a quantity of about the same dimensions as we have found for ragweed pollen protein.

Summary.

1. Ragweed pollen has the following composition: Alcohol soluble, 42.9%; moisture, 5.3%; crude fiber, 12.2%; pentosans, 7.3%; ash, 5.4%; dextrin, 2.1%; protein, 24.4%. Of the protein, about 7.5% could not be extracted, while 6.75% was extracted with dilute alkali, and only about 5% with 10% salt solution. The albumin and globulin fraction is therefore quite small. The analytical figures indicate the presence of proteoses. The nitrogen in the alcoholic extract (1.08%) is probably a base, and the nitrogen in the saline extract after alcohol had precipitated the proteins (1.9%) probably contains this base, and also some proteose.

The alcoholic extract (42.9%) contains fat, 10.8%; lecithin, 0.75%; ether soluble but not ligroin soluble, 1.75%; sucrose, 0.4%; glucose, 1.6%; resin, 17.4%; and a nitrogenous base.

2. A characteristic ophthalmic test could be obtained in the case of two average hay fever subjects with quantities of ragweed pollen protein amounting to 0.0_51 to 0.0_55 g.

The writer is indebted to Mr. Clayre Pomeroy and Mr. J. F. Staley for assistance in this analysis, and to his colleague, Dr. L. H. Harvey, for the botanical description.

KALAMAZOO, MICH.

[CONTRIBUTION FROM THE CHEMICAL LABORATORIES OF COLUMBIA UNIVERSITY, NO. 299.]

INFLUENCE OF CERTAIN ELECTROLYTES UPON THE COURSE OF THE HYDROLYSIS OF STARCH BY MALT AMYLASE.

BY H. C. SHERMAN AND JENNIE A. WALKER.

Received May 8, 1917.

The influence of electrolytes and of hydrogen-ion concentration upon enzyme action is now so well recognized as to require no elaboration of the statement that experiments conducted with malt extract or commercial "diastase" containing unknown kinds and amounts of organic and inorganic material cannot be regarded as conclusive.

Osborne's highly purified malt amylase preparations[1] were devoted to determinations of diastatic power and especially to the study of the chemical nature of the enzyme. Recent investigations in this laboratory upon methods of purification[2] and the influence of various electrolytes upon the activity of the purified enzyme[3] have made possible the present study of the general course of the hydrolysis of starch by malt amylase

[1] THIS JOURNAL, 17, 587 (1895); 18, 536 (1896).

[2] Sherman and Schlesinger, *Ibid.*, 35, 1617 (1913); 37, 643 (1915).

[3] Sherman and Thomas, *Ibid.*, 37, 623 (1915).

under known conditions and with special reference to the influence of certain activating electrolytes.

The experiments of Thomas[1] established the optimum concentrations of several acids and acid salts for the conditions used in the determination of diastatic power[2] wherein the enzyme is allowed to act for 30 minutes upon 2% "soluble" starch at a temperature of 40°. In the present investigation the temperature employed for the action of the enzyme has always been 40° and the activating electrolytes have been chosen from among those studied by Thomas; but concentrations of electrolytes, of substrate, and of enzyme, and the time allowed for the hydrolysis, have been varied as described below.

Materials and Methods Employed.

Enzyme Preparation.—The active material employed in all of the experiments described in this paper is Malt Amylase Preparation No. 146 prepared in this laboratory by the method of purification previously described.[3]

The diastatic power of this preparation expressed on the scale in use in this laboratory since 1910[4] was 1060. This implies that it formed about 2700 times its weight of maltose from soluble starch in 30 minutes at 40°. The corresponding diastatic power on Lintner's scale would be about 1600. In other words its activity was 16 times that of Lintner's most active enzyme preparation and about 640 times that of a Lintner standard malt. At the time of the experiments here described the preparation was between one and two years old and had about five-sixths of its original diastatic power.

Weighed portions of this enzyme preparation were dissolved in specially purified triple-distilled water; kept cold (7–8° C.); and used always within 24 hours to avoid deterioration of the enzyme in solution.

Substrate.—The substrate used throughout the experiments was "Lintner Soluble Starch," purchased from Merck and purified further as described in previous papers from this laboratory.

Water and Reagents were also specially purified as in previous work.[4]

Apparatus and Laboratory Methods and Precautions were as described in connection with previous work upon the activation of malt amylase.[4]

Technique of Experiments.—Each experiment or set of experiments was conducted according to the following general plan of manipulation: Air-dry "soluble" starch equivalent to the desired number of grams of anhydrous material was "dissolved" (dispersed) by boiling in water,

[1] Sherman and Thomas, THIS JOURNAL, 37, 623 (1915).

[2] Sherman, Kendall and Clark, *Ibid.*, 32, 1073 (1910).

[3] Sherman and Schlesinger, *Ibid.*, 37, 643 (1915).

[4] *Loc. cit.*

the desired amount of activating solution added and the whole brought to a definite volume (usually 100 cc. per gram of starch) at a temperature of 40°. To each 100 cc. of this substrate was added 1 cc. of a solution made by dissolving 15 mg. (or other weighed amount) of the enzyme preparation in 100 cc. of water, the enzyme solution being measured into the digestion flask (or "Non-sol" bottle) and the solution containing the substrate and activator poured upon it. The temperature was maintained at 40° by immersing the flask or bottle in a water bath controlled by a thermostat and at the end of each desired interval of time 25 cc. of the mixture were withdrawn by means of a pipet and run at once into mixed Fehling solution to which had been added sufficient water so that the introduction of the measured portion of digestion solution would yield the correct dilution for the determination of the reducing sugar produced by the action of the enzyme upon the starch, suitable corrections being applied for the reducing action of the starch itself and for the dilution occasioned by the addition of the enzyme solution to the measured solution of substrate and activator. This general plan was followed throughout the investigation, quantities and dilutions being modified to meet the requirements of the case as the concentrations of enzyme and substrate were varied.[1]

In order to economize space in the tabulation of results only the corrected weight of reducing sugar, calculated (in accordance with experimental evidence previously described[2]) as maltose, per 100 cc. of the digesting solution, and the yield of maltose in percentage of the theoretical, or the latter alone are ordinarily given in the tables which follow.

The General Course of the Hydrolysis.

