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STUDIES ON AMYLASES. II. A STUDY OF THE ACTION OF PANCREATIC AMYLASE.

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In an examination of methods for the determination of diastatic power[1] it was found particularly difficult to obtain concordant results with pancreatin, and the peculiar behavior of the pancreatic amylase made plain the desirability of a special study of its action. The main results of this study are here given in as condensed and systematic a form as possible and not necessarily in the chronological order of the experiments.

Materials and Methods.

Enzyme Preparations.—In the experiments described in this paper the pancreatic amylase has been used in the form of commercial pancreatin. Seven samples purchased in New York City at various times during 1907–9 showed, when tested in the favorable conditions worked out during the investigation, the following relative diastatic powers: No. 1, 16; No. 2, 151; No. 3, 9; No. 4, 105; No. 5, 75; No. 6, 78; No. 7, 66. The stronger preparations which presumably contained the smaller amounts of impurities were used in the experiments described beyond.

Starch.—The soluble starch used was prepared by the Lintner[2] method with attention to the details discussed by Ford.[3] After completion of the acid treatment and removal of most of the acid by washing, the starch was transferred to a large glass bottle and washed about 65 times by decantation with about twice its bulk of water. At each washing the starch was vigorously mixed with the water by rolling the bottle back and forth on a table, after which the starch was allowed to settle thoroughly and the water siphoned off. This was repeated 3 or 4 times daily for about 3 weeks. The starch was then brought upon a Büchner funnel and the wash water removed as thoroughly as possible by suction, the slight acidity of the starch determined by titrating a weighed portion with $N/100$ alkali, using rosolic acid as indicator. The starch was then given a final washing with water containing the very small amount of sodium hydroxide calculated as necessary for its neutralization, filtered with thorough suction and air-dried. Four different preparations made in this way showed 12 to 17 per cent. of moisture, required 0.1 to 0.3 cc. of N/100 acid or alkali per gram for neutralization, and showed reducing powers of 27 to 30.5 mg. cuprous oxide per gram of anhydrous starch.

[1] Sherman, Kendall and Clark, THIS JOURNAL.

[2] *J. prakt. Chem.*, [2] 34, 378 (1886).

[3] *J. Soc. Chem. Ind.*, 23, 414 (1904).

Water.—Ordinary distilled water was found to be a cause of irregular results, especially when working without added electrolyte, nor did neutralization of such water render it satisfactory. In all subsequent work we used water which had been redistilled from alkaline permanganate, the steam being passed through a long column of glass wool to filter out any possible spray.

Procedure.—Unless otherwise stated the procedure was that of the gravimetric method for diastatic power proposed in the preceding paper except for the activating agents. The results are expressed in milligrams of cuprous oxide weighed, corrected for the reducing power of the particular soluble starch used in each experiment. In case it was impossible to determine at once the reducing sugars formed in an experiment, so that a means of stopping the enzyme action and preserving the solution was necessary, the addition of hydrochloric acid enough to make 0.1 per cent. stopped the action of the amylase at once and did not affect the reducing power of the solution, even on standing 24 hours.

In cases in which the amylase acted more than one hour, a mixture of toluene and chloroform was added to prevent bacterial growth and evaporation. This was effective and had no influence upon the amount of copper reduced.

Influence of Added Electrolytes.

Neutral Salts.—As early as 1875, Nasse[1] held that there is an important and specific dependence of the activity of ferments upon the presence of salts. In his experiments the addition of chlorides, nitrates, and sulphates of sodium, potassium, and ammonium raised the diastatic power of pancreatin by 7 to 31 per cent.

Grützner[2] compared the effects of sodium chloride, bromide, iodide, and fluoride.

Vernon[3] found that pancreatic extracts suffered less loss of diastatic power when diluted with tap water than with distilled water and that the addition of a small quantity of salt increased the activity, and also diminished the reduction of activity occasioned by dilution. The activity of his preparation was 30 times as great in 0.1 per cent. salt solution as in distilled water.

Preti[4] reported that the amylases obtained from the pancreas, urine, blood serum, malt and cryptogams were all rendered practically inert by dialysis and restored to activity by the addition of a neutral electrolyte.

Bierry and Graja[5] working in conjunction with Victor Henri found a similar result with pancreatin.

[1] *Arch. ges. Physiol.*, 9, 138 (1875).
[2] *Ibid.*, 91, 195 (1902).
[3] *J. Physiol.*, 27, 174 (1901); 28, 156, 375 (1902).
[4] *Biochem. Z.*, 4, 1 (1907).
[5] *Compt. rend. soc. biol.*, 60, 479; 62, 432 (1906–7); *Compt. rend.*, 143, 300 (1906).

Slosse and Limbosch[1] also confirmed Preti's observations.

We have confirmed the observation that if both the pancreatin and the starch be first dialyzed in collodion sacks the pancreatic amylase becomes inactive but is reactivated by the addition of salt. Further experimentation showed that if the starch was dialyzed so that the only salts present were those of the enzyme preparation a small amount of high grade pancreatin was inactive; but was rapidly activated by the addition of very small amounts of salt.

Thus 0.35 mg. of pancreatin No. 2 in 50 cc. of 1 per cent. dialyzed starch at 40° for 1 hour showed after various additions of salt the activities indicated by the weights of cuprous oxide found as follows:

Sodium chloride milligrams.....	0.01	0.1	1.0	10	30	60	90	121
Cuprous oxide milligrams.....	0	10	51	87	91	86	85	85

In another series of experiments 1 mg. of pancreatin No. 4 acted for 1 hour at 40° upon 100 cc. of 2 per cent. starch which had not been dialyzed with the following results:

NaCl. Mg.	Cuprous oxide. Mg.	NaCl. Mg.	Cuprous oxide. Mg.
0	13	60	183
0.1	34	100	182
0.5	90	200	182
1	119	400	167
5	171	600	168
10	178	800	158
20	186	..	..
25	180	..	..
30	187	..	..
35	193	..	..
40	194	..	..

