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THE EFFECT OF ACETYLSALICYLIC ACID ON CARBONIC ANHYDRASE *IN VITRO*

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Abstract—Carbonic anhydrase was treated with neutral (pH 7) aspirin solutions and also with solutions of aspirin and other acids of pH 3–5. In subsequent enzyme activity determinations no inhibition by the neutral solutions could be demonstrated, whereas the more acid solutions caused a progressive loss of activity with decreasing pH. This effect appeared to be non-specific with respect to the acid used and to depend only upon the hydrogen ion concentration of the carbonic anhydrase solution. The results obtained provide no support for the suggestion that inhibition of carbonic anhydrase may be a factor in aspirin-induced erosion of gastric mucosa.

THE oral administration of acetylsalicylic acid to man and laboratory animals can result in erosion of the gastric mucosa. The cause of this is unknown. Lish *et al.*[1] reported the inhibition of carbonic anhydrase by acetylsalicylic acid *in vitro* and though they failed to demonstrate inhibition *in vivo*, they suggested that this might under certain conditions be a factor in the erosive action of acetylsalicylic acid.

Davies and Longmuir[2] and Davies and Edelman[3] observed that when isolated frog gastric mucosa was stimulated to secrete acid, erosion of the mucosa occurred when carbon dioxide or bicarbonate was excluded from the external medium, or when carbonic anhydrase inhibitors were present. According to these workers, carbon dioxide is utilised in maintaining the acid–base balance during acid secretion. Carbonic anhydrase, normally present in oxyntic cells, is considered necessary to catalyse the conversion of carbon dioxide to carbonic acid in sufficient amounts to prevent the accumulation of alkali during acid secretion.[4]

Davenport and Jensen[5] have reported the suppression of acid secretion in the excised mouse stomach by certain carbonic anhydrase inhibitors and Janowitz *et al.*[6] observed a reduction in gastric acid secretion in man after the administration of the carbonic anhydrase inhibitor, acetazolamide.

Reports concerning the effect of acetylsalicylic acid on gastric acid secretion in man are contradictory but many workers have reported that acid secretion is reduced in animals.[1, 7, 8] Anderson[9] gave graded doses of acetylsalicylic acid to guinea-pigs and found that basal secretion was suppressed when the dose exceeded 100 mg/kg body weight.

The fact that acetylsalicylic acid and carbonic anhydrase inhibitors appear to have some pharmacological activities in common, prompted us to re-examine the effect of acetylsalicylic acid upon carbonic anhydrase activity *in vitro*. Experimental conditions were selected to test the effect of acetylsalicylic acid upon carbonic anhydrase catalysis

of the reaction $H_2CO_3 \rightleftharpoons H_2O + CO_2$, using a wide range of acetylsalicylic acid to substrate ratios.

EXPERIMENTAL

Materials

All water used in the experiments was de-ionized and CO_2 free. Reagents were of Analar grade; reagent and enzyme solutions were prepared freshly as required. Aqueous solutions of purified carbonic anhydrase (Light & Co., Colnbrook) were used. Certain experiments were repeated using a 1:50 dilution of rat blood as the source of the enzyme.

Methods

Aqueous solutions of acetylsalicylic acid (aspirin) were prepared by dilution of 0·4 M aspirin in ethanol to the required concentration. Two series of solutions were used—(a) Neutral (pH 7) solutions, obtained by dilution with water or buffer and adjustment to pH 7 with 0·2 N NaOH. (b) Simple aspirin solutions of pH 3–4·5 depending upon the aspirin concentrations.

Solutions of salicylic acid and benzoic acid were prepared similarly.

All solutions were cooled to 0–0·5° and carbonic anhydrase activity was determined at this temperature by measuring—(a) The rate of hydration of CO_2 by the indicator method of Maren *et al.*,[10] (b) the rate of CO_2 evolution from bicarbonate solutions by (i) the manometric method of Krebs and Roughton,[10] (ii) an adaption of the method described by Palmer.[12]

The reaction times were determined in triplicate, (a) with no carbonic anhydrase or aspirin present (uncatalysed reaction), (b) with carbonic anhydrase but no aspirin present (enzyme catalysed reaction), (c) in the presence of carbonic anhydrase after treatment with aspirin. The aspirin solutions were maintained in contact with carbonic anhydrase at room temperature for a minimum of 10 min before the enzyme activity was determined. Concentrations reported in the text refer to the concentration of aspirin during this period of contact with the enzyme.

