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Some Observations on the Conversion of Three Different Human Pepsinogens to their Respective Pepsins*

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Fractionation of human gastric mucosal extracts on DEAE-cellulose (diethylaminoethyl-cellulose) yields pepsinogens I, II, and III. (Seijffers, M. J., Segal, H. L., and Miller, L. L., *Am. J. Physiol.*, in press). Acidification of pepsinogens I, II, and III to *p*H 2 for 8 minutes followed by incubation at varied *p*H values (1.1–7.3) for 45 minutes, and subsequent alkalinization by dialysis vs. buffer at *p*H 7.8 leads to formation of compounds intermediate in behavior between pepsinogen and pepsins, designated as human pepsin-pepsin inhibitor (HPPI) complexes. In contrast to pepsin, HPPI complexes have no milk-clotting activity at *p*H 5.5 and are stable to alkali at *p*H 7.8. In contrast to pepsinogens, HPPI complexes are unstable at or above *p*H 9.2. The amount of HPPI complex formed depends on the pepsinogen, on the duration of incubation, and on *p*H. Thus preliminary incubation of pepsinogen II at *p*H 4 yields the least amount of HPPI complex, presumably as a result of proteolysis of peptide inhibitor released in initial acid activation of pepsinogen. Availability of peptide inhibitor and combination with pepsin at *p*H greater than 5.3 are both necessary to form HPPI complex.

We have recently subjected human gastric mucosal extract to chromatography on DEAE-cellulose[1] and found three distinct pepsinogen fractions designated as pepsinogens I, II, and III (Seijffers *et al.*, 1963a). Each of the pepsinogens was then acidified to ascertain whether each would yield its own pepsin (Seijffers *et al.*, 1963a). Since the conversion of swine pepsinogen to pepsin at *p*H 2.0 has been reported as almost instantaneous (Ege and Menck-Thygesen, 1933) it was of considerable interest that DEAE-cellulose-column chromatography of acidified human gastric mucosal extract revealed a peak of proteolytic activity not seen during chromatography of the acid juice secreted by the same gastric mucosa. This peak of proteolytic activity preceded on the chromatogram the peaks of proteolytic activity corresponding to the pepsins found in gastric juice, persisted even after prolonged acidification of the mucosal extract at *p*H 2.0, and was alkali resistant (Seijffers *et al.*, 1963b). This prompted a study under controlled conditions of the conversion of the three human pepsinogens to pepsins including a scrutiny of the *p*H optimum for this conversion. It became clear that this fraction, although more alkali resistant than the corresponding pepsin, was not identical with pepsinogen, and was probably similar to the pepsin-pepsin inhibitor complex described for swine pepsin by Herriot (1938). Accordingly these alkali-resistant fractions will be referred to hereafter as HPPI (human pepsin-pepsin inhibitor) complexes (see Discussion). The following paragraphs report observations on the formation of these HPPI complexes from their precursors, human pepsinogens I, II, and III.

MATERIALS AND METHODS

Human Pepsinogen I, Pepsinogen II, and *Pepsinogen III.*—Four different human stomachs were obtained at surgical operation and served as the source of human gastric mucosal extracts used in this study. Solutions of pepsinogens were prepared by means of column chromatography of gastric mucosal extracts on DEAE-cellulose as described elsewhere in detail (Seijffers *et al.*, 1963a). Pepsinogen solutions were dialyzed against 0.001 M sodium acetate buffer, *p*H 5.4, and refrigerated until use for periods up to 2 weeks, during which time no significant conversion to pepsin occurred.

Assay of Proteolytic Activity.—Substrate for assay of proteolytic activity was acid hemoglobin consisting of 1 part 0.29 N HCl and 4 parts of 2.5% (w/v) bovine hemoglobin (Fisher Scientific Company). Five ml of acid hemoglobin and 1 ml of enzyme solution, with appropriate blanks, were incubated at 37° for 60 minutes. The *p*H of these incubation mixtures ranged between 2.2 and 2.6. The reaction was terminated by the addition of 9 ml 0.3 M trichloroacetic acid. A tyrosine standard consisting of 5 ml acid hemoglobin and 1 ml of 500 μg/ml tyrosine was incubated during

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[1] Abbreviations used in this work: DEAE-cellulose, diethylaminoethyl-cellulose; HPPI, human pepsin-pepsin inhibitor.

