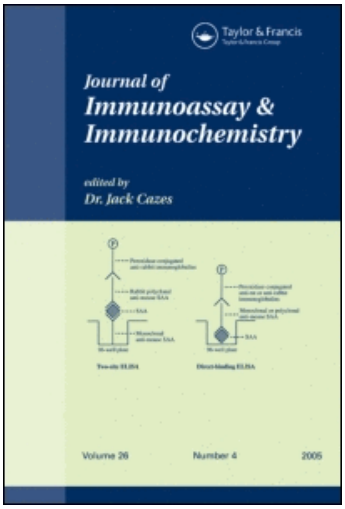

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THE CYTOCHEMICAL SECTION-BIOASSAY OF GASTRIN-LIKE ACTIVITY

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ABSTRACT

A cytochemical section-bioassay of gastrin-like activity is described in which such activity in plasma is assayed by its stimulation of carbonic anhydrase activity in serial, 18 μm-thick, sections of suitably prepared gastric fundus of the guinea-pig. The index of precision was 0.1±0.05 (mean±SEM; n=8). Fiducial limits were 75-134%. Intra-assay variation was ±6.4% (n=4); inter-assay variation was ±16.3% (n=3). The mean gastrin-like activity in the plasma of 15 fasting normal subjects was $5.1\pm0.49\times10^{-12}$M (range $1.4-18.2\times10^{-12}$M).

INTRODUCTION

It is becoming increasingly apparent that it is sometimes helpful to be able to assign a biological potency to a result obtained by radioimmunoassay. This view was expressed as a firm

recommendation by the World Health Organization Expert Committee on Biological Standardization (1), which recommended "that emphasis be placed on the development of biological microassays, which preferably have a sensitivity comparable with radioimmunoassays, with which they should be run in parallel." The reason for this emphasis on checking radioimmunoassay by bioassay was that "a limitation on the use of immunoassays for evaluating hormonal bioactivity is that the methods measure a composite of antigenic activity, which is not necessarily related to the bioactivity of the hormone." This problem is particularly marked in the assay of gastrin which can occur in plasma in various forms, not all having identical bioactivity (2,3). These difficulties led Walsh and Grossman (4) to conclude that "Because of the heterogeneity of circulating gastrin and because different molecular forms vary in biologic activity, total gastrin activity determined by radioimmunoassay cannot be more than a crude index of bioactivity." Although Dockray and Taylor (5) developed a radioimmunoassay which is specific for the heptadecapeptide gastrin, this does not preclude the need for a highly sensitive bioassay.

A sensitive cytochemical bioassay (6), done on segments of guinea-pig fundus, was too slow for routine purposes. This communication deals with an equally sensitive bioassay that, because it is done on sections of the fundus, allows many samples to be assayed simultaneously in a within-animal procedure.

MATERIAL

The hormones used for this study were obtained from the following sources. Human synthetic gastrin I (G_{17}), batch no. 68/439 with a potency of 1000 units/mg, was obtained from the National Institute for Biological Standards and Control, Hampstead, London NW3 6RB. Human synthetic big gastrin I (G_{34}) was obtained as a gift from Professor R.A. Gregory (Department of Physiology, University of Liverpool, Liverpool L69 3BX). Pentagastrin was obtained as Peptavlon from Imperial Chemical Industries Ltd., Blackley, Manchester M9 3DA. Natural porcine cholecystokinin (CCK), 95% pure (potency: 500 Ivy Dog Units/mg), was prepared by Professor V. Mutt, GIH Research Unit, Karolinska Institute, Stockholm. The C-terminal octapeptide of CCK (CCK-OP) was synthesized and provided by Dr. Miguel Ondetti, Squibb Institute for Medical Research. The sample of natural porcine secretin (95% pure) was prepared by Professor V. Mutt and had a potency of 3,500 units/mg. Rabbit anti-gastrin antibody (G/R/26-PF) was kindly provided by Dr. B. Morris of the Supra-Regional Assay Service, Guildford, Surrey.

ASSAY PROCEDURE

Female guinea-pigs (Hartley strain, about 400 g body weight) were killed by asphyxiation in nitrogen and the fundus of the stomach was removed and cleaned of debris. Strips of fundus (3x2 mm) were maintained *in vitro* at 37°C for 5 h on a defatted lens tissue on a table made from stainless steel mesh with Trowell's (7) T8 medium,

pH 7.6, added up to the level of the lens tissue. The culture chambers were sealed with lanoline and the atmosphere of the chambers was replaced by 95% oxygen:5% carbon dioxide. After 5 h at 37°C the strips were primed by exposing them to 2.3×10^{-16}M of G_{17} gastrin for 5 min. They were then chilled to -70°C in n-hexane ("free from aromatic hydrocarbons" grade, boiling range 67-70°C). After 30 s the tissue was removed with cold forceps and stored in dry glass tubes at -70°C. Within three days the tissue was sectioned at 18 μm in a Bright's cryostat with a cabinet temperature of -25°C and the knife cooled to -70°C. The methods of chilling, sectioning and transferring the sections on to the glass slides were those described in detail by Chayen et al. (8).

