expected to give maximum rates of carbohydrate oxidation (Wenner, Dunn & Weinhouse, 1954).

All these results show that an adaptive increase in the oxidation of galactose in erythrocytes and liver takes place in rats when the diet contains 20 % of lactose; this increase is lowered when lactose content of the diet is increased to 40 %. Galactose oxidation by liver and erythrocytes in group 1 of series 2 was higher than that of group 1 of series 1. This indicated that the galactose-oxidizing capacity increased with age. Glucose oxidation was decreased in erythrocytes and liver of rats receiving 20 % of lactose in the diet and such decrease was still greater in erythrocytes of rats receiving 40 % of lactose (Table 3).

The increased oxidation of galactose might be due to an increase in enzyme concentrations, permeability effects or alterations in cofactor concentrations. It has not been possible to differentiate between these causes in the present work. The control rats used by Fiegelson & Conte (1954) showed high galactose oxidation and the oxidation rate in these adult rats could not be increased by galactose administration, whereas in our experiments young rats were used where galactose oxidation in the control rats was low and lactose feeding increased the galactose oxidation. The galactose-oxidation capacity of our control rats increased with age, which is in conformity with the initial high galactose-oxidation capacity of the adult rats used by Fiegelson & Conte (1954).

SUMMARY

1. Three groups (1-3) of albino rats (30-35 g.) were placed on diets in which respectively 0, 20 and 40 % of lactose was substituted for sucrose.
2. After 45 days' feeding, galactose-oxidizing

activity in the erythrocytes had increased considerably in group 2, but not in group 1.

3. After 3 months' feeding, galactose-oxidizing activity of suspensions of liver in Ringer-phosphate had increased in group 2 but not in group 1.

4. After 6 months' feeding the rates of consumption of oxygen (/mg. of N of erythrocytes or /mg. dry wt. of liver) with galactose as substrate had increased in group 2 compared with group 1.

5. In group 3 this increase was less in the erythrocytes and insignificant in liver. The rates of consumption of oxygen by erythrocytes and liver with glucose substrate were lower in group 2 than in group 1, and still lower in group 3.

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Studies on Gastric Proteolysis

4. PROTEINASE ACTIVITY OF GASTRIC JUICE AND GASTRIC-MUCOSAL EXTRACTS AT pH 6 TO 8*

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There have been several reports of the existence in gastric juice and gastric-mucosal extracts of a weak proteinase with maximal activity at neutral pH (Willstätter & Bamann, 1929; Klein & Wilkinson, 1933; Griffiths, 1934; Emerson & Helmer, 1936; Taylor, Castle, Heinle & Adams, 1938; Fox, 1949;

Glass & Boyd, 1954; Janowitz & Hollander, 1954). Except for that of Willstätter & Bamann, these reports have been concerned with gastric mucolysine (Glass & Boyd, 1954; Janowitz & Hollander, 1954), an enzyme which liberates tyrosine-containing products from human gastric mucus, or with the intrinsic anti-pernicious anaemia factor of Castle (1929). This factor occurs in gastric juice and

* Part 3: Taylor (1959c).

gastric-mucosal extracts, and several attempts have been made to show that its action upon ox muscle at pH 7.0, to yield a product which will cause a remission of pernicious anaemia, is proteolytic. Taylor *et al.* (1938) found a parallel variation between the intrinsic-factor activity of various preparations and their proteolytic activity at neutral pH, and showed that the gastric juice of patients with pernicious anaemia possessed neither action. Since the discovery of vitamin B₁₂, the hypothesis that intrinsic factor is a proteolytic enzyme has been largely abandoned, but the nature of the observed proteolytic activity of gastric juice at neutral pH remains obscure. It is not definitely established whether this activity results from true gastric secretion or whether it arises from traces of pancreatic or duodenal enzymes which have been regurgitated into the stomach, or from saliva, leucocytes or desquamated cells. Even if it results from gastric secretion, the question has been raised whether the activity could not be accounted for by a weak action of pepsin at neutral pH (Jones, Grieve & Wilkinson, 1938). Experiments concerned with these problems are now described.