In earlier experiments demonstrating the favorable influence of acids and acid salts upon the activity of malt amylase, the time of action of the enzyme has usually been thirty minutes or one hour. In those previously made in this laboratory to determine the optimum concentrations of the activators here used, the time was always thirty minutes. In such cases malt amylase shows much greater activity in the presence of a proper small concentration of acid or acid salt than in its absence. Earlier work had shown that pancreatic amylase as tested in half-hour digestions is more active in the presence of alkali; but when the experiments were sufficiently prolonged it was found[3] that ultimately (in a typical experiment by the end of 16 hours) more hydrolysis had taken place in the

[1] The data given in this paper were all determined in the absence of any antiseptic. Several parallel experiments with added toluene gave slightly lower and no more regular results. That no antiseptic was found necessary may be due to the fact that throughout this investigation only highly purified materials were employed.

[2] Sherman and Punnett, THIS JOURNAL, 38, 1877 (1916).

[3] Kendall and Sherman, *Ibid.*, 32, 1097–1100 (1910).

neutral than in the alkaline solution. It was therefore important to determine early in the present investigation whether a similar "crossing of the time curves" would be found when malt amylase acted through long periods with and without the addition of an activating acid, or whether the yield of maltose would at all stages be found higher in the presence than in the absence of the acid. This question was first tested in a series of experiments in which neutral solutions without added electrolyte were compared with those to which had been added hydrochloric or phosphoric acid or acid phosphate in the concentration used in determinations of diastatic power.[1] In all such comparisons the solutions containing the acid or acid salt have shown a higher yield of reducing sugar than the neutral solutions without added electrolyte, whatever the stage of hydrolysis at which the comparison was made. Table I and Fig. 1 show the results of a typical series of experiments of this sort (Sets 33–36). Here the substrate was 1% starch and the concentration of enzyme preparation was 0.00015%.

TABLE I.—SHOWING THE GENERAL COURSE OF THE HYDROLYSIS OF 1% SOLUBLE STARCH BY 0.00015% OF THE MALT AMYLASE PREPARATION NO. 146 (DIASTATIC POWER 1060, "NEW SCALE" OF 1910), WITH AND WITHOUT THE ADDITION OF ACTIVATING ELECTROLYTES.

	Neutral (Set 33). Maltose formed.		HCl 0.0002 *M* (Set 34). Maltose formed.		H_3PO_4 0.0005 *M* (Set 36). Maltose formed.		KH_2PO_4 0.06 *M* (Set 35). Maltose formed.	
Time.	G.	% of theory.	G.	% of theory.	G.	% of theory.	G.	% of theory.
1 hour.....	0.1773	16.80	0.5008	47.46	0.4924	46.67	0.5572	52.81
2 hours.....	0.3130	29.66	0.6620	62.75	0.6876	65.17	0.7384	69.99
3 hours.....	0.4184	39.65	0.7140	67.67	0.7196	68.20	0.7604	72.07
4 hours.....	0.4876	46.21	0.7340	69.57	0.7428	70.40	0.7776	73.70
5 hours.....	0.5356	50.77	0.7396	70.10	0.7580	71.84	0.7792	73.85
6 hours.....	0.5888	55.81	0.7560	71.65	0.7648	72.49	0.7852	74.42
22 hours.....	0.7396	70.10	0.7784	73.77	0.7648	72.49	0.7924	75.10
28 hours.....	0.7816	74.08	0.8000	75.82	0.8112	76.88	0.8360	79.23
46 hours.....	0.7980	75.63	0.8112	76.89	0.8176	77.49	0.8384	79.46
52 hours.....	0.8236	78.06	0.8300	78.67	0.8400	79.61	0.8592	81.43
72 hours.....	0.8184	77.57	(0.8100)	...	0.8264	78.32	0.8660	82.08

It should be noted that in this case the concentration of enzyme preparation was 0.00015% which is about the maximum amount that could be used in the determination of diastatic powers by ordinary methods when dealing with preparations of such enzymic activity. With much smaller concentrations of enzyme the yield of maltose in neutral solution may constantly remain considerably below that in the "activated" solutions even when the experiments are continued until the rate of hydrolysis in both cases becomes too slight for measurement.

Other conditions being uniform, the use of a larger amount of enzyme

[1] Kendall and Sherman, THIS JOURNAL, 37, 623 (1915).

regularly results not only in a more rapid production of reducing sugar but also in a larger yield. This may be attributed in part to the unavoidable deterioration or inactivation of the amylase which is relatively more prominent the more dilute the enzyme solution. But it also indi-

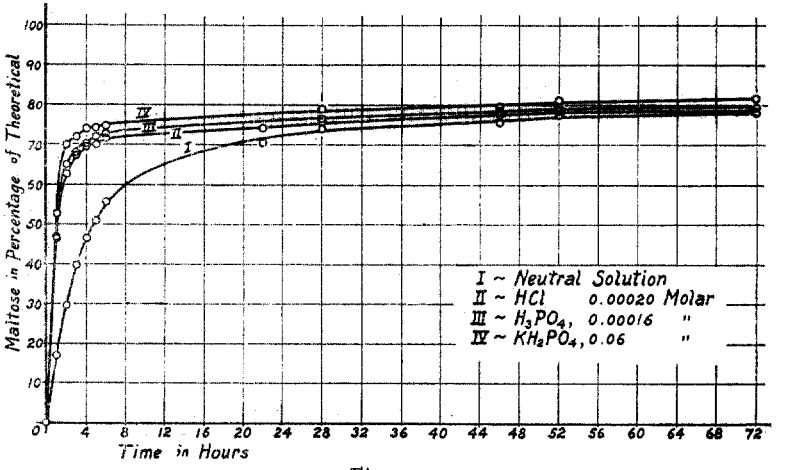

Fig. 1.

cates that the so-called "resting point" is not a definite point and is not to be interpreted as cessation of hydrolysis nor as a true equilibrium but rather as a stage of the process in which the substrate now remaining is in the form of a dextrin or dextrins much more "resistant" to enzyme hydrolysis than was the original starch. This may be explainable as due to the adsorption or surface concentration of the products of hydrolysis diminishing the opportunity for (efficiency of) contact between enzyme and dextrin. Starch apparently possesses in much more marked degree

