The Function of Carbonic Anhydrase in the Stomach

By R. E. DAVIES AND J. EDELMAN

*Medical Research Council Unit for Research in Cell Metabolism,
Department of Biochemistry, The University, Sheffield 10*

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Berend (1937) suggested that part of the mechanism of gastric hydrochloric acid production might be the catalysed hydration of carbon dioxide to carbonic acid by carbonic anhydrase, but he failed to find significant quantities of the enzyme in the stomach. However, Davenport & Fisher (1938) showed conclusively that there are large amounts in gastric mucosa, and Davenport (1939, 1940*a*) found that most of the gastric carbonic anhydrase is in the oxyntic cells.

Davenport (1940*b*) discovered that thiocyanate could inhibit both carbonic anhydrase activity and acid secretion in dogs, but Feldberg, Keilin & Mann (1940, 1948) found that concentrations of thiocyanate, which strongly inhibited gastric acid secretion in decerebrate cats, inhibited gastric carbonic anhydrase by only about 10%. They also found that concentrations of sulphanilamide sufficient to inhibit the enzyme between 80% and 'completely' did not decrease the rate of acid secretion. Similar failures to inhibit acid secretion in dogs, cats or turtles with sulphanilamide or the more powerful inhibitor, thiophene-2-sulphonamide were reported by Davenport (1941, 1946*a*) and by Anderson & Wilbur (1948).

Since there is an uptake of one molecule of carbon dioxide to produce a bicarbonate ion for each hydrogen ion secreted, hydration of carbon dioxide certainly occurs in oxyntic cells (Davies, 1948). Moreover, Davies & Roughton (1948) have calculated that (a) this hydration is so fast that carbonic anhydrase activity is needed within mammalian and amphibian oxyntic cells, and (b) the amount of enzyme activity in the cells is apparently in great excess of the requirements. Only about 0.3% or less of the enzyme activity extractable from oxyntic cells is actually required to catalyse the uptake of carbon dioxide even during high rates of hydrochloric acid secretion.

The failure of previous attempts to inhibit acid secretion by inhibitors of carbonic anhydrase can be explained as follows. Roughton (1943) has shown that complete inhibition of this enzyme in the blood should lead to the speedy death of the animal. Since all the animals used in these experiments were respiring normally, there must have been sufficient residual carbonic anhydrase activity in the red cells

and, since there is about five times as much of this enzyme in oxyntic cells as in red cells (Davenport, 1939), the gastric enzyme could not have been completely inhibited *in vivo*.

This difficulty has been avoided in the experiments described below by the use *in vitro* of tied bags of isolated frog and toad gastric mucosa. After virtually complete inhibition of the carbonic anhydrase, acid secretion ceased and the mucosa was damaged. Such damage did not occur in similar experiments with non-secreting gastric mucosa.

A part of the present work was communicated to the Biochemical Society (Davies & Edelman, 1948).

EXPERIMENTAL

Methods. The saline media, manometric procedure, treatment of tissues and methods of estimation of the rates of acid secretion were those described by Davies (1948) and Davies & Longmuir (1948).

The muscle layers were removed from the well washed stomach of a frog or toad, and the tube of gastric mucosa formed was tied with silk at both ends. The tied bag was incubated in a glucose-containing physiological saline solution in a double-armed Warburg cup containing histamine (final concentration 5×10^{-6} M) in one arm, and a solution of the inhibitor in the other. Acid secretion commenced either spontaneously or following the addition of histamine, and the inhibitor was then added to see if it interfered with the normal course of secretion.

All experiments were performed at least in triplicate, and usually in quintuplicate. Concordant results were always obtained. Values of Q_{O_2} and Q_{HCl} are in μ l./mg. dry wt./hr.

Inhibitors. The carbonic anhydrase inhibitors used were sulphanilamide (Mann & Keilin, 1940), thiophene-2-sulphonamide (Davenport, 1945), *p*-toluenesulphonamide, *p*-sulphonamidobenzoic acid and 4'-sulphonamidophenyl-2-azo-7-acetamido-1-hydroxynaphthalene-3:6-disulphonic acid (prontosil soluble) (Krebs, 1948). They were all neutralized with NaOH before use.

Estimation of carbonic anhydrase activity. At the end of the incubation the gastric mucosa was ground with sand and the carbonic anhydrase activity of aqueous extracts estimated at 0° by the manometric method of Krebs & Roughton (1948). Sufficient inhibitor was added to the phosphate and bicarbonate solutions used in this method to keep the concentration the same as that in the medium during the original incubation of the mucosa.

RESULTS

Effects on acid secretion. Preliminary experiments showed, as anticipated, that a relatively high concentration of carbonic anhydrase inhibitor was needed to affect acid secretion by the oxyntic cells.

