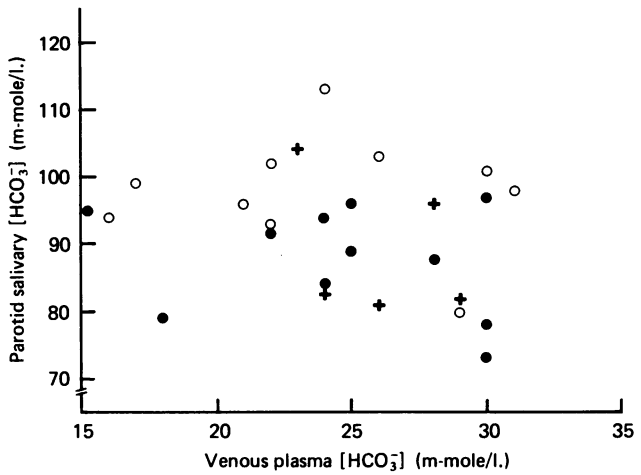

Fig. 3. Relation between parotid venous blood [HCO_3^-] and salivary [HCO_3^-]. ○, control; ●, carbonic anhydrase inhibitors (high dose); +, carbonic anhydrase inhibitors (high dose) + stimulation.

altered by the presence of inhibitors. In the presence of acetazolamide (six experiments), saliva flow rate rose from 0·27 ± 0·05 to 2·9 ± 0·8 ml./min. Changes in salivary pH, P_{CO_2}, Na and K concentrations were not significant. Salivary bicarbonate concentration did not increase significantly during stimulation of Moussu's nerve, as it had before acetazolamide infusion. Mean bicarbonate concentration was 90 ± 4 m-mole/l. at resting flow and 93 ± 4 m-mole/l. at stimulated flow. Salivary phosphate concentration fell ($P < 0·01$) from a mean value of 21·0 ± 2·0 to 15·3 ± 1·2 m-mole/l. The trend to increased chloride concentration from 30 ± 3 m-mole to 36 ± 4 m-mole/l. was not significant. When experiments with all inhibitors were pooled, the change in bicarbonate concentration from 87 ± 3 to 91 ± 3 m-mole/l. during Moussu's nerve

TABLE 2. Relations of bicarbonate and phosphate in saliva and blood. Rates of appearance in saliva and removal from blood (μmole/min)

			Removal from plasma and erythrocytes			
	n	Appearance in saliva	Apparent disappearance from plasma	Estimated loss from red blood cells	From secreted plasma H_2O	Total
			(A) Bicarbonate			
(1) Before inhibitors						
Basal salivary flow	11	29 ± 4	12·6	1·9	8·4	22·9
(2) After inhibitors						
(*a*) Basal salivary flow	11	22 ± 4	15·5	2·3	6·5	24·3
(*b*) Stimulated salivary flow	11	238 ± 51				
Blood sampled	5	194 ± 37	111	16	66	193
			(B) Phosphate			
(1) Before inhibitors						
Basal salivary flow	11	4·4 ± 0·6	3·1	0·7	0·4	4·2
(2) After inhibitors						
(*a*) Basal flow	11	5·2 ± 0·6	4·3	1·0	0·4	5·7
(*b*) Stimulated salivary flow	11	43 ± 8				
Blood sampled	5	35 ± 8	22	5·3	3·2	31

* These calculations were based on: (1) measured concentrations of inorganic phosphate in sheep red blood cells; (2) the assumption that the ratio $[HCO_3^-]$ red blood cells/$[HCO_3^-]$ plasma equals that for man; (3) the assumption that the % extraction of HCO_3^- and inorganic phosphate from red blood cells is the same as observed for plasma.

stimulation was not significant, the fall in phosphate concentration from 22·1 ± 1·6 to 17·0 ± 1·2 m-mole/l. was still highly significant ($P < 0·01$), and the rise in chloride concentration from 29 ± 2 to 34 ± 3 m-mole/l., reached significance ($P < 0·05$).