Method of Maren et al.[10] Four millilitres aqueous aspirin (0·05–2 mM) were added to 1 ml carbonic anhydrase solution (8 mg/100 ml water).

Carbonic anhydrase activity was determined by adding 1 ml of either water, or enzyme solution, or enzyme–aspirin solution to 3·5 ml indicator solution (12·5 mg Phenol Red/l. of 2·6 mM $NaHCO_3$) and 1·6 ml water; CO_2 was bubbled through at a constant rate and 0·9 ml buffer solution (30 ml M Na_2CO_3, 20·6 ml M $NaHCO_3$, water to 100 ml) added. The reaction was timed until the indicator had the same colour by visual comparison as that of a standard.

The enzyme catalysed reaction time was 16–20 sec and the uncatalysed reaction time 80 sec.

Method of Krebs and Roughton.[11] Two millilitres 0·04 M aspirin in 0·1 M phosphate buffer (pH 7) and 0·5 ml carbonic anhydrase solution (4 mg/100 ml) were placed in a Warburg flask and 1 ml 0·1 M sodium bicarbonate placed in the side arm. After mixing, the rate of CO_2 evolution was measured by recording the manometer readings after 30 sec and thereafter at 15 sec intervals for 3 to 4 min. The molar ratio of aspirin to substrate (bicarbonate) was approximately 1:1.

Method of Palmer.[12] This method provided an aspirin to substrate ratio of approximately 20:1. A 0·33 M solution of aspirin was prepared in 0·033 M phosphate buffer (pH 7·38), 3 ml of this solution and 0·5 ml carbonic anhydrase solution (10 mg/100 ml) were placed in the reaction chamber of the apparatus which was essentially as described by Palmer, and 10 ml 0·0005 N NaOH solution and one drop of phenolphthalein solution (0·2 per cent) were placed in the collecting vessel. A current of CO_2-free air at constant pressure was passed through the solution, 0·5 ml 0·1 M sodium bicarbonate was added to the reaction vessel and the time recorded for the phenolphthalein in the collecting vessel to be decolourised. The enzyme catalysed reaction time was 1·5 min, and the uncatalysed reaction time about 10 min.

RESULTS

Inhibition of carbonic anhydrase by neutral (pH 7) solutions of aspirin could not be demonstrated by any of the three experimental methods used, with concentrations of aspirin up to 0·28 M and molar ratios of aspirin to bicarbonate up to 20:1. Under the same experimental conditions inhibition of carbonic anhydrase by accepted inhibitors, sulphanilamide and acetazolamide, could readily be demonstrated at $1{\cdot}2 \times 10^{-5}$ M and 1×10^{-7} M respectively, concentrations similar to those reported by other workers.[10, 13, 14]

The effect upon carbonic anhydrase of simple aqueous solutions of aspirin was then studied at the unadjusted pH of these solutions. Reduced carbonic anhydrase activity was observed; the results are given in Table 1. These findings contrast with the lack of

TABLE 1. THE REACTION TIMES FOR THE HYDRATION OF CO_2 IN THE PRESENCE OF CARBONIC ANHYDRASE PREVIOUSLY TREATED WITH AQUEOUS SOLUTIONS OF ASPIRIN, DETERMINED BY THE METHOD OF MAREN *et al.*[10]

Aspirin content of carbonic anhydrase solution		Reaction time*
$10^5 \times$ Conc. (M)		Sec
160		60
80		45
40		34
20		32
16		30
8		28
4		25
0	(Enzyme catalysed reaction)	20
0	(Uncatalysed reaction)	80

* The mean of three determinations.

effect observed with neutral (pH 7) aspirin solutions and suggest that the loss of enzyme activity may be related to the hydrogen ion concentration of the aspirin–enzyme solutions.

Solutions of aspirin (4 mM) were therefore adjusted to pH 3, 4 and 5 with 0·2 M NaOH, and their effect on carbonic anhydrase activity was determined by the method of Maren *et al.*[10] The results are given in Table 2.

To examine the possibility that loss of carbonic anhydrase activity was related only to pH and not to a specific effect of aspirin, the effects of equimolar solutions of aspirin, salicylic acid and benzoic acid upon carbonic anhydrase activity were compared by the same procedure. The effect of acidifying the enzyme solution to pH 3–4 by the addition of HCl was also studied. The results of these experiments (Table 3) show a close correspondence between pH and the degree of reduction of carbonic anhydrase activity.