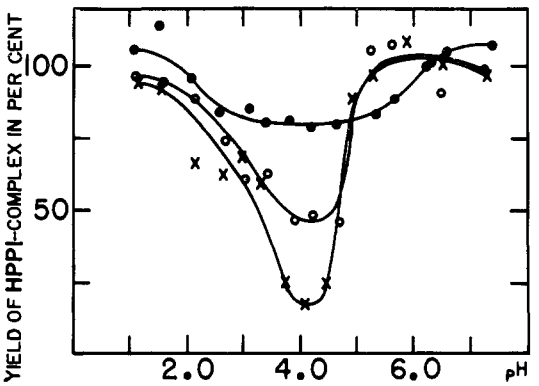

FIG. 1.—Yield of HPPI complex I, II, and III from pepsinogen I, II, and III at various *p*H values. Residual proteolytic activity after dialysis against buffer *p*H 7.8 of aliquots of pepsinogen solutions incubated at various *p*H values (as described under Experimental A) is expressed as a percentage of remaining proteolytic activity of corresponding aliquots dialyzed against buffer *p*H 5.3. Calculations are based on data presented in Tables I, II, and III. Per cent yield of HPPI complex I, ●—●; per cent yield of HPPI complex II, X—X; Per cent yield of HPPI complex III, O—O.

the same period of time and served, after the addition of 9 ml trichloroacetic acid, as standard. The trichloroacetic acid supernatant solutions were fed into an Autoanalyzer (Technicon Instrument Co.) which analyzed for tyrosinelike substances according to the method of Anson and Mirsky (1932). Results were expressed as μg tyrosinelike substance released by 1 ml enzyme solution from 5 ml acid hemoglobin during 60 minutes incubation at 37° in reference to the tyrosine standard of 500 μg.

EXPERIMENTAL

A. Conversion of Pepsinogens to HPPI Complexes.—Four-ml aliquots of ice-cold pepsinogen solution (*p*H 5.4) were pipetted into test tubes. One ml 0.077 N HCl was added to give a *p*H of 2.0. The test tubes were placed immediately in a water bath at 24° and left for 8 minutes. Thereafter, 10 ml of either dilute HCl or appropriate McIlvaine's standard buffers (Hodgman, 1950) were added to give a final *p*H ranging from *p*H 1.0 to *p*H 7.0. The test tubes were left for an additional 45 minutes at 24°, after which the reaction was terminated by pouring the contents (minus 3 ml used for a *p*H determination) of the test tube into a dialysis sac which was placed in a beaker with ice-cold 0.1 M potassium phosphate buffer, *p*H 7.8; the buffer solution was changed after 2 and 5 hours. A control experiment was run exactly as described above except that 0.1 M acetate buffer, *p*H 5.3, was used as dialysis fluid instead of phosphate buffer, *p*H 7.8. After 20 hours' dialysis as described above, the contents of the dialysis sacs were assayed for proteolytic activity.

In each experiment a solution of the corresponding pepsin was dialyzed along with the enzyme aliquots described above to ascertain that all pepsin in the aliquots was inactivated by the dialysis against buffer *p*H 7.8. For this purpose pepsin I, II, and III corresponding to pepsinogen I, II, and III were obtained from chromatography of acidified gastric mucosal extract (Seijffers *et al.*, 1963b).

B. Alkali Inactivation of Pepsinogens and HPPI Complexes.—To 4 volumes of pepsinogen solution (*p*H 5.4) was added 1 volume of dilute HCl to give a *p*H of 2.0. After 8 minutes' incubation at 24° the mixture was transferred to a dialysis sac which was dialyzed against 0.1 M phosphate buffer, *p*H 7.8. The proteolytic activity demonstrable after prior acidification and subsequent dialysis against buffer of *p*H 7.8 has been designated HPPI-complex activity. For comparison, aliquots of the corresponding unacidified pepsinogen solutions were dialyzed against the same (*p*H 7.8) buffer. At the end of 20 hours' dialysis vs. buffer *p*H 7.8, an alkali inactivation experiment was done as follows: 15 ml of the dialyzed material was mixed with 2 ml 0.5 M tris(hydroxymethyl)aminomethane (final *p*H, 9.2) and the mixture was incubated at 24°. At 0, 10, and 30 minutes after the addition of the Tris solution residual proteolytic activity was assayed by pipetting 1 ml into 5 ml of the acid hemoglobin mixtures as described above. Alkali inactivation at 24° does not differentiate between HPPI complex I and pepsinogen I; incubation at 37° was therefore performed as well.