Fig. 1. Effect of *p*-toluenesulphonamide on acid secretion by isolated frog gastric mucosa. Histamine added first, followed by the inhibitor to final concentrations of 3×10^{-3} or 1.7×10^{-2} M at arrows marked *p*-TS.

Fig. 2. Effect of 10^{-3} M-prontosil soluble and 10^{-2} M-*p*-sulphonamidobenzoate on histamine-stimulated acid secretion by isolated toad gastric mucosa.

The addition of *p*-toluenesulphonamide to final concentrations of 10^{-5} , 10^{-4} , 10^{-3} or 3×10^{-3} M had no observable effect on respiration or acid secretion by isolated gastric mucosa, but at 10^{-2} M the Q_{HCl} was lowered. In every case 1.7×10^{-2} M-*p*-toluenesulphonamide caused complete inhibition of acid

secretion, but this always occurred after a lag period. There was usually only slight inhibition during the first 0.5 hr. after addition, but acid secretion was

Fig. 3. Lack of effect of 1.7×10^{-2} M-*p*-toluenesulphonamide, added at arrows marked *p*-TS, on unstimulated respiration of some isolated toad tissues.

Fig. 4. Inhibition of respiration of toad-kidney slices following addition of 1.7×10^{-2} M-*p*-toluenesulphonamide at arrow marked *p*-TS.

Fig. 5. Inhibition of histamine-stimulated respiration of isolated acid-secreting toad gastric mucosa following addition of 1.7×10^{-2} M-*p*-toluenesulphonamide at arrow marked *p*-TS.

completely stopped within 1–1.5 hr. (Fig. 1). This delayed, but complete, inhibition of acid secretion was also observed with 10^{-2} M-*p*-sulphonamidobenzoate, 10^{-2} M-thiophene-2-sulphonamide and 10^{-3} M-prontosil soluble (Fig. 2), but not with 10^{-3} or 7×10^{-3} M-sulphanilamide. Not only was acid

secretion stopped, but the acid already secreted into the mucosal bag began to leak outwards (Figs. 1 and 2).

Effects on respiration and aerobic glycolysis. It was important to find out whether this inhibition of acid secretion was caused by some non-specific toxic action of these substances. The unstimulated respiration of isolated toad-liver slices, sartorius muscle, back skin, pancreas or gastric mucosa was found to be unaffected by 10^{-5} , 10^{-4} , 10^{-3} , 3×10^{-3} , 10^{-2} or 1.7×10^{-2} M-*p*-toluenesulphonamide (Fig. 3). In contrast with this the respiration of toad-kidney slices was found to be inhibited by 1.7×10^{-2} M-*p*-toluenesulphonamide (Fig. 4). The histamine-stimulated respiration of gastric mucosa was inhibited by 1.7×10^{-2} M-*p*-toluenesulphonamide in the way expected if the respiration of the oxyntic cells were abolished after a lag period in a similar fashion to the inhibition of acid secretion (Fig. 5). Experiments with open sheets of histamine-stimulated gastric mucosa in bicarbonate saline showed that 1.7×10^{-2} M-*p*-toluenesulphonamide increased the aerobic glycolysis. This increase in aerobic glycolysis also occurred in the presence of 10^{-2} M-thiophene-2-sulphonamide and 10^{-2} M-*p*-sulphonamidobenzoate, but was much smaller with prontosil soluble. Such increases in aerobic glycolysis are a common sign of cell damage.

If the incubation was continued for more than 1 hr. after the addition of these powerful carbonic anhydrase inhibitors to actively acid-secreting gastric mucosa, ulcerations and perforations occurred which were indistinguishable from those described by Davies & Longmuir (1948) for active mucosa stimulated to secrete hydrochloric acid in the absence of external supplies of carbon dioxide.

Effects on carbonic anhydrase activity. The carbonic anhydrase content of the gastric mucosa of frogs and toads used was found to be from 0.1–0.2 Davenport unit/mg. wet wt. This is similar to the values found for frogs by Davenport (1946b). Numerous estimations were made of the carbonic anhydrase activity of dilute and concentrated extracts prepared by grinding the gastric mucosa with sand after the incubations with inhibitor. In no case where acid secretion had been abolished was any trace of activity found. This failure to detect any activity, by the method of Krebs & Roughton (1948), in concentrated extracts of gastric mucosa means that the carbonic anhydrase must have been inhibited more than 99%.

Although prontosil soluble is highly coloured and is an exceedingly powerful inhibitor of carbonic anhydrase, it was not found possible to use it to determine the distribution of this enzyme in the oxyntic cells, because this compound stains proteins unspecifically and was diffusely distributed in the mucosa.