Similar though less extended experiments upon four other samples of pancreatin gave confirmatory results.

If the enzyme preparation does not contain sufficient neutral electrolyte to completely activate the amylase, and no salt is added, a peculiar relation between rate of conversion and amount of enzyme is found. Doubling the amount of enzyme will more than double the rate of conversion, because doubling the amount of enzyme at the same time doubles the electrolyte in solution. Some results obtained with neutral solutions containing no added salt are:

Mg. Enzyme.	Mg. Cuprous oxide.
10	42
20	86
30	137
40	204
60	283
90	418

[1] *Bull. soc. roy. sci. med. nat. Bruxelles*, 1908, 80.

It is evident that salt not only helps the diastatic action but is essential to it. The salts accidentally present in commercial pancreatin and in very carefully prepared soluble starch are sufficient to enable the amylase to act to some extent, but any results so obtained are abnormal and entirely misleading as indications of real diastatic power, since pancreatic amylase acts in nature in a medium containing salt and must have an appreciable amount of some salt in order to function normally.

Experiments with potassium and ammonium chlorides gave similar results.

Alkalies.—Several investigators[1] of pancreatic and other amylases have reported alkalies harmful and some have found activity increased by the presence of a minute amount of acid, while others maintain that strict neutrality is the best condition for amylolytic action.

Tested without added electrolyte 5 mg. of pancreatin No. 5 acting upon 100 cc.[2] of 2 per cent. undialyzed starch for 1 hour at 21° gave the following results:

Cubic centimeters 0.01 *N* NaOH added.....	0	1	2	3	4	6	8[1]
Milligrams cuprous oxide found.....	71	61	49	42	24	14	4

These results were obtained by Meyer in the preliminary part of this study. No further experiments were made upon the influence of alkali alone because it was felt as already explained that any results obtained in the absence of sufficient salt to activate the enzyme must be regarded as abnormal.

Addition of Salt and Alkali.—Ebstein, in 1893,[3] showed that a degree of alkalinity which would otherwise stop the action of pancreatic amylase did not prevent a vigorous action when "blood salts" were present in the solution and this helpful influence of the blood salts appeared to be due chiefly to the sodium chloride. This observation does not seem to have been appreciated or followed up.

Our work has included a considerable number of observations of the same enzyme working in solutions containing sufficient salt but of varying alkalinity. In the following table are brought together results obtained at different times but which are fairly comparable in representing in each case the mg. of cuprous oxide reduced by the sugar formed

[1] Chittenden and Ely, *Am. Chem. J.*, 4, 107 (1882); Chittenden and Smith, Studies Yale Laboratory of Physiological Chemistry, 1, 36 (1884–5); Duggan, *Am. Chem. J.*, 8, 211 (1886); Schierbeck, *Skand. Arch. Physiol.*, 3, 344 (1891–2); Ebstein, *Virchow's Archiv.*, 134, 475 (1893); Wood, *Am. Chem. J.*, 15, 663 (1893); Vernon, *J. Physiol.*, 27, 174 (1901); Grützner, *Arch. Physiol.*, 91, 195 (1902); Cole, *J. Physiol.*, 30, 202 (1904); Effront, *Compt. rend. soc. biol.*, 57, 234 (1904); Ford, *J. Soc. Chem. Ind.*, 23, 414 (1904); Maquenne, *Compt. rend.*, 142, 285, 1059 (1906).

[2] In these and all similar experiments the reaction volume stated was adjusted after the addition of acid or alkali and is therefore the total volume.

[3] *Virchow's Archiv*, 134, 475.

from the action of 0.125 mg. of pancreatin No. 2 upon 0.25 grams of soluble starch[1] at 40° for the length of time stated.

EFFECT OF ADDED ACID AND ALKALI ON SOLUTIONS CONTAINING NEUTRAL ELECTROLYTE.

Added acid or alkali.	Time. 10 min.	30 min.	1 hr.	2 hrs.	3 hrs.	5 hrs.	25 hrs.
8 cc. 0.01 *N* H_2SO_4 per 100 cc.	..	..	0	..	..	..	..
6 " " "	..	..	0	..	..	..	..
4 " " "	..	..	3	..	7	..	..
3 " " "	..	..	87	151	..	222	277
2 " " "	..	..	153	218	..	251	272
1 " " "	..	..	223	242	..	254	271
Neutral	..	..	227	243	..	254	270
1 cc. 0.01 *N* NaOH per 100 cc.	143	207	235	..	240	252	..
2 " " "	156	204	226	..	244	250	..
3 " " "	124	191	214	..	241	244	..
4 " " "	..	..	200	222	..	239	256
6 " " "	..	..	154	196	..	232	250
8 " " "	..	..	124	186	..	227	250
20 " " "	..	..	19	..	40	163	..
30 " " "	..	..	11	..	18	40	..
40 " " "	..	..	3	..	9	11	..
50 " " "	..	..	0	..	4	6	..

