Br. J. Pharmac. (1980), **68**, 413–423

ESTIMATION OF pK_B VALUES FOR HISTAMINE H_2-RECEPTOR ANTAGONISTS USING AN *in vitro* ACID SECRETION ASSAY

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1 Histamine H_2-receptor antagonism by burimamide, metiamide and cimetidine was analysed under apparent equilibrium conditions in the lumen-perfused isolated stomach preparation of the mouse.

2 The behaviour of these compounds was not incompatible with simple competitive antagonism but the estimated pK_B values ($-\log K_B$) were all significantly lower, by about 1 log unit, than reference values reported for guinea-pig atrium or rat uterus.

3 There seems no need to propose that the parietal cell receptors are somehow different from the others; metiamide continually appears in the gastric juice so that a steady-state but not equilibrium can presumably be reached with respect to the bath concentration and this could keep the antagonist concentration artificially low in the region of the receptors.

Introduction

Histamine H_2-receptors were originally defined as those membrane sites mediating some histamine responses, e.g. tachycardia, relaxation of rat uterus, dilatation of blood vessels and stimulation of gastric acid secretion (Black, Duncan, Durant, Ganellin & Parsons, 1972). Tests for simple competition and estimations of dissociation constants were performed for a few antagonist/H_2-receptor interactions in *in vitro* atrial and uterine muscle preparations. However, no satisfactory *in vitro* assay for gastric acid secretion was available to test for simple competitive antagonism and to estimate pK_B values ($= -\log K_B$). The selective H_2-receptor antagonists had similar qualitative activity on histamine-stimulated acid secretory responses which were refractory to H_1-receptor blockade by mepyramine (suggesting a homogeneous H_2-receptor population). Nevertheless, the ability of these compounds to inhibit the secretory actions of vagal stimulation or gastrin raises questions about the specificity of their antisecretory actions.

The introduction of the *in vitro* lumen-perfused rat stomach preparation by Parsons (1975) allowed quantitative estimations of agonist-antagonist relationships to be investigated. However, in this preparation, Bunce & Parsons (1976) found that for the assay of the metiamide/histamine interaction the Schild plot had a slope of 0.73 which was significantly less than unity. Apparently one of the necessary conditions for simple competitive antagonism had not been met. Before concluding that metiamide is not a competitive antagonist and therefore probably a non-specific inhibitor of histamine-induced acid secretion, other factors have to be considered which may distort the Schild plot and obscure competitive antagonism. Bunce & Parsons (1976) had examined the antagonist activity of metiamide over only a relatively small (10 fold) concentration range, close to the apparent K_B concentration. Furchgott (1972) has shown that uptake or metabolism of agonist may reduce the effective concentrations at receptor sites. Distortion of the location and slope of the Schild regression can then occur either when the removal processes become saturated at high dose-ratios or when the antagonist inhibits the removal processes as well as the receptors. Furchgott has also drawn attention to the effects of experimental design on the shape of the Schild plot.

The most generally used method of estimating pK_B values from a Schild plot regression depends upon estimation of dose-ratios. The usual experimental design is based on a 'within-preparations' experiment, in which the tissue is exposed to agonist concentrations by sequential or cumulative dose schedules before and after equilibration of the tissue with a concentration of antagonist. With this design, 'between-preparations' variations in sensitivity to the agonist may be eliminated from the estimation. However, within each tissue there may also be some desensitization with time, as was disclosed in pilot experiments for the assay described here.

0007-1188/80/030413-11 $01.00

This time factor cannot be removed by using a balanced design because the procedure of giving the antagonist after the control study cannot be reversed due to the uncertainty of completely washing out the antagonist before starting a new control curve. One way of overcoming this time-dependent desensitization is to correct the shifted dose-response curve by the amount of shift observed in a 'paired' control preparation taken from the same animal (Furchgott, 1967). Although this procedure still leaves some theoretical difficulties (assessment of satisfactory matching, imbalance in treatment schedule and definition of correct control line), the paired-tissue design had to be rejected here because it could not be applied to the whole-stomach preparation.

We have used a different experimental design in which each stomach has only one treatment with an agonist. Employing replicates of each treatment and three different agonist concentrations allows the estimation of parallel concentration-response lines, one for each antagonist level including control. This experimental design and subsequent analysis avoids using the same control data with the data from each antagonist level to estimate dose-ratios, a weakness of the usual 'within-preparation' design because the control data have been obtained no more precisely than the data for the other antagonist concentrations. We have used an iterative computer method to estimate the pK_B by non-linear least-squares as first described by Waud (1975).

In this paper, the assay technique and analysis used to estimate the antagonist pK_B values of burimamide, metiamide and cimetidine against histamine- or dimaprit-stimulated acid secretion in the isolated whole stomach of the mouse preparation are evaluated.