DISCUSSION

Although sulphonamides inhibit carbonic anhydrase solutions instantaneously (Mann & Keilin, 1940), it is of interest that the effects on acid secretion became apparent only after a lag period. Carbonic anhydrase is needed to catalyse the hydration of carbon dioxide and hence prevent the accumulation of alkali in acid-secreting oxyntic cells. The resultant bicarbonate ions are then exchanged for chloride ions from the medium. Complete inhibition of the enzyme should thus lead to the disorganization of acid-secreting oxyntic cells after a time lag, and this actually occurs.

Mann & Keilin (1940) showed that the type of sulphonamide used in these experiments does not inhibit respiration, glycolysis or a variety of enzyme systems, even in very high concentrations. They wrote: 'These inhibitors therefore make it possible to abolish the activity of carbonic anhydrase in cells and organisms without affecting the activity of other known catalytic systems.' The unstimulated respiration of frog-liver slices, sartorius muscle, back skin, pancreas or gastric mucosa was unaffected by the inhibitors, and in these cases carbonic anhydrase was either absent or non-functional. The respiration of acid-secreting gastric mucosa was lowered by the amount expected if damage to the oxyntic cells followed the inhibition of carbonic anhydrase (Fig. 5). These results, together with the increase in glycolysis and the appearance of ulceration and perforations in mucosae which had been actively secreting acid, show that the inhibitors only affect gastric mucosa when it is secreting acid. This strongly supports the view that their action is entirely dependent on their ability to inhibit carbonic anhydrase. It is of interest that the ulcerations and perforations observed when actively acid-secreting mucosae were deprived of external supplies of carbon dioxide can also appear when the external supplies of carbon dioxide are made unavailable to the oxyntic cells by the inhibition of carbonic anhydrase.

The inhibitors are needed in very high concentrations judged by their effectiveness in free solution at 0° (Davenport, 1945; Krebs, 1948). This is expected for several reasons. Carbonic anhydrase activity is present in many cells in great excess and high concentrations of inhibitor have been needed to demonstrate the effects of the inhibition of carbonic anhydrase on carbon dioxide evolution from blood *in vitro* and *in vivo* (Keilin & Mann, 1941; van Goor, 1944; Jacobs & Stewart, 1942; Anderson & Thomson 1948) and on acid secretion and ammonia excretion by kidney (Höber, 1942; Pitts & Alexander, 1944; Pitts & Lotspeich, 1946; Ferguson, 1951). For blood *in vitro*, Davenport (1946a) has calculated that if less than 97% of the enzyme is inhibited the cata-

lysed reaction is entirely uninhibited, and to reduce the catalysed rate by 90 % more than 99.8 % of the enzyme must be inhibited. The fact that it is more difficult to show effects of inhibiting carbonic anhydrase in the stomach than in the kidney and lungs may be partly because 'in the kidney as in the lungs the rate of flow of a fluid through a tube sets a time limit within which the required series of reactions must occur' (Davenport, 1946*a*). Another factor is the large excess of carbonic anhydrase activity even in oxyntic cells which are secreting acid at high rates (Davies & Roughton, 1948), and the fact that the uncatalysed hydration of carbon dioxide within oxyntic cells is sufficiently fast on its own to maintain the acid-base balance during rates of acid secretion of the order of one-tenth the maximum observed rates.

In the non-secreting oxyntic cells, as in other cells, carbon dioxide evolution occurs because the cytoplasmic pH is lower than the equilibrium value: in the acid-secreting oxyntic cell carbon dioxide uptake occurs because the cytoplasmic pH is higher than the equilibrium value, since hydrogen ions are continuously removed to form hydrochloric acid. The amount of carbonic anhydrase activity needed within acid-secreting oxyntic cells depends on the extent to which the pH of the cytoplasm is alkaline to the equilibrium value. Davies & Roughton (1948) have calculated that with a Q_{HCl} of 500 in mammalian cells at a pH difference of 0.01 a magnification of the uncatalysed uptake of 400-fold is required, whereas at a pH difference of 0.1 this is diminished to 40-fold. However alkaline the cell might become, a magnification of about ten is required. Since the carbonic anhydrase present is sufficient to produce a magnification of the order of 15,000-fold, a very high degree of inhibition (> 99.7 %) is needed to raise the cytoplasmic pH far enough to interfere with the enzymes involved in acid secretion.

Further reasons for needing high concentrations of these inhibitors are that the presence of protein lowers the effectiveness of these sulphonamides (Krebs, 1948), and that the carbonic anhydrase in the oxyntic cell may be less accessible to the inhibitors than carbonic anhydrase in free solution. Although no direct evidence is available regarding the percentage inhibition of the enzyme in the intact cells, there was no detectable enzyme activity liberated when the mucosae, in which acid secretion had been stopped, were ground with sand.