It will be seen from the data in the above table:

(1) That in solutions sufficiently acid, where the concentration of hydroxyl ion may be regarded as practically zero, there was no action; (2) that in passing from acid through neutral to faintly alkaline solutions the increasing concentration of hydroxyl ion is accompanied by an increased amylolytic activity up to the degree of alkalinity obtained by adding 2 cc. of 0.01 *N* sodium hydroxide per 100 cc. of solution; (3) that greater additions of alkali have a retarding effect but the action is not stopped except by the addition of relatively large proportions of 0.01 *N* solution; (4) that although the alkalinity of the solution has a great influence upon the speed of the reaction still there is a tendency toward a constant final yield of reducing sugar provided sufficient time be allowed.

In other experiments where a larger excess of starch was present the optimum amount of added alkali was found to be higher.

Experiments were also made in which varying amounts of sodium chloride and sodium carbonate were added. The following table shows the milligrams of cuprous oxide obtained by the action of equal amounts of pancreatin No. 2 upon equal volumes of 2 per cent. starch solution for 1 hour at 40°.

[1] One-half milligram pancreatin acted on 100 cc. of 1 per cent. starch solution (containing the indicated amount of acid or alkali) of which 25 cc. were withdrawn and diluted to 100 cc. before treating with Fehling solution. The amount of added salt varied from 125–300 mg. and was sufficient in each case to activate the enzyme.

EFFECT OF ADDED SODIUM CHLORIDE AND SODIUM CARBONATE.

Sodium chloride. Mg.	Sodium carbonate, 00.1 N solution. 1 cc.	2 cc.	4 cc.	5 cc.	6 cc.	7 cc.	8 cc.	9 cc.	15 cc.
60– 100	234	248	280	290	284	278	250	244	84
120– 200	..	253	..	292	289	279	..	..	..
300– 400	..	..	..	290	291	278	256	261	96
500–1000	..	..	..	280	..	263	241	230	81

Comparison of these data with those previously given indicates that the amount of salt necessary to activate the enzyme is comparatively small (in none of these cases was it over 0.3 per cent.) and that moderate variations in the amount of salt added do not materially influence the optimum alkalinity so that even in alkaline solutions the helpful effect of the salt appears to be due to a direct activation of the enzyme rather than to its influence upon the ionization of the alkali.

If, however, the salt is insufficient to fully activate the amylase, the optimum alkalinity[1] rises with the concentration of salt. In a case of this sort the following results (expressed in mg. of cuprous oxide) were obtained:

EFFECT OF SALT UPON OPTIMUM ALKALINITY.

Added NaOH.	0.	1.	2.	3.	4.	5 cc. 0.01 N.
1 milligram NaCl	110	103	85	61	..	..
5 milligrams NaCl	214	243	233	202	..	..
500 milligrams NaCl	..	..	400	427	430	296

That the optimum alkali was higher here than in the preceding table is due to the fact that more starch was used. This relation will be fully explained later in this paper.

The results of some of the experiments made to compare the effects of different alkalis in the presence of a uniform amount of salt are shown in the following table.

As would be expected the optimum is reached more quickly and the retarding effect of excess is more pronounced with carbonate that with phosphate and with hydroxide that with carbonate. The activity at optimum concentration is, however, very nearly the same in all cases except with ammonia where the lower results may very probably be due to a solvent effect upon the cuprous oxide. It should also be noted that the unit of comparison is fiftieth-molar for phosphate while the hydroxide and carbonate solutions were 0.01 N by titration with standard acid.

[1] The above results show plainly the increase of activity produced by adding alkali in the presence of sufficient salt. Since Cole (*loc. cit.*) has shown that the activity of ptyalin is increased by acid it is suggested that some amylases may use the hydroxyl and others the hydrogen ion to accomplish the hydrolysis of starch. This point should be studied further upon other amylases sufficiently activated by salt.

The conditions for all these determinations were: One-half mg. pancreatin No. 4. Sodium chloride, 700 mgs. Temperature, 40°. Time, 60 min. Starch, 2 per cent.

EFFECT OF DIFFERENT KINDS OF ALKALI.

Alkali added.	Cuprous oxide, mg. NaOH N/100.	KOH N/100.	NH_4OH N/100.	Na_2CO_3 N/100.	K_2CO_3 N/100.	Na_2HPO_4 M/50.
None	56	..	..	56	..	..
1 cc. 0.01 *N* per 100 cc.	153	..	..	132	..	..
2 " "	229	..	..	198	..	260
3 " "	279	280	255	242	..	..
4 " "	309	313	281	278	277	301
5 " "	307	289	273	290	..	..
6 " "	196	151	206	301	302	304
7 " "	85	..	..	308	..	..
8 " "	49	..	..	314	297	306
9 " "	..	..	..	305	..	..
10 " "	..	..	..	273	222	313
11 " "	..	..	..	215	..	317
12 " "	..	..	..	159	..	304
13 " "	..	..	..	118	..	..
14 " "	..	..	..	102	..	305
15 " "	..	..	..	78	..	..
20 " "	..	..	..	40	..	273
25 " "	..	..	..	29	..	..
30 " "	..	..	..	23	..	264
35 " "	..	..	..	18	..	..
50 " "	..	..	..	12	..	224
75 " "	..	..	..	9	..	..