EXPERIMENTAL

The methods and materials used have been described previously (Taylor, 1957, 1959*a*). Saliva was collected from normal human subjects by simple expectoration without stimulation by chewing. Factor B (Welch & Heinle, 1951) was donated via the Nuffield Department of Clinical Medicine by Professor A. D. Welch. Glandular mucoprotein was prepared by the method of Glass, Boyd, Rubinstein & Svigals (1952).

Enzyme-substrate mixtures were brought to a suitable pH for digestion by addition of 67 mM-disodium hydrogen phosphate-potassium dihydrogen phosphate buffer. The change of pH which occurred during proteolysis was less than 0.1 unit.

RESULTS

Proteolytic activity of human gastric juice

Action on plasma protein. Twenty-two observations were made upon fourteen normal subjects, over the range pH 6.0-8.0 (Table 1). These specimens of gastric juice were free from contamination with duodenal contents, as judged by the criteria described subsequently. Nine subjects showed activity as measured by an increased formol titration, and five did not. Definite peaks of activity were detected in only seven subjects, all of whom showed in one or more samples of gastric juice a maximum at pH 6.8-7.2 (Fig. 1). Three subjects showed activity at pH 7.5-7.7 as well.

The amount of proteolysis at the maximum at pH 6.8-7.2 varied from one-tenth to one-quarter of that at the maximum at pH 1.7-2.4, and digestion at the maximum at pH 7.5-7.7 was of the same order.

Action on other proteins. Two samples of gastric juice which digested plasma at neutral pH also digested casein with maxima at pH 6.9 and 7.5 respectively (Table 1). The amount of proteolysis was about one-eighth of that at pH 1.8-2.2. Human serum albumin was slightly digested by gastric juice from two other subjects but no clearly defined peaks of activity could be determined. Egg albumin was not digested at neutral pH by the gastric juice from three subjects which had previously been shown to digest plasma protein. Subjects whose juice failed to digest plasma protein also failed to digest casein and human serum albumin.

Further investigation of inactive juices. Griffiths (1934), Emerson & Helmer (1936), Taylor *et al.* (1938) and Fox (1949) found the proteolytic activity of gastric juice at neutral pH to be much more easily demonstrable by techniques involving the estimation of increase of non-precipitable nitrogen than by those estimating liberated amino or carboxyl groups directly. The failure to detect activity in five of fourteen normal subjects might perhaps be thus explained. The five inactive juices were therefore incubated with plasma protein and with casein. After precipitation of proteins by adding trichloroacetic acid to a final concentration of 5% (w/v), the total N of the filtrate was determined by the micro-Kjeldahl method and compared with that from the usual control tubes, mixed after separate incubation. In no case had significant amounts of digestion occurred.

Proteolytic activity of gastric-mucosal extracts

Extracts of normal human fundic mucosa from two subjects showed slight digestion of plasma protein over the range pH 6.4-7.2 (Table 1). An extract of human pyloric mucosa was inactive.

An extract of dried swine mucosa (Pepsac) in 0.01N-HCl digested plasma protein with a maximum at pH 6.7 and casein with one at pH 6.4. An extract in 2% NaCl solution digested human serum albumin with a maximum at pH 6.6. An extract of 'Gaster siccata' B.P. Codex in 2% NaCl showed proteolytic activity at pH 6.5-7.2 upon plasma protein and casein, although a significant maximum could not be established. This extract was, however, inactive on egg albumin.

Commercial rennet (a NaCl-glycerol extract of calf gastric mucosa) digested plasma protein with a pH maximum in the range pH 7.0-7.2 (Table 1) but failed to digest egg albumin.

Table 1. *Proteinase activity of human gastric juice and of human, swine and calf gastric-mucosal extracts over the range pH 6.0-8.0*

The amount of liberated amino acid nitrogen in this table and in the accompanying figures is that in the whole digest, which consisted usually of 3 ml. of buffer, 1 ml. of gastric juice or enzyme extract and 1 ml. of plasma or 5% (w/v) human serum albumin or 5% (w/v) egg albumin or 2 ml. of 2% (w/v) casein. Temp. 37°; 3 hr. 'Insulin juice' and 'histamine juice' refer to the gastric secretions obtained after intravenous injection of insulin and subcutaneous injection of histamine respectively.

