3. The isoelectric point of the haemolytic factor was determined by an electrophoretic-fractionation method and found to be at pH 6.4.

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Studies on Gastric Proteolysis

1. THE PROTEOLYTIC ACTIVITY OF HUMAN GASTRIC JUICE AND PIG AND CALF GASTRIC MUCOSAL EXTRACTS BELOW pH 5

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Sørensen (1909), Michaelis (1914) and Northrop (1922) found that purified pepsin preparations digest proteins *in vitro* with a single maximum in the region of pH 2. Because of this, most contemporary physiological texts state that gastric proteolysis *in vivo* in mammals occurs most readily near this pH. It was observed as early as 1909, however, that gastric juice and mucosal extracts exhibited relatively greater proteolytic activity at pH 4 than did preparations of pepsin (Takemura, 1909; Hirayama, 1910), and Willstätter & Bamann (1929) reported the existence in swine gastric mucosal extracts of a second proteolytic enzyme, a cathepsin, which was not present in gastric juice and which digested gelatin and egg albumin with a pH maximum at 3.5–4.0. A proteolytic peak at this pH was also demonstrated with both Cudahy and Northrop pepsin, when acting on casein, gelatin and edestin (Dyckerhof & Tewes, 1933), and it was again suggested that a second enzyme was responsible. Norris & Elam (1940) found that crystalline salmon pepsin, when prepared by different methods, gave pH-activity curves with either one or two maxima. These observations strongly suggested that gastric mucosal extracts might exhibit two proteolytic pH maxima, one of which may sometimes disappear during the purification of pepsin.

Not until 1940 was it first demonstrated that human gastric juice may digest proteins with two pH maxima, near 2 and 4 (Freudenberg, 1940).

Because activity at the second maximum could be increased by incubating with hydrogen sulphide or hydrogen cyanide, the presence of a cathepsin was again postulated. Buchs (1947) extended Freudenberg's observations, working mainly with human gastric juice on edestin, and concluded that gastric juice contained two enzymes, pepsin and cathepsin. The latter was found, unlike pepsin, to be activated by potassium cyanide, hydrogen sulphide and Co^{3+} and Mn^{2+} ions, to be relatively resistant to ultraviolet radiation and to heating at 70°, and to be inhibited by uranyl and Al^{3+} ions. Two pH maxima, near 2 and 4, have also been observed for proteolysis by human gastric juice by Merten & Ratzer (1949), Milhaud & Epiney (1951), and Bramstedt & Krüger (1954), for proteolysis by swine pepsin (Pope & Stevens, 1951; Ramer, 1954) and for proteolysis by dog gastric juice (Penitschka, 1953; Pfisterer, 1955).

In the calf also, gastric mucosal extracts have been shown to digest proteins with two pH maxima near 2 and 3.5 (Berridge, 1945). The second maximum is attributed, however, to rennin, which when crystallized from crude extracts undoubtedly digests proteins with a pH maximum near 3.5 (Berridge, 1945; De Baun, Connors & Sullivan, 1953). Rennin might thus seem to be taking the place of the 'cathepsin' of other mammals, but the absence of a second pH maximum from rennin-free extracts of calf gastric mucosa has never been demonstrated.

The existence of two pH maxima and of a separate gastric cathepsin have been challenged by several workers. Geilenkirchen & Elbers (1950), using hydrochloric acid extracts of swine mucosa, found two pH maxima with edestin as substrate, but only one with each of four purified plasma-protein fractions. They concluded that the two maxima were the property of the substrate, not of the enzyme. Crebolder, Engel & Otten (1951), working with human gastric juice and the substrates edestin, haemoglobin and plasma protein, contested several of Buchs's observations about the different effects of activators and inhibitors on cathepsin and pepsin. Milhaud & Epiney (1951), although finding two pH maxima, obtained no activation at pH 4 by $-SH$ groups or $-CN^-$ ions. Tolckmitt (1954) concluded that the occurrence of two peaks in the pH-activity curves of Buchs was an artifact caused by changing from hydrochloric acid to acetic acid for control of pH. More recently, Masch & Huchting (1955) and Christensen (1955) have failed to find evidence of more than one pH maximum. Acceptance of the existence of a separate cathepsin in gastric juice or mucous membrane is made still more difficult by the failure of several workers (Buchs, 1947, 1954; Milhaud & Lepine, 1949; Merten, Schramm, Grassmann & Hannig, 1952; Heinrich, 1953) to isolate it separately in solution from gastric mucosa by means of chemical, electrophoretic or ultracentrifugal methods.

There would thus appear to be doubt whether gastric juice and pepsin preparations display one or two proteolytic pH maxima *in vitro*; whether, when two occur, one of them is an artifact associated with the use of certain substrates and buffers; and whether the second maximum, if genuine, is caused by a second enzyme, a cathepsin. The relationship of the proteolytic activity of rennin to the alleged catheptic activity also needs clarifying. An investigation bearing on some of these problems is now described.