(*c*) *Relation between plasma and salivary concentrations of bicarbonate and phosphate at basal and stimulated saliva flow* (*Table 2*)

Stimulation of Moussu's nerve before infusion of carbonic anhydrase inhibitors increased bicarbonate output in saliva from 42 ± 10 to 488 ± 67 μmole/min and phosphate output rose from 5·9 ± 2·0 to 49 ± 9 μmole/min. Just before the infusion of carbonic anhydrase inhibitors, bicarbonate output in saliva was 29 ± 4 μmole/min

and phosphate output was $4{\cdot}4 \pm 0{\cdot}6$ μmole/min. From Table 2, the loss of bicarbonate from the blood was 23 μmole/min before inhibitors and 24 μmole/min during infusion of inhibitors. The concomitant values for phosphate are 4·2 and 5·7 μmole/min respectively. When salivary flow was stimulated the output of bicarbonate and phosphate increased to 194 ± 37 and 35 ± 8 μmole/min respectively in the five experiments for which blood samples were collected and the losses from the blood rose to 193 and 31 μmole/min.

In view of the very small differences between the bicarbonate appearing in the saliva and that lost from the blood the probable CO_2 produced was calculated on the basis of blood flow, arterial and venous P_{O_2}, haematocrit and R.Q. 0·86. The mean values which resulted were (*a*) control – basal flow, 9·8 μmole/min, (*b*) low dose inhibitor – basal flow, 8·0 μmole/min, (*c*) high dose inhibitor – basal flow, 7·9 μmole/min, (*d*) high dose inhibitor – stimulated flow, 17·9 μmole/min.

DISCUSSION

Maren (1977) reported that 10^{-4} M-acetazolamide and 10^{-5} M-ethoxzolamide are sufficient for full inhibition of carbonic anhydrase activity in most tissues. The small effect of concentrations up to 10 times these levels on the secretion of bicarbonate by the sheep's parotid gland raised the possibility that carbonic anhydrase in this gland might be insensitive to the inhibitors. Fernley, Coghlan & Wright (1979) demonstrated two substances with carbonic anhydrase activity. By anatomical separation and by histochemical demonstration (Hansson, 1968) carbonic anhydrase appears only in the end-pieces of the gland (G. B. Ryan, personal communication). The predominant enzyme has the same activity characteristics, molecular weight and K_I values as the ubiquitous one in erythrocytes. The other has 10 times the molecular weight but $\frac{1}{6}$–$\frac{1}{3}$ the sensitivity to inhibitors.

The small effect on HCO_3^- transport was not due to the inhibitors infused not gaining access to all compartments of the parotid gland – vascular, cellular and salivary. Evidence for penetration of the gland by the inhibitor was obtained from concentrations of inhibitors in contemporaneous plasma and saliva samples during inhibitor infusion (cf. Rasmussen, 1964). Saliva:plasma concentration ratios were 0·53 for acetazolamide, 0·35 for ethoxzolamide and 0·01 for benzolamide, i.e. concentrations up to 5×10^{-3} M for acetazolamide and 10^{-3} M for ethoxzolamide in saliva. Thus, the slightly higher degree of resistance of the second enzyme to inhibitors does not explain the high degree of insensitivity to them of the HCO_3^- transport by the gland. The concentration ratios are consistent with the pK_a, binding to plasma proteins, and lipid solubility of these three inhibitors (Maren, 1963*a*, 1977). The low ratio for benzolamide is consistent with evidence of its relative exclusion from most tissues (Maren, 1977). This also related to the proposition of Rector, Carter & Seldin (1965) that acetazolamide acts at the luminal surface of the proximal convoluted tubules of the kidney.

Another possible reason for the small effect of carbonic anhydrase inhibition of $CO_2 + HCO_3^-$ transport via a molecular CO_2 step might be that the reactions $CO_2 + H_2O \rightarrow H_2CO_3$ or $HCO_3^- \rightarrow OH^- + CO_2$, uncatalysed, could support the traffic. The possible pathways (*A* and *B*) are shown in Fig. 4. The uncatalysed reaction rates for

the several steps are known. The pH of the plasma and saliva was measured; the pH of intracellular content is not likely to be higher than that of plasma (Kostyuk, Sorokina & Kholodova, 1969). The contact time of blood with secretory tissue was estimated from the preparations previously reported (Blair-West *et al.* 1969) at 0·4 sec at maximal stimulation. Metabolic production of CO_2 was estimated, and P_{CO_2} and $[HCO_3^-]$ in blood and saliva and the flow rates of these were measured. With these data, the possible total transfer of bicarbonate through the molecular

