IN-VITRO STUDIES ON THE BACTERICIDAL PROPERTIES OF NATURAL AND SYNTHETIC GASTRIC JUICES

ROBERTA DARE*, J. T. MAGEE† AND G. E. MATHISON‡

*Department of Microbiology, Queen Elizabeth College (University of London),
Campden Hill Road, London, W8 4AH*

ALTHOUGH the bactericidal effects of gastric juice are well established (Garrod, 1937), little is known of the role of components other than hydrochloric acid. The experiments reported here were designed to supply some of the required information. *Shigella sonnei* was selected as a test organism, since penetration of an infective dose beyond the "acid barrier" of the stomach is a clear requirement for pathogenesis in bacillary dysentery, and Garrod found that *Shigella* spp. were the least susceptible to the lethal effects of stomach acid among a range of organisms that infect by the oral route. The action of two samples of natural gastric juice, at pH 1.4 and pH 2.7, and of an artificial gastric juice upon the organism were compared, and the relative bactericidal activity of each of the various components of the artificial gastric juice was assessed.

MATERIALS AND METHODS

Natural gastric juice. Fasting samples of natural gastric juice were obtained with a Ryle's tube from volunteers. Mucous material was removed by filtration through glass wool. Samples were tested for obvious bacterial contamination and found to yield no growth on direct culture under aerobic conditions at 37°C. They were stored at -20°C. Subject A provided gastric juice at pH 1.4 and subject B at pH 2.7.

Artificial gastric juice. The formulation used for this semi-synthetic preparation was adapted from that of Spector (1965) and is set out in table I. Fucose and sialic acid were not included, and egg-white lysozyme was substituted for "gastric" lysozyme. The final pH was adjusted to 1.4 in order to simulate that of the gastric secretion of subject A.

Pepsin estimation. The method of Anson and Mirsky as modified by Herriot (1955) was employed, with haemoglobin as substrate and under the standard conditions recommended.

Test strain and studies of bactericidal rate. For all experiments a saline suspension of *Shigella sonnei* no. NCTC8220 was used as an inoculum. No animal passage of the stock culture was attempted and it behaved as a rough-colonied strain at all stages of the experimental procedures. An 18-hr broth culture was centrifuged and resuspended in saline (0.9 per cent. NaCl) to give a concentration of 2.5×10^8 cells per ml. Gastric juice (9.0 ml) was placed in a 50-ml flask and equilibrated at 37°C in a slow-shaking waterbath. Bacterial suspension (1.0 ml) was added and the flask shaken for 15 s before the first sample was withdrawn. Further samples of 0.5 ml were withdrawn at suitable times and each was transferred immediately to 4.5 ml of neutralising buffer. Dilution and plating for viable counts by the Miles-Misra technique followed without delay. Plates were incubated at 37°C and colonies counted after 24 hr, counts being expressed as the mean of five replicates per

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* Present address: Department of Bacteriology, St George's Hospital, Hyde Park Corner, London, S.W.1.

† Present address: Aspro Nicholas Ltd, 225 Bath Road, Slough, Bucks.

‡ Requests for reprints should be sent to G. E. M.

dilution. Control preparations containing saline in place of gastric juice were held in flasks and sampled as above.

Neutralisation of samples. Samples were neutralised by transferring them to a buffer consisting of 0.1M disodium hydrogen phosphate and 0.1M sodium dihydrogen phosphate. The final pH after the addition of a 0.5-ml sample of gastric juice to 4.5 ml buffer was pH 7.1.

TABLE I

Formula for artificial gastric juice (adapted from Spector, 1956)

(i) *Salt solution*, 96 ml per 100 ml

CaCl ₂	0.24 g per l
NaCl	2.79 g per l
KCl	8.74 g per l
HCl	105 g moles per l

This was sterilised by autoclaving and stored at 4°C.

