TABLE II.—PERCENTAGE COMPOSITION OF INOSITE-PHOSPHORIC ACID ACCORDING TO DIFFERENT INVESTIGATORS.

Investigator.	Proposed formula.	C.	H.	P.
Posternak and others	$C_2H_8P_2O_9$	10.08	3.36	26.07
Verbrodt	$C_{12}H_{40}P_{11}O_{46}$	11.42	3.17	27.04
Rising	$C_6H_{20}P_5O_{22}$	12.02	3.34	25.88
This article	$C_{12}H_{41}P_9O_{42}$	12.68	3.61	24.56

Our formula is $C_{12}H_{41}P_9O_{42}$; Rising's, multiplied by 2, would be $C_{12}H_{40}P_{10}O_{44}$; Verbrodt's is $C_{12}H_{40}P_{11}O_{46}$, while the formula proposed by Posternak, and agreed to by other investigators, multiplied by 6, would be $C_{12}H_{48}P_{12}O_{54}$. Both the formula and the percentage composition of our acid agree fairly well with that of Rising. The carbon content of Verbrodt's acid is 1.34% above that of the Posternak acid, and the results of Patten and Hart, Hart and Tottingham, and Anderson were always higher than their theory called for.

We believe our formula, $C_{12}H_{41}P_9O_{42}$, more nearly represents the truth, and propose it as the empirical formula of inosite-phosphoric acid, or the so-called phytic acid of feeding materials.

Summary.

The method for the preparation of inosite-phosphoric acid, or so-called phytic acid, on which rests the theory that this acid corresponds to the formula $C_2H_8P_2O_9$ or a multiple of it, produces an impure product containing notable amounts of inorganic impurities.

The inosite-phosphoric acids of wheat bran and cottonseed meal are identical and correspond to the formula $C_{12}H_{41}P_9O_{42}$.

We have no evidence that wheat bran contains an inosite-phosphoric acid with pentose in the molecule.

The formula $C_{12}H_{41}P_9O_{42}$ is proposed for the inosite-phosphoric acid, or so-called phytic acid, of feeding materials.

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ON SOME CONDITIONS AFFECTING THE ACTIVITY AND STABILITY OF CERTAIN FERMENTS.

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The behavior of soluble ferments, or enzymes, toward other ferments and the action of foreign bodies, especially acids, alkalies and salts, on ferments, have been the subjects of a great number of investigations, some of which have a purely theoretical interest, while others have a more direct practical importance as bearing on questions of digestion and other actions in the animal body.

One of these queries touches the question of the mutual action of pepsin, trypsin and amylopsin and the behavior of each in presence of a great variety of chemical compounds. A special phase of this problem relates

to the action of pepsin and hydrochloric acid on the mixture of enzymes known as pancreatin, a question which has been discussed in the literature through several decades, and which is yet far from settled, apparently. From the standpoint of therapeutics the question possesses no little importance, since the value of pancreatin administered as a remedy must depend on how it reacts with the gastric juice of the stomach through which it must pass before reaching a medium in which it can act.

In several recent researches conflicting answers have been given to this seemingly simple question.[1] In our laboratory it has come up in connection with an enquiry begun under the auspices of the Council on Pharmacy and Chemistry of the American Medical Association, which has gone far enough to lead to some positive conclusions regarding the action of pepsin on trypsin, and which will be discussed elsewhere.

It is not our intention to report this work here beyond saying that the action of the gastric hydrochloric acid seems to be somewhat intensified by the presence of pepsin. In other words, while hydrochloric acid of a certain concentration is destructive of trypsin and amylopsin this action is more marked when the acid is accompanied by pepsin. Certain subsidiary questions coming up in this practical investigation have led to other studies, and some of these form the subject of this paper. The most important of these questions are concerned with the action of weak acids, alkalies and water on trypsin and amylopsin, since it is useless to discuss the behavior of pepsin on these enzymes until the action of the inorganic medium itself is better understood.

It will be recognized at once that these are not new questions; much work has been done on each one of them, but to the whole series of enquiries as well as to the one concerned with diastatic action the words of Oppenheimer[2] may be well applied: "Differences in methods of observation and other causes have unfortunately brought us to this pass that on many questions no agreement at all has been reached. Each new investigator has only succeeded in increasing the immense literature, without bringing a definit solution to the question, since another investigator in turn comes forward to dispute his conclusions." At the risk of adding to this confusing mass of literature the following experiments are offered. We shall consider first some points in the behavior of the pancreatic diastase, and as we are concerned at the outset with the starch used in the experiments on diastases something about this product will be said first.

The Preparation of Standard Starch.

In our opinion a large part of the confusion referred to in the above

[1] See for example a paper by Zimmerman, *J. Ind. Eng. Chem.*, **3**, 750 (1911) and one by Wroblewski, Bednarski and Wojczynski, *Hofmeister's Beiträge*, **1**, 289.

[2] "Die Fermente und ihre Wirkungen," first edition, p. 162.

quotation comes from the irregularities in the methods of making the standard paste. The difficulties in this direction have been referred to by writers from the earlier ones, as Lintner,[1] to the most recent, Sherman,[2] for example. The last named, with others, insists on the necessity for long washing of the starch and for long boiling of the paste. The marked absorbing power of starch for certain salts makes a washing necessary where crude products are used, as would be suggested by the experiments of Hoyes Lloyd[3] on the adsorption of salts, acids and alkalies by starches.