Serial sections were placed in a trough immediately prior to exposure to gastrin. Duplicate sections were treated for 75 sec with one of four graded logarithmic doses of gastrin (2.3×10^{-15}-2.3×10^{-12}M; 0.005 pg/ml-5 pg/ml G_{17}) or to a dilution of the test sample of plasma (usually diluted 1/100 and 1/1000). The gastrin and dilutions of the test sample were made up in 0.1 M Hepes buffer, pH 7.0, containing gum tragacanth (0.005% w/v). The apparatus shown in Fig. 1 allows up to 28 sections to be exposed simultaneously to the hormone so that 5 test samples and 4 graded concentrations of G_{17} can be measured each on duplicate sections and at two dilutions. After 75 s the sections were reacted for carbonic anhydrase activity by the method of Loveridge (9). The reaction medium was as follows:

Solution A: 9 ml 0.5 M sulphuric acid; 9 ml 0.1 M cobalt sulphate ($CoSO_4.7H_2O$); 3 ml 0.067 M potassium dihydrogen ortho-

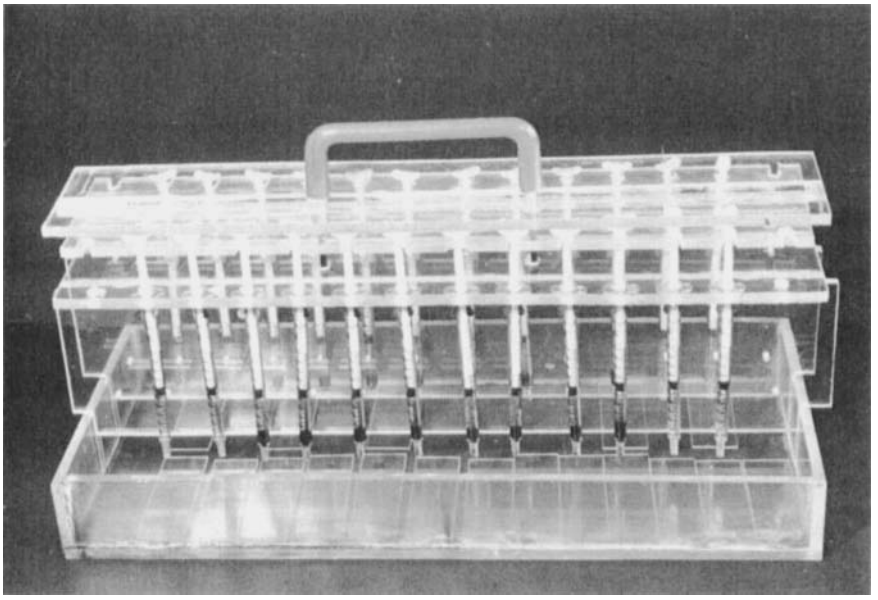

FIGURE 1. A photograph of the apparatus used for the section-assay. The superstructure consists of a rack of 1-ml syringes held so that they can be filled and emptied by raising or lowering the handle. They are positioned so as to fit above the sections on the glass slides, laid horizontally under each of the syringes. After exposure of the sections to the hormone, the superstructure is removed and the trough is flooded with the calculated volume of the reaction-medium so as to achieve a depth of 0.7 mm.

phosphate (all these reagents are B.D.H. 'Analar' grade); 4.5 ml distilled water.

Solution B: 1.125 g sodium hydrogen carbonate (B.D.H. 'Analar' grade) in 60 ml 0.1 M HEPES (Sigma) buffered to pH 7.4 with sodium hydroxide. The two solutions were prepared fresh and mixed immediately before use. After mixing, the solution was stirred until the pH had risen to pH 6.8 (1-2 min). The reaction was done for 60 s at 37°C.

Carbonic anhydrase catalyzes the following reaction (10):

$$HCO_3^- \rightleftharpoons OH^- + CO_2$$

The hydroxyl ions are trapped by cobalt as a precipitate of cobalt hydroxide. The accumulation of CO_2 inhibits activity so that this gas must be allowed to diffuse readily from the site of the reaction (9). To assist this diffusion the depth of the reaction medium was kept minimal (0.8 mm) and the trough was agitated gently throughout the reaction time. After the reaction the sections were washed in running tap water (30 s), immersed in a saturated solution of hydrogen sulphide, rinsed in distilled water and mounted in Farrants' medium. The hydrogen sulphide converts the cobalt hydroxide to the intensely coloured cobalt sulphide.