TABLE I
CONVERSION OF PEPSINOGEN I TO HPPI COMPLEX I

	Proteolytic Activity	
*p*H[a]	After Dialysis vs. Buffer *p*H 5.3[b]	After Dialysis vs. Buffer *p*H 7.8[c]
1.06	607	642
1.48	642	732
2.09	714	678
2.60	678	571
3.10	652	553
3.40	652	518
3.80	642	518
4.20	660	518
4.63	669	535
5.30	652	544
5.61	660	580
6.21	607	607
6.55	580	607
7.35	526	562
Pepsin I aliquot	950	17

[a] The *p*H to which aliquots were adjusted after 8-minute incubation at *p*H 2.0. [b] Residual proteolytic activity in micrograms tyrosinelike substance of aliquots after prior incubation for 45 minutes at various *p*H (as indicated in first column) and dialysis vs. buffer *p*H 5.3. [c] Residual proteolytic activity in micrograms tyrosinelike substance of aliquots after prior incubation for 45 minutes at various *p*H (as indicated in first column) and dialysis vs. buffer *p*H 7.8.

C. Milk-clotting Activity.—HPPI complex was prepared from pepsinogen by acidification and subsequent dialysis against buffer *p*H 7.8 as described under B. HPPI complex was activated to give pepsin as follows: To 2 ml HPPI-complex solution was added 0.1 ml 2 N HCl to give *p*H 2.9. This activation mixture was incubated for 60 minutes at 37° after which 0.1 ml was pipetted into 2 ml of 0.1 M acetate buffer, *p*H 5.3. The milk-clotting reaction was started by adding 0.5 cc of a mixture of 5 parts homogenized fresh milk, 5 parts 0.1 M *p*H 5.3 acetate buffer and 1 part of 0.1 M $CaCl_2$ and incubation at 37°. The final *p*H of the reaction mixture was 5.5. Unactivated HPPI complex (0.1 ml) was assayed in a similar way. The clotting time expressed in minutes is the time lapse between the addition of milk and the grossly visible aggregation of protein particles.

RESULTS

A. Conversion of Pepsinogens to HPPI Complexes.—The experimental data for the conversion of pepsinogens to corresponding HPPI complexes are presented

TABLE II
CONVERSION OF PEPSINOGEN II TO HPPI COMPLEX II

pH[a]	Proteolytic Activity: After Dialysis vs. Buffer pH 5.3[b]	Proteolytic Activity: After Dialysis vs. Buffer pH 7.8[c]
1.15	283	266
1.52	300	275
2.10	316	208
2.58	277	175
2.92	258	175
3.29	250	150
3.72	266	67
4.06	258	42
4.45	283	67
4.92	266	233
5.24	258	250
5.90	275	300
6.50	266	266
7.30	217	208
Pepsin II aliquot	325	0

[a] The pH to which aliquots were adjusted after 8-minute incubation at pH 2.0. [b] Residual proteolytic activity in micrograms tyrosinelike substance of aliquots after prior incubation for 45 minutes at various pH (as indicated in first column) and dialysis vs. buffer pH 5.3. [c] Residual proteolytic activity in micrograms tyrosinelike substance of aliquots after prior incubation for 45 minutes at various pH (as indicated in the first column) and dialysis vs. buffer pH 7.8.