Methods

Acid secretion

Mice of either sex (Charles-River, 25 to 30 g), having had free access to food and water, were anaesthetized with ether or killed directly. Silastic cannulae with short polyethylene tips were tied into the pyloric and fundic parts of the stomach. The oesophagus was ligated and the stomach contents then washed out with 5 ml warm, oxygenated, unbuffered solution (mM: NaCl 135, KCl 4.8, $MgSO_4$ 1.2, $CaCl_2$ 1.3 and glucose 31.6). The stomach was placed in an organ bath containing 40 ml buffered solution at 37°C (mM: NaCl 118, KCl 4.8, $MgSO_4$ 1.2, KH_2PO_4 0.14, $NaHPO_4$ 15.9, $CaCl_2$ 0.65 and glucose 31.6) and gassed with 95% O_2 and 5% CO_2. The stomach lumen was continuously perfused from the pyloric cannula at 1 ml/min with warm unbuffered solution. The perfusate was passed over a flow-type pH electrode system, raised 18 cm above the stomach to increase intragastric pressure and distend the stomach. The pH was continuously displayed by a digital pH meter (Corning, 125) and recorded on single channel potentiometric recorders (Bryans 28000 or Smith Servoscribe). Six organ baths were used concurrently. Generally, each stomach was allowed to stabilize for 20 min before the antagonist was added to the bath (serosal surface). The agonist was added to the bath 40 min later and the pH was recorded until the plateau response was evident (20 to 40 min) (Figure 1). Each stomach was used for only one measurement of response to the agonist, then a fresh tissue was set up. With this protocol, experiments on up to 24 stomachs could be completed in one day.

Assay designs

For each H_2-antagonist, a randomised design with 90 stomachs was used to estimate a pK_B value. Five levels of antagonist concentration were used including zero (control level). For each concentration of antagonist three levels of agonist concentration were used, thereby generating 15 treatments in all. On any experimental day, 15 stomachs were used, one stomach being allocated to each treatment. This design was repeated on 6 experimental days to give a total of 6 stomachs at each treatment. Since only one dose of agonist was used in each stomach, preliminary experiments were conducted to estimate agonist concentrations required to obtain responses in the presence of antagonists.

To estimate the shift in dose-response line for just one concentration of the antagonist, a randomised 24 stomach 2 + 2 bioassay design (Colquhoun, 1971) was completed on one experimental day. Six stomachs were given a low and 6 stomachs a high dose of histamine in both the control and the antagonist-treated stomachs.

[^{14}C]-metiamide assay

Stomachs were lumen-perfused as outlined above and the pH of the perfusate continuously recorded before the perfusate was passed to an automatic fraction collector (LKB) programmed to collect 3 ml samples over 3 min. As usual, a 20 min stabilization period was allowed before addition of [^{14}C]-metiamide (3.7 μCi) to the bath giving a bath concentration of 10^{-4} M. After a further 40 min, a large dose of histamine (10^{-3} M) was added to the bath. Aliquots (100 μl) of bathing solution were sampled periodically before and every 5 or 10 min after addition of [^{14}C]-metiamide to the bath. From each 3 ml sample of perfusate, a 500 μl aliquot was taken for counting.

Drugs

Drugs used were atropine sulphate (Sigma), histamine diphosphate (Sigma) and tetrodotoxin (Sigma). Burimamide, metiamide and dimaprit were generous gifts from Dr M. E. Parsons, Smith Kline and French Laboratories Limited. [^{14}C]-metiamide (specific activity 3.78 μCi/mg, approx. 95% radiochemical purity) was a kind gift from Dr D. J. Morecombe, Smith Kline and French Laboratories Limited.

Statistical methods

These have been described in complete working detail by Stone & Angus (1978). Briefly summarised, they employ the standard statistical computing system GLIM 3 (General Linear Interactive Modelling) and a PDP11 computer (although adaptation to other types of computer is straightforward).

The methods have three stages: (a) preliminary exploratory analyses involving choices of measurement of a response (response metameter), dose-response curves and experimental design variables (such as time of day or bath factors) which assess the acceptability of parallelism of the dose-response curves at different antagonist levels; (b) tests of competitive antagonism and estimation of pK_B values using the interactive, non-linear, least-squares curve fitting approach of Waud (1975); (c) a graphical display called the 'Clark plot' (Stone & Angus, 1978), which shows how the spacings of the dose-response lines conform to the theory of simple competitive antagonism.

These stages were applied to the data from the 90 stomachs in each of the four main assays and also to the data from Bunce & Parsons (1976). For the 2 + 2 bioassays, analyses of variance were used to test for linearity of regression, parallelism and difference between control and test stomachs and 95% confidence limits were calculated for the dose-ratios (Colquhoun, 1971).

Responses measured

The pH of the lumen perfusate was read from the continuous record at three points: (i) just before the addition of antagonist, pH_1; (ii) just before the addition of the agonist, pH_2; and (iii) when the response to the agonist had reached a plateau, pH_3 as indicated in Figure 1.

The phenomenon of the reduction of basal secretion after addition of antagonist, as indicated in Figures 1 and 2, raises the important question of choice of response variable. Three obvious choices are available: (i) to take just the plateau secretion response, pH_3, observed after the agonist was added to the bath; (ii) to take the difference, $pH_3 - pH_2$, i.e. the plateau response to agonist minus the immediate base line value (responses derived in this way are widely used in pharmacology); or (iii) the plateau response minus the basal secretion measured prior to addition of the antagonist, $pH_3 - pH_1$. We analysed the choices pH_3, $pH_3 - pH_2$ and $pH_3 - pH_1$ for each assay.