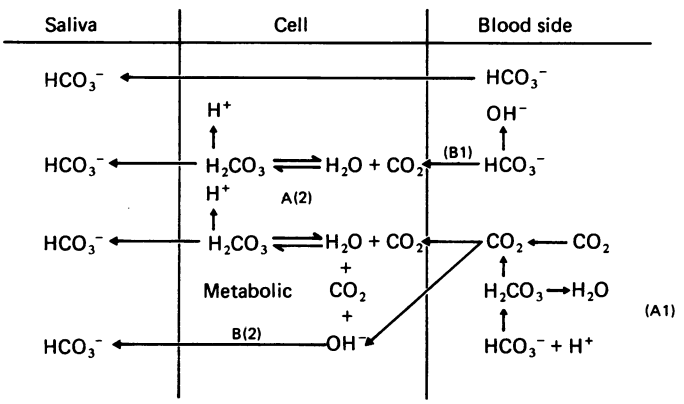

Fig. 4. Schema of possible pathways for HCO_3^- and CO_2 from blood or cells to parotid saliva. The top pathway indicates direct transport of HCO_3^- from blood to saliva. The lower pathways indicate transfers via molecular CO_2.

CO_2 steps at 38 °C was estimated at only 10 % of the HCO_3^- appearing in the saliva (see Appendix for details of calculations).

A further point to note is that carbonic anhydrase qua enzyme cannot contribute to the free energy change of the system which is of the order of 900 cal per mole of bicarbonate raised from plasma concentration 27 to 118 m-mole/l. at 38 °C. There was no correlation between the P_{CO_2} in plasma and the $[HCO_3^-]$ in saliva and therefore there must be an energy link to the transfer mechanism.

The proposition that transfer of HCO_3^- as such is the major method of secretion requires a depletion of plasma HCO_3^- equivalent to a high proportion of that appearing in the saliva. At basal flow, the calculated net removal of bicarbonate from arterial blood was 23 μmole/min compared with a mean output of 29 μmole/min in saliva (Table 2). During inhibitor infusion, the calculated removal was 24 μmole/min at basal flow and 193 μmole/min at stimulated flow compared with salivary outputs of 22 ± 4 and 194 ± 37 μmole/min respectively.

It seems reasonable therefore to propose that the major bicarbonate ion transfer system in the sheep's parotid gland when producing a bicarbonate concentration of approximately 100 m-mole/l. and secreting under stimulation about eleven times as much bicarbonate as when unstimulated does not involve molecular CO_2. This transfer is reduced by only about 10 % by acetazolamide and ethoxzolamide at blood levels up to 10^{-2} M. The only evidence of rate limitation by carbonic anhydrase inhibition at high secretion rates during secretomotor nerve stimulation is that the rise in

[HCO_3^-] in these circumstances does not occur, even though the amount of bicarbonate secreted increases by a factor of 10. There is also a fall of bicarbonate concentration by 10% at basal secretion rates. There appears, therefore, to be a component of the bicarbonate transfer system, accounting for about 10% of the transfer and susceptible to these inhibitors.

Related to this argument is the evidence for interrelated transfer of bicarbonate, phosphate and chloride ions in the formation of sheep parotid saliva. At basal flow rate, the small fall in bicarbonate concentration that followed infusion of carbonic anhydrase inhibitors was associated with a rise in phosphate concentration. In the absence of inhibitors, neural stimulation increased bicarbonate concentration and this was associated with falls in phosphate and chloride concentrations so that the sum of these anionic concentrations remained nearly constant. In the presence of inhibitors, neural stimulation did not increase bicarbonate concentration and the fall in phosphate concentration, which was associated with a reduction of plasma phosphate from an arterial value of $1{\cdot}48 \pm 0{\cdot}09$ to $0{\cdot}40 \pm 0{\cdot}14$ m-mole/l. in the venous outflow, i.e. 73% extraction, was balanced by a rise in chloride concentration. In these exiguous bicarbonate and phosphate conditions the discrepancy between bicarbonate + phosphate concentrations and Na + K concentrations is compensated with the copiously available chloride ion. The observation that parathyroid hormone (PTH) also causes a reciprocal change of bicarbonate and phosphate concentrations in sheep's parotid saliva (Clark, French, Beal, Cross & Budtz-Olsen, 1975), i.e. a lowering of bicarbonate and an increase of phosphate concentration, is a further indication of a common or linked mechanism. This similarity of action of carbonic anhydrase inhibitors and PTH in the sheep's parotid gland may be related to (i) their common action of increasing renal excretion of phosphate (Maren, 1967; Goldberg, 1973; Mudge, Berndt & Valtin, 1973) and (ii) the ability of carbonic anhydrase inhibitors to inhibit the PTH induced increase in plasma Ca concentration in nephretomized rats (Waite, 1972).