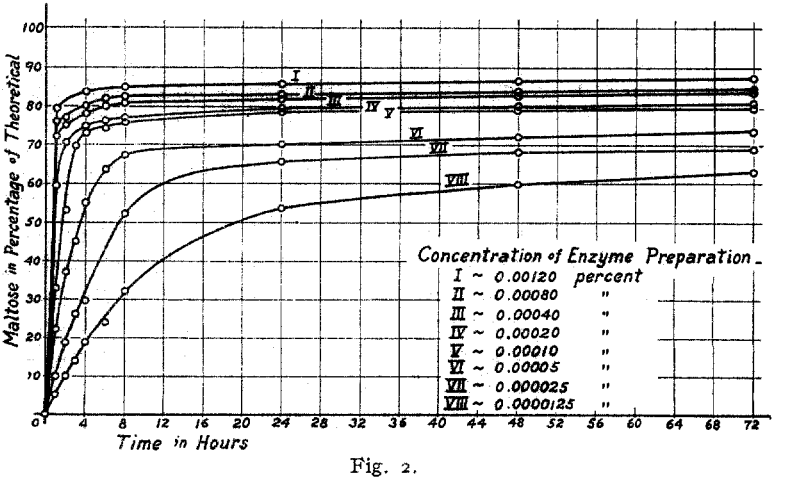

Fig. 2.

than does dextrin the properties of an emulsoid colloid and therefore would be expected to be much more readily adsorbed. Hence the initial rapid hydrolysis and the relatively slight effect of the products until the starch has been digested and given place to dextrin which is not preferentially adsorbed by the enzyme to anything like the same extent as is the starch.

The experiments summarized in Table II and Fig. 2 show that the so-called resistant dextrin does undergo a slow hydrolysis under the influence of the amylase, the yield of maltose being higher in the presence of a higher concentration of enzyme throughout the entire measurable course of the hydrolysis, that is, until the rate of production of maltose has become too small for quantitative determination.

TABLE II.—INFLUENCE OF ENZYME CONCENTRATION UPON THE COURSE AND EXTENT OF THE HYDROLYSIS OF 1% SOLUBLE STARCH IN PRESENCE OF PRIMARY POTASSIUM PHOSPHATE (0.06 *M*).

	Enzyme concentrations (in percentage of the total solution) and maltose formed (in percentage of theoretical).									
Time of hydrolysis.	0.0000125%.	0.000025%.	0.00005%.	0.00010%.	0.00015%.	0.00020%.	0.00030%.	0.00040%.	0.00080%.	0.00120%.
1 hr.	5.23	9.75	21.90	32.85	52.96	59.18	68.61	72.37	76.30	79.58
2 hrs.	9.73	18.49	36.98	52.82	70.18	70.44	74.87	75.06	76.97	83.70
3 hrs.	13.48	26.28	45.03	69.83	72.28	...	76.46	...	...	...
4 hrs.	18.94	34.32	55.18	72.93	73.59	74.56	77.11	78.27	80.07	...
6 hrs.	24.00	(34.63)	63.40	73.73	75.29	75.77	78.70	80.74	82.25	84.92
8 hrs.	32.27	52.13	67.21	75.75	...	77.31	...	81.25	82.65	...
24 hrs.	53.43	65.51	70.05	78.88	78.43	79.09	80.56	82.21	83.09	85.96
48 hrs.	59.80	67.38	72.49	79.09	80.19	80.71	...	83.29	83.90	86.72
72 hrs.	63.33	68.81	73.64	79.91	81.77	81.62	...	83.76	84.12	87.57
96 hrs.	64.93	69.79	74.71	...	...	82.72	...	84.47	84.85	...
120 hrs.	...	...	75.30	...	...	...	...	85.57	85.31	90.22

The data here given were obtained from solutions containing acid phosphate (0.06 *M*). Similar but somewhat lower results were obtained in solutions activated by hydrochloric acid (0.00020 *M*) and phosphoric acid (0.00016 *M*). In neutral solutions the final yield of maltose with relatively large amounts of enzyme were nearly as high as in the activated solutions, but with small amounts of enzyme the yields of maltose were much smaller. Thus in the presence of as much as 0.0012% of the enzyme preparation here used the speed of hydrolysis was so great (Fig. 3) that the "activating" effect of the added acid or acid salt was not perceptible and the yield of maltose both in the neutral and in the activated solutions was much greater than that (80% of theoretical) which has frequently been referred to in earlier discussions as a final yield or as constituting a resting point of the diastatic hydrolysis. But in the presence of only 0.000025% of the enzyme preparation the yield in the solution activated

by acid phosphate was only 70% of the theoretical while in the neutral solution the very small amount of enzyme exerted only a fraction of its possible catalytic effect (Fig. 3).

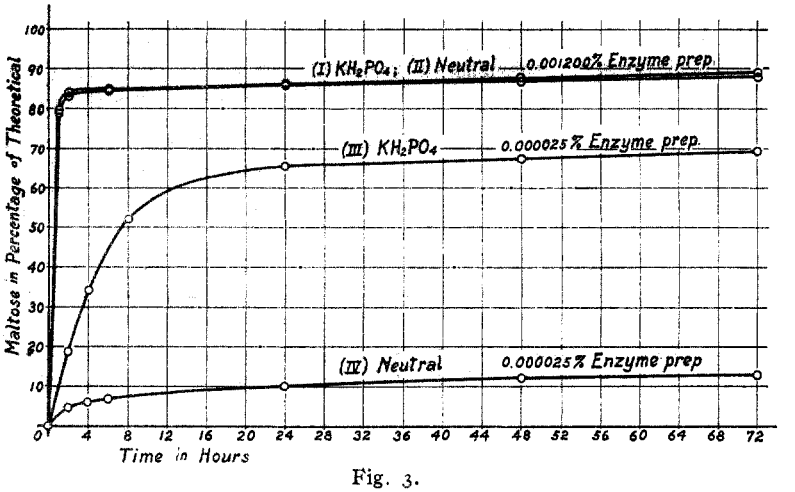

Fig. 3.

Influence of the Concentration of Electrolyte.

Experiments were made with 1% starch and various concentrations of electrolytes, not only to establish the amount of electrolyte necessary to cause the most rapid conversion of a 1% starch solution, but also to determine whether a solution of 1% starch containing that concentration of activator which appears to be optimum at the beginning of the conversion would continue to yield the largest amount of maltose as the reaction proceeded or whether this quantity of electrolyte would later

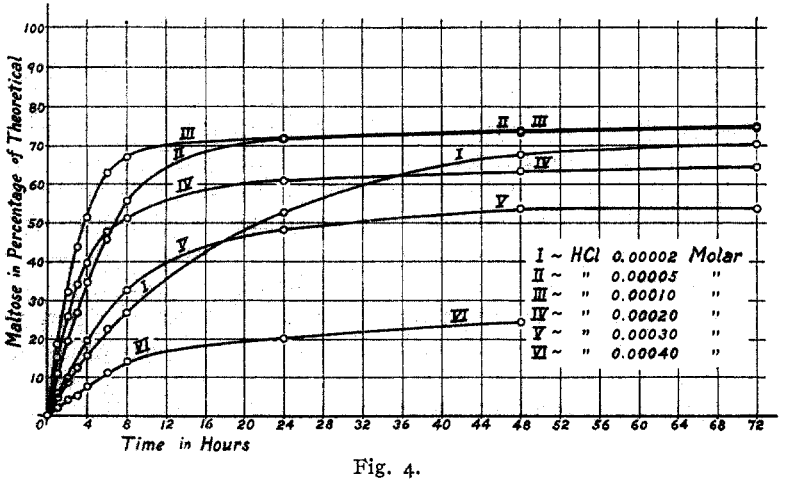

Fig. 4.