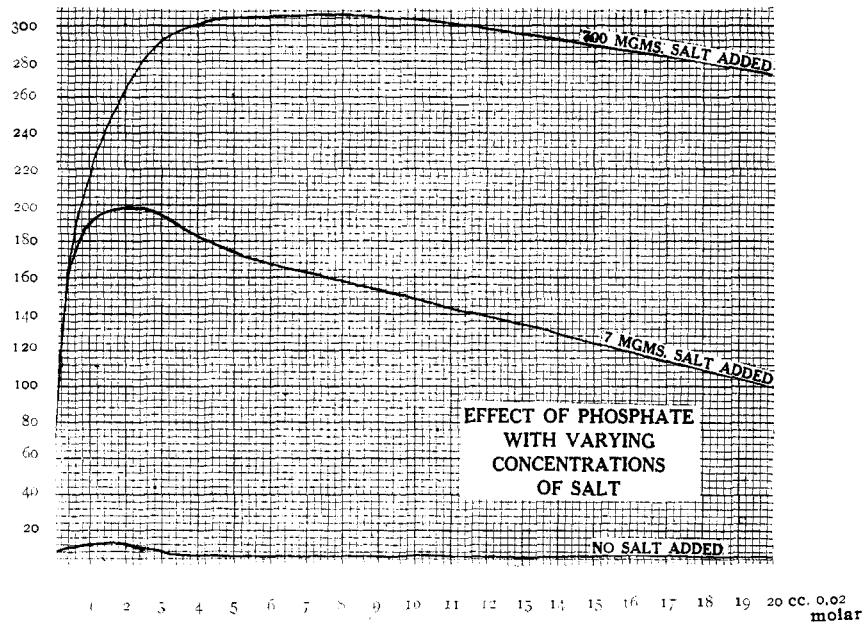
Disodium phosphate was also tried alone and with the addition of small and large amounts of sodium chloride. The weights of cuprous oxide obtained were as follows:

EFFECT OF PHOSPHATE AND DIFFERENT AMOUNTS OF SALT.

Cc. M/50. Na_2HPO_4.	Pancreatin No. 6, 10 mg. Added NaCl. None.	Pancreatin No. 4, 0.5 mg. Added NaCl. 7 mg.	Pancreatin No. 4, 0.5 mg. Added NaCl. 700 mg.
0	161	72	51
1	231	190	..
2	193	198	260
3	159	194	..
4	..	182	301
5	118	..	..
6	..	163	304
8	..	150	306
20	53	100	273
30	..	77	264
40	35	69	236

Where no salt was added 20 times as much enzyme was used for the experiment in order to get a comparable yield of reducing sugar, hence the partial activation by the salts accidentally present.

Since the figures in one column are for a much larger weight of pancreatin than in the others, the effect may be more readily seen from the following curves:



When the phosphate alone was added in small amount it caused a rapid rise in speed of conversion but in slightly larger amounts its effect was depressing. At the optimum concentration of the phosphate alone the enzyme was very incompletely activated.

With the addition of a small amount of salt, sufficient largely, but not completely, to activate the enzyme, small additions of phosphate greatly increased the speed of the reaction while slightly larger amounts had a marked retarding action.

With the addition of an abundance of sodium chloride (0.7 per cent.) the phosphate continued to increase the speed of the reaction up to a much greater concentration, giving a maximum much higher than with the smaller amount of chloride, and beyond the maximum there was only a very gradual retardation as the additions of phosphate were still further increased.

The following results were obtained with 0.35 mg. of pancreatin 2, with 5 cc. of 0.01 normal sodium carbonate in 100 cc. of a solution of 2 per cent. starch.

EFFECT OF SALT ON THE TIME CURVE.

Time. Minutes.	Cuprous oxide with NaCl. 1.5 mg.	5 mg.
20	12	21
40	23	42
60	33	66
80	44	88
100	56	111
120	68	133
140	79	154
160	92	178

Quantitative Relations of Enzyme, Starch Salt and Alkali.—In the experiments described in this paper we have usually employed (as will be seen from the descriptions accompanying the data) from 0.1 to 1 mg. of high grade commercial pancreatin. Our recent experiments upon the purification of pancreatic amylase give evidence that the pancreatin here used contained at least 90 per cent. of material other than actual amylase and as the volume has usually been 100 cc., we have a concentration not greater than 1 : 1,000,000 to 1 : 10,000,000 of actual amylase in the solution. Considerations of actual quantities relate, therefore, to starch, salt and alkali.

In discussing the influence of added salt and alkali, experiments have already been described in which the diastatic action was seen to rise rapidly at first with increasing concentrations of salt, after which a much greater increase of salt had very little effect. As further examples the following three pairs of determinations may be cited:

All other conditions being the same for the two tests in each set,

50 milligrams of salt gave 280 milligrams cuprous oxide.
1000 milligrams of salt gave 279 milligrams cuprous oxide.

100 milligrams of salt gave 84 milligrams cuprous oxide.
1000 milligrams of salt gave 81 milligrams cuprous oxide.

90 milligrams of salt gave 238 milligrams cuprous oxide.
2000 milligrams of salt gave 228 milligrams cuprous oxide.

It is also apparent from the table of results showing effect of added sodium chloride and sodium carbonate that the optimum alkalinity did not increase with increasing additions of salt. On the other hand there is an evident tendency to a higher optimum of alkalinity with an increasing concentration of starch. Thus 1.05 mg. pancreatin No. 2 acting for 20 minutes at 40° upon 100 cc. solutions containing 500 mg. salt and different amounts of starch and alkali gave the following results expressed in milligrams of cuprous oxide.

These results, as well as other data obtained under different conditions, including smaller and larger concentrations of starch, indicate

that the amount of alkali to be added in order to obtain the most rapid conversion (*i. e.*, the optimum addition of alkali) increases with the concentration of the starch and that this optimum alkalinity is rather sharply defined for low concentrations of starch and becomes much less sharply defined as the concentration of the starch is increased.