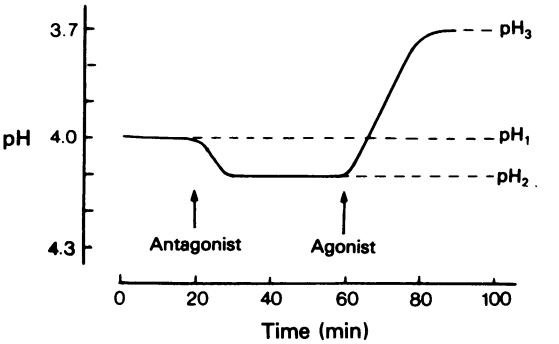

Figure 1 Schematic diagram of the continuous pH recording of the lumen perfusate from an *in vitro* mouse stomach preparation. The H_2-antagonist was added to the bath 20 min after setting up the preparation followed by the agonist a further 40 min later. After the plateau secretion had been observed the stomach was replaced with a fresh tissue. pH values were read from the trace at the times indicated (pH_1, pH_2, pH_3).

Results

Stability of preparation

The lumen-perfused mouse stomachs secreted acid from the time they were placed in the organ bath. The stability of the preparation was assessed by recording the pH of the perfusate for 120 min in 12 stomachs in the absence of any addition of agonist or antagonist (Figure 3). For each record, the pH value was read off at 20, 60 and 90 min after setting up each stomach, i.e. at times corresponding to the three measurements in the assay of H_2-antagonism (Figure 1). The pH of the perfusate varied widely between stomachs and within each stomach over the first 10 min but this variation decreased with time and a steady pH was maintained for the remainder of the experiment (Figure 3). Analysis of variance of the three values for the 12 stomachs showed a significant variation in pH between stomachs ($F_{11,22} = 10.8$, $P < 0.001$). Although some increase in acid secretion was observed between 20 and 60 min there was no significant overall within-preparations difference between the three pH means ($F_{2,22} = 0.87$, NS) suggesting that the preparation was indeed quite stable over 2 h.

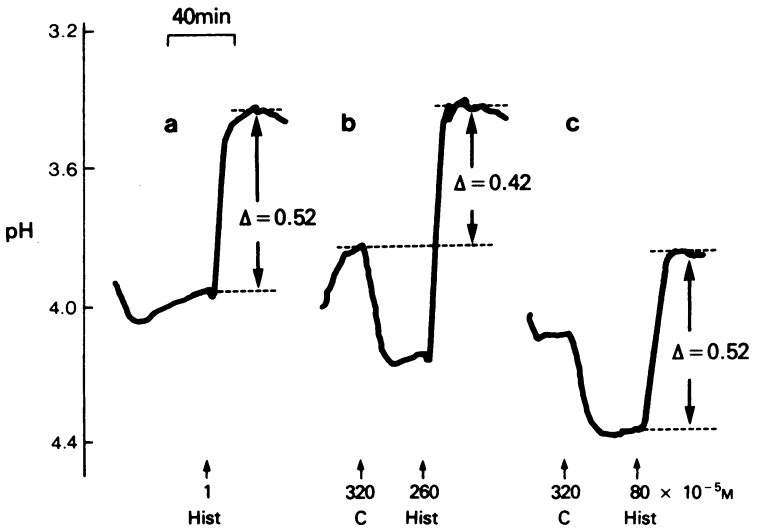

Figure 2 pH recordings from the lumen-perfused mouse stomachs taken from the histamine/cimetidine assay. (a) Control preparation without equilibration of antagonist; (b) and (c) stomachs equilibrated with a high concentration of cimetidine (C), 40 min before addition of histamine (Hist) to the bath. Dotted lines indicate the pH levels taken to measure the different delta response metameters: (a), $pH_3 - pH_2$; (b) $pH_3 - pH_1$; (c) $pH_3 - pH_2$.

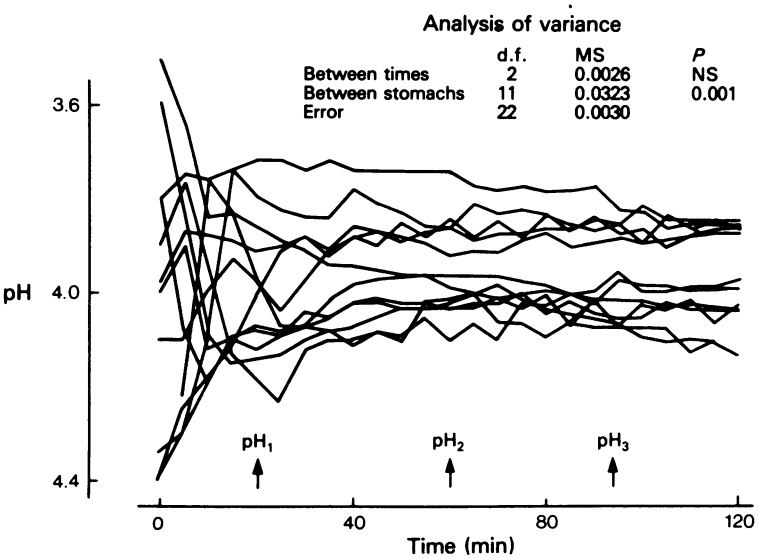

Figure 3 Stability of 12 lumen-perfused mouse stomach preparations where pH values of lumen perfusate were measured over 120 min without addition of agonist or antagonist. Lines show individual values. Insert: analysis of variance table for 3 pH values measured at times 20, 60 and 90 min after setting up each stomach.