EXPERIMENTAL

Gastric juice. Normal human gastric juice was obtained by intragastric tube from healthy human subjects or from hospital patients in whom there was no evidence of anaemia, gastrointestinal disease or disease of the gall-bladder. Subcutaneous histamine or intravenous insulin were the usual stimulants. During collection of gastric juice, saliva was expectorated into a separate vessel. Samples of gastric juice containing bile were discarded.

Gastric mucosal extracts. Hog mucosa was obtained from stomachs removed within 15 min. of death. Extracts were made in 2% (w/v) NaCl unless otherwise stated. For experiments on calf-mucosal extracts, commercial rennet—a glycerol-saline extract of calf mucosa—was used. Extracts were also made from dried hog stomach by using

two commercial sources, Pepsac (Boots Pure Drug Co., Ltd.) and Gaster siccata B.P.C. (British Drug Houses Ltd.).

Pepsin. Crystalline swine pepsin (Armour) was used. Pepsin A was prepared from it by the method of Herriott, Desreux & Northrop (1940) as modified by Baker (1951).

Substrates. All but two were obtained commercially. In each instance the purest available product was sought and used, unchanged. The number of components in protein substrates was ascertained by paper electrophoresis at pH 8.6 in 0.1M-diethyl barbiturate buffer and at pH 7.4 in 0.067M-phosphate buffer. Human plasma was obtained in batches of about 500 ml. after separation of red cells from blood donated to the Transfusion Service. Benzyloxy-carbonyl-L-glutamyl-L-tyrosine was kindly prepared by Dr P. W. Kent. Plasma was used as substrate in the earlier experiments because, as it consists of several different proteins, there seemed more likelihood of detecting with it all the proteolytic activity of gastric juice than if a single protein substrate were used.

Buffer solutions. Enzyme-substrate mixtures were brought to a suitable pH for digestion by addition of one of the buffers, 0.1M-glycine-HCl for pH 1-4, or 0.2M-sodium acetate-HCl for pH 1.5-5. These buffers caused no detectable activation or inhibition of proteolysis when compared with digestion at similar pH in dilute HCl. It was found that at all practicable concentrations of buffer and substrate protein, the pH of the digestion mixture was different from that of the buffer alone, i.e. the substrate becomes a part of the buffer system.

Determination of pH. The pH of the digestion mixtures was determined by the Cambridge or Marconi pH meters with a calomel half-cell and glass electrode. A standard pH of 3.97 at 15° was given by an aqueous 0.05M-solution of potassium hydrogen phthalate (10.211 g./l.). All proteolytic experiments were carried out at 37°, but the pH of digestion mixtures was determined at room temperature without correction, for the error thus introduced was small (Clark, 1928) compared with the change of pH during proteolysis.

Change of pH during proteolysis. This depends on the time of digestion and on the amount of buffer solution used, but with excessive buffer the subsequent formol titrations become large and unmanageable. In practice it was found best to add sufficient buffer to limit the change of pH to less than 0.1 unit.

Determination of amount of proteolysis. The liberation of carboxyl groups from peptide bonds was determined by formol titration. Maximal accuracy was achieved by titrating, without preliminary neutralization before adding formaldehyde, to an end point of pH 8.5 determined by phenolphthalein (Taylor, 1956, 1957). The final formaldehyde concentration was 6-9% (w/v) (Levy, 1934).

Detailed experimental procedure. Boiled gastric juice was found impracticable for control experiments because its cloudiness prevented accurate comparison with the digest at the end point and because on boiling there was a demonstrable loss of $-NH_2$ groups. Unboiled gastric juice has itself a perceptible formaldehyde titration, which increases when gastric juice is incubated alone at acid pH (Taylor, 1956). Thus the use of a digestion mixture at zero time as a control is also unacceptable. An attempt to overcome these difficulties was made by incubating gastric juice and substrate separately in buffer for the same length of time as the digest and using their combined formol

titration as control. This combined value was, however, consistently higher than that of the 'digest' when there was no digestion, and investigation showed the cause to be a small but definite loss of titratable groups on mixing gastric juice and protein substrates before incubation (Taylor, 1956).

The following experimental procedure was finally found acceptable—for each point on the pH-activity curve, seven boiling tubes were prepared and incubated at 37°.

Tube	Substrate (ml.)	Enzyme (ml.)	Buffer (ml.)
1, 6	<i>a</i>	—	<i>x</i>
2, 7	—	<i>b</i>	<i>y</i>
3, 4, 5	<i>a</i>	<i>b</i>	<i>x+y</i>