The measurements were made at 550 nm with a x20 objective, a scanning spot of 1 μm diameter and a mask of 20 μm diameter, which optically isolated each parietal cell. The activity in ten parietal cells was measured in each of two duplicate sections and the mean of these readings was converted to mean integrated extinction by reference to a standard calibration graph. To correct for non-specific adsorption of cobalt and for spurious light-scattering by the tissue, ten similar-sized fields were measured in the muscle and the mean of these readings was subtracted from that obtained from the parietal cells. In general, the extinction in the muscle was up to 50% of that found in the non-stimulated parietal cells and did not vary with increasing concentration of the hormone. Thus it became a smaller proportion of the activity measured in the parietal

cells, with increasing concentration of the hormone, being only 20-26% at higher concentrations of G_{17}.

RESULTS

In sections incubated with 2.3×10^{-12}M G_{17} an increase in carbonic anhydrase activity could be detected by 20 s, was significant by 40 s ($p < 0.005$) and was maximal by 75 s ($p < 0.001$) and then decreased slightly from 75 to 120 s (Fig. 2). Thus the carbonic anhydrase activity increased from 16.9 units of basal activity to 25 units at 75 s. In contrast, in sections incubated with the vehicle alone, the activity rose from 16.9 to 18.1 units which was just not significant ($0.25 > p > 0.1$), estimated from all the readings for the duplicate slides). In sections incubated for 75 s with various

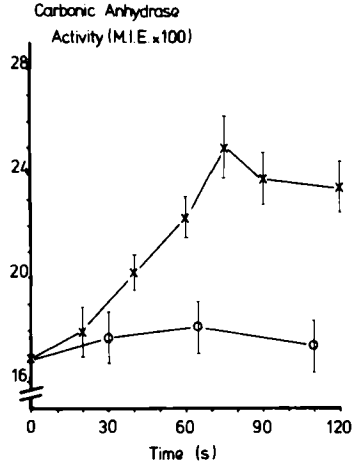

FIGURE 2. The time-course (mean±SEM) of the carbonic anhydrase activity (MIEx100) induced in the parietal cells in sections of guinea-pig fundus by 2.3×10^{-12}M of G_{17}. The lower graph (circles) shows the effect of the vehicle alone.

concentrations of G_{17}, a significant increase ($p < 0.01$) in carbonic anhydrase activity could be detected at 2.3×10^{-14} M G_{17}. The increase in enzyme activity was maximal with 2.3×10^{-12} M G_{17} and the activity decreased at higher concentrations. Sections exposed to vehicle alone showed activity that was usually below that obtained with 2.3×10^{-15} M G_{17}.

Suitable dilutions of plasma, collected in lithium-heparin tubes, normally gave a response that was parallel to that obtained with dilutions of the standard reference preparation (Fig. 3); such parallelism was frequently not obtained with samples of serum, even

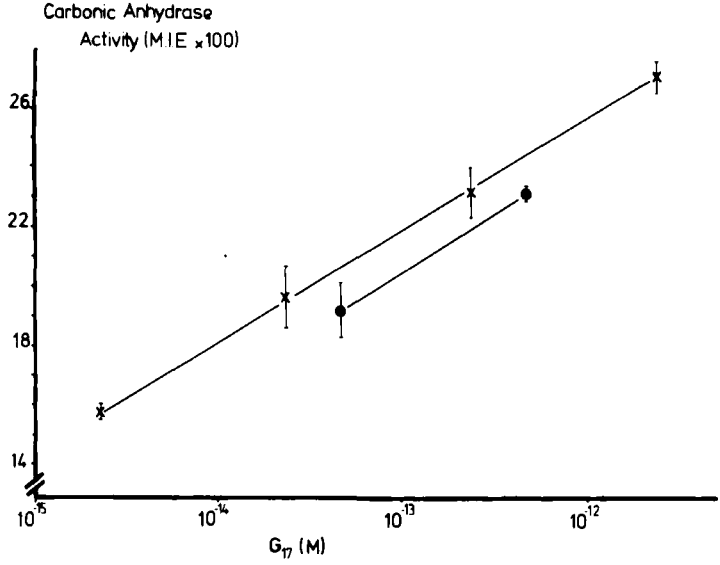

FIGURE 3. The calibration graph (crosses) relating the carbonic anhydrase activity (mean±SEM) induced in parietal cells in sections exposed to four concentrations of a standard preparation of G_{17}, and the response to two concentrations of plasma (filled circles): the upper concentration was $1:10^2$; the lower, $1:10^3$. The response to dilutions of plasma was parallel to the calibration graph.

from the same samples of blood (Fig. 4). In samples of plasma the gastrin-like activity was largely (90%) or completely abolished by the addition of a specific antibody at $1:10^4$ dilution.