While agreeing fully with these writers in regard to the importance of a pure starch for the tests, we have come to somewhat different conclusions in respect to the method of preparing it. There is but little choice between corn and potato starch as a standard, if both are pure and normal, but there is a practical advantage on the side of the potato product as it comes into the market. We have used both starches with equally good results, but this does not mean that good results may be obtained with any kind of corn or potato starch. In fact, some starches are quite unsuitable and no amount of washing will render them usable. Our experience, extending over a number of years in the preparation of starch pastes for standards, has convinced us that the best results may be expected from a laboratory-made potato starch, obtained by grating the potato to a pulp and straining this through muslin by the aid of water alone, no alkali or acid being used. The settled starch liquor is decanted a number of times, being replaced always by clean distilled water, condensed in block tin. But water of the high degree of purity recommended by some writers, pure enough for conductivity experiments, seems to us a quite unnecessary refinement in the washing of the starch or in the subsequent preparation of the paste.

The potato itself, however, is the important factor in the process. It is a matter of common experience that some potatoes as ordinarily boiled in the household, with the skins, soon become soft or "mealy," and mash to a smooth uniform mass. Other potatoes require a much longer time for cooking and even then are lumpy. This difference may be due to the ripeness of the potatoes, but it is more often due to the variety itself. There are certain kinds of potatoes which are not suitable for table use and which do not work up to a good starch in the manufacture. In the examination of commercial potato and other starches we have often found great differences in behavior, and are inclined to refer them to the original material. Starch made from green potatoes or from frozen potatoes is always irregular in its action. A great deal of such starch naturally finds its way into the market. But we have seen commercial potato starch

[1] Lintner, *J. prakt. Chem. N. F.*, **34**, 378.

[2] Sherman and colleagues, THIS JOURNAL, **32**, 1073, 1087.

[3] THIS JOURNAL, **32**, 1213.

which is quite as pure and clean as our home-made starch, and which is with one or two washings quite suitable for use. With clean starch of this kind, it is possible to make a clear paste with short boiling, twenty minutes being sufficient. In recent experiments we have found essentially identical results from pastes made in this way, after boiling twenty minutes, and after boiling four hours under a reflux condenser. Very long boiling should be avoided, as it brings about some little dextrinization, especially with some commercial corn starches.

Method of Observation.

In following out the action of diastases on such clear starch paste, made up usually to the strength of ten grams of anhydrous starch to the liter, we employ the form of the iodine test worked out in this laboratory, and reported by one of us.[1] In the digestions, always at 40°, we carry the reaction to the colorless end point, when 5 drops of the digesting fluid are added to 5 cc. of a dilute iodine solution, made by dissolving 1 gram of iodine and 2 grams of potassium iodide in 125 cc. of water and diluting 2 cc. of this solution to 1 liter, finally, for use.

When the dilute starch paste is added to this iodine solution the blue color comes out sharply. As digestion proceeds the blue changes to a purple tint, then a purplish red, a rather deep red, a light red or reddish, a yellowish shade and finally colorless, or at most the faint shade of the weak iodine solution. It may be advantageous to use even a weaker iodine to note the final tint, but usually we do not do this. When used with pancreatic preparations Sherman[2] objects to this method of following the reaction because of the appearance of the red shades. But it is just this appearance and the gradual changes which constitute the most desirable features of the iodine test. Its advantages have been pointed out by others.[3] The conversion of starch to sugar is far from being an immediate process, and whether the dextrins are definit and sharply definable bodies or not, we must admit that there are various stages in the change from the original starch to the final malt sugar. The purple color which succeeds the blue corresponds to the so-called amylo-dextrin of some authors and its appearance is a valuable indication of the progress of the action. This stage is followed by that of other mixtures, not definit of course, but all clearly suggesting the rapidity of the transformation. It is desirable to employ a method in which this transformation may be conveniently followed, and this is true of the iodine method as used by us. At the end we have just as much information as may be derived from the copper reduction tests, which we have often used for controls, and much additional information, besides, which may be lost by making the

[1] Johnson, THIS JOURNAL, **30**, 798.

[2] *Loc. cit.*, p. 1081.

[3] See Roberts, *Proc. Royal Soc.*, **32**, 145; Detmer, *Z. physiol. Chem.*, **7**, 1.

copper reduction in presence of the excess of starch. If the object of the investigation is merely to determin the value of a given diastase in the conversion of starch in some technical process, in the fermentation industries, for example, the determination by the cuprous oxide formation is undoubtedly the most satisfactory. Here the reaction may be carried to practical completion and there need be no confusion as to how much of the observed reduction is due to sugar and how much to the complexes included under the different kinds of dextrins. In a physiological enquiry, however, the time element is often the most important, and the effect here is much more clearly discernible through the application of the iodine method, as it is possible to begin tests within a minute after mixing the starch paste and the ferment and to repeat them at short intervals, as desired.

The Effect of Weak Alkalies on the Diastatic Activity.

That a diastase