The amounts *x* and *y* of buffer are such as to bring the gastric juice and substrate to the same pH, within 0.1 pH unit. Tube 5 is removed half-way through incubation for the determination of pH. Four other tubes are prepared for each point on the pH-activity curve, each containing sufficient formaldehyde, adjusted to pH 8.5 with 0.1N-NaOH, to give a formaldehyde concentration in the titration mixture at the end point of 6–9% (w/v). At the end of the digestion period, tubes 1 and 2 are removed together, tube 2 is emptied into tube 1 and approximately one-third of the contents of a formaldehyde tube added rapidly. The remaining two-thirds of the formaldehyde is used to rinse out tube 2 twice into tube 1. In this way a control tube is prepared, each of the constituents of which has been treated exactly as in the digest, even to the initial mixing before the reaction commences. The contents of the control tube are then titrated rapidly with 0.1, 0.04, or 0.0286N-NaOH until pH 8.5 is obtained. The tube is then stoppered. Neutralized formaldehyde and phenolphthalein are next added to tube 3 and the contents titrated to the colour of tube 1. Tube 4 is treated like tube 3 and tubes 6 and 7 like tubes 1 and 2. Each point on the pH-activity curve is thus determined in duplicate. A little formaldehyde solution is left behind in tubes 2 and 7, but this does not introduce an error since the formaldehyde was initially brought to pH 8.5, i.e. to the final pH of the titration. Duplicates usually agree to within 0.02 ml. and any experiment in which they differ by more than 0.04 ml. is discounted. Known amounts of different amino acids and peptides have been determined under conditions simulating proteolysis (Taylor, 1957) and the standard deviation has never exceeded 0.01 ml. of NaOH.

Criteria for establishing the existence and significance of a pH maximum. In the tables and figures which follow, the number, position and magnitude of the pH maxima have been ascertained from pH-activity curves, which have been constructed by drawing smoothly flowing curves through the experimental data. 'Shoulders' have rarely been encountered in these curves, and when they have occurred they have not been counted as maxima. The significance of a maximum when determined in this way may be assessed as follows: The maximum standard deviation of a point on a pH-activity curve, based on a single observation, is 0.01 ml. of 0.1N-, 0.04N- or 0.0286N-NaOH. The standard error of the difference of two means of two observations each may therefore be calculated and is 0.01 ml. It was thus considered significant if the maximum digestion differed from the minima on either side by more than four times this amount, and this criterion has been adopted throughout.

Reproducibility of pH maxima. When several proteolytic pH-activity curves are obtained by incubating the same gastric juice or mucosal extract with the same substrate under identical conditions on different occasions, the positions of the resulting pH maxima do not vary by more than 0.2 pH unit and often by less. This is illustrated by the first three observations of Table 3.

Extent of substrate hydrolysis. When determining a pH-activity curve it is essential that excess of substrate over enzyme be present, lest maximal hydrolysis should occur over a wide pH range, resulting in a plateau rather than in one or more maxima. Care has been taken to see that the liberated amino acid nitrogen did not usually exceed 15% of that of the substrate, so that even if pepsin can only release about one-third of the peptide nitrogen in proteins (Northrop, Kunitz & Herriott, 1948) an ample excess of substrate has always been present.

RESULTS

Proteolytic activity of human gastric juice

Action on plasma. Digestion of the proteins and peptides of plasma at 37° for periods of ½–6 hr. has been followed over the pH range 1.3–5.0 on twenty-four occasions in seventeen normal subjects, all of whom secreted gastric juice containing free acid. Representative curves relating pH to proteolytic activity are shown in Figs. 1 and 2, and Table 1 indicates the observed pH maxima and the activity in terms of liberated amino acid nitrogen at these maxima for digestion periods of 3 hr. No maxima occurred between pH 4 and 5.

It will be seen that, in all but three subjects, two separate peaks of activity were observed, one at pH 1.7–2.4 and another at pH 3.3–4.0. Of the three exceptions, two subjects showed a third peak at pH 2.7 and 2.8, and the third subject showed a similar peak at pH 2.8 but no peak between pH 3.3 and 4.0. Of the two main maxima, that at pH 1.7–2.4 was the higher in ten subjects but the lower in four. The ratio of maximal activity at the pH 1.7–2.4 peak to that at pH 3.3–4.0 varied from 0.79 to 2.4, mean 1.21. The variation in pH of the maxima from subject to subject is noteworthy. Differences between the batches of plasma used as substrate may partly account for this, but is unlikely to be the main factor because, in subjects 1 and 3 for example, the differing pH maxima were obtained with the same sample of plasma, and because a similar variation of pH maximum occurred with other protein substrates (Table 2) of which the same sample was used throughout. Samples of gastric juice collected at different times from the same individual gave pH maxima which sometimes differed more widely from each other (e.g. subjects 2 and 4) than would be expected from the known range of reproducibility of the pH maxima when using a single sample of gastric juice. No consistent differences of pH maximum could be detected

Fig. 1. pH-activity curves for the digestion of proteins by human gastric juice at 37° for 3 hr. O, Human plasma protein; ●, human serum albumin; △, egg albumin.

Fig. 2. pH-activity curve, showing 3 maxima, for the digestion of human plasma protein by normal human gastric juice at 37° for 3 hr.