(ii) *Amino acid solution*, 1 ml per 100 ml

Alanine	2.25 g per l
Arginine	3.45 g per l
Aspartic acid	2.0 g per l
Cystine	2.75 g per l
Histidine	1.65 g per l
Glycine	1.45 g per l
Isoleucine	1.05 g per l
Leucine	1.7 g per l
Lysine	1.6 g per l
Methionine	1.15 g per l
Phenylalanine	1.3 g per l
Proline	2.45 g per l
Tyrosine	1.15 g per l
Serine	1.95 g per l
Threonine	1.65 g per l
Tryptophan	1.05 g per l
Glutamic acid	2.6 g per l

This was sterilised by filtration and stored at 4°C.

(iii) "*Sugar*" solution, 1 ml per 100 ml

Glucose	77 g per l
Glucuronic acid	2.0 g per l
Glucosamine	33 g per l
Ascorbic acid	0.95 g per l

This was sterilised by filtration and stored at 4°C.

(iv) *Lysozyme solution*, 0.5 ml per 100 ml

Chick egg-white lysozyme chloride (BDH) 1.52 g per l

(v) *Pepsin solution*, 0.5 ml per 100 ml

Crystallised pepsin (Armour Pharmaceutical Company) 10.0 g per l

1 ml of sterile distilled water was added to give 100 ml total volume; final pH was adjusted to 1.4.

Pepsin, peptone and starch. The pepsin used was crystalline pepsin (Armour Pharmaceutical Company). The peptone was Oxoid Bacteriological Peptone and the starch, was Lintner's starch (British Drug Houses).

RESULTS

Bactericidal properties of artificial and natural gastric juice

Results of tests with (i) artificial gastric juice, pH 1.4, (ii) natural gastric juice, pH 1.4 and (iii) natural gastric juice, pH 2.7, showed that all three were

FIG. 1.—Effect of dilution of normal and artificial gastric juices upon their bactericidal properties. ●—● Natural gastric juice, pH 1.4; ○—○ artificial gastric juice, pH 2.7; ▲—▲ natural gastric juice, pH 1.4.

extremely bactericidal under the conditions of the experiment. In no case did detectable survivors persist in mixtures in which the test *Shigella sonnei*

FIG. 2.—Bactericidal effect of natural and artificial gastric juices at a dilution of 1 in 32. ●—● Natural gastric juice, pH 2.8; ○—○ artificial gastric juice, pH 2.8; ▲—▲ natural gastric juice, pH 4.1.

organisms were exposed for longer than 45 s. In an attempt to develop kinetic studies, various dilutions of the gastric juices were tested for their bactericidal

effect in the system. Parallel series of twofold dilutions were tested against the organism for a fixed exposure of 10 min. The results of this experiment are

FIG. 3.—The effect of pH on the bactericidal activity of diluted artificial gastric juice.

summarised in fig. 1. It can be seen that the killing patterns of the artificial (pH 1.4) and the natural (pH 1.4) samples are similar but that the two natural

gastric juices (pH 1.4 and pH 2.7) behave quite differently. The reasons for the observed differences in killing patterns between the two natural gastric juices are elaborated in the *Discussion* (q.v.), but it should be noted at this point

FIG. 4.—The bactericidal effects of incomplete diluted artificial gastric juices. ●—● Control—complete artificial gastric juice; ●—● the enzyme component omitted; ○—○ pepsin omitted; ▲—▲ lysozyme omitted; △—△ enzymes, “sugars” and amino acids omitted, i.e., HCl and electrolytes only were present; ○—○ amino acids omitted; ●—● “sugars” omitted.

that, as a result of dissociation, the *pH* of the natural (*pH* 1·4) and artificial (*pH* 1·4) gastric juices changed to *pH* 2·8 on being diluted 1 in 32, whereas that of the other natural gastric juice (*pH* 2·7) changed to *pH* 4·1. Accordingly, a dilution of 1 in 32 was selected for all further experiments as the bactericidal activity of each of the samples then seemed to be comparable under the test conditions. Fig. 2 shows plots of the time course of killing with each of the three samples at this dilution. It can be seen that the artificial and normal gastric juices behave in a similar manner when they are at the same *pH* but that the less acid sample is much less bactericidal. The death rate appears to be exponential up to 20 minutes' exposure.