The mean index of precision (λ) for the section procedure was 0.102±0.05 (n = 8) which compares with 0.14 (n = 36) for the segment procedure. Fiducial limits of an assay (P = 0.95) were 75 to 134% which is comparable to a range of 72-140% to 98-103% for the segment procedure. Inter-assay variation was ±16.3% (n = 3); intra-assay variation was ±6.4% (n = 4).

The accuracy of the assay was tested by the recovery of exogenous standard gastrin added to plasma. Two samples of plasma

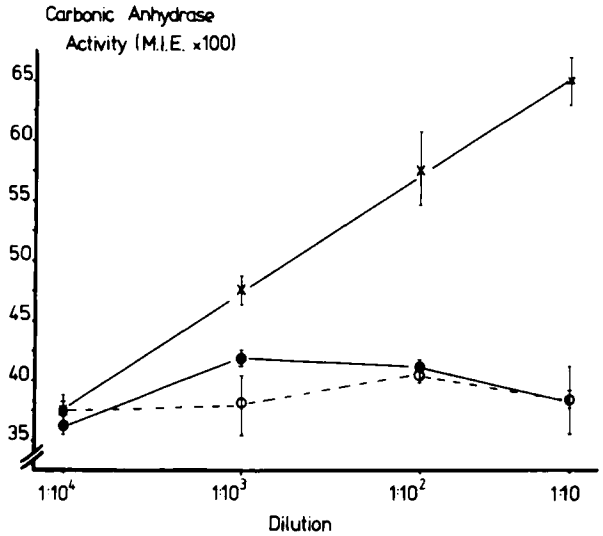

FIGURE 4. The carbonic anhydrase activity (MIEx100) found in sections exposed to four dilutions of a plasma (crosses; mean±SEM) was linearly related to the concentration of the plasma. This relationship did not pertain to dilutions of the serum from the same sample of blood, whether taken into glass (filled circles) or plastic (open circles) tubes.

were first assayed in the usual way to establish the endogenous concentration of the hormonal activity. Then G_{17}-gastrin was added to each sample to a concentration of 50 pg/ml and the samples were assayed again. The recovery in the two samples was 96% and 112%.

In a group of 15 normal volunteers the fasting levels of gastrin-like activity assayed by this procedure were 5.1×10^{-12}M ± 0.49. The range was 1.4 to 18.2×10^{-12}M and showed a logarithmic normal distribution.

On a molar basis, pentagastrin was equipotent to G_{17} (potency ratio of 1.02 ± 0.25, mean ± SEM; n = 5) whereas G_{34} was relatively inactive and gave a flat, non-parallel response when tested under these conditions, namely at the time at which the activity of G_{17} was maximal.

At a concentration of 2.5×10^{-11}M, CCK evoked increased carbonic anhydrase activity that was maximal at between 60 and 90 s and equivalent to 52% of the activity induced by 2.3×10^{-12}M G_{17}. The effects over a range of concentrations expressed relative to the maximal response in serial sections evoked by 2.3×10^{-12}M G_{17} indicated that, when acting alone, CCK was approximately 1000 times less potent than G_{17} (Fig. 5). To examine the combined effects of G_{17} and of CCK, three samples were prepared, each containing 2.3×10^{-11}M (50 pg/ml) G_{17}. CCK was then added to each sample at concentrations of 3.75×10^{-11}M (150 pg/ml), 1.25×10^{-11}M (50 pg/ml) and 2.5×10^{-12}M (10 pg/ml) respectively. The carbonic anhydrase activity elicited by 1/100 and 1/1000 dilutions of each sample was

then measured and compared with that caused by G_{17}, so allowing an estimate of the gastrin-like activity in each sample to be made. The first two samples, containing 3.75×10^{-11}M and 1.25×10^{-11}M of CCK, gave responses which were not parallel to the standard graph for G_{17} alone (Table 1). The response produced to the two dilutions of the third sample, containing 2.5×10^{-12}M CCK, was almost parallel to that of G_{17} alone. When corrected for dilution only the third sample assayed at a level equivalent to the concentration of gastrin (2.3×10^{-11}M) present in the sample (Table 1). Thus at the higher of these concentrations of CCK, which in the absence of gastrin would give only a small elevation of carbonic anhydrase activity (see Fig. 5), CCK caused some inhibition of the normal response to gastrin causing the dose-response graph to be non-parallel to that of gastrin alone.