Alkali added. Cc. of 0.01 *N* NaOH.	Starch, grams. 0.5	1.0	2.0	3.0
2	203	271	276	269
3	87	170	285	271
4	45	86	245	293
5	35	47	115	259

Thus the quantity of starch not only influenced the optimum of added alkali, but the enzyme was largely protected from the retarding effects of alkali in excess of the optimum by moderate increase in the concentration of the starch, whereas a proportionate increase in the amount of salt added had no noticeable effect.

Velocity and Equilibrium of the Reaction.

Influence of Concentration of Starch.—The following experiments bear upon the relation between amount of starch and the speed of its hydrolysis. Different amounts of pancreatin No. 6 were allowed to act at 40° upon a uniform volume (100 cc.) of starch solution whose strength was varied in the different cases from 0.1 per cent. to 4 per cent. In all cases 700 mg. of sodium chloride and 10 cc. of 0.02 molar disodium phosphate per 100 cc. of solution were added. The time of reaction was one hour. The figures are mg. of cuprous oxide reduced by the sugar formed under the given conditions.

Starch. Per cent.	Pancreatin, mg. 0.15.	0.30.	0.45.	0.60.
4	78	156	235	313
3	78	152	225	299
2	76	147	217	286
1	69	132	196	259
0.5	66	123	172	223
0.3	60	108	145	177
0.2	58	100	125	142
0.1	48	70	78	83

In another series the amount of starch present was so small that in no case was the increase in speed of conversion directly proportional to the weight of enzyme used, but it is seen that the rate of conversion is more nearly proportional to the enzyme in the higher concentrations of starch. The conditions were: Time, 5 minutes. Temperature, 40°. Disodium phosphate, 1.5 cc. 0.02 molar and sodium chloride, 200 mg. per 100 cc.

Starch. Per cent.	Pancreatin, mg. 0.1	0.2	0.3	0.4
0.1	12	19	28	35
0.2	12	19	28	37
0.5	12	23	33	41

It will be seen from the above tables that with sufficient amounts of starch the speed of conversion was directly proportional to the amount of enzyme present, while with less starch the increase in speed was not in proportion to the increase of enzyme. The sufficiency of the starch is not a matter of the percentage strength of the starch solution, but of the ratio of starch to enzyme. This point will be considered more fully below.

Influence of Alkalinity.—It has been pointed out that the effect of increasing alkalinity is, first to accelerate the speed of conversion and then to retard it. It has also been shown that the optimum alkalinity varies with the percentage of starch and for this reason the effect of a given amount of alkali on the time curve may be, first, acceleration, and then retardation. Therefore, if the time curve was determined, *e. g.*, for a solution of 2 per cent. starch containing the optimum concentration of alkali at the start, as the conversion proceeded, the alkali, which would remain constant, would have a greater retarding influence as its ratio to the amount of starch present increased.

If, on the other hand, the concentration of hydroxyl ion were much below the optimum at the start the speed of conversion would be greatly retarded, but as the action continued there would be a constantly accelerating influence (due to the diminishing percentage of starch) and finally the rate of conversion in this solution would equal, or exceed, that of solutions having a greater initial concentration of hydroxyl ion. The following data and chart illustrate this effect of alkali on the time curve, the results being given in mg. of cuprous oxide.

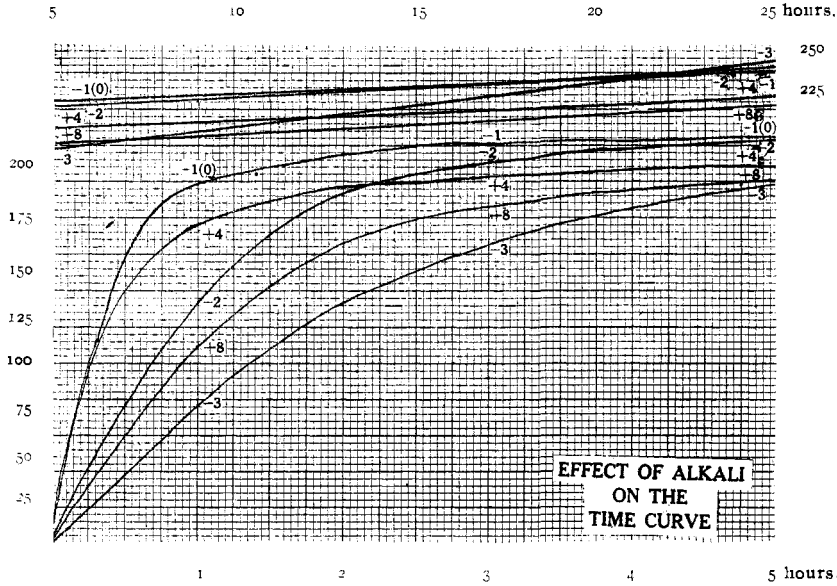
EFFECT OF ALKALI ON THE TIME CURVE.

Added acid or alkali.	Time, in hours. 1.	2.	5.	25.
3 cc. 0.01 *N* H_2SO_4	77	133	198	246
2 cc. 0.01 *N* H_2SO_4	135	193	222	242
1 cc. 0.01 *N* H_2SO_4	198	214	225	240
Neutral	201	215	225	238
3 cc. 0.01 *N* NaOH	199	212	218	230
4 cc. 0.01 *N* NaOH	177	196	211	227
6 cc. 0.01 *N* NaOH	136	173	205	221
8 cc. 0.01 *N* NaOH	109	165	201	221

The conditions for these determinations were: Starch, 1 per cent. Temperature, 40°. Volume (inclusive of added acid or alkali), 113 cc. Salt, 125 mg. Pancreatin No. 2, 5 mg.