TABLE III—INFLUENCE OF CONCENTRATION OF ELECTROLYTE UPON ACTIVITY OF MALT AMYLASE IN 1% STARCH, THE CONCENTRATION OF ENZYME PREPARATION BEING 0.00005% IN ALL CASES SHOWN IN THIS TABLE. THE RESULTS ARE EXPRESSED AS MALTOSE FORMED IN PERCENTAGE OF THE AMOUNT THEORETICALLY OBTAINABLE FROM THE STARCH.

	Kind and concentration of added electrolyte.																			
		Hydrochloric acid.						Phosphoric acid.							Primary potassium phosphate.					
Time.	None (neutral solution).	0.00002 *M.*	0.00003 *M.*	0.0001 *M.*	0.0002 *M.*	0.0003 *M.*	0.0004 *M.*	0.00002 *M.*	0.00004 *M.*	0.00006 *M.*	0.00008 *M.*	0.00016 *M.*	0.00025 *M.*	0.00033 *M.*	0.005 *M.*	0.01 *M.*	0.03 *M.*	0.06 *M.*	0.12 *M.*	0.18 *M.*
30 min.	...	(4.46)	6.82	10.68	...	...	...	2.69	4.19	8.07	11.18	...	...	...	8.50	10.22	...	...	...	...
1 hr.	3.58	5.76	10.75	18.43	15.36	4.77	1.72	3.20	7.47	13.21	19.09	17.36	9.54	2.02	14.53	22.52	34.17	33.81	32.98	32.51
2 hrs.	5.85	8.25	19.31	31.90	25.43	9.83	3.76	6.16	14.32	23.17	33.22	29.03	17.30	5.76	26.98	36.04	53.89	52.21	53.27	50.17
3 hrs.	7.34	12.61	26.87	43.49	33.84	...	4.92	8.53	...	30.87	42.50	38.49	...	8.60	35.54	...	...	...	...	...
4 hrs.	9.46	15.64	34.38	51.12	39.44	19.81	7.33	12.47	26.14	38.39	51.94	43.73	28.76	12.87	44.11	59.76	68.75	65.75	62.96	60.28
6 hrs.	14.44	22.34	45.93	62.97	47.47	27.38	11.24	20.17	34.99	49.49	63.90	50.52	36.72	18.17	56.81	70.38	71.35	67.44	64.73	65.15
8 hrs.	16.06	26.53	55.87	67.04	50.98	32.55	14.17	23.04	41.48	57.72	68.13	54.83	40.88	22.34	66.35	74.29	73.96	69.02	67.37	68.41
24 hrs.	26.19	52.41	71.84	71.86	60.66	47.98	19.94	40.42	56.85	72.08	74.29	64.54	49.46	32.87	74.69	76.21	74.84	72.16	68.16	68.60
48 hrs.	37.83	67.48	73.27	74.10	63.53	52.80	24.60	55.82	61.44	73.07	75.93	67.00	52.22	36.54	75.30	77.30	77.78	74.84	70.90	69.36
72 hrs.	43.22	70.65	...	74.71	...	54.30	24.70	61.63	62.54	73.72	76.47	67.16	53.84	...	76.53	78.00	78.02	75.06	71.59	70.48
96 hrs.	44.94	73.02	75.28	75.79	65.07	55.64	26.24	62.16	...	73.91	77.81	67.99	54.26	...	78.52	79.94	79.48	75.28	73.74	...
120 hrs.	...	...	...	76.60	65.75	...	27.36	...	...	...	...	68.13	...	...	...	...	...	76.21	76.21	...

show a retarding influence as the concentration of starch diminished and the ratio of acid to starch increased.[1]

Typical or average results of such experiments are shown in Table III and Figs. 4, 5, and 6.

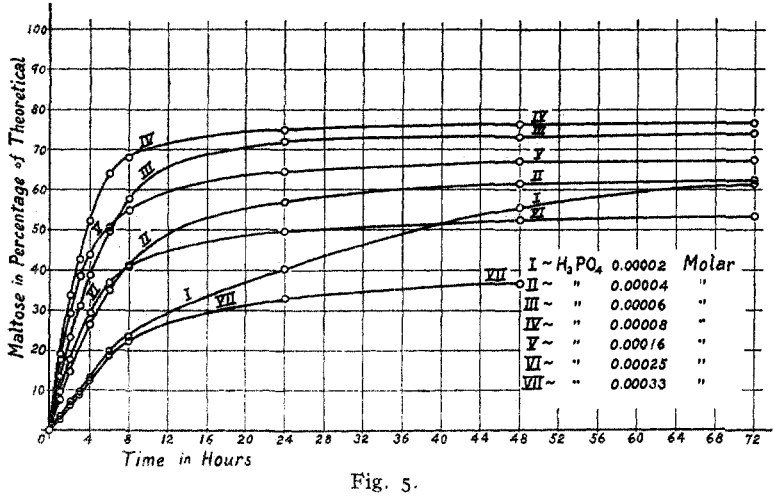

Fig. 5.

It will be seen that the effects of the three electrolytes tested show considerable differences.

Hydrochloric Acid.—With hydrochloric acid as activator the optimum concentration for the conditions obtaining in these experiments was found to be 0.0001 *M*.