TABLE 2. THE REACTION TIMES FOR THE HYDRATION OF CO_2 IN THE PRESENCE OF CARBONIC ANHYDRASE PREVIOUSLY TREATED WITH AQUEOUS ASPIRIN SOLUTIONS OF pH 3, 4 AND 5; DETERMINED BY THE METHOD OF MAREN *et al.*[10]

Aspirin content of carbonic anhydrase solution $10^5 \times$ Conc. (M)	pH of solution	Reaction time* Sec
320	3	67
320	4	30
320	5	28
0	(Enzyme catalysed reaction)	20
0	(Uncatalysed reaction)	75

TABLE 3. THE REACTION TIMES FOR THE HYDRATION OF CO_2 IN THE PRESENCE OF CARBONIC ANHYDRASE PREVIOUSLY TREATED WITH EQUIMOLAR SOLUTIONS OF ASPIRIN, SALICYLIC ACID, OR BENZOIC ACID, AND THE EFFECT OF ACIDIFYING THE ENZYME SOLUTION TO pH 3–4 WITH HYDROCHLORIC ACID. THE METHOD OF MAREN *et al.*[10] WAS USED

	Aspirin content and pH of carbonic anhydrase solution		Reaction time*
	$10^5 \times$ Conc. (M)	pH	Sec
Aspirin	320	3·15	70
	160	3·32	59
	80	3·52	31
	40	3·62	26
Salicylic acid	320	2·94	74
	160	3·15	68
	80	3·35	43
	40	3·52	26
Benzoic acid	320	3·37	46
	160	3·55	33
	80	3·73	28
	40	3·90	25
Hydrochloric acid		3·0	76
		3·3	48
		3·6	30
		4·0	25
	0	(Catalysed reaction)	24
	0	(Uncatalysed reaction)	75

* Mean of three determinations.

DISCUSSION

Aspirin solutions of pH greater than four have not been observed to have any effect upon carbonic anhydrase activity *in vitro*, whereas aspirin solutions having a pH less than four have been shown to cause a loss of enzyme activity. This effect appears to be non-specific with regard to the acid used and to depend only upon the hydrogen ion concentration of the carbonic anhydrase solutions.

Keller and Gottwald[15] reported that carbonic anhydrase is irreversibly inactivated by acidification to below pH 3·5 and that the degree of inactivation is proportional to the pH of the enzyme solution.

Lish and co-workers[1] report that aspirin (0·182 mM in the final reaction mixture) inhibited 89·7 per cent of the original carbonic anhydrase activity of rat blood *in vitro*. The pH of the aspirin solution which was in contact with the enzyme was not stated. The direct addition of aspirin to diluted rat blood may well have exposed the enzyme to acidic conditions for long enough to cause its inactivation. However, if the enzyme was only in contact with neutral solutions of aspirin, their results are at variance with those obtained in the present work; a comparable loss of carbonic anhydrase activity having been observed only with aspirin solutions of approximately pH 3.

The results of the present investigation are substantially in agreement with the findings of Keller and Gottwald,[15] but appear to conflict with the findings of Lish and others.[1]

It is considered that for a pharmacological effect of aspirin upon the gastric mucosa to be mediated by the inhibition of carbonic anhydrase, the inhibition must be virtually complete. No such degree of inhibition of carbonic anhydrase by aspirin could be demonstrated *in vitro* within the physiological pH range. Thus, the results of the present study do not support the suggestion that aspirin-induced erosion of the gastric mucosa involves the inhibition of carbonic anhydrase.

If an acid such as aspirin is absorbed from the stomach in its undissociated form, as indicated by the work of Schanker *et al.*,[16] and if the same mechanism is also operative for the removal of the acid from the mucosal cells, the pH gradient between the gastric lumen (pH $\simeq$ 2), and the mucosal cells (pH $\simeq$ 7) will tend to produce an intracellular accumulation of the acid anion.[17] An accumulation of a foreign anion in a mucosal cell could lead to its disorganisation and possibly to its disruption. This would offer an explanation for the observation of Anderson[9] that many acids which are absorbed from the stomach can cause an erosion of the gastric mucosa in experimental animals, similar to that produced by aspirin.

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