Table 1. pH maxima for the digestion of plasma protein by gastric juice from seventeen normal subjects

In this and succeeding tables, the amounts of liberated amino acid nitrogen are those in the whole digest, which usually consisted of 3 ml. of buffer, 1 ml. of enzyme extract or gastric juice, and 1 ml. of plasma or other substrate. With casein and gelatin 2 ml. of a 2% solution was normally taken. Thus with 5% serum or egg albumin as substrate the initial substrate concentration in the digest was normally 1%.

Subject	Gastric juice	pH 1.5-2.4		pH 2.5-3.2		pH 3.3-4.0	
		Maximum pH	mg. of amino acid N	Maximum pH	mg. of amino acid N	Maximum pH	mg. of amino acid N
1	Resting juice	1.9	0.60	—	—	3.5	0.25
	Insulin juice	2.1	0.87	—	—	3.8	0.68
2	Resting juice	2.1	0.72	—	—	4.0	0.67
	Resting juice	2.0	0.94	—	—	3.7	1.06
	Insulin juice	2.2	1.00	—	—	3.7	0.85
	Insulin juice	2.2	0.92	—	—	3.5	0.92
3	Insulin juice	1.7	1.01	—	—	3.7	1.11
4	Resting juice	1.8	0.83	—	—	3.5	0.77
	Insulin juice	2.3	0.74	—	—	3.8	0.55
	Insulin juice	2.2	0.41	—	—	3.8	0.36
	Insulin juice	2.4	0.43	—	—	3.6	0.29
5	Histamine juice	2.2	0.73	—	—	3.5	0.63
6	Histamine juice	1.9	0.62	2.8	0.60	Declining activity no maximum	
7	Histamine juice	2.3	0.48	—	—	3.4	0.48
8	Resting juice	1.8	0.57	2.8	0.53	3.8	0.49
9	Histamine juice	2.3	0.73	—	—	3.6	0.83
10	Histamine juice	2.0	0.73	—	—	3.5	0.63
11	Histamine juice	1.8	0.49	—	—	3.5	0.43
12	Histamine juice	1.7	0.45	2.7	0.49	3.6	0.41
13	Histamine juice	1.8	0.53	—	—	3.6	0.67
14	Histamine juice	2.4	0.77	—	—	3.3	0.84
15	Histamine juice	1.9	0.63	—	—	3.4	0.60
16	Histamine juice	1.9	0.59	—	—	3.3	0.57
17	Histamine juice	1.8	0.50	—	—	3.4	0.44

between gastric juice collected after resting, and after administration of histamine or of insulin. The duration of digestion from $\frac{1}{2}$ to 6 hr. did not significantly affect the position and relative magnitude of the pH maxima. The positions of the maxima were unaffected also within experimental error by changes of substrate concentration up to eightfold.

Action on casein, gelatin and edestin. The digestion of casein below pH 5 was followed in three subjects, all of whom showed two maxima, at pH 1.8-2.2 and pH 3.5-3.6 (Table 2). The variation from subject to subject of the pH of the former maximum should be noted although the same sample of casein was used throughout. This sample contained two main components on paper electrophoresis. Gelatin and edestin similarly contained two main electrophoretic components and were each digested with two pH maxima (Table 2). The liberation of titratable groups from gelatin was small compared with that from serum albumin or plasma, in agreement with the results of Northrop (1932).

Action on human and bovine serum albumin. These substrates contained only one protein component when examined by paper electrophoresis, but were each digested with two pH maxima, which occurred within the same range as those for plasma (Fig. 1, Table 2).

Action on egg albumin. This substrate was found on paper electrophoresis to contain two major and two minor components. Its digestion by gastric juice was observed in seven subjects with results somewhat different from those so far obtained (Table 2). Only one maximum of proteolysis, at

pH 1.5-1.8, occurred in five subjects (Fig. 1). The maximum at pH 3.3-4.0, obtained with other substrates, did not occur. In the other two, two maxima were observed, one at pH 1.5 and 1.6 and the other at pH 2.5 and 2.8. These were the two subjects who had shown three maxima for the digestion of plasma protein.

Action on benzyloxycarbonyl-L-glutamyl-L-tyrosine. This compound is one of the few synthetic

Fig. 3. pH-activity curves for the digestion of benzyloxycarbonyl-L-glutamyl-L-tyrosine by normal human gastric juice (●) and by an extract of normal human fundal mucosa (○) at 37° for 21 hr.