The concentration of starch was here 1%. As previously determined[2]

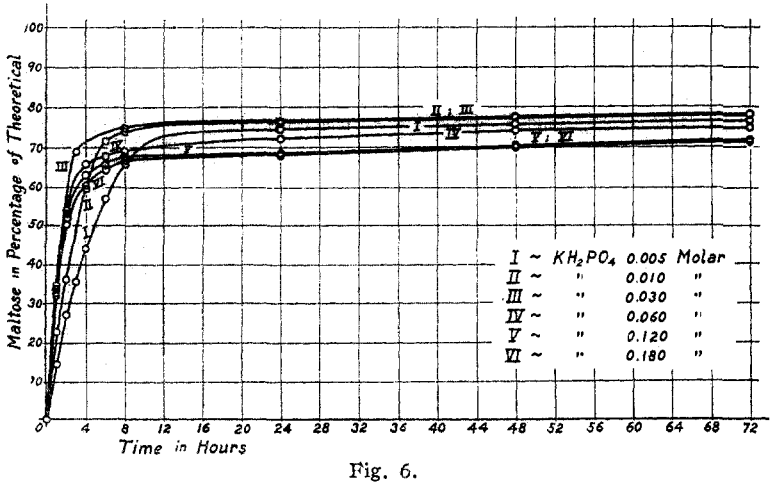

Fig. 6.

[1] Compare Kendall and Sherman, *loc. cit.*

[2] THIS JOURNAL, 37, 623 (1915).

the optimum concentration in 2% starch is 0.0002 *M*. Thus the concentration of the substrate has a marked effect upon the amount of acid which must be introduced in order to secure optimum activity of the enzyme. This appears to be due to fixation of hydrogen ion by the starch itself or the alkali previously added to the starch to render it neutral,[1] for the effective hydrogen-ion concentration as measured electrometrically was found to be practically alike ($C_H 10^{-4.4}$, or $P_H{}^+$ 4.4 in the Sorensen notation) for a 2% starch containing 0.0002 *M*, and for a 1% starch containing 0.0001 *M* hydrochloric acid.

With twice the optimum concentration of this acid the hydrolysis was only slightly retarded in the earlier stages but was more seriously inhibited later, the yield of maltose in the presence of 0.0002 *M* hydrochloric acid reaching only about 65% of theoretical as compared with about 75% obtained by the action of the same amount of enzyme in the presence of the same acid at the optimum concentration (for 1% starch) of 0.0001 *M*.

As compared with twice the optimum (0.0002 *M*), a concentration of half the optimum (0.00005 *M*) hydrochloric acid resulted in a rate of hydrolysis slightly lower in the early stages but much better sustained, so that the curve for 0.00005 *M* (II in Fig. 4) is seen to cross that for 0.0002 *M* when about 45% of the theoretical amount of maltose had been formed, and to coincide with the curve for 0.0001 *M* at and beyond 72%. Similarly the yield of maltose in the solution containing only 0.00002 *M* passes that in 0.0003 *M* at 47% and that in 0.0002 *M* at 63%.

The inhibitory effects of concentrations of hydrochloric acid 2, 3 and 4 times the optimum will be seen to be very marked, both absolutely and as compared with the effects of analogous concentrations of acid phosphate (Fig. 6).

Phosphoric Acid.—In the solutions containing various concentrations of phosphoric acid (Fig. 5) from one-fourth optimum (0.00002 *M*) to four times optimum (0.00033 *M*) the enzyme activities are seen to vary in the same manner as in the corresponding experiments with hydrochloric acid but to a less extent, probably because the capacity for secondary ionization gives this acid a certain measure of "buffer effect."[2]

Primary Potassium Phosphate.—The acid phosphate solutions show the "buffer effect" in pronounced degree, the addition of this acid salt in excess, even up to concentrations of 4 and 6 times the optimum, having only a slight inhibitory action. Comparison of Figs. 4 and 6, or of the

[1] Soluble starch made by the Lintner method always shows slight acidity even after prolonged washing with purified water. This acidity is neutralized with 0.01 molar sodium hydroxide, using rosolic acid as indicator, before the activating electrolyte is added.

[2] Possibly the starch adsorbs hydrogen ions which are subsequently liberated when the starch is hydrolyzed.

corresponding data in Table III, renders further elaboration of this point unnecessary, and demonstrates strikingly the advantage of the acid phosphate as an activating electrolyte. The optimum hydrogen-ion concentration (as measured electrometrically) is found to be practically alike (P_{H^+} 4.3 to 4.5) for the three electrolytes and for the two concentrations of substrate; but to obtain this effective hydrogen-ion concentration in 1% starch solutions requires the addition of only half as much of the acid or acid salt as is required in the case of 2% starch solutions.

Influence of the Concentration of the Substrate.

Experiments were also made to show the course of the hydrolysis when a constant concentration of enzyme acted upon different concentrations of starch in the presence of acid phosphate and of hydrochloric acid, respectively. The concentration of electrolyte in each case was that found optimum, in 30-minute experiments,[1] for the intermediate concentration of starch (2%). In each case therefore the 1% solution contained more, and the 4% solution presumably less, than its optimum concentration of acid or acid salt. Nevertheless in the presence of acid phosphate the rate of formation of maltose by a given amount of enzyme is remarkably constant up to a point corresponding (for each concentration of starch) to a production of at least one-half the theoretical yield of maltose. This regularity is undoubtedly due to the "buffer effect" of the acid phosphate maintaining a hydrogen-ion concentration approximating the optimum notwithstanding the variations in the substrate. In the presence of the hydrochloric acid, on the other hand, the hydrolysis is retarded both in the 1% and in the 4% starch solutions, the former having more, and the latter less than an optimum acidity. In the 4% starch solution the hydrolysis continues (as in the cases of underactivation, shown in Figs. 4, 5 and 6) steadily though at a less than optimum rate, and as the amount of maltose theoretically obtainable is here twice that from the 2% starch, the curves cross after the latter has passed into the stage in which the rate of hydrolysis is always greatly diminished.

In the Earlier Stages of the Hydrolysis Is the Time Curve Linear or Logarithmic?

Earlier investigators experimenting with malt extract or commercial preparations of malt amylase have reached various conclusions as to the nature of the quantitative relationship between concentrations of enzyme or time of its action and the amount of maltose produced. Some have reported a direct linear relation while others find that the relationship is logarithmic, the hydrolysis proceeding in accordance with the unimolecular reaction law. A linear relation has been argued because, as it is only the catalytic effect of the enzyme which causes the hydrolysis to proceed at a measurable rate, the speed of hydrolysis might be expected to be determined by the number of contacts between enzyme and substrate; and, while the ratio of substrate to enzyme is very large, the number of contacts and the rate of hydrolysis might be expected to

[1] *Loc. cit.*

increase directly with the concentration of enzyme or with the time. On the other hand, since the chemical reaction by which starch is changed to maltose is an hydrolysis occurring in water and hence essentially a unimolecular reaction, and since the enzyme simply affects the speed of hydrolysis and is not itself used up in the process, the chemical change is a reaction of the first order and might therefore be expected to follow the unimolecular reaction law.