The mean pH for the three time intervals was 3.96 which corresponds to a resting H^+ secretion rate of 109.6 nmol/min which is significantly higher than the 42 ± 3 nmol/min found for lumen-perfused rat stomachs (Bunce & Parsons, 1976). The resting or basal acid secretion could be reduced by lowering the height of the outflow perfusion cannula, thereby lowering intragastric pressure and wall distension. However, this wall distension is necessary for this preparation to be sensitive to secretagogues as shown by others (Bunce & Parsons, 1976; Wan, 1977).

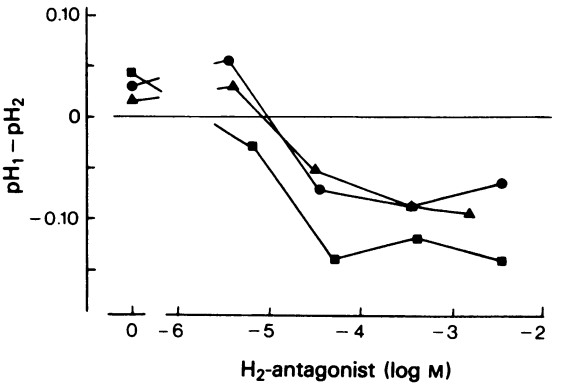

Figure 4 Effect of three H_2-antagonists on basal acid secretion in the isolated mouse stomach. Change in basal pH was determined for 90 stomachs in each assay where each point shown is the mean $pH_1 - pH_2$ values for 18 stomachs. Symbols differentiate H_2-antagonist used: (●) metiamide, assay I; (■) cimetidine, assay III; (▲) burimamide, assay IV. Standard errors for means have been omitted for clarity but were in the range 0.017 to 0.047 pH units.

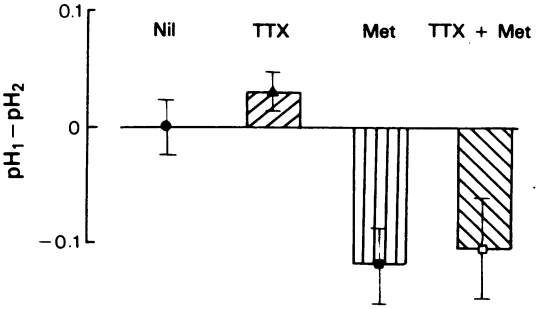

Figure 5 Effect of tetrodotoxin (TTX) and metiamide (Met) on basal acid secretion ($pH_1 - pH_2$) in the isolated mouse stomach. Mean ± s.e. are shown for each treatment: (●) no antagonist, $n = 14$; (▲) tetrodotoxin 10^{-7} M, $n = 12$; (■) metiamide 10^{-3} M, $n = 6$; (□) metiamide 10^{-3} M and tetrodotoxin 10^{-7} M, $n = 6$ (n = number of experiments).

Analysis of basal acid secretion

In preliminary experiments it was noticed that incubation of the stomachs with any H_2-antagonist could cause a fall in resting acid secretion (Figure 2). In each of the four large assays which followed we have examined the effect of H_2-antagonist on resting secretion in the relationship observed between $pH_1 - pH_2$ and the antagonist dose (Figure 4). Resting secretion fell when the H_2-antagonist concentration was higher than 10^{-5} M in each assay. However, the magnitude of the fall was quite similar for the higher concentrations suggesting that only a part of the resting acid secretion was sensitive to these drugs.

In another assay the effect of abolishing neural influences on basal secretion was examined by equilibrating the stomachs with tetrodotoxin (10^{-7} M). This neurotoxin has been shown to block electrical field stimulation of gastric acid secretion in this preparation (Angus & Black, 1978). However, tetrodotoxin did not significantly affect resting acid secretion nor did it potentiate the fall in resting secretion observed with metiamide (10^{-3} M) (Figure 5). Stomachs equilibrated with atropine, 10^{-7} M or 10^{-6} M did not show any significant trend in resting acid secretion.

pK_B for H_2-antagonists and graphical display

Results were obtained for the four assays: histamine/metiamide, assay I; dimaprit/metiamide, assay II; histamine/cimetidine, assay III; and histamine/burimamide, assay IV. The computer analysis used for these assays has been previously reported in detail and will be briefly summarised here (Stone & Angus, 1978). For each assay, concentration-response lines with common slope could be fitted to the data; for example, results for assay III are shown in Figure 6 with response variable pH_3. Table 1 summarises the computer-fitted values for parameters of n and pK_B (where n is equivalent to the slope in the Schild plot) for three choices of response metameter in four assays.

In general the behaviour of the three H_2-antagonists was not incompatible with simple competitive antagonism to histamine or dimaprit as the n values all contained unity in the 95% confidence intervals. However, the pK_B values were lower than expected by as much as one unit compared to values reported for other H_2-receptor mediated responses, such as tachycardia in guinea-pig atrium and relaxation in rat uterus (Black *et al.*, 1972; Parsons, 1973).