Effect of pH on the bactericidal activity of gastric juice

To determine the effect of *pH*, tests were made in a series of diluted artificial gastric juices in which only the hydrochloric acid content varied. The inoculum was exposed for a fixed 10-min. period at *pH* values in the range 1·5 to 7·0. From *pH* 3·0 to *pH* 7·0 there is little difference, but below *pH* 3·0 there is a marked increase in the lethal effect (fig. 3).

The effect of other components in gastric juice at pH 2·8

Various samples of diluted artificial gastric juice were prepared, each lacking one of the major components. The bactericidal effect of each was determined by constructing death curves (fig. 4). Pepsin, lysozyme and the amino-acid mixture appear to exert separate bactericidal effects, since the omission of any or all of these reduces the death rate. The sugars present in this artificial system appear to have a protective influence.

The effect of pepsin concentration

The above results indicated that pepsin was second in importance to hydrochloric acid in conferring bactericidal activity upon the artificial gastric juice. The concentration of pepsin used was initially determined by estimating the amount of pepsin in the natural gastric juice of subject A and then adding this amount to the artificial gastric juice. In the gastric juice from subject A, the concentration of pepsin was in the range 115–200 units per ml, and that of subject B contained 40 units per ml; and a 0·01 per cent. solution of the crystalline pepsin that we used contained 25 units per ml.

Samples of artificial gastric juice were prepared in which the pepsin concentration varied and their bactericidal properties were compared. The results (fig. 5) suggest that bactericidal activity is not directly proportional to the pepsin concentration over the limited range tested.

The effect of added nutrients upon the bactericidal activity of artificial gastric juice

Samples of diluted artificial gastric juice containing various amounts of peptone were tested for bactericidal activity during single exposures of 10 min.

The results (fig. 6) show a slight protective effect that was proportional to the amount of peptone added over the range tested. Starch had a much greater protective effect. When starch was added at a final concentration of 1 mg per

FIG. 5.—The effects of varying the pepsin concentration in diluted artificial gastric juice. ▲—▲ Complete artificial gastric juice—pepsin content 0.5 g per l; ○—○ pepsin content, 0.25 g per l; ●—● pepsin omitted; △—△ pepsin content 2.5 g per l. The concentrations of pepsin given are the amounts before dilution 1 in 32.

ml of gastric juice, no killing effect was detectable unless undiluted gastric juice was used (table II).

FIG. 6.—The effects of supplementation with peptone on the bactericidal activity of diluted artificial gastric juice.

TABLE II

The protective effect of starch against the bactericidal action of artificial gastric juice

Test solution	Percentage viability after exposure for 10 min.
Artificial gastric juice diluted 1 in 32 (pH 2.8)	0.01
Artificial gastric juice, diluted 1 in 32, plus 1 per cent. starch	100

DISCUSSION

An important factor to be considered in the interpretation of these results is the dilution effect. A dilution of 1 in 32 proved to be operationally convenient, but the pH 1.4 and pH 2.7 samples were affected in different ways. On dilution, the pH of the gastric juice changes as a result of dissociation of HCl. The difference in bactericidal activity on diluting the two samples could be partially explained on the basis of the requirement for HCl in the conversion of pepsinogen

to pepsin (Bell, Davison and Scarborough, 1968). The peptic activity of the pH 2.7 samples used in our experiment was a fifth of that of the pH 1.4 sample. Thus this generally "weaker" character of the pH 2.7 secretion is consistent with the observed effects of dilution on bactericidal activity.

The interpretation of the results obtained with modified semi-synthetic gastric juices depends upon the reliability and reproducibility of the death curves. All show a deviation from exponential kinetics. Microscopical evidence suggests that this is due to a pH-dependent con-agglutination of the bacteria that exerts a protective effect, rather than to the survival of a more insensitive fraction. The effect was particularly pronounced in pilot experiments performed without mechanical agitation. The results presented are otherwise reproducible.