This accelerating and retarding action of the alkali depending on the percentage of starch and therefore on the amount of conversion, explains one cause why previous investigators have found such varying conditions of alkali and acid for the optimum conversion of the starch.

An example taken from our work will show the great variation which may exist. When 4 per cent. starch was used and the time of conversion was limited to 20 minutes, 5 cc. of 0.01 *N* alkali per 100 cc. of solution were required for maximum speed of conversion. When 1 per cent. starch was used and 25 hours allowed for the time of conversion the greatest amount converted was in a solution containing 3 cc. of 0.01 *N* acid per 100 cc. of solution.



In discussing the curves we will designate the cc. of 0.01 *N* acid used as minus alkali; *e. g.*, —1, —2, or —3 will mean 1, 2, or 3 cc. of 0.01 *N* acid and +3, +4, +8 will mean cc. of 0.01 *N* sodium hydroxide.

The chart shows that a relatively high concentration of hydroxyl ion is required at first to give maximum speed to the reaction, but that a much lower concentration of hydroxyl ion will eventually cause the rate of conversion to equal, and finally exceed, that of the higher concentration. This is shown in the accompanying curves. At the end of 1 hour the amount of conversion is in the order 0, +3, —1, +4, +6, —2, +8, —3 cc. 0.01 *N* alkali. At the end of 5 hours the order is 0, —1, —2, +3, +4, +6, +8, —3, and at the end of 25 hours the order is —3, —2, —1, 0, +3, +4, +6, +8.

The following set of determinations with varying amounts of alkali also illustrate this fact.

	5 min.	16 hrs.
0 cc. of 0.01 normal NaOH per 100 cc.	13	261
1 cc. of 0:01 normal NaOH per 100 cc.	15	251
2 cc. of 0.01 normal NaOH per 100 cc.	17	245
3 cc. of 0.01 normal NaOH per 100 cc.	11	240

If we take the amount of the conversion in the first stages of the reaction we find the greatest speed in the solution containing 2 cc. of hydroxide per 100 cc., while after 16 hours the neutral solution shows the highest result.

A series of results where the time interval was less shows the effect of alkali in the first part of the conversion. The total time of digestion was only 5 hours, so the last set of figures does not represent the final equilibrium.

EFFECT OF VARIOUS CONCENTRATIONS OF ALKALI.

	Cc. 0.01 *N* NaOH per 100 cc.			
Minutes.	0.	1.	2.	3.
10	124	143	156	124
20	185	195	194	177
30	195	207	204	191
60	217	235	226	214
120	222	240	235	231
180	229	241	244	241
240	231	248	246	243
300	232	252	250	244

The conditions were: Temperature, 40°. 5 mg. of pancreatin 2. 300 mg. of sodium chloride per 100 cc. 1 per cent. starch.

Here, although the increments of alkali were very small, their influence was apparent throughout the five hours of the experiment.

To test the effect of a less caustic alkali sodium hydroxide and disodium phosphate were compared with the following results (expressed in mg. of cuprous oxide) in experiments on 1 per cent. starch containing 300 mg. sodium chloride per 100 cc.

Time. Minutes.	With NaOH.[1]	With Na_2HPO_4.[2]
5	48	49
10	85	88
15	119	124
20	145	151
25	164	170
30	174	180

The depressing effect of the alkali as the reaction proceeds is shown to be less with the phosphate than with the hydroxide.

[1] 2 cc. 0.01 *N* NaOH.

[2] 2 cc. 0.02 molar disodium phosphate.

Our results indicate that the point of final equilibrium in the conversion of starch by pancreatic amylase is but little influenced by variations in hydroxyl ion concentration within the limits which permit the action to take place with measurable speed. In the experiments already described in which the conditions varied from an addition of 3 cc. of 0.01 N H_2SO_4 to 8 cc. of 0.01 N NaOH the great difference of hydroxyl ion concentration was accompanied by a difference of only ten per cent. in the amount of reducing sugar found at the end of 25 hours.

In another series of experiments 10 mg. of pancreatin were allowed to act upon 4 grams of starch in 350 cc. of water containing 125 mg. salt per 100 cc. for about 16 hours. The solution was then divided into four equal parts, 5 mg. more of enzyme and the desired amounts of alkali were added to each part, and the reducing sugar found in the different flasks. The amount of conversion was found after the flasks had stood 1, 5 or 45 hours.

While the results do not agree exactly they show that there was no decided change in any of the flasks. The results are expressed in mg. of cuprous oxide.

	Cc. 0.01 N NaOH per 100 cc.			
	0.	10.	20.	30.
When divided	255	255	255	255
1 hour later	257	259	256	255
5 hours later	261	259	255	253

Another set with larger amounts of alkali gave:

	Cc. 0.01 N NaOH per 100 cc.			
	20.	30.	40.	50.
When divided	250	250	249	246
45 hours later	258	253	255	254

While there was a slight change in these solutions, still there was no decrease in reducing sugar to indicate reversion and within the limits of experimental error they all attained the same equilibrium.

Further, the equilibrium was not influenced by the amount of enzyme present. After standing 20 hours a solution containing 3 mg. of enzyme reduced 264 mg. of cuprous oxide and the same solution with 12 mg. of enzyme reduced 266. Two other solutions containing 3 and 12 mg. of enzyme reduced 246 and 249 mg. cuprous oxide respectively.