Since the enzyme and substrate are both colloidal, the case is plainly more complicated than that of a simple unimolecular reaction in a homogeneous system, and is also different from that of the hydrolysis of sucrose by invertase recently discussed by Nelson and Vosburgh.[1]

We are uncertain at the present time whether in reactions of this type diffusion processes or purely chemical reactions play the predominant part.[2] In either case we would obtain the same formula ($1/t \log 1/1 - x = k$) so that the constancy of k gives no indication as to which of these predominates.

Whatever the conception of the mechanism[3] by which the catalytic effect of the enzyme is exerted the net result measured in the experiments now under discussion is the formation of reducing sugar (maltose) through hydrolysis. Hence the comparison of the values of k in the familiar formula $k = 1/t \log 1/1 - x$ may be conveniently employed to indicate to what extent the rate of change is governed by the concentration of the substrate and to compare the speed of formation of maltose at various stages in the general course of the hydrolysis.

In this connection it must also be kept in mind that the maltose is produced largely through the intermediate formation of dextrin, *i. e.*, the maltose is formed from the starch largely through consecutive hydrolyses. This in itself would tend toward a rising value of k in the initial stages of such an hydrolysis of starch to maltose which might give to this part of the reaction an appearance of a linear rather than a logarithmic relationship.

Another difficulty is introduced into the discussion of the question whether in the early stages of the hydrolysis the relationship is linear or logarithmic, by the fact that within the region for which a linear relationship has sometimes been claimed, *i. e.*, up to a yield of about 30% of the theoretically possible amount of maltose, the numerical difference between the results to be expected from a linear or a logarithmic relationship are hardly larger than might in individual cases be covered by experimental errors. In order to minimize the effect of such errors we have performed a much larger number of experiments than could be recorded in detail without unduly prolonging this report. In several cases substantially identical experiments have been carried out repeatedly at considerable intervals of time (weeks or months); more often the experimental

[1] THIS JOURNAL, **39**, 790 (1917).

[2] Cf. Lewis, "A System of Physical Chemistry," I, 507–10.

[3] If one accepts the evidence that starch molecules are of the order of magnitude of colloidal particles (Lobry de Bruyn and Wolff; Bayliss) the adsorption hypothesis and the hypothesis of an intermediate chemical compound may be regarded as synonymous in this case. It may also be noted that we are here dealing with border-line phenomena so far as the ordinary distinctions between homogeneous and heterogeneous systems are concerned.

conditions have been varied in order to ascertain whether such variations are responsible for the divergent results reported by previous investigators.

In neutral solutions containing no added electrolyte we find that, unless the amount of enzyme is relatively large, the speed of hydrolysis is low from the beginning and falls off earlier and in more pronounced degree than when the enzyme acts (as it normally should) in the presence of favorable amounts of electrolytes. In the solutions activated by acid or acid phosphate it was feasible to study the course of the reaction in detail for such concentrations of enzyme as did not cause an hydrolysis too rapid for quantitative measurement in earlier stages or so slow as to be unduly complicated by the decay or inactivation of enzyme which (in the case of amylase at least) seems always to result when the ratio of solvent or substrate to enzyme becomes too enormous. Of the enzyme preparation here used it was found best to employ concentrations of 0.000025% or 0.00005% in the study of the form of the time curve. Tables IV and V

TABLE IV.—TYPICAL DATA BEARING UPON THE PROBLEM OF LINEAR OR LOGARITHMIC RELATIONSHIPS.

	2% starch 0.00005% enzyme preparation 0.06 *M* KH_2PO_4 (Set 8).		1% starch 0.00005% enzyme preparation 0.06 *M* KH_2PO_4 (Set 119).		1% starch 0.00005% enzyme preparation 0.0002 *M* HCl (Set 129).		1% starch 0.000025% enzyme preparation 0.03 *M* KH_2PO_4 (Set 219).		1% starch 0.000025% enzyme preparation 0.0001 *M* HCl (Set 220).	
Time.	Maltose in percentage of theory.	k*$\times 10^5$.	Maltose in percentage of theory.	$k \times 10^5$.	Maltose in percentage of theory.	$k \times 10^5$.	Maltose in percentage of theory.	$k \times 10^5$.	Maltose in percentage of theory.	$k \times 10^5$.
20 min.	3.28	72	7.52	169	5.23	116	3.86	85	3.55	78
40 min.	6.46	72	13.54	157	10.20	116	7.79	88	7.72	87
60 min.	9.64	72	19.10	153	14.55	113	10.11	77	11.02	84
80 min.	11.65	67	24.43	152	19.31	116	14.73	84	14.45	84
100 min.	14.78	71	31.03	163	22.22	109	16.74	79	16.52	78
120 min.	17.49	69	35.57	158	25.57	106	19.28	77	19.75	79
140 min.	...	..	39.86	157	28.92	105	22.43	78	22.27	78
160 min.	...	..	42.41	149	30.98	100	25.14	78	25.71	80
180 min.	...	..	47.00	153	34.28	101	27.63	78	27.09	76
200 min.	...	..	50.20	151	35.78	96	30.47	78	30.58	79
220 min.	...	..	52.82	148	38.23	95	31.77	75	31.09	73
240 min.	...	..	53.81	139	39.64	91	34.99	77	33.82	74
260 min.	...	..	57.53	143	40.89	87	...	..	...	..
280 min.	...	..	58.56	136	41.66	83	...	..	...	..
300 min.	...	..	60.73	135	43.55	82	40.53	75	38.58	70
320 min.	...	..	61.03	127	...	...	...	..	...	..
340 min.	...	..	62.05	124	...	...	...	..	...	..
360 min.	...	..	62.66	118	47.93	78	46.70	75	45.17	72
420 min.	...	..	64.63	107	...	...	...	..	...	..
480 min.	...	..	65.67	97	51.88	66	53.96	70	51.89	66
24 hrs.	...	..	68.48	34	58.68	27	72.12	38	72.15	38
48 hrs.	...	..	70.88	18	63.09	15	75.94	21	75.00	20
72 hrs.	...	..	71.44	12	62.98	10	76.45	14	77.85	15

* From the formula $k = 1/t \log 1/1 - x$, in which t equals time in minutes and logarithms are to the base 10.

TABLE V.—TYPICAL AND AVERAGE DATA BEARING UPON THE FORM OF THE TIME CURVE AND THE INFLUENCE OF ENZYME CONCENTRATION.