This work was supported by a grant-in-aid from the National Health and Medical Research Council of Australia. Benzolamide was generously supplied by Lederle Laboratories Division, Cyanamid Australia Pty Ltd, and ethoxzolamide was generously donated by The Upjohn Co., Kalamazoo, Michigan, U.S.A. The authors wish to thank Dr J. G. Mackenzie, Melbourne Diagnostic Group, Richmond, Victoria, for chemical assay of ethoxzolamide.

APPENDIX

Introduction

The purpose of this note is to calculate the maximum possible rate of transfer of $CO_2 + HCO_3^-$ from the blood and gland tissue to saliva, in the stimulated sheep's parotid gland, via molecular CO_2 as a stage of the processes *A* and *B* of the schema (Fig. 4) under complete carbonic anhydrase inhibition. Using values which are known, or the maximal biologically feasible and favourable for the conditions on which the rate of these reactions are dependent, the results give an extreme value of $\simeq$ 10% of the observed value for these processes. This indicates that at least 90% of the transfer occurs as transport of ionic HCO_3^-.

Stimulated flow rates and other relevant data for both plasma and saliva under conditions of carbonic anhydrase inhibitors are given in Table 3. Two points of particular significance are as follows.

(1) The net rate of salivary $CO_2+HCO_3^-$ secretion is about 25% of the rate of $CO_2+HCO_3^-$ delivery to the gland by the arterial blood.

(2) Even if all the CO_2 present in equilibrium with HCO_3^- in arterial blood were transported as such into the saliva, this could account for no more than one fifth of the observed $CO_2+HCO_3^-$ secretion rate in the saliva.

TABLE 3. Data for stimulated saliva flow under condition of carbonic anhydrase inhibition

	Flow rate (P = cm^3 min^{-1})	pH	[HCO_3^-] = m-mole l.$^{-1}$	[CO_2] = m-mole l.$^{-1}$
Arterial plasma	22·74	7·46	31·4	1·7
Venous plasma	20·6	7·40	26	1·6
Saliva	2·14	8·06	89	1·2

If the mechanism of transport of $CO_2+HCO_3^-$ is completely via CO_2, this would require an initial dehydration of arterial HCO_3^- to CO_2, followed by transport of CO_2 and its subsequent re-hydration to HCO_3^-. This argument depends on the maximum rate of these dehydration-rehydration processes. In the presence of carbonic anhydrase inhibitors, these rates can be calculated from known rate constants in enzyme-free systems. Table 3 shows the values needed for these calculations. The calculations show that even if all CO_2 which could be formed by the uncatalyzed dehydration of HCO_3^- in arterial plasma during its time of contact with the secretory tissue of the salivary gland were transported into the saliva, this could account for only $\sim$ 1% of the observed rate of appearance of HCO_3^- in saliva, i.e. reactions $A(1)$ and $B(1)$ could provide $\sim$ 1% of the observed rate.