The difference in activity of the pH 1.4 and pH 2.7 natural gastric juices and of artificial gastric juice at different pH values points to the major role of the acid component. When only acid and electrolytes were present, the lethal effect was not much less than that of the complete artificial system or of natural gastric juice. Of all the "non-acid" components in the diluted artificial system at pH 2.8, pepsin had the greatest antibacterial influence. Lysozyme exerted an effect also, but only slightly enhanced the activity of pepsin when the two were present together. However, the lysozyme used was egg-white lysozyme which has an optimum of pH 6.0. Gastric lysozyme may well be more effective, and it is likely that lysozyme would play a greater part in the destruction of Gram-positive bacteria in the stomach. Of the non-enzymic components, the sugars and organic acids appeared to exert a protective effect and the amino-acid mixture to be slightly bactericidal.

The relevance of investigations of this nature may be open to debate. The use of diluted gastric juice is obviously justified since dilution and buffering effects operate during the passage of food and water through the stomach, so that the pH of its contents then differs from that of fasting gastric secretion. Moreover, the secretion itself changes in composition after initial stimulation. With prolonged stimulation, the pH of the stomach contents may rise to a value as high as pH 5 or 6 as a result of dilution (Drasar, Shiner and McLeod, 1969) and this would permit survival of our test strain of *Shigella sonnei*. Clearly the condition of the stomach contents at the time of ingestion of shigellae, and probably of other organisms, is a crucial factor in determining the degree of their survival. The effects of starch and peptone in our in-vitro system suggest that food in the stomach would exert a major protective influence. That the stomach contents are not as harsh an environment for bacteria as is generally supposed is evidenced by the study of Drasar *et al.*, who examined the bacterial flora of fasting gastric secretions from normal and achlorhydric subjects. These workers found that the size and variety of the bacterial population increased with the pH of the secretions. The importance of pH has also been established in studies with oral vaccines against dysentery and typhoid fever (Dupont *et al.*, 1971) in which high oral doses were coupled with pre-treatment with oral sodium bicarbonate, and in establishing cholera in human volunteers (Music *et al.*, 1971). In the latter investigation it was found that

diarrhoea was induced by an oral dose of 10^4 *Vibrio cholerae* organisms with bicarbonate, but a dose of 10^8 organisms was required if bicarbonate was not given. Moreover, the duration of the buffering effect could be correlated with susceptibility to disease.

When the pH of the stomach contents is high, factors other than stomach acid may play a greater role. Hauschild, Hilsheimer and Thatcher (1967) studied the antibacterial effect on *Clostridium perfringens* of an artificial gastric juice consisting of electrolytes and HCl. They showed that not only proteins in general but also pepsin had a protective effect. This contrasts with our findings, and may reflect differences in cell wall composition between the test organisms. It would be unwise to extrapolate our findings to other organisms in the absence of appropriate data. The artificial gastric juice we used seemed to be an adequate substitute for natural gastric juice in comparative studies with *Shigella sonnei*, but experiments with other test organisms, preferably freshly isolated strains, should now be performed.

Other factors that we have not considered, but which could be tested in the artificial system, might influence the survival of bacteria in gastric secretions. For example, the specific effects of antibodies, particularly IgA which is present in gastric secretions, and the role of gastric phagocytes, might be examined.

SUMMARY

Artificial gastric juice was prepared and shown to be comparable with natural gastric juice in its bactericidal activity against a stock strain of *Shigella sonnei*. The acid component of natural gastric juice was the major bactericidal component, but pepsin increased the bactericidal effect significantly. Other components such as lysozyme and amino acids contributed to the lethal properties of the artificial system, whereas sugars and organic acids collectively exerted a protective influence. Peptone or starch added to the gastric juice reduced its bactericidal activity. The artificial gastric juice was most effective in killing *Shigella sonnei* in tests at pH 1.5–pH 3.0; at values above pH 3 the organism was able to survive in this system.

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