The choice of response metameter clearly influences the estimated pK_B value. In each assay I, II, III, $pH_3 - pH_2$ consistently gave lower pK_B values than did either pH_3 or $pH_3 - pH_1$ (Table 1). The major difference between the three response metameters is the effect of the fall in acid secretion caused by the antagonist and this appears to affect the $pH_3 - pH_2$ response metameter and subsequent analysis. So far, our choice of response metameter has been restricted to the use of the pH scale but other workers have used the hydrogen ion secretion rate in their analysis (Bunce & Parsons, 1976). For comparison, therefore, we have further analysed the data from assay I using eight different choices of response metameter including hydrogen ion secretion rate with or without logar-

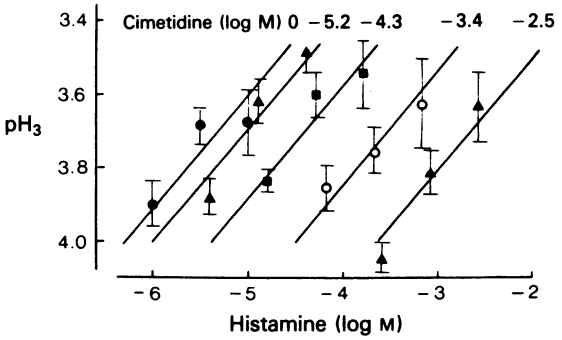

Figure 6 Concentration-response lines for assay III (histamine/cimetidine) with the response metameter pH_3. Each point is the mean ± s.e. for 6 stomachs where different symbols indicate concentration of cimetidine equilibrated with the stomach 40 min before addition of histamine. Lines fitted are regression lines with common slope.

ithms (used to adjust for non-homogeneity in variance). The results shown in Table 2 indicate that the response metameter containing the equivalent of the pH_2 measurement for hydrogen ion secretion rate are the lowest in rank order of pK_B values. Further, the range of possible pK_B estimates spans 0.8 pK_B units indicating again that the choice of response metameter will significantly affect the estimated parameter value.

A Schild plot of the relationship between histamine dose-ratios and concentration of H_2-antagonist (assay III) is linear with a slope not different from unity (0.99) and $pK_B = 5.17$ (Figure 7). This confirms the computer-fitted values of n = 0.99 and $pK_B = 5.14$. However, the Schild plot is not particularly suitable for estimating these parameters given the experimental design of these assays. The heavy use of the location of the control histamine dose-response line in relation to measurement of dose-ratios is weighting the subsequent analysis in the Schild regression.

Table 1 Computer-fitted pK_B values and n values, equivalent to the slope in the Schild plot, for H_2-receptor antagonists in isolated lumen-perfused mouse stomachs

Assay	*Agonist/antagonist*	*Response metameter*	*pK_B (95% c.l.)*	*n (95% c.l.)*
I	Histamine/metiamide	pH_3	5.07 (4.7, 5.4)	0.96 (0.7, 1.2)
		$pH_3 - pH_1$	4.92 (4.5, 5.4)	0.83 (0.6, 1.1)
		$pH_3 - pH_2$	4.40 (4.1, 4.7)	0.90 (0.6, 1.2)
II	Dimaprit/metiamide	pH_3	5.23 (5.0, 5.5)	1.17 (0.9, 1.4)
		$pH_3 - pH_1$	5.35 (5.0, 5.7)	1.30 (0.9, 1.7)
		$pH_3 - pH_2$	4.78 (4.5, 5.0)	Non-convergence
III	Histamine/cimetidine	pH_3	5.14 (4.8, 5.5)	0.99 (0.8, 1.2)
		$pH_3 - pH_1$	5.38 (5.2, 5.6)	0.88 (0.8, 1.0)
		$pH_3 - pH_2$	4.76 (4.5, 5.0)	0.90 (0.8, 1.0)
IV	Histamine/burimamide	pH_3	4.59 (4.4, 4.8)	0.98 (0.7, 1.2)

pH_1 was measured just before giving the antagonist, pH_2 was measured just before giving the agonist and pH_3 was measured during the plateau response to the agonist. 95% c.l. = 95% confidence limits.

Table 2 pK_B values ±95% confidence limits for a variety of response metameters in assay I; histamine/metiamide

Response metameter	*pK_B*	*95% confidence limits*
$\ln (R_3 - R_1)$	5.20	4.9, 5.5
R_3	5.08	4.7, 5.4
pH_3	5.07	4.7, 5.4
$R_3 - R_1$	5.05	4.7, 5.4
$pH_3 - pH_1$	4.92	4.7, 5.2
$R_3 - R_2$	4.86	4.6, 5.1
$\ln (R_3 - R_2)$	4.72	4.5, 5.0
$pH_3 - pH_2$	4.40	4.1, 4.7

ln = natural logarithm ($\log_e$); pH_1, R_1 measured before giving antagonist; pH_2, R_2 measured before giving agonist; pH_3, R_3 measured at plateau response to agonist; R scale = hydrogen ion secretion rate; nmol/min.