Model calculations

A very simple model of the salivary gland system is adequate for calculation of the maximum possible rate of transport of CO_2 due to the uncatalysed rate of dehydration of HCO_3^-. Subscripts a, v and s depict a quantity characteristic of arterial plasma, venous plasma and saliva respectively; flow rates are represented by P. For the whole gland

$$P_a = P_v + P_s.$$

Ignoring metabolic production of CO_2 the rate of delivery, R_d, of $HCO_3^- + CO_2$ to the gland is given by

$$[HCO_3^-]_a P_a + [CO_2]_a P_a + R_b = R_d,$$

where R_b is an estimated value of the HCO_3^- delivered by the red blood corpuscles (see Table 2). Using data from Table 3,

$$R_d = 0{\cdot}714 + 0{\cdot}041 + 0{\cdot}016 = 0{\cdot}77 \text{ m-mole min}^{-1}.$$

Note that the CO_2 constitutes $\sim$ 5% of the total $CO_2+HCO_3^-$ in arterial plasma.

The rate of removal of $HCO_3^- + CO_2$ by salivary secretion, R_s, is given by

$$R_s = [HCO_3^-]_s P_s + [CO_2]_s P_s.$$

$$\therefore \quad R_s = 0{\cdot}19 + 0{\cdot}0026 = 0{\cdot}19 \text{ mmole min}^{-1}.$$

This is nearly 5 times the actual rate of CO_2 delivery by the arterial blood.

Finally, the rate of removal of HCO_3^- and CO_2 in the venous blood should be

$$R_d - R_s = 0{\cdot}77 - 0{\cdot}19 = 0{\cdot}58 \text{ m-mole min}^{-1}.$$

The data of Table 3 confirm this figure.

Clearly, if transport of $HCO_3^- + CO_2$ is to occur via CO_2 transport only, substantial amounts of the HCO_3^- in arterial blood must first be dehydrated to CO_2.

The dehydration of HCO_3^- can be represented schematically as

$$H^+ + HCO_3^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} H_2CO_3 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} CO_2 + H_2O,$$

$$HCO_3^- \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} CO_2 + OH^-.$$

Since it is well known that the second reaction makes no significant contribution to the dehydration rate at pH values less than 7·5 (Garg & Maren, 1972) it will henceforth be neglected.

To calculate the maximum possible rate of production of CO_2 the rehydration of CO_2 is ignored as if all the CO_2 formed were removed instantly from the reaction site. We shall also assume that *all* the CO_2 formed in this dehydration is successfully transported from the blood, into the saliva, and there re-hydrated. Clearly this will set an *absolute upper limit* to the rate of $CO_2 + HCO_3^-$ available for transfer via CO_2 transport.

The net rate of HCO_3^- dehydration is then given by

$$-\frac{d}{dt}[HCO_3^-] = k'[HCO_3^-] = \frac{k_2 k_1}{k_{-1}}[H^+][HCO_3^-]$$

All the rate constants have either been directly measured, or can be calculated exactly from other known rate constants and equilibrium constants. Thus, at 38 °C

$$k_2 = 49{\cdot}5 \text{ sec}^{-1} \qquad \text{(Garg \& Maren, 1972)},$$

$$k_{-1}/k_1 = K_a = 3{\cdot}22 \times 10^{-4} \text{ M},$$

where K_a is the apparent acidity constant of carbonic acid, H_2CO_3, corrected to an osmolality of 0·29 from the true thermodynamic value of $1{\cdot}58 \times 10^{-4}$ (Garg & Maren, 1972).

At the arterial blood pH of 7·46, and allowing for an activity coefficient of 0·7 for H^+ at an osmolality of 0·29, we have

$$[H^+] = 4{\cdot}95 \times 10^{-8} \text{ M},$$

so that

$$\frac{k_2 k_1}{k_{-1}}[H^+] = 0{\cdot}0076 \text{ sec}^{-1},$$

i.e. $k' = 0{\cdot}0076 \text{ sec}^{-1}$.

In order to calculate the maximum possible rate of CO_2 delivery by dehydration of HCO_3^- in arterial blood, we must also know the contact time, t_c, during which the CO_2 transfer is possible. This can be estimated from the known dimensions and density of the capillaries in the gland, and has been calculated to be 0·4 sec or 0·006 67 min (see Discussion). This figure can be used to calculate a contact volume, V_c, i.e. the volume of blood in effective contact with the secretory part of the gland at any given time:

$$\begin{aligned} V_c &= P_a . t_c \\ &= 0{\cdot}000\,15L. \end{aligned}$$

Thus, the absolute maximum rate of delivery of CO_2 to saliva by dehydration of arterial HCO_3^- will be

$$\begin{aligned} k' \times [\dot{H}CO_3^-]_a \times V_c &= 0{\cdot}0076 \times 0{\cdot}0314 \times 0{\cdot}000\,15 \times 60 \\ &= 2{\cdot}1 \times 10^{-6} \text{ mole min}^{-1} \\ &= 0{\cdot}0021 \text{ m-mole min}^{-1}. \end{aligned}$$

This represents about 1 % of the observed total rate of $CO_2 + HCO_3^-$ transfer from arterial blood into the saliva.