Subject or extract	Substrate	Proteinase activity	
		Maximum pH	mg. of amino acid N
Human			
1. Resting juice	Plasma	6.9	0.18
2. Resting juice	Plasma	Active, no maximum	
Resting juice	Plasma	6.8	0.18
Insulin juice	Plasma	7.5	0.18
3. Insulin juice	Plasma	Active, no maximum	
4. Resting juice	Plasma	6.9	0.14
		7.5	0.12
Resting juice	Plasma	7.5	0.30
Insulin juice	Plasma	Active, no maximum	
Insulin juice	Plasma	Active, no maximum	
Insulin juice	Plasma	7.7	0.20
Insulin juice	Plasma	7.6	0.15
5. Resting juice	Plasma	Inactive	
6. Histamine juice	Plasma	Active, no maximum	
7. Histamine juice	Plasma	Inactive	
8. Histamine juice	Plasma	Inactive	
9. Histamine juice	Plasma	7.1	0.06
10. Histamine juice	Plasma	7.1	0.09
11. Histamine juice	Plasma	Inactive	
12. Histamine juice	Plasma	Inactive	
Histamine juice	Plasma	Inactive	
13. Histamine juice	Plasma	6.9	0.08
		7.6	0.06
14. Histamine juice	Plasma	7.2	0.09
4. Insulin juice	Casein	7.5	0.04
10. Histamine juice	Casein	6.9	0.08
6. Histamine juice	Human serum albumin	Active, no maximum	
9. Histamine juice	Human serum albumin	Active, no maximum	
1. Resting juice	Egg albumin	Inactive	
9. Histamine juice	Egg albumin	Inactive	
13. Histamine juice	Egg albumin	Inactive	
Fundic mucosal extract 1	Plasma	Active, no maximum	
Fundic mucosal extract 2	Plasma	Active, no maximum	
Pyloric mucosal extract 1	Plasma	Inactive	
Swine			
Pepsac (0.01 N-HCl extract)	Plasma	6.7	0.09
Pepsac (0.01 N-HCl extract)	Casein	6.4	0.05
Pepsac (2% NaCl extract)	Human serum albumin	6.6	0.12
Gaster siccata (2% NaCl extract)	Plasma	Active, no maximum	
Gaster siccata (2% NaCl extract)	Casein	Active, no maximum	
Gaster siccata (2% NaCl extract)	Egg albumin	Inactive	
Calf			
Commercial rennet 1	Plasma	7.0	0.06
Commercial rennet 2	Plasma	7.3	0.05
Commercial rennet 1	Egg albumin	Inactive	

Paper chromatography of digestion products

Table 2 shows that digests of plasma protein and gastric juice at pH 6.9 contain products staining with ninhydrin which were not present before digestion. This confirms that proteolysis has in fact taken place.

Investigation of possible sources of error

Effect of gastric urease. This enzyme is known to act over the range pH 6.0–8.0. If ammonia were liberated by it during the present experiments, a spuriously high formol-titration would result from the subsequent interaction of formaldehyde and NH_4^+ ions. Inaccuracy in the determination of pH maxima might thus arise. Direct determination of ammonia in the digestion mixtures with Nessler's reagent revealed, however, that there was no excess liberation of ammonia above the small amounts occasionally found in controls. This is probably because gastric urease is rapidly inactivated (Martin, 1933; Hollan, 1947) by the high H^+ -ion concentrations at which the juices were secreted (pH 2.0) and stored (pH 3.0–4.0). Experiments with unacidified resting gastric juice showed that gastric urease exhibits a pH maximum at pH 6.6 but that, at the low substrate concentrations likely to be present in digests containing plasma, the amount of ammonia formed even under these favourable conditions would not significantly affect the proteolytic pH-activity curves.

Effect of duodenal contents. The duodenum may contain trypsin, chymotrypsin and several peptidases, all of which exert maximal proteolytic activity in the range pH 6.0–8.0. If its contents were to be regurgitated into the stomach in the absence of bile, contamination would occur which could not be spotted by the naked eye.