Table 1

The effect of CCK on the measurement of gastrin-like activity

Concentrations of hormones in sample (M)		Gastrin-like activity corrected for dilution (M) in	
G_{17}	CCK	1:100 dilution	1:1000 dilution
2.3×10^{-11}	3.75×10^{-11}	0.55×10^{-11}	1.7×10^{-11}
2.3×10^{-11}	1.25×10^{-11}	0.83×10^{-11}	1.8×10^{-11}
2.3×10^{-11}	0.25×10^{-11}	1.61×10^{-11}	2.3×10^{-11}

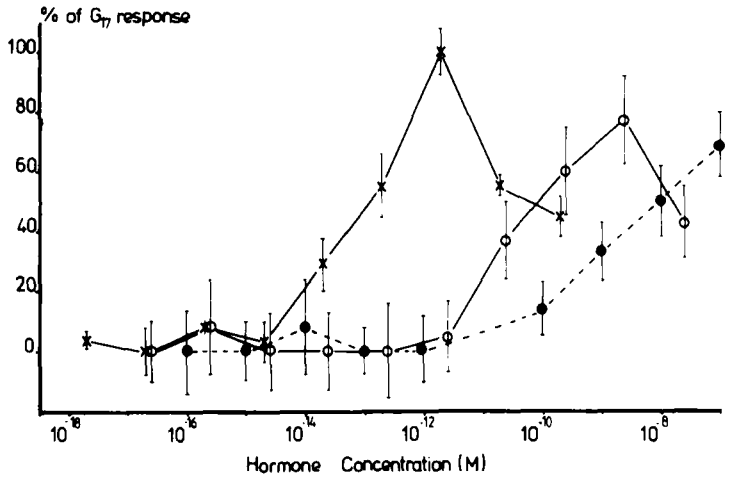

FIGURE 5. The relative responses elicited by various concentrations of secretin (filled circles), CCK (open circles) and G_{17}, in sections from the same gastric fundus, taking the maximal response to G_{17} (at 2.3×10^{-12}M) as 100% response.

As might be expected from its structural homology with the C-terminus of G_{17}, when tested alone the octapeptide of CCK had a molar potency ratio, relative to G_{17}, of 0.68 ± 0.11 (mean ± SEM; n = 5). Secretin was 100 times less potent than CCK and therefore 10^5 times less potent than G_{17} (Fig. 5); this finding might reflect contamination of the sample by CCK. To examine the combined effects of secretin and gastrin, the activity of 10^{-13}M gastrin was tested in the presence of increasing concentrations of secretin (10^{-13}-10^{-10}M). At 10^{-11} and 10^{-12}M the inhibition of G_{17}-activity was 21% and 16% respectively, with no inhibition with 10^{-13}M secretin; at 10^{-10}M, secretin completely abolished the stimulation of carbonic anhydrase activity by G_{17}.

DISCUSSION

These results seem to indicate that it is possible to measure gastrin-like activity in samples of plasma by exposing suitably prepared and primed sections of guinea-pig fundus to adequate dilutions of the samples. The recovery of exogenously added G_{17} of close to 100% indicates that these sections can recognize this hormone adequately; the index of precision of 0.1 is adequate as are the intra- and inter-assay variations (6.4% and 16.3% respectively). The activity on these sections of CCK alone was 1000 times less than that of G_{17} and the influence of secretin alone was negligible. Because of the complex interactions on the target-cell by various gastrointestinal hormones it is impossible to ensure, in all cases, that the gastrin-like bioactivity represents the sole and total influence of gastrin itself. However, it has been shown that secretin, at ten times the molar concentration of G_{17}, caused only 16% inhibition of the effect of G_{17}; even at 100 times the molar concentration of G_{17} the inhibiting effect of secretin was only 21%. For most practical purposes, these degrees of inhibition, and thus of uncertainty as to the true concentration of G_{17}, may not be serious impediments to the assay of gastrin-like activity. More serious is the effect of the intact CCK molecule. Undoubtedly the real solution to this problem is to develop a separate bioassay of CCK which has equivalent sensitivity to the present method for gastrin-like activity. However, it may be noted that the presence of appreciable concentrations of intact CCK caused marked deviation from parallelism with increasing dilution (Table 1); such lack of

parallelism would, and should, indicate that no value for gastrin-like activity can be assigned to that sample.

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