Table 2. pH maxima for the digestion of protein substrates by gastric juice from normal subjects

Substrate	Gastric juice	pH 1.5-2.4		pH 2.5-3.2		pH 3.3-4.0	
		Maximum pH	mg. of amino acid N	Maximum pH	mg. of amino acid N	Maximum pH	mg. of amino acid N
2% casein	Histamine juice	2.0	0.31	—	—	3.5	0.36
	Histamine juice	1.8	0.21	—	—	3.5	0.11
	Insulin juice	2.2	0.32	—	—	3.6	0.29
5% human serum albumin	Histamine juice	1.9	0.53	—	—	3.5	0.67
	Histamine juice	2.0	0.27	—	—	3.5	0.31
	Histamine juice	2.2	0.62	—	—	3.4	0.50
	Resting juice	2.3	0.24	—	—	3.7	0.27
5% bovine plasma albumin	Histamine juice	2.3	0.67	—	—	3.3	0.63
	Histamine juice	2.0	0.60	—	—	3.3	0.66
2% gelatin	Histamine juice	2.1	0.08	—	—	3.3	0.06
	Histamine juice	2.4	0.11	—	—	3.9	0.07
2% edestin	Histamine juice	1.6	0.14	—	—	4.0	0.10
5% egg albumin	Resting juice	1.8	0.27	Slowly declining activity, no peak			
	Histamine juice	1.6	0.35				
	Histamine juice	1.7	0.34				
	Histamine juice	1.5	0.42				
	Histamine juice	1.7	0.38				
	Histamine juice	1.6	0.35	2.5	0.34	—	—
	Histamine juice	1.5	0.35	2.8	0.20	—	—

dipeptides which pepsin will attack (Fruton & Bergmann, 1939; Harington & Pitt-Rivers, 1944). Significant splitting takes place only after several hours and these authors used incubation periods of as long as 24 hr. in order to demonstrate it. They found that pepsin preparations split this substrate at an optimum pH of 4.0. The effect of gastric juice has not previously been investigated. Fig. 3 shows that the pH optimum for the action of gastric juice on this substrate is at pH 4.0 also. The small amount of hydrolysis over a period of 21 hr. resembles the activity found with pepsin.

Action on other substrates. Gastric juice failed to hydrolyse, at pH values below 6, clupeine, peptone,

Fig. 4. pH-activity curves for the digestion of proteins by pig gastric mucosal extracts at 37° for 3 hr. ●, Human serum albumin and fresh mucosal extract; ○, egg albumin and dried mucosal extract; △, casein and dried mucosal extract.

glycylglycine, L-leucylglycine, L-tyrosylglycine, L-alanylglycine, glycylglycylglycine, L-leucylglycylglycine, L-alanylglycylglycine, glutathione and pteroyl tetraglutamate.

Fig. 5. pH-activity curves for the digestion of human plasma protein by commercial rennet (○) and by the supernatant material after rennin has been removed from rennet (●) at 37° for 3 hr.

Table 3. pH maxima for the digestion of protein substrates by extracts of swine and calf gastric mucosa

Substrate	Extract	pH 1.5-2.4		pH 2.5-3.2		pH 3.3-4.0	
		Maximum pH	mg. of amino acid N	Maximum pH	mg. of amino acid N	Maximum pH	mg. of amino acid N
Plasma	Pepsac in 0.01N-HCl	—	—	2.8	1.08	3.6	1.04
		—	—	2.8	1.12	3.6	1.05
		—	—	2.8	0.99	3.6	0.92
	Gaster siccata in 0.01N-HCl	—	—	2.7	0.46	3.6	0.35
5% human serum albumin	Commercial rennet	1.9	0.22	—	—	3.3	0.27
	Commercial rennet	1.6	0.42	—	—	3.3	0.36
	Fresh swine mucosa in 2% NaCl extract	1	1.8	—	—	3.6	1.32
		2	1.9	—	—	3.5	1.04
		3	1.9	—	—	3.8	0.85
	Pepsac in 2% NaCl	1.9	0.20	—	—	3.3	0.22
2% casein	Pepsac in 0.01N-HCl extract	2.0	0.73	—	—	3.3	0.90
		2.1	0.15	—	—	3.3	0.18
	Gaster siccata in 0.01N-HCl	1.9	0.21	—	—	3.3	0.23
		—	—	2.6	0.25	3.7	0.38
5% egg albumin	Gaster siccata in 2% NaCl	1.5	0.51	Declining activity without maxima			
	Gaster siccata in 0.01N-HCl	1.7	0.20	Declining activity without maxima			
	Commercial rennet	1.6	0.14	—	—	3.7	0.03

Proteolytic activity of swine and calf mucosal extracts

Extracts of fresh pig mucosa and of dried whole stomach, in sodium chloride solution or in 0.01N-hydrochloric acid, digested plasma, casein and human serum albumin with two pH maxima, at pH values ranging from 1.8 to 2.8 and from 3.5 to 3.8 (Fig. 4) (Table 3). Egg albumin was digested with only a single maximum at pH 1.5. These results resemble those with human gastric juice. Two samples of commercial rennet also digested plasma with two pH maxima at pH 1.6 and 3.3, and 1.9 and 3.3 respectively (Fig. 5). Unlike human gastric juice and pig mucosal extracts, rennet digested egg albumin with two pH maxima, at pH 1.6 and 3.7 (Table 3). Digestion at this last maximum was slight. This maximum is at a different pH from the second maximum with this substrate which was observed at pH 2.5 and 2.8 in a minority of normal human subjects.