The only sources of molecular CO_2, available for transfer into saliva, are the CO_2 present in equilibrium with HCO_3^- in arterial plasma and the CO_2 produced by gland metabolism. As stated in the main report, O_2 use indicates a metabolic yield of 0·0179 m-mole min^{-1} of molecular CO_2 which could be hydrated uncatalysed in the tissue water ($\sim$ 9 ml. at pH $<$ 7·4 and 38 °C; direct measurements of intracellular pH have not been observed above 7·3 (Kostyuk *et al.* 1969)). On the assumption that the concentration of CO_2 in plasma in the secretory area does not drop below its equilibrium venous value of 1·6 m-mole l.$^{-1}$, the maximum possible rate of transfer of $HCO_3^- + CO_2$ via a CO_2 stage in the circumstances of these experiments with stimulated salivary flow was 0·0179 + 0·0021 = 0·020 m-mole min^{-1}, i.e. 10 % of the 0·19 m-mole min^{-1} appearing in the saliva.

REFERENCES

Blair-West, J. R., Coghlan, J. P., Denton, D. A., Nelson, J. F., Wright, R. D. & Yamauchi, A. (1969). Ionic, histological and vascular factors in the reaction of the sheep's parotid to high and low mineralocorticoid status. *J. Physiol.* **205**, 563–579.

Brusilow, S. W. & Diaz, C. L. (1962). Effect of acetazolamide on dog parotid saliva. *Am. J. Physiol.* **202**, 158–160.

Chauncey, H. H. & Weiss, P. A. (1958). Composition of human saliva. Parotid gland secretion: flow rate, pH and inorganic composition after oral administration of a carbonic anhydrase inhibitor. *Archs int. Pharmacodyn. thér.* **113**, 377–383.

Clark, R. C., French, T. J., Beal, A. M., Cross, R. B. & Budtz-Olsen, O. E. (1975). The acute effects of intravenous infusion of parathyroid hormone on urine, plasma and saliva in the sheep. *Q. Jl exp. Physiol.* **60**, 95–106.

Coats, D. A., Denton, D. A., Goding, J. R. & Wright, R. D. (1956). Secretion by the parotid gland of the sheep. *J. Physiol.* **131**, 13–31.

Coats, D. A., Denton, D. A. & Wright, R. D. (1958). The ionic balances and transferences of the sheep's parotid gland during maximal stimulation. *J. Physiol.* **144**, 108–122.

Coats, D. A. & Wright, R. D. (1957). Secretion by the parotid gland of the sheep: the relationship between salivary flow and composition. *J. Physiol.* **135**, 611–622.

Denton, D. A. (1956). The effect of Na^+ depletion on the Na^+:K^+ ratio of the parotid saliva of the sheep. *J. Physiol.* **131**, 516–525.

FERNLEY, R. T., COGHLAN, J. P. & WRIGHT, R. D. (1979). A high molecular weight carbonic anhydrase from ovine parotid tissue. *Proc. XIth Int. Congr. Biochem.* (in the press).

GARG, L. C. & MAREN, T. H. (1972). The rates of hydration of carbon dioxide and dehydration of carbonic acid at 37 °C. *Biochim. biophys. Acta* **261**, 70–76.

GOLDBERG, M. (1973). The renal physiology of diuretics. In *Handbook of Physiology*, section 8, *Renal Physiology*, ed. ORLOFF, J. & BERLINER, R. W., pp. 1003–1032. Washington, D.C.: American Physiological Society.

HANSSON, H. P. J. (1968). Histochemical demonstration of carbonic anhydrase activity in some epithelia noted for active transport. *Acta physiol. scand.* **73**, 427–434.