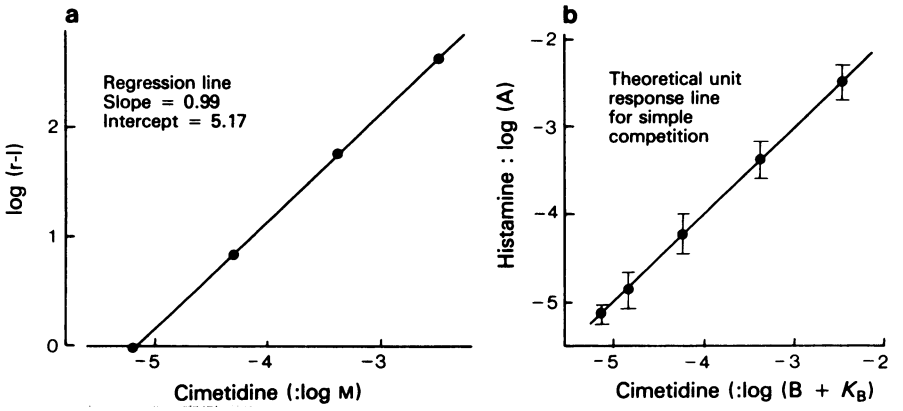

Figure 7 (a) Schild plot for the histamine-cimetidine assay in lumen-perfused mouse stomach. Ordinate scale: (r − 1), where r is the dose-ratio of concentrations of histamine producing equal responses determined from parallel-fitted lines (log scale). Abscissae: concentrations of cimetidine (log scale). Linear regression line has slope of 0.99 and $pK_B = 5.17$. Response metameter used was pH_3. (b) 'Clark plot' for histamine-cimetidine assay in lumen perfused mouse stomach. Ordinate scale: histamine concentration (log scale) producing equal responses in the absence or presence of cimetidine. Abscissae: sum of cimetidine concentration (B) and computer-estimated dissociation constant (K_B) on log scale. Points shown display the degree of displacement of the individual histamine dose-response lines from the model of simple competitivity. Error bars show ±95% confidence limits. Note: Lowest point on line displays the histamine concentration for the control dose-response line when cimetidine = 0, i.e. at $-\log K_B = pK_B$.

An alternative method of calculating the pK_B (Waud, 1975) and of displaying the agonist-antagonist interaction (Stone & Angus, 1978) has been applied to the same assay as described above. This plot is derived from Clark's (1926) demonstration that the ratio between the concentrations of agonist and antagonist for equal responses is constant over a wide range of antagonist concentrations. Deviations from linearity were found only at low levels of antagonism. The plot is based on the necessary consequence of simple competitivity theory that the difference between log (agonist) and log (antagonist $+K_B$) is constant for equal responses and free from the deviations seen by Clark (1926). This graphical display shows how well the displacement of the experimentally-determined histamine dose-response lines, as shown in Figure 6, fit the overall model of simple competition. If a point lies within the 95% confidence limits for that level of log (B + K_B) determined from the family of dose-response lines, then that dose-response curve has been displaced from control and from the other lines (equal weighting) according to the model of simple competition. A point displayed above (or below) the unit response line indicates that the antagonist has been slightly more (or less) active than predicted from an overall fit of all the dose-response lines by the iterative method of non-linear least-squares. The 'Clark' plot for the cimetidine assay departs so little from simple competition that the points do not appear to lie away from the isobol unit response line at all (Figure 7).

Therefore the three H_2-receptor antagonists behave not incompatibly with simple competitive antagonism of acid secretion even though the pK_B values are up to 1 unit lower than expected. This finding contrasts with that of Bunce & Parsons (1976) where although they reported a slope significantly lower than unity (0.73) they found a pA_2 of 5.9 which was not significantly different from that previously found in atrium and uterus. The dose-ratios found for this calculation were obtained from three separate 3 + 3 assays in contrast to the procedure reported here where the data are analysed as a whole. Unlike the mouse stomach assay, where only a single measurement is made in each preparation, the Bunce & Parsons (1976) assay used six measurements in each preparation and therefore introduced the problem of time-dependent changes. They had tackled this problem by showing that two three-point dose-response curves could be replicated in each stomach without significant displacement. We have re-analysed their data, using the GLIM system, for fitting the Schild equation to the logarithms of the dose-ratios (r) determined after fitting parallel lines to each preparation with response metameter $R_3 - R_2$:

$$\log(r - 1) = b \log B - \log K_B. \qquad (1)$$

We confirmed Bunce & Parsons' analysis of a significantly low slope b of 0.60 (s.e. 0.13) thereby apparently revealing evidence of departure from simple competition. However, although their experimental design does not allow any *explicit* adjustment for

time-dependent changes as do the designs proposed by Furchgott (1967) it is possible to modify the GLIM analysis to incorporate such changes. We suppose that the changes take the form of a dose-ratio, r_0, expressing the change between the three control observations and the three antagonist observations that is a constant for all preparations and that would counteract the time effect. The following re-arrangement of the competitive Schild equation (1) with b = 1 incorporates the adjustable parameter, r_0.

$$\log r = \log\left\{1 + \frac{B}{K_B}\right\} + \log r_0 \qquad (2)$$

The GLIM system gave the least-squares estimate of log (r_0) for fitting equation (2) to Bunce & Parsons' data as well as the residual sum of squares (RSS). The joint least-squares estimates of pK_B and log (r_0) that minimize the RSS were 4.98 and 0.40 respectively i.e. a time-dependence dose-ratio of 2.5. The pK_B value and its 95% confidence limits (4.5, 5.5) determined under these conditions was similar to that determined in our mouse assay and the data were not incompatible with simple competition, with slope in the Schild plot not significantly different from unity. Therefore, we have considered other factors known to distort this analysis.

Equilibrium conditions

Measurement of pK_B values in whole tissues assumes that the antagonist concentration in equilibrium with receptors is equal to the bath concentration. In assay III cimetidine 5.08×10^{-5} M, equilibrated for 40 min in the bath before adding the agonist, gave a dose-ratio of 6.8, whereas the expected dose-ratio calculated from a $pK_B = 6$ in guinea-pig heart should have been about 50. A number of assays were conducted in an attempt to raise the concentration of antagonist in equilibrium with the receptors by prolonging incubation time and by applying the antagonist to both surfaces of the stomach wall *in vitro*, i.e. to the bath (outside surface) and to the lumen perfusate (inside surface). Dose-ratios estimated by 2 + 2 parallel line assays for one concentration of cimetidine were used to assess the activity of the antagonist on histamine-stimulated secretion. However, prolonged equilibration of the stomach with the antagonist in the bath with or without the antagonist in the lumen perfusate failed to increase the dose-ratio significantly (Table 3). When cimetidine was placed only in the lumen perfusate for 1 h before histamine was added to the bath in the usual way, the dose-ratio was not significantly different from unity.