Effect of substrate and substrate components on pH-activity curves

The optimum near pH 2 for the digestion of proteins by the same sample of gastric juice or pepsin is known to vary slightly with the substrate (Northrop *et al.* 1948) and the present work confirms this (compare maxima with egg albumin and edestin with those with other substrates). That the nature of the substrate may also affect the shape of the pH-activity curve between pH 3 and 4 is already apparent, for, whereas most proteins are digested with a second maximum in this region, egg albumin is not. It is possible that pH-activity curves with two maxima might arise if the substrate were to contain more than one protein component, each being digested at a single but different optimum pH. Although no protein or protein component has yet been found which, when digested by gastric juice, shows a solitary optimum at pH values high enough to account in this way for the observed second maximum at pH 3.3-4.0, the possibility that different substrate components may be responsible for the two maxima must be more positively excluded.

The number of protein components in each of the substrates was determined by paper electrophoresis. Whereas plasma, edestin, casein, gelatin and egg albumin consisted of more than one component, human serum albumin, crystalline bovine plasma albumin and bovine plasma albumin fraction V (Cohn & Hughes, 1946) each appeared to be homogeneous. It is apparent therefore that substrates with only one component give digestion curves with two pH maxima; that plasma, with at least four

components, gives similarly two maxima; but that egg albumin with two major electrophoretic components has a single digestion maximum. It would seem therefore that the number of pH maxima varies independently of the number of substrate components.

Even when a protein substrate is electrophoretically homogeneous it is still possible, if some of the molecules become denatured before or during digestion, for two components to be available, the native protein and the denatured, for hydrolysis by gastric juice or mucosal extracts. If each component were to undergo optimum proteolysis at a different pH, the resulting pH-activity curve for the apparently homogeneous substrate might show two maxima. This possibility was investigated by denaturing a 4% solution of plasma albumin fraction V, and a 4% solution of crystalline bovine plasma albumin by boiling for 10 min. at pH 3.0. The resulting solutions contained only one electrophoretic component on paper and were used as substrates for gastric juice and for pepsin A. Both were still digested with two pH maxima below pH 5.0 (Fig. 6). Further evidence that single-component substrates may be digested with two pH maxima is furnished by Fig. 7, which shows the auto-digestion curve of pepsin A. This enzyme preparation has only one protein component on paper electrophoresis and by solubility measurements (Fig. 8), yet digests itself with two pH maxima at 1.8 and 3.3. It would seem therefore that, although the precise maximal points vary with different

Fig. 6. pH-activity curves for the digestion of heat-denatured bovine plasma albumin fraction V by normal human gastric juice (○) and pepsin A (●) at 37° for 3 hr.

substrates, the number of proteolytic maxima is not related to the number of substrate components or to the presence of denatured substrate.

Effect of buffer anions on pH-activity curves

Tolckmitt (1954) claimed that the existence of two pH maxima in the pH-activity curves for the hydrolysis of proteins by gastric juice is an artifact caused in the experiments of several investigators by substitution of acetic acid for hydrochloric acid for control of pH above 2.5. Springer (1950) and Masch & Huchting (1955) have also demonstrated

Fig. 7. pH-activity curve for the auto-digestion of pepsin A, prepared from crystalline swine pepsin, at 37° for 3 hr.

Fig. 8. Solubility curve of pepsin A in 0.2M-acetate buffer at pH 4.8 and 60% saturation with magnesium sulphate, at room temperature. Nitrogen was estimated by micro-Kjeldahl technique.

that different anions slightly alter the shape of pH-activity curves.

The principal buffer that has been used in the present experiments is glycine-hydrochloric acid. Fig. 9 shows that when a fresh pig mucosal extract digests plasma albumin V in the presence of hydrochloric acid only, a curve with two maxima is obtained. An almost identical curve is obtained when the same materials are incubated in glycine-hydrochloric acid buffer. The use of the latter buffer is not therefore the cause of the two maxima. Tolckmitt himself, although finding only a single pH-optimum, observed no important differences between digestion curves obtained with hydrochloric acid alone and with glycine-hydrochloric acid. The only other buffer occasionally used in the experiments described here was sodium acetate-hydrochloric acid for pH values above 3.8. Despite the use of this buffer at higher pH values it was still possible to obtain curves with single pH maxima when egg albumin was used as substrate (Figs. 1, 3). Further, whenever double pH maxima were found, they never occurred at pH values related to those over which the second buffer was in use.

Role of rennin

In Fig. 5 are shown the pH-activity curves of crude rennet and of the material remaining after precipitation from it of rennin. Rennin was removed by saturating three times with sodium chloride. After the final saturation a fine opalescence was removed by centrifuging at 38 000 g for 15 min. in the Spinco ultracentrifuge. The clear supernatant was free of rennin activity but was nevertheless able to digest plasma protein with

Fig. 9. pH-activity curves for the digestion of bovine plasma albumin fraction V, by a fresh extract of pig fundal mucosa in media containing HCl alone (○) and glycine-HCl (●) for control of pH, at 37° for 3 hr.

maxima at pH 2.0 and 3.5. The single pH optimum of rennin is experimentally well-founded, but in the absence of the enzyme calf mucosal extracts still exhibit two proteolytic pH maxima, which resemble those of human gastric juice and swine mucosal extracts.