KOSTYUK, P. G., SOROKINA, A. & KHOLODOVA, YU. D. (1969). Measurement of hydrogen, potassium and sodium ions in striated muscle fibres and nerve cells. In *Glass Microelectrodes*, ed. LAVALLÉE, M., SCHANNE, O. F. & HÉBERT, N. C., pp. 322–348. New York: Wiley.

KRAINTZ, L., BLAIR-WEST, J. R., COGHLAN, J. P., DENTON, D. A. & WRIGHT, R. D. (1972). Aldosterone action on the parotid gland: sodium replete sheep. *Acta endocr., Copenh.* **70**, 510–514.

MCDOUGALL, E. I. (1948). Studies on ruminant saliva. 1. The composition and output of sheep's saliva. *Biochem. J.* **43**, 99–109.

MAREN, T. H. (1963*a*). The relation between enzyme inhibition and physiological response in the carbonic anhydrase system. *J. Pharmac. exp. Ther.* **139**, 140–153.

MAREN, T. H. (1963*b*). Carbonic anhydrase kinetics and inhibition at 37°: an approach to reaction rates *in vivo*. *J. Pharmac. exp. Ther.* **139**, 129–139.

MAREN, T. H. (1967). Carbonic anhydrase: chemistry, physiology and inhibition. *Physiol. Rev* **47**, 595–781.

MAREN, T. H. (1977). Use of inhibitors in physiological studies of carbonic anhydrase. *Am. J. Physiol.* **232**, *F*291–297.

MUDGE, G. H., BERNDT, W. A. & VALTIN, H. (1973). Tubular transport of urea, glucose, phosphate, uric acid, sulfate and thiosulfate. In *Handbook of Physiology*, section 8, *Renal Physiology*, ed. ORLOFF, J. & BERLINER, R. W., pp. 587–652. Washington, D.C.: American Physiological Society.

NIEDERMEIER, W., STONE, R. E., DREIZEN, S. & SPIES, T. D. (1955). Effect of 2-acetylamino-1,3,4-thiadiazole-5-sulfonamide (Diamox) on sodium, potassium, bicarbonate and buffer content of saliva. *Proc. Soc. exp. Biol. Med.* **88**, 273–275.

PITTS, R. F. (1963). *Physiology of the Kidney and Body Fluids*, p. 165. Chicago: Year Book Medical Publishers.

RASMUSSEN, F. (1964). Salivary excretion of sulphonamides and barbiturates by cows and goats. *Acta pharmac. tox.* **21**, 11–19.

RECTOR, F. C. JR. (1973). Acidification of the urine. In *Handbook of Physiology*, section 8, *Renal Physiology*, ed. ORLOFF, J., & BERLINER, R. W., pp. 431–454. Washington, D.C.: American Physiological Society.

RECTOR, F. C. JR., CARTER, N. W. & SELDIN, D. W. (1965). The mechanism of bicarbonate reabsorption in the proximal and distal tubules of the kidney. *J. clin. Invest.* **44**, 278–290.

SOMMER, H. M., KAISER, D. & DRACK, E. (1975). pH and bicarbonate excretion in the rat parotid gland as a function of salivary rate. *Pflügers Arch.* **355**, 353–360.

UNITED STATES PHARMACOPEIA (1975). P. 190. Rockville: U.S. Pharmacopeial Convention Inc.

WAITE, L. C. (1972). Carbonic anhydrase inhibitors, parathyroid hormone and calcium metabolism. *Endocrinology* **91**, 1160–1165.

YAKATAN, G. J., MARTIN, C. A. & SMITH, R. V. (1976). Enzymatic determination of acetazolamide in human plasma. *Analytica chim. Acta* **84**, 173–177.

YOSHIMURA, H., IWASAKI, H., NISHIKAWA, T. & MATSUMOTO, S. (1959). Role of carbonic anhydrase in the bicarbonate excretion from salivary glands and mechanism of ionic excretion. *Jap. J. Physiol.* **9**, 106–123.

YOUNG, J. A., MARTIN, C. J., ASZ, M. & WEBER, F. D. (1970). A microperfusion investigation of bicarbonate secretion by the rat submaxillary gland. *Pflügers Arch.* **319**, 185–199.