Sulphanilamide is from ten to one hundred times less effective than the other sulphonamides which were used (Krebs, 1948), and Feldberg *et al.* (1940) found that it did not inhibit acid secretion at $7 \times 10^{-3} M$ concentration in decerebrate cats. This concentration is also ineffective in isolated frog gastric mucosa (Davies & Edelman, 1948). These

experiments were carried out simultaneously with those of Davenport & Jensen (1948) who studied the effects of sulphanilamide and thiophene-2-sulphonamide on hydrochloric acid secretion by isolated mouse stomachs. They obtained a small but significant diminution of acid secretion with $0.0014 M$ thiophene-2-sulphonamide and concluded that '...if carbonic anhydrase is necessary for acid secretion the quantity required is extremely small' (see also Davenport, 1946*a*). These observations are all in accordance with the theory advanced previously (Davies, 1948) that carbonic anhydrase plays a role in maintaining the acid-base balance in oxyntic cells during acid secretion.

Forster (1948), by observing the colour changes of phenol red, showed that isolated frog-kidney slices secrete acid even when unstimulated, and this finding has been confirmed in this laboratory. Davenport & Wilhelmi (1941) found large amounts of carbonic anhydrase in kidney, and it is known that inhibitors of this enzyme stop acid secretion in the frog kidney (Höber, 1942). The effect of *p*-toluenesulphonamide on respiration and acid secretion of frog and toad kidney slices is therefore expected (Davies, 1949).

SUMMARY

1. The presence of carbonic anhydrase in frog and toad gastric mucosa is confirmed.
2. Complete inhibition of hydrochloric acid secretion was produced, after a lag period, in isolated frog and toad gastric mucosa by the following concentrations of carbonic anhydrase inhibitors: $1.7 \times 10^{-2} M$ -*p*-toluenesulphonamide, $10^{-2} M$ -*p*-sulphonamidobenzoate, $10^{-2} M$ -thiophene-2-sulphonamide, and $10^{-3} M$ -prontosil soluble, but not by lower concentrations or by $7 \times 10^{-3} M$ -sulphanilamide.
3. The unstimulated respiration of frog and toad liver slices, sartorius muscle, back skin, pancreas or gastric mucosa was unaltered by these concentrations of the inhibitors.
4. The respiration of acid-secreting frog and toad gastric mucosa was inhibited by these compounds; also, after a lag period, by the amount expected if the acid-secreting cells were damaged following the accumulation of alkali within them. A similar inhibition of respiration occurred in toad kidney slices.
5. No trace of carbonic anhydrase activity, even in concentrated extracts of frog and toad gastric mucosa, could be found after these experiments: the extracted enzyme was inhibited more than 99 %.
6. The results are in accordance with the view that carbonic anhydrase plays a role in maintaining the acid-base balance in oxyntic cells during hydro-

chloric acid secretion, but that the amount of carbonic anhydrase required is only a very small proportion of the total potential activity.

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Isolation of Unequivocal Uroporphyrin III

A FURTHER STUDY OF TURACIN

By R. E. H. NICHOLAS AND C. RIMINGTON

Department of Chemical Pathology, University College Hospital Medical School, London, W.C. 1

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No authentic specimen of uroporphyrin III has until now been prepared either synthetically or from natural material.

The first uroporphyrin to be isolated was that from urine of the congenital porphyria patient Petry. Fischer (1915) originally considered it to be a heptacarboxylic porphyrin, but later (Fischer & Hilger, 1925) established that it was an octacarboxylic acid which could be partially decarboxylated, losing four acid groups and yielding coproporphyrin I, by heating with hydriodic acid in acetic acid or with 1% hydrochloric acid at 180–190° (Fischer, 1916; Fischer & Zerweck, 1924). This reaction established that it belonged to the isomeric series derivable from aetioporphyria I, and Fischer concluded that it had the structure, porphin-1:3:5:7-tetra-acetic acid-2:4:6:8-tetrapropionic acid since comparison with a synthetic 'isouroporphyrin I' having four methyl and four methylmalonic acid

β side chains and with a similar porphyrin having succinic acid instead of methylmalonic acid groups (Fischer & Zischler, 1937; Fischer & Hofmann, 1937) eliminated these structures as constitutional possibilities.

Uroporphyrin I, isolated from bones and urine of congenital porphyria patients and from pearl oyster shells, *Pteria vulgaris* (Fischer & Haarer, 1932), and the pigment from turacin, stated to be uroporphyrin I (Fischer & Hilger, 1924), was the only uroporphyrin known (although the melting points of the methyl esters of different preparations were widely variable from about 273 to 293°) when almost simultaneously, Waldenström, Fink & Hoerbürger (1935; see also Waldenström, 1934, 1935, 1936) and Mertens (1936, 1937) claimed that the urine of sufferers from acute porphyria contained another uroporphyrin, the octamethyl ester of which melted at about 258°, and in the products of the decarboxylation of which