DISCUSSION

The results presented above indicate that, under the defined experimental conditions, by the use of unmodified gastric juice or simple gastric mucosal extracts and a method which estimates total liberated carboxyl groups directly, proteins other than egg albumin are digested with two pH maxima below pH 5. Two observations require special emphasis: first, the occasional occurrence of pH-activity curves with three proteolytic pH maxima, which have not been described previously; secondly, the unique position of egg albumin among the substrates so far investigated in yielding, usually, only a single pH maximum.

It has proved possible to exclude the chemical properties of the buffer as the cause of the two maxima. Tolckmitt's contention (1954) that the double pH maxima of previous workers have resulted from changing buffers may be examined more closely. He observes the pH optimum of gastric juice upon edestin to be about 3.0 and finds that at pH 2.5 in *m*-acetic acid proteolytic activity is about 20 % less than in 0.009*M*-hydrochloric acid. If a change is made from one to the other at this point, a minimum appears which has the effect of creating a more acid maximum at about pH 2 in addition to the maximum at about pH 3 which would in any case occur. These results should be interpreted with caution, for the observed pH optimum of 3.0 for the digestion of edestin is unusually high, and most observers from the time of Sørensen have observed optimum activity at pH values below this, usually between 1.5 and 2.5. Even more striking is the fact that, on changing from hydrochloric acid for control of pH below pH 2.5 to acetic acid above pH 2.5, a new maximum at about pH 2 appears as an artifact. Thus if Tolckmitt be correct, the characteristic pH maximum of gastric juice and pepsin at about pH 2, which has been recognized and repeatedly confirmed over many years, is artifactual. The conditions under which Tolckmitt's artifact appears are not in any case applicable to the experiments that have been described above. A further strong point against either maximum arising through a change of buffer is the occurrence under exactly comparable experimental conditions of only a single optimum with egg albumin and with benzyloxycarbonyl-L-glutamyl-L-tyrosine.

From the evidence presented here multi-com-

ponent substrates can also be excluded as the cause of a double pH maximum, but the role of the individual substrate is clearly important, otherwise egg albumin and benzyloxycarbonyl-L-glutamyl-L-tyrosine would not behave differently from the others. Equally, however, would it appear that the enzyme source is important, for Norris & Elam (1940) found when using the same substrate that the number of maxima depends on the method of preparation of crystalline salmon pepsin.

The experiments with rennet indicate that calf mucosal extracts, in the absence of rennin, possess a proteolytic action similar to that of human gastric juice. Rennin would thus appear to be present in the calf as a proteolytic enzyme additional to that (or those) responsible for peptic and 'catheptic' activity. The 'catheptic' activity of human gastric juice and of pig mucosal extracts is not therefore the physiological counterpart of rennin in the calf, as the similarity of their proteolytic pH maxima might at first suggest.

It may be wondered how it has happened that so many observers before 1930, and several after that date, have reported pH-activity curves with only a single pH maximum, usually in the neighbourhood of pH 2. There are seven reasons for this, the first five of which have been mentioned by Buchs (1947, 1954): (1) Many experiments in the literature have been carried out on preparations made from commercial pepsin (in which 'catheptic' activity may possibly have been destroyed) and not upon gastric juice. (2) pH-activity curves have often not been continued to as high as pH 4 and, even when they have, insufficient points are available to reveal the maximum between pH 3.3 and 4.0 (see Sørensen, 1909; Rona & Kleinmann, 1924). (3) Different methods of extraction of pepsin may cause differing types of curve in which the maximum between 3.3 and 4.0 is often masked (see Norris & Elam, 1940). (4) An obvious second maximum is sometimes ignored (see Michaelis & Mendelsohn, 1914). (5) Willstätter & Bamann stated in 1929 that cathepsin was not secreted in the gastric juice, although found in the mucous membrane of the stomach, and since that time cathepsin has been regarded as a more or less unwelcome contaminant of pepsin preparations and has been systematically disregarded. (6) Some of the earlier experiments were carried out with egg albumin as substrate (e.g. Sørensen, 1909) and could not, in view of the observations presented here, be expected to yield pH-activity curves with double maxima. (7) There is evidence from the work of Masch & Huchting (1955) that the method of estimation of proteolysis affects the shape of the pH-activity curve. Although most of their pH-activity curves show single maxima, many of those which have been determined for gastric juice

(rather than pepsin) by the biuret method appear to show two. With the Folin and nephelometric methods only single maxima are shown. It therefore becomes particularly important to determine proteolysis by methods such as formol titration, which estimate hydrolysed peptide bonds quantitatively, rather than to rely on methods that estimate only a particular hydrolytic product or the precipitation of undigested substrate.