[^{14}C]-metiamide in lumen perfusate

In the lumen perfusion assay used in this study, equilibration between bath concentration and the receptors may be prevented if there was a steady loss of antagonist into the secretion. Bases will tend to partition into the acid medium of the parietal secretion. We have analysed this loss of antagonist by using radiolabelled metiamide. Approximately 9 to 12 min after adding [^{14}C]-metiamide (10^{-4} M) to the bath, radiolabel was detected in the lumen perfusate (Figure 8). The perfusion dead space was approx. 3 ml from the stomach to the collecting vials so that time for passage of [^{14}C]-metiamide from outer bathing surface to stomach lumen was about 6 min. The radiolabel in the perfusate was assumed to be [^{14}C]-metiamide and not a metabolite. This seemed a reasonable assumption because, even after whole body dosing of the drug, between 60% to 90% of metiamide was recovered in the urine unchanged (Hesselbo, 1973).

The level of radiolabel in the perfusate was low, but the levels in each sample of perfusate increased with time to reach a steady rate of loss (equivalent to a transfer rate constant of 3×10^{-3} min^{-1}) at about 40 min (Figure 8). There was a slight decline in mean levels of the *bath* concentration of radiolabel with time but this trend was not significant (Figure 8). After 60 min the total mean loss of radiolabel in the perfusate was 11×10^3 d/min, i.e. 0.23% of the bath radiolabel, 5×10^6 d/min.

Discussion

At first sight, acid secretion by the isolated lumen-perfused mouse stomach appears to be suitable for

Table 3 Effect of different equilibration times and of different sites of cimetidine (5.08×10^{-5} M) administration on histamine dose-response lines (in each assay the response metameter pH_3 was used)

	Cimetidine incubation time (min)			
Assay design	*bath*	*lumen perfusate*	*Dose-ratio*	*95% c.l.*
3 + 3	40	—	6.76	(1.1, 19.8)
2 + 2	40	60	6.70	(2.3, 15.1)
2 + 2	120	140	11.75	(6.3, 20.6)
2 + 2	—	60	2.52	(0.9, 10.6)

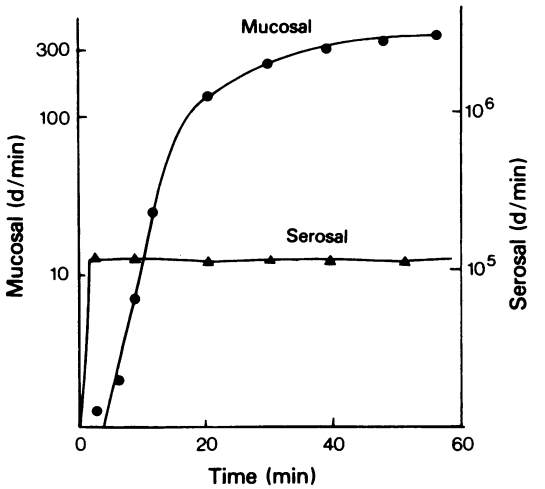

Figure 8 Left ordinate scale: radioactivity (d/min, log scale) of [^{14}C]-metiamide in 1 ml samples of mucosal solution (●) after lumen perfusion of the isolated mouse stomach. Right ordinate scale: radioactivity (d/min, log scale) of [^{14}C]-metiamide in 1 ml samples of bath fluid (serosal solution ▲) determined periodically. At time zero, 3.7 μCi [^{14}C]-metiamide (10^{-4} M) was added to the bath (serosal solution). Points are mean values from five experiments. Standard errors lie within the symbols in each case.

measurement of antagonist equilibrium constants. The preparations are robust and can be set up rapidly. They maintain a stable level of acid secretion, when distended, for some hours. In stomachs from young adult mice, oxygenation of the oxyntic gland area, through the thin stomach wall, appears adequate. These stomachs are sensitive to secretagogues such as histamine, choline esters and pentagastrin and respond rapidly to reach a plateau of secretion.

In this preparation, we have shown that the behaviour of burimamide, metiamide and cimetidine is not incompatible with simple competitive antagonism at H_2-receptors over a wide concentration range irrespective of whether dimaprit or histamine was used as agonist. The definition of simple competitive antagonism was based on the parallel shift of concentration-response lines such that the displacement (dose-ratios) did not deviate from the model of a one-to-one reversible molecular interaction. However, accepting the apparent pK_B values from these assays immediately raises the question of why we found a significantly lower pK_B value compared to other isolated tissue preparations of heart and uterus, and especially compared to the assay on immature rat isolated stomach preparations used by Bunce & Parsons (1976). Considering the within-preparations assay design of Bunce & Parsons, it was apparent that a small time-dependent factor could have influenced their estimation of dose-ratios, a factor not relevant to our mouse assay design. This time-dependent factor, considered as a dose-ratio, was surprisingly small (2.5) in terms of how accurately dose-ratios can be measured. The associated estimate of pK_B was not different from that determined in the mouse assay and the data were not incompatible with simple competitivity. This relatively small adjustment to the dose-ratios determined by Bunce & Parsons has, therefore, led to quite substantial effects on the Schild plot especially when they only considered a small 10-fold range of antagonist concentration.