In Fig. 2 are compared the pH-activity curves upon plasma of a colourless and a heavily bile-stained gastric juice obtained during the same histamine test meal in a normal subject. The colourless juice has a plateau of activity from pH 6.2 to 7.4 and virtually no activity at pH 7.8, whereas the contaminated juice has an optimum at pH 7.8. Subtraction of the former curve from the latter, on the assumption that activity equal to that of the former curve is present in the latter, yields a curve with a maximum at pH 7.9. This may be regarded, approximately, as the difference in proteolytic activity brought about by contamination with bile-stained duodenal contents. This curve is in many respects similar to that of Fig. 3, in which bovine trypsin is digesting plasma with only slight activity at pH 6.0 and with a maximum at pH 7.9. Colourless juices which gave activity in the region pH 7.5–8.0 were therefore regarded with suspicion, lest there had been a regurgitation of non-bile-stained duodenal materials. In such juices the hydrolytic action upon chloracetyl-L-tyrosine, which is a specific substrate for pancreatic carboxypolypeptidase, was investigated. Any juice in which this enzyme was thus detected was considered to be contaminated with duodenal contents and its proteolytic activity at neutral pH was

Fig. 1. pH-Activity curves for the digestion of plasma protein at pH 6.0–8.0 by gastric juice from three normal subjects (■, ●, ▲). Digestion mixtures consisted of 3 ml. of buffer, 1 ml. of gastric juice and 1 ml. of plasma. The isolated points (○) were obtained by doubling the concentrations of gastric juice (●) and substrate in the digestion mixture. Temp. 37°; 3 hr.

Table 2. Paper chromatography of digestion products from two samples of normal human gastric juice incubated with plasma protein

Temp. 37°, for 2 hr. at pH 6.9. 78% (w/w) Phenol in water. Ninhydrin stain.

Gastric juice A		Gastric juice B	
R_F	Amount of product	R_F	Amount of product
0.06	+	—	—
0.17	+++	0.17	++
0.25	+	0.25	+
0.33	+	—	—
0.42	+	—	—
0.54	++	0.54	+
0.77	+	—	—

ignored. The absence of bile, the absence of hydrolysis of chloracetyl-L-tyrosine and the absence of proteolytic maxima at pH 7.9 were thus the criteria upon which freedom from contamination with duodenal material was established. The pH maxima at pH 7.5-7.7 shown in Table 1 are considered not to be of duodenal origin in so far as all these criteria are obeyed.

Fig. 2

Fig. 3

Fig. 2. pH-Activity curves for the digestion of plasma protein at pH 6.0-8.5 by clear (●) and bile-stained (○) juices from the same normal subject. A curve representing the difference between the two curves is also given (Δ). Temp. 37°; 3 hr. Digestion mixtures were as described for Fig. 1.

Fig. 3. pH-Activity curve for the digestion by bovine trypsin of plasma protein. Temp. 37°; 3 hr. Digestion mixtures consisted of 5 ml. of buffer, 1 ml. of crystalline trypsin solution (10 mg./100 ml.) and 1 ml. of plasma.

Fig. 4. pH-Activity curves for the digestion of plasma protein at pH 6.0-8.0 by saliva from two normal subjects. Temp. 37°; 3 hr. Digestion mixtures consisted of 2 ml. of saliva, 1 ml. of plasma and 5 ml. of buffer.

Effect of saliva. Saliva is an inevitable contaminant of most samples of gastric juice. When saliva and plasma protein are incubated together, an increase of formol titration occurs. This has been demonstrated in each of five subjects and is maximal at about pH 7.5 (Fig. 4). Upon centrifuging saliva it is found that activity is present in both supernatant and residue (Table 3). It would appear that approximately a quarter of the activity was removed in the centrifuged deposit, the volume of which is about one-hundredth of that of the original saliva. Since the activity of both the supernatant and the salivary deposit has the same pH maximum, it is possible that the proteolytic action of saliva is really that of broken-down cells from the buccal mucosa rather than that of a true enzyme from salivary glands.