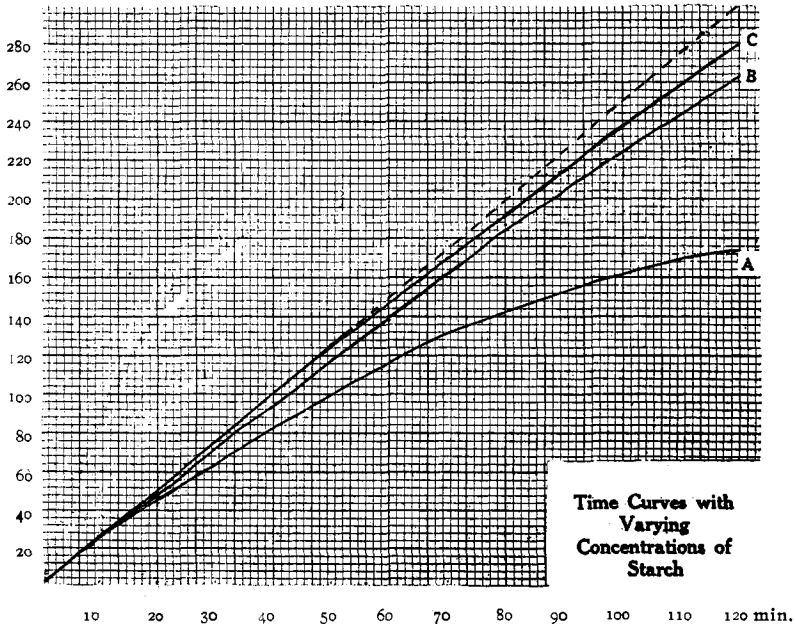
Velocity of Reaction.—Our principal data bearing upon the velocity of the reaction for different concentrations of starch were obtained in 4 series of experiments. In each experiment of the first 3 series (A, B, C) 0.6 mg. pancreatin No. 6 acted for the time indicated upon starch solutions the strengths of which were for series A, 0.5 per cent.; for B, 2 per cent.; for C, 4 per cent.; and which contained in all cases 700 mg. sodium chloride and 10 cc. of fiftieth-molar disodium phosphate per 100 cc. In series

D 5 mg. of pancreatin No. 2 acted upon 1 per cent. starch solution containing 700 mg. sodium chloride and 1.5 cc. of 0.01 *N* sodium hydroxide per 100 cc.

The results of the four series expressed in mg. of cuprous oxide were as follows:

Time. Min.	Curve. A.	Time. Min.	Curve. B.	Curve. C.	Time. Min.	Series. D.
20	47	10	25	25	5	81
40	83	20	48	50	10	140
60	117	30	72	74	15	172
80	142	40	94	100	20	184
100	160	50	118	125	25	194
120	174	60	138	147	30	198
140	179	70	160	170	40	204
160	187	80	185	192	55	212
180	189	90	204	215	70	214
200	193	100	224	237	100	221
220	197	110	243	259	130	225
240	199	120	263	280	190	230

The results of series A, B, and C are also shown graphically in the accompanying curves:



It will be seen from the data and curves (1) that the *initial* speed of conversion for a constant amount of enzyme was the same for the three different concentrations of starch; (2) that the speed of the reaction

diminished more rapidly the smaller the initial concentration of the starch.

By a mathematical consideration of the relation between the original concentration of the starch and the amount of reducing sugar estimated as maltose formed, the following differential and integral equations were deduced:

$$\frac{dx}{dt} = K\left(\frac{C-x}{C+x} - \frac{x}{PC}\right)$$

and

$$\frac{PC(1-P+\sqrt{R})}{2\sqrt{R}M}\left(\log_{10}\frac{\sqrt{R}C+PC+C}{\sqrt{R}C+PC+C+2x} + \frac{\sqrt{R}+P-1}{\sqrt{R}-P+1}\log_{10}\frac{\sqrt{R}C-PC-C}{\sqrt{R}C-PC-C-2x}\right) = Kt.$$

In these equations:

C = original concentration of starch;

x = varying concentration of reducing sugar;

P = a constant;

R = $(P^2 + 6P + 1)$;

M = the logarithm of e to the base 10.

The value for P is determined by substituting the experimental results of series B and C in the equation and finding a number which gives constant values to K for the entire series of observations. The value thus found for P was 12. When the numerical data of our experiments were substituted for these letters the equation reduced to the form

$$\left(\log_{10}\frac{13.865\,C}{13.865\,C+x} + 6.9\log_{10}\frac{0.865\,C}{0.865\,C-x}\right) = Kt.$$

It is by means of this equation that the values of K given in the preceding paper were established.

Influence of Temperature.—Vernon[1] pointed out the fact that Roberts found an optimum temperature at 30°. The activity remained at its maximum until 45°, when it rapidly diminished to 70°, where it ceased. Vernon himself gives figures (with tap water) indicating 35° as the optimum temperature and the activity still existing at 65°. When he used 0.2 per cent. salt the results were widely different. The maximum activity was then at 50° instead of 35° C., but the action ceased between 65° and 70°.

Our results on the effect of temperature are as follows:

When no added electrolyte is present the effect of increasing the temperature from 21° to 40° is to depress the activity. The following Lintner figures were obtained with 4 samples of pancreatin:

Lintner figure		
at 21° C.	at 40° C.	Per cent. 40°/21°.
6.98	5.78	83
1.27	1.16	91
13.79	12.22	88
1.38	0.83	60

[1] *J. Physiol.*, **27**, 174 (1901).

If, however, the proper amounts of salt and alkali are present the activity at 40° is nearly four times as great as at 21°.

The following figures were obtained with pancreatin No. 6, by the regular gravimetric method followed throughout the work. There were 10 cc. of 0.02 molar disodium phosphate and 700 mg. of salt present in every 100 cc. of solution. One-half mg. of enzyme was allowed to act for 1 hour in each case.