TABLE III
CONVERSION OF PEPSINOGEN III TO HPPI COMPLEX III

pH[a]	Proteolytic Activity: After Dialysis vs. Buffer pH 5.3[b]	Proteolytic Activity: After Dialysis vs. Buffer pH 7.8[c]
1.12	491	481
1.58	482	458
2.15	500	443
2.66	482	355
3.05	500	310
3.45	473	296
3.89	473	219
4.27	500	240
4.68	482	267
5.29	446	473
5.62	500	543
6.28	518	516
6.51	464	424
7.28	411	405
Pepsin III aliquot	727	12

[a] The pH to which aliquots were adjusted after 8-minute incubation at pH 2.0. [b] Residual proteolytic activity in micrograms tyrosinelike substance of aliquots after prior incubation for 45 minutes at various pH (as indicated in the first column) and dialysis vs. buffer pH 5.3. [c] Residual proteolytic activity in micrograms tyrosinelike substance of aliquots after prior incubation for 45 minutes at various pH (as indicated in first column) and dialysis vs. buffer pH 7.8.

in Tables I, II, and III. Residual proteolytic activity of the dialysis vs. buffer pH 7.8, which is ascribable to HPPI complex, is obviously minimal in aliquots which had been incubated at pH 3.0–5.0 prior to dialysis. Proteolytic activity of aliquots is seen not to be affected by dialysis vs. buffer pH 5.3. These tables show furthermore that the corresponding pepsin solution dialyzed against buffer pH 7.8 was in every instance completely inactivated. Yield in per cent of HPPI complex I, II, and III from corresponding pepsinogens (proteolytic activity after dialysis vs. buffer pH 7.8/ proteolytic activity after dialysis vs. buffer pH 5.3 × 100) at various pH values was calculated from data in Tables I, II, and III and is graphed in Figure 1.

B. Alkali Inactivation of Pepsinogens and HPPI Complexes.—Table IV presents residual proteolytic activity 0, 10, and 30 minutes after alkalinization of the HPPI-complex solution to pH 9.2. Corresponding pepsinogen solutions (lower half of the table) are obviously more alkali resistant. HPPI complex I is more alkali resistant than HPPI complexes II and III, and is more perceptibly inactivated at 37°.

C. Milk-clotting Activity.—Unactivated HPPI complexes showed no milk clotting or a greatly prolonged milk-clotting time as compared to the activated HPPI complex which effected clotting in several minutes.

TABLE IV
ALKALI INACTIVATION OF PEPSINOGENS AND HPPI COMPLEXES AT pH 9.2 AND 24°

Period of Alkali Inactivation (min)	Residual Proteolytic Activity (micrograms tyrosinelike substance): HPPI Complex I	HPPI Complex II	HPPI Complex III	HPPI Complex I[a]
0	658	579	318	652
10	670	163	122	439
30	658	70	4	288
	Pepsinogen I	Pepsinogen II	Pepsinogen III	Pepsinogen I[a]
0	610	1094	504	650
10	624	1085	457	596
30	644	1030	437	610

[a] Results of alkali inactivation of Pepsinogen I and HPPI complex I at 37°.

DISCUSSION

We have described elsewhere (Seijffers *et al.*, 1963a) the isolation of three distinct pepsinogen fractions from human gastric mucosa. In the course of studying conversion of the pepsinogens to the corresponding pepsins, it was found that controlled acidification followed by dialysis vs. buffer pH 7.8 results in the formation of compounds which are not pepsins since the latter are completely inactivated by this treatment (Tables I, II, and III). The compound is similar to pepsinogen in its lack of milk-clotting activity at pH 5.5 as described above but differs from the corresponding pepsinogen in being more alkali labile (Table IV).

Study of the literature revealed that a compound, intermediate between pepsinogen and pepsin, called the pepsin-pepsin inhibitor complex, had been described by Herriot (1938). In his studies on the kinetics of conversion of swine pepsinogen to pepsin a discrepancy between disappearance of pepsinogen and appearance of pepsin was noted. These observations led to the formulation of a dissociable pepsin-pepsin inhibitor complex. The pepsin inhibitor, a polypeptide which is split from the pepsinogen molecule during the conversion of pepsinogen to pepsin, was crystallized by Herriot (1941). In solution of pH greater than 5.4 the inhibitor combined with pepsin to form a complex which had no milk-clotting activity at pH 5.7. This inhibitor was shown to be digested maximally at pH 3.6 by pepsin.