	1% starch and 0.06 *M* KH_2PO_4.						1% starch and 0.03 *M* KH_2PO_4.					
	0.000025% enzyme preparation (Set 110).		0.00005% enzyme preparation (Set 111).		0.00010% enzyme preparation (Set 112).		0.000025% enzyme preparation (average).		0.00005% enzyme preparation (average).		0.00015% enzyme preparation (average).	
Time.	Maltose in percentage of theory.	$k \times 10^5$.	Maltose in percentage of theory.	$k \times 10^5$.	Maltose in percentage of theory.	$k \times 10^5$.	Maltose in percentage of theory.	$k \times 10^5$.	Maltose in percentage of theory.	$k \times 10^5$.	Maltose in percentage of theory.	$k \times 10^5$.
1 hr.	8.7	64	20.8	168	32.9	288	10.5	80	18.5	148	53.6	555
2 hrs.	16.7	65	31.3	135	52.8	271	18.9	75	33.2	145	70.2	437
3 hrs.	..	..	..	...	..	...	26.8	75	45.3	145	74.2	327
4 hrs.	30.9	66	52.1	133	69.8	216	33.8	74	55.3	143	75.3	252
5 hrs.	..	..	..	...	..	...	39.1	71	..	...	..	...
6 hrs.	42.3	66	64.2	123	72.9	157	45.3	72	65.3	127	76.6	175
8 hrs.	52.6	67	72.8	117	73.7	120	53.0	68	69.3	106	78.3	138
24 hrs.	..	..	..	...	..	...	68.8	35	73.2	39	80.1	48
30 hrs.	68.7	27	73.5	32	75.8	34	..	..	..	...	..	..
48 hrs.	71.3	18	76.0	21	78.9	23	71.8	19	74.6	20	82.8	26
72 hrs.	71.4	12	76.4	14	79.1	17	72.4	12	75.5	14	83.2	17
96 hrs.	72.7	9	77.8	11	79.9	12	..	..	..	...	84.0	13

show typical and average results for such concentrations of enzyme and for differing concentrations of hydrochloric acid and of acid potassium phosphate.

In Set 8 (Table IV) with 2% starch and 0.06 *M* acid phosphate (the optimum for a 2% starch solution) the maltose increases with the time in a manner which might easily be interpreted as showing a "practically" linear relationship. Actually, however, the relationship is more strictly logarithmic, as is shown by the constancy of the values of k. For so much of the hydrolysis as is covered by this experiment it will be seen that identical data might readily be interpreted either as indicating a linear or a logarithmic relationship.

In Set 119 with 1% starch and the same concentrations of enzyme and activator, the rate of hydrolysis, expressed as k, is approximately twice as great as in Set 8 and the relationship of product to time steadily falls away from the linear but remains approximately logarithmic up to a yield of about half the theoretical amount of maltose.

In Set 129 with 1% starch and 0.0002 *M* hydrochloric acid the rate of hydrolysis is lower and falls more rapidly, doubtless because of the excessive hydrogen-ion concentration in this solution.

In Sets 219 and 220, in which 0.000025% enzyme in 1% starch was activated by the optimum concentrations of acid phosphate and hydrochloric acids, respectively, the relationship is again seen to be logarithmic rather than linear (although a casual examination of curves based on these data might indicate that they are linear for the first 30% of the hydrolysis).

The same is true of experiments summarized in Table V in so far as these show sufficient data during the early stages of the hydrolysis to have a bearing upon the present question.

Of the results as a whole it may be said that even in the presence of the most favorable electrolyte there is no constant evidence of a strictly linear relationship, the time curve being much more nearly logarithmic than linear and appearing linear only in so far as the linear and logarithmic relationships would give nearly identical results in the earliest stages of the hydrolysis.

The Speed of the Hydrolysis at Different Stages.

From the data given in Tables IV and V, as well as many other experiments here omitted for the sake of brevity, it appears that in the hydrolysis of soluble starch by malt amylase in the presence of a favorable concentration of an electrolyte such as hydrochloric or phosphoric acid or acid potassium phosphate the rate of hydrolysis as indicated by k in the formula $(1/t \log 1/1 - x = k)$ remains nearly constant until about half of the theoretical amount of maltose has been formed. Beyond this point the value of k falls more or less rapidly, depending somewhat upon the acidity of the solution and the concentration of the enzyme. Examination of the data shows that the rate of hydrolysis was so much better maintained at the lower acidity of Sets 219 and 220 than in Sets 119 and 129 where twice the optimum concentrations of acid phosphate and hydrochloric acid were used, that in the solution of lower acidity although only half as much enzyme is present the production of maltose finally passes that in either of the others.

Calculating the speed of hydrolysis for each time interval separately by the formula $k = 1/t_2 - t_1 \log C_1/C_2$ brings out nothing new in the relationships above discussed and necessarily makes the results appear less regular, since it exaggerates the effects of the unavoidable experimental errors.

Because of the fact that under the conditions obtaining in earlier investigations, the diastatic action becomes almost inappreciable when about 80% of the theoretical amount of maltose had been formed, it has sometimes been held that the value of k in the formula $k = 1/t \log 1/1 - x$ should be calculated on the basis of an assumption that an 80% yield of maltose represents completion of the enzymic hydrolysis. Data given earlier in this paper show that no such assumption is correct. Nevertheless, it is true that all of the starch has usually been hydrolyzed before 80% of the theoretical yield of maltose has been obtained so that by the time this point has been reached the nature of the substrate is quite different from, and much more resistant than, that with which the process was begun. For this reason it seemed of interest to calculate values of k on this "80% basis," but as the results did not bring out anything in addition to what has already been shown, it seems unnecessary to give space to the data here. On this latter basis of calculation the values of k necessarily become higher than those calculated in the regular way; they are more likely to show a slight rise in the early stages of the hydrolysis; but they do not alter the conclusion that the relationship approximates the logarithmic rather than the linear.

That the decreasing value of k after 50–55% of the theoretical amount of maltose has been formed is not due simply to the deterioration of the enzyme with time may be seen from the data given in the last columns of Table V, where this stage in the hydrolysis was reached in an hour but was nevertheless followed by a decided decrease in the speed of the reaction during the second hour.

From the fact that, at least in solutions containing 0.03 M to 0.06 M acid phosphate, the rate of maltose production as expressed by k of the formula $1/t \log 1/1 - x = k$ holds constant up to a yield of about 50–55% and is directly proportional to the enzyme concentration, it follows that through this range, which is much greater than that ever regarded as even approximately linear, there exists a simple inverse relationship between the enzyme concentration and the time required to form a given amount of maltose—assuming, of course, that other conditions, including the initial concentration of substrate (soluble starch), are constant.