Other factors were, therefore, considered which are known to distort analysis of antagonism such as uptake and tissue metabolism of the agonist in the region of the receptor compartment. However, there is no evidence that a histamine uptake process exists in the stomach (Cross, 1973) and, in fact, when we used SKF 91581, a compound shown to inhibit [^{14}C]-histamine uptake in heart muscle, no significant change in the histamine 2-point assay was observed. As for tissue metabolism, if there was indeed an active histamine-methyltransferase affecting this assay, Furchgott's (1972) analysis predicts that the Schild regression would deviate from unit slope over some part of the plot. This was not observed for any H_2-antagonist assay over 1000 fold concentration range.

Another possible reason for the low pK_B values could be that the H_2-receptors mediating acid secretion on the parietal cells were different from those in heart and uterus. However, Scholes, Cooper, Jones, Major, Walters & Wilde (1976) have characterized the adenylate cyclase system in a cell suspension from dog gastric mucosa as being sensitive to H_2-receptor stimulation. They reported that the behaviour of metiamide was not incompatible with simple competitive antagonism with a pK_B (6.46) value certainly not lower and maybe even higher than the reference measurements.

Apparently, the discrepancy is not between histamine receptors on parietal cells and histamine receptors on other tissues, but between secretory preparations and non-secretory preparations of parietal cells. The discrepancy is not confined to histamine receptors because a similar inconsistency was found for the atropine/bethanechol and related interactions (Angus & Black, 1979). These findings point to the secretory process *per se* as the source of error. The lumen-perfusion used in the isolated whole stomach technique will carry away any substances which appear in the parietal cell secretion. The loss of agonists into the perfusate would not interfere with the assay unless the process was saturable or the antagonist was capable of preventing the loss. If, on the other hand, the antagonists appear in the effluent, this

will prevent the preparation coming into concentration equilibrium although a steady-state will be achievable. [^{14}C]-metiamide (assuming minimal metabolism in the stomach wall), has been shown to appear in the effluent from the mouse stomach preparation. The transfer rate constant appears to be about 3×10^{-3} min^{-1}. Although this loss was not enough to lower the bath concentration of metiamide during a 1 h incubation, the loss could be sufficient to produce a steep concentration gradient at the receptor/parietal cell interface, if restricted diffusion through a suitably thin unstirred layer is assumed. Consequently the bath concentration would overestimate the concentration in equilibrium with the receptors and the assay would underestimate the degree of antagonism. Addition of antagonists to the lumen perfusate did not alter the estimated pK_B values and probably did *not* alter the interfacial concentration gradient either; like fresh water rivers, continuous hydraulic flow in the gastric glands from intracellular canaliculi to the mucosal surface would be expected to prevent back diffusion of lumenal solutes and, in addition, the low pH of the parietal cell secretion will alter the ionic species of weak bases compared to that in the bath fluid. The question cannot be settled by methematical modelling but an answer based on measurement may be possible in the future.

Finally, these assays have raised a question about how to choose the response metameter in comparative bioassays. The mouse isolated stomach has a basal secretion of acid which can be reduced by a maximum of 0.15 pH units by metiamide. If this result means that part of the basal acid secretion is associated with the endogenous release of histamine, then this local histamine will sum with the exogenous histamine added to the bath and the bath concentration will underestimate the concentration at the receptors. Under conditions of simple competition, equilibrium concentration-response curves (as for pH_3 or $pH_3 - pH_1$) will be parallel when plotted against the logarithm of the true agonist concentration. The curves actually drawn against the logarithm of the exogenous agonist concentration will be biased to the left by the difference between the logarithms, i.e. by the logarithm of the ratio of the true concentration to the exogenous concentrations. This bias may be expected to affect at most the points on the control curve that have the lowest exogenous concentration; the true control curve could be shifted to the left (with some flattening of slope) resulting in possible *overestimation* of pK_B. The overestimation would not be detectable if the endogenous histamine concentration were small compared with the lowest exogenous concentration used. Whether or not there is significant overestimation from this, the observed sizeable reduction of basal secretion (pH_2) by added antagonist can account for the lower dose-ratios and pK_B estimates associated with $pH_3 - pH_2$. This can be appreciated on the grounds that, in the presence of antagonist, the exogenous concentration will have to be lowered to reduce pH_3 so that the difference $pH_3 - pH_2$ matches that of control (Figure 3).

If we accept that endogenous histamine is not present in sufficient amount to result in overestimation of pK_B from the pH_3 (or $pH_3 - pH_1$) values, we must interpret the lower estimates with $pH_3 - pH_2$ (the Δ method) as underestimates. The results in Table 1 show that measurements involving pH_2 or its metameters lead to significantly low pK_B estimates and the previous argument has been used to conclude that the higher estimates are preferable.

We wish sincerely to thank Drs M.E. Parsons and K.T. Bunce for providing us with their data from the rat secretion assay and for their comments on our analysis. We thank Mrs Caroline Harrison, Mr Peter Randall and Miss Marion Lowe for expert technical assistance. J.A.A. is a holder of a National Health and Medical Research Council of Australia C.J. Martin Fellowship.

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(Received December 7, 1978.
Revised May 11, 1979.)