Several of these seven factors may have operated together to cause the single pH maxima that have been observed by earlier workers. It is difficult, however, to reconstruct in retrospect exactly what has happened, and some observations of curves with only one maximum, such as those with samples of Northrop pepsin, are not easily accounted for by any combination of these factors. The present investigation provides evidence that more than one pH maximum is normally to be found with most substrates, and that the presence of more than one maximum cannot be adequately explained as an artifactual effect of buffer anions or of differing substrate components.

Finally the influence of these observations on contemporary ideas of *in vivo* gastric proteolysis may be considered. From the tables and figures of the text it is apparent that, *in vitro*, proteolysis at the maximum between pH 3.3 and 4.0 could be approximately as effective as that at pH 1.6–2.4. There is therefore no physiological reason why intragastric proteolysis should not begin at as high as pH 4.5 and proceed swiftly at pH values above 3.5. It would thus be possible for significant gastric digestion of proteins to occur in the absence of 'free acid'. Since it takes an appreciable time for the gastric contents to achieve a pH as low as 2.0, it is possible that when the stomach empties early the lower pH maximum of gastric proteolysis is never reached, but this need not mean that significant gastric proteolysis has not occurred. Similarly, when bile-stained duodenal contents are regurgitated into the stomach, gastric proteolysis will not cease unless the pH rises above 4.5.

SUMMARY

1. The gastric juice of twenty-five normal subjects was found to digest one or more of the substrates plasma protein, casein, human serum albumin, gelatin, bovine plasma albumin and edestin with two pH maxima below 5. The two maxima range from pH 1.6 to 2.4 and from pH 3.3 to 4.0 for different individuals and with different substrates.

2. The pH maxima of gastric juices from different subjects and of samples collected at different times from the same individual sometimes differed more widely, when tested on the same

sample of substrate, than could be accounted for by the range of reproducibility of the pH maxima as determined with the same sample of gastric juice.

3. The two optima are unaffected, within experimental error, by time of digestion from $\frac{1}{2}$ to 6 hr. or by changes of substrate concentration up to eight-fold.

4. The gastric juice of two normal subjects digested blood plasma with three pH maxima, the third occurring at pH 2.8 and 2.9.

5. In contrast with the action on most substrates, the gastric juice of five normal subjects digested egg albumin with only one maximum at pH 1.5–1.8. The gastric juice of the two subjects who digested plasma with three maxima digested egg albumin with two, the second maximum occurring at pH 2.5 and 2.8.

6. The gastric juice of two normal subjects hydrolysed the specific pepsin substrate, benzyl-oxy-carbonyl-L-glutamyl-L-tyrosine, with one optimum at pH 4.0.

7. Swine and calf gastric mucosal extracts possessed proteolytic properties similar to those of human gastric juice, except that calf extracts digested egg albumin with two maxima.

8. The proteolytic activity of calf mucosal extracts at pH 3.5 is not caused wholly by rennin, which has a pH maximum in this region, but also by another enzyme which is present in extracts from which rennin has been removed.

9. The two proteolytic pH maxima are shown to occur independently of the number of substrate components and of the buffer anions present in the digestion mixture.

10. Gastric proteolysis *in vivo* might occur readily at pH 3.5–4.5. It is not necessary that an intragastric pH as low as 2.0 or 1.5 be achieved.

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Studies on the Proteins of Fish Skeletal Muscle

6. AMINO ACID COMPOSITION OF COD FIBRILLAR PROTEINS*

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Recent work (Connell, 1958*b*) has shown that the peculiar instability of cod as compared with rabbit myosin cannot be explained with reference to the picture of the molecular weight or shape of the fish protein revealed by sedimentation, diffusion and viscosity. It was therefore decided to see whether any features of the amino acid composition of cod myosin distinguished it from rabbit myosin.

The other main fibrillar protein, actin, has been included in the investigation since it was also of interest to compare the amino acid composition of this protein from two different species. In addition, cod 'natural' actomyosin, that is actomyosin prepared directly from cod muscle, has been analysed both because of its importance as a model substance and because an idea of the relative amounts of myosin and actin in the complex can be obtained by comparing their amino acid compositions. Since the method of Hasselbach & Schneider

(1951) cannot be applied to fish because the solution used to extract myosin also extracts considerable amounts of actin (J. J. Connell, unpublished work), the indirect determination of myosin and actin in natural actomyosin would seem to be the only way of measuring the amounts of myosin and actin in fish muscle.

MATERIALS AND METHODS

Proteins. Myosin, actin and actomyosin were prepared from the skeletal muscle of cod (*Gadus callarias* L.) killed just beforehand as described by Connell (1958*b*). Myosin and actomyosin were precipitated from the final buffer solution by dialysis at 0° against many changes of distilled water for several days, and the precipitates were dried with acetone, ethanol-ether (1:3, v/v) and finally in air at room temperature. The actin was purified twice by removing unpolymerized material in the preparative ultracentrifuge, the final pellet of F-actin being depolymerized in 0.1 M-adenosine triphosphate solution, pH 8 (1 ml./10 mg. of protein), and salt removed by dialysis against distilled

* Part 5: Connell (1958*b*).