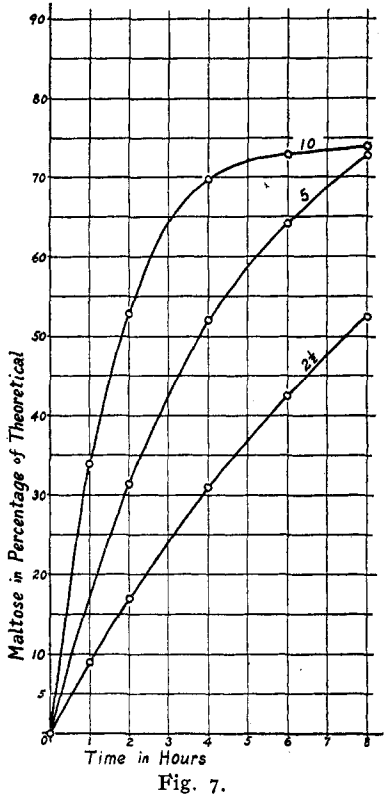

Fig. 7.

Fig. 7 shows the early part of the time curves for the first half of Table V (Sets 110, 111, 112). The three experiments here represented were carried out side by side with all conditions alike except enzyme concentration which was 0.000025, 0.00005 and 0.00010%, respectively, or in the proportions $2^1/_2 : 5 : 10$ or $1 : 2 : 4$. At any point up to a yield of about 52% of the theoretical amount of maltose it may readily be seen from the graphs (Fig. 7) that the time required to form a given amount of maltose is inversely as the amount of enzyme present.

The establishment of the relationship between enzyme concentration and rate of hydrolysis not simply for the region in which the time curve approximates the linear but through a much wider range will greatly facilitate the extension of investigations which involve quantitative comparisons of diastatic activity or amylase concentration.

Summary.

The rate of formation of reducing sugar (maltose) from soluble starch by purified malt amylase, both in neutral solution containing no added

electrolyte and with the addition of regulated amounts of hydrochloric or phosphoric acid or of primary potassium phosphate, has been investigated from the beginning of the reaction to completion or until the hydrolysis is no longer measurable.

When the activating electrolyte was added in such amount as to give optimum or nearly optimum concentration of hydrogen ion, the action of the enzyme was increased not only in the earlier stages but throughout the entire range investigated. The greater the concentration of enzyme the less the effect of the added electrolyte. The conclusions which follow refer, unless otherwise stated, to 1% starch "solutions" containing electrolytes and small amounts of enzyme.

The same optimum hydrogen-ion concentration, $C_H 10^{-4.4}$, was found to hold for each of the acid electrolytes tested and throughout the course of the hydrolysis. (With neutralized starch substrate used in this laboratory the amount of acid or acid phosphate required for optimum activation is about half as much for 1% as for 2% starch.)

When more than the optimum amount of acid was added the hydrolysis proceeded at less than the optimum rate throughout; when less, the initial rate was better sustained. This difference was most pronounced in the case of hydrochloric acid; less with phosphoric acid; least in the case of acid phosphate ("buffer effect").

The beneficial "buffer" effect of the acid phosphate was also apparent in experiments in which the enzyme was made to act upon different concentrations of starch (1, 2 and 4%) in the presence of the same concentration of added electrolyte.

Throughout the first half of the hydrolysis, or up to a yield of half the theoretical amount of maltose, the rate of maltose formation from soluble starch was found to be proportional to the concentration of substrate, at least in solutions containing favorable amounts of acid or acid phosphate.

When, in similar experiments, enzyme concentration is varied within limits suitable for such quantitative study, the rate of maltose formation is found to be directly proportional to the enzyme concentration, up to a yield of about half the theoretical amount of maltose. This broadens the range within which diastatic activities may be compared quantitatively.

In the action of malt amylase upon soluble starch, we find no "region of linear relationship" in which the yield of reducing sugar is directly proportional to time.

Experiments with widely varied enzyme concentration show that there is no cessation of hydrolysis nor true equilibrium at 80% as claimed by some previous investigators.

We desire to express our indebtedness to the Carnegie Institution of

Washington for the use of a malt amylase preparation which had been purified in connection with investigations conducted by aid of its grants.

NEW YORK CITY.

[CONTRIBUTION FROM THE LABORATORY OF THE NORTHWESTERN UNIVERSITY MEDICAL SCHOOL.]

ON THE ASSUMED DESTRUCTION OF TRYPSIN BY PEPSIN AND ACID. III. OBSERVATIONS ON MEN.[1]

BY J. H. LONG AND MARY HULL.

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In two earlier publications we have given data on this problem obtained from experiments *in vitro* and from observations on dogs.[2] From both lines of investigation it appeared that trypsin is much more resistant to the action of pepsin under certain conditions than was formerly supposed. We are now attempting to duplicate some of the experiments carried out on the dogs through somewhat similar experiments carried out on the human subject.

The plan observed is essentially this. Following a test meal the whole stomach contents, as far as possible, are drawn off after a proper interval and examined for acid, bile, pepsin and possible tryptic action. If the stomach is found to behave normally then, in the observation proper, some powdered trypsin is given along with the test meal and after a time the contents are drawn off as before and examined for the persistence of the tryptic action. In the preliminary experiments where no trypsin is ingested the tryptic proteolysis found is very slight, usually, and may depend on a behavior normal to the stomach, or it may come from the regurgitation of intestinal contents holding small amounts of the ferment, The presence of bile would suggest the possibility of this. The proof of the presence of trypsin in the stomach contents is found in the ability of the collected liquid to digest fibrin under conditions where pepsin present can not act, that is, in a medium of a slight degree of alkalinity. The extent of digestion through a period of three hours is measured, finally, by the formaldehyde amino acid titration.

As subjects for our observations we have used some cases from the hospitals connected with the School and some students who were willing to submit to the tests through periods long enough to have value. Several series of tests were begun and abandoned because the subjects found it irksome to take the Rehfuss tube daily. The results of the observations satisfactorily completed are given below in tabular form. While most

[1] This investigation has been made with the assistance of a grant from the Committee on Therapeutic Research, Council on Pharmacy and Chemistry, American Medical Association.

[2] THIS JOURNAL, 38, 1620 (1916); 39, 162 (1917).