Considering these observations it seems likely that the alkali-resistant compounds herein described are human equivalents of the porcine pepsin-pepsin inhibitor complex described by Herriot; accordingly, we have referred to these compounds as human pepsin-pepsin inhibitor complexes, although strictly speaking they

have admittedly not been proved to be such complexes. Proof would require at least formation of an identical alkali-resistant compound from human pepsin and human pepsin inhibitor. Since human pepsin inhibitor has not been isolated, this cannot be demonstrated at present.

The reaction in our experiments under A is therefore probably as follows: Pepsinogens are completely converted to pepsins during 8 minutes' incubation at 24° at *p*H 2.0. During this conversion of pepsinogen to pepsin a polypeptide is split off which is slowly digested by pepsin during the ensuing 45-minute period with a *p*H optimum of about 4.0. This digestion is effectively stopped by putting the reaction mixture in ice-cold buffer, *p*H 7.8, which diffuses into the dialysis bag and promotes a combination of the pepsin with the remaining undigested polypeptide to form HPPI complex; in this complex the pepsin is stable toward a *p*H of 7.8. Conversely pepsin, for which no alkali-protective polypeptide is available, is destroyed by buffer, *p*H 7.8.

After incubation at various *p*H values, dialysis of identical control aliquots against buffer *p*H 5.3 shows clearly that the potential proteolytic activity in all aliquots is the same. This rules out any artifact such as denaturation or autodigestion of proteolytic enzymes in the *p*H range of 3.0–5.0 during the 45 minutes' incubation at 24°. Our experiments do not as yet allow any conclusion as to whether an intermediate such as HPPI complex is formed as a first step during the degradation of pepsinogen, as postulated by Herriot, or whether pepsin itself is formed as a first step.

Throughout this study it has been presumed that at least three different HPPI complexes, I, II, and III exist. This would be in accord with the proven existence of at least three different human pepsinogens and pepsins (Seijffers *et al.*, 1963a). Chromatographic separation of three different HPPI complexes, analogous to our separations of human pepsins and pepsinogens, has not yet been achieved; however, the fact that HPPI complex I is more alkali resistant than HPPI complexes II and III (Table IV) tends to support this view.

The experiments described in this report are of interest in several respects. They show that the yields of HPPI complex, i.e., the amounts of proteolytic activity demonstrable after acidification and subsequent dialysis against buffer *p*H 7.8, are under the conditions used greatest for pepsinogen I and smallest for pepsinogen II (Figure 1). A likely interpretation is that this is a reflection of the ease with which the respective pepsins digest the alkali-protective polypeptide referred to, or possibly of the varying resistance of these (different?) polypeptides to digestion.

It might be noted (Figure 1) that the *p*H optimum for this process is not around *p*H 2.0 but near *p*H 4.0 for all three human pepsin fractions; this *p*H is close to that reported for the digestion of crystalline swine pepsin inhibitor by swine pepsin. It is also close to the *p*H optimum for the digestion of a number of synthetic substrates by swine pepsin (Fruton and Bergmann, 1939).

The slight loss of activity seen in aliquots incubated at *p*H greater than 6.0 (Tables I, II, III) probably reflects destruction of free pepsin before association with the protective polypeptide occurs.

It is noteworthy that the procedure of acidification and subsequent alkalinization of pepsinogens described affords a means of isolation of HPPI complex for study. The porcine pepsin-pepsin inhibitor complex of Herriot has been studied immunologically presumably after its formation from crystalline pepsin inhibitor (VanVunakis and Levin, 1963). Some data are available about the porcine pepsin inhibitor itself (VanVunakis and Herriot, 1956), but no further data are available about the properties of the pepsin-pepsin inhibitor complex. Its relative alkali stability seems, until this study, to have escaped attention.

The above observations have practical implications for quantitative studies of the conversion of pepsinogen to pepsin. If the amount of pepsinogen in an aliquot is presumed to be the alkali-resistant fraction, then it may contain not only pepsinogen but also pepsin-pepsin inhibitor complex unless the *p*H before assay was brought to 9.2 at 37°.

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