Temperature.	Cuprous oxide. Mg.
21°	65
30°	122
40°	238
45°	298
50°	345
55°	378
60°	256
65°	66

Between 20° and 40° the speed is about doubled every 10°, in accordance with van't Hoff's rule for normal chemical reactions; between 40° and 55° the acceleration is less, but temperature still has a great effect. Beyond 55°, where the maximum activity was obtained, the rate of change decreases very rapidly.

The depressing effect of increasing the temperature on amylase in solutions without added salt and alkali may be due to the fact that the water itself has a greater paralyzing effect at the higher temperature. We know that pure water, acting on pancreatic amylase free from neutral electrolyte, gradually destroys it, but if a trace of salt and alkali are present it will remain active for a long time.

Influence of Asparagine.—Marked differences of opinion exist as to whether asparagine really increases the diastatic power of amylases or only helps to give them favorable conditions for activity by regulating the reaction of the solution.

We have experimented upon the effect of asparagine in two ways:

First, by determining the time curve for solutions containing alkali with and without the addition of asparagine. The results of these showed no increase of activity in the presence of the asparagine.

The results are as follows:

Minutes.	Cuprous oxide, mg. A.	B.	C.	D.
5.....	48	46	49	47
10.....	85	81	88	86
15.....	119	118	124	122
20.....	145	142	151	149
25.....	164	162	170	168
30.....	174	175	180	179

Solution A contained 2 cc. 0.01 N sodium hydroxide per 100 cc.
Solution B contained 2 cc. 0.01 N sodium hydroxide + 50 mg. asparagine.
Solution C contained 2 cc. 0.02 molar disodium phosphate.
Solution D contained 2 cc. 0.02 molar disodium phosphate + 50 mg. asparagine.

In a second series we added asparagine to solutions containing increasing amounts of alkali as phosphate.

So long as the amount of phosphate was below the optimum the addition of asparagine depressed the activity of the amylase. When so much phosphate was added that its alkalinity depressed the action, the addition of asparagine helped to counteract the effect of the excess of alkali and to allow the enzyme to act normally. The results follow:

Alkalinity of solution. cc. 0.02 molar Na_2HPO_4 per 100 cc.	Mg. cuprous oxide	
	without asparagine.	with asparagine.
2	260	227
3	(290)	281
4	301	291
5	(304)	309
6	307	312
7	(308)	327
10	313	329
16	296	329
20	273	324
30	264	316
40	236	307

In the presence of 10 cc. of 0.02 molar disodium phosphate increasing the amount of asparagine had no effect.

Asparagine. Mg.	Cuprous oxide. Mg.
10	321
100	329
150	328
200	330
300	324

Summary.

1. Pancreatic amylase when tested in the form of high grade commercial preparations upon soluble starch prepared by the Lintner method and purified by dialysis, was inactive, but was activated by the addition of a small amount of neutral electrolyte and was still further activated by the addition of both salt and alkali.

2. Until the enzyme was fully activated the optimum concentrations of salt and alkali appeared to depend upon each other; beyond this point the optimum concentration of alkali appeared to depend primarily upon the concentration of the starch.

3. With a given amount of enzyme, properly activated, the initial rate of hydrolysis was not dependent upon the amount of starch; as the re-

action proceeded its rate diminished but less rapidly the greater the amount of starch present.

4. Working with 1 per cent. starch, however favorable the conditions of salt and alkalinity and however large the amount of enzyme, the hydrolysis tended to come to equilibrium when the weight of maltose reached about 85 per cent. of the initial weight of starch.

5. In the presence of more than the optimum amount of alkali the point of equilibrium was approximately the same if sufficient time was allowed and fresh enzyme added during the reaction.

6. The data obtained have been applied in a method of determining and expressing diastatic power based upon the optimum conditions of salt and alkalinity as found for pancreatin and the quantitative relation between the amount of amylase and the reducing sugar it produces as shown by the velocity curve. The description of this method has been given in the preceding paper.

7. Between 20° and 40° the action of pancreatic amylase (properly activated by sodium chloride and sodium phosphate) showed a temperature coefficient approximating van't Hoff's rule for normal chemical reactions, since it nearly doubles for a rise of 10°.

8. Tested in solutions of varying alkalinity containing sodium chloride and sodium hydroxide or sodium phosphate, asparagine showed little, if any, effect not explainable by its influence upon the alkalinity of the solution.

NOTE.

Note: Preparation of ortho- and para-Nitrophenols.—The preparation of the nitrophenols as ordinarily carried out is accompanied by the production of much tar and is a somewhat unpleasant operation. The yield of the para compound is small, 50 grams of phenol giving according to Gattermann only from 5 to 10 grams of para-nitrophenol. Inexperienced operators often get no more than one or two grams of the compound, if any at all. This justifies the publication of the improvement recommended in the present note.

The production of tar is completely avoided and a yield of 18 per cent. of para-nitrophenol (13 grams from 50 grams of phenol) is obtained uniformly, *if the nitric acid is vigorously stirred during the addition to it of the phenol.* The results given below were obtained by carrying out the operation as follows: A solution of 80 grams sodium nitrate and 100 grams of strong sulphuric acid in 200 cc. of water was energetically stirred with the aid of a small water motor and kept at about 25°. To this was added drop by drop, at the rate of about 30 or 35 drops a minute, out of a separatory funnel, a solution of 50 grams crystallized phenol in 5 grams alcohol. The stirring was continued for about one-half hour