Further information about the contaminating effects of saliva may be obtained by investigating the activity of gastric juice or of mucosal extracts under circumstances where the presence of saliva is rigorously excluded. Swine pyloric juice, from an isolated pouch, was found to exert proteolytic activity at neutral pH with a maximum at pH 7.2. Commercial rennet and extracts of human and swine gastric mucosa were maximally active between pH 6.4 and 7.3 but not at higher pH. These results indicate that although proteolytic activity may occur above pH 7.3, when a maximum is found above this limit it is usually with material in which the presence of saliva cannot be excluded.

Effect of bacteria, leucocytes and desquamated cells. The possibility that these contaminants may contribute to the proteolytic activity of gastric juice at neutral pH has been investigated by centrifuging samples of active juice and determining the activity of the precipitate. In each of five subjects it was inactive at pH 6.8-7.2. Even if the bulk of the activity had escaped into the gastric juice, some residual activity of the contaminants might have been expected in the precipitate. These observations would support the view that the enzyme causing proteolytic activity at neutral pH is secreted by the mucous membrane. The possibility that it is derived from bacteria proliferating

Table 3. Liberation of titratable amino groups from 1 ml. of plasma

Plasma was in 67 mM-phosphate buffer, pH 7.5. Temp. 37°; 3 hr.

Enzyme preparation	Formol titration (ml. of 0.04N-NaOH)
Saliva (1 ml.)	0.21
Centrifuged supernatant (1 ml.) of saliva	0.15
Centrifuged deposit (1 ml.), dissolved in half original volume	0.10

in the deeper layers of the mucosa, as appears to be the case with gastric urease (Davies & Kornberg, 1955), cannot be excluded.

Effect of pepsin at neutral pH

The evidence that has been presented indicates that gastric juice is sometimes capable of proteolytic action at neutral pH when care is taken to exclude, or allow for, urease action, and for contamination by duodenal contents, saliva, bacteria, leucocytes and desquamated cells. A possible explanation of this proteolytic activity may be that it is caused by pepsin. The activity is not, however, a simple prolongation of that of pepsin at pH 4.0, for it arises as a distinct maximum, and a different pepsin-substrate union from those already considered (Taylor, 1959*b*) would be implied.

Attempts to show that pepsin exerts a proteolytic action at neutral pH have been made by incubating crystalline swine pepsin, pepsin A and electrophoretically separated human pepsins with plasma protein, casein and egg albumin. In no

instance was activity between pH 6.0 and 8.0 detected. The fact that some gastric juices of Table 1 have marked peptic activity below pH 5.0, yet do not show activity at pH 6.0–8.0, is further evidence against pepsin being the responsible enzyme, as is the observation that sometimes patients with pernicious anaemia and chronic hypochromic anaemia secrete gastric juice with no peptic activity but with proteolytic activity at neutral pH (Taylor, 1956).

Further properties of the enzyme responsible for activity at neutral pH

The isolation of a specific substance with activity at neutral pH in solid or crystalline form has not been achieved, but the data of Fig. 5 indicate that the activity may be removed more or less completely from gastric juice by 50–100% saturation with ammonium sulphate. Attempts at further purification have been unsuccessful, as there is considerable loss of activity when sub-fractionation procedures are attempted. The activity has been found to be stable at neutral pH on storage, as long as the gastric juice remains free from bacterial contamination. Activity is, however, lost when gastric juice is stored for any appreciable length of time (e.g. 1 week) at below pH 3.0 at 0–5°. These properties provide further evidence that the activity at neutral pH is distinct from that of pepsin, which is destroyed at alkaline pH but not below pH 3.0.

Relationship to intrinsic factor

Three active preparations of intrinsic factor have been investigated for proteinase activity over the range pH 6.0–8.0. Of these factor B (Welch & Heinle, 1951) showed activity, but glandular mucoprotein (Glass *et al.* 1952) and substance E (Taylor, 1955; O'Brien, Taylor, Turnbull & Witts, 1955) possessed none.

DISCUSSION

The experiments described above show that the gastric juice of a majority of normal human subjects, and human and swine gastric-mucosal extracts, possess a weak proteinase with maximal activity at pH 6.8–7.2. The responsible enzyme is precipitated by 50–100% saturation with ammonium sulphate. Proteinase activity with a pH maximum at pH 7.5–7.7 is also sometimes present in gastric juice, but not in mucosal extracts. It is probably caused by contamination with saliva, which contains, particularly in the centrifuged deposit, a proteinase which is active at this pH.

The experiments also indicate the caution with which any claim that gastric juice exerts a proteolytic action at neutral pH should be received. It is suggested that such a claim is valid only (1) if

Fig. 5. Effect of ammonium sulphate fractionation upon the proteolytic activity at pH 6.0–8.0 of normal human gastric juice (▲). ○, Precipitate from 50 to 100% saturation, dialysed free of ammonium sulphate and re-dissolved in half the original volume; ●, supernatant after 100% saturation, similarly dialysed. Plasma protein as substrate. Temp. 37°; 3 hr. Digestion mixtures in each instance consisted of 1 ml. of gastric juice or enzyme extract, 1 ml. of plasma and 3 ml. of buffer.

there is an increase of titrable amino or carboxyl groups, accompanied by (2) qualitative evidence of ninhydrin-reactive products on paper chromatograms of the digest, (3) if these occur in the absence of bile and saliva, and of trypsin, chymotrypsin or carboxypeptidase activity, as tested for by absence of hydrolysis of a substrate specific for one of these enzymes, and (4) if separate controls of substrate and enzyme are incubated for the same length of time as the digest (Taylor, 1959*a*). Controls with boiled enzyme, or the reaction mixture at zero time, do not correct for the error of substrate or enzyme autodigestion. When working with small amounts of activity or with sensitive methods of analysis, such as paper chromatography, neglect of this precaution may give a spurious impression of activity.

If these criteria are adopted, much of the previous work on the proteinase activity at neutral pH may be of doubtful significance. The only pH-activity curves for the enzyme that have been published previously (Taylor *et al.* 1938) show an optimum in the region pH 7.5–8.0. The present observations indicate that this activity was caused by duodenal or salivary contamination rather than by the true gastric enzyme.

The previous evidence that gastric-proteinase activity at neutral pH and intrinsic-factor activity are associated depended mainly on the similar behaviour of the two activities to various procedures of fractionation and adsorption. In both glandular mucoprotein (Glass *et al.* 1952) and substance E (O'Brien *et al.* 1955) the two activities are dissociated. It does seem therefore that the two activities are caused by different molecules or occur at different sites on the same molecule. In the latter case, loss of proteinase activity would be explained by fragmentation of the molecule or by inactivation at one site.

The physiological role of the enzyme would seem, on the available evidence, to be unimportant, for under normal circumstances the pH in the stomach when food is present is much more acid than that at which activity of the enzyme *in vitro* can be demonstrated, and even at favourable pH this activity is slight. The apparent absence of the enzyme without obvious ill-effect in about one-third of the subjects who have been investigated would also suggest that its physiological role is slight, as well as perhaps explaining why some earlier workers have doubted its presence. The possibility that the enzyme arises not from gastric cells, but from bacteria proliferating in the deeper layers of the mucosa, remains to be investigated.

SUMMARY

1. Slight proteinase activity, with a pH maximum in the range pH 6.8–7.2, has been found in

the gastric juice of nine out of fourteen normal subjects.

2. Similar activity occurred with extracts of normal human, swine and calf gastric mucosa and with swine pyloric juice.

3. This activity was present when contamination with saliva or duodenal contents was excluded and was not concentrated in the desquamated cells and leucocytes of the centrifuged deposit. It did not result from a slight perpetuation at neutral pH of the action of pepsin.

4. The responsible enzyme is precipitated from gastric juice at about pH 5.0 by 50–100 % saturation with ammonium sulphate. Castle's intrinsic factor may be concentrated similarly but the two substances are different.

5. Saliva, from five normal subjects, possessed weak proteinase activity with a pH maximum near pH 7.5. This activity is concentrated in the centrifuged deposit which contains mainly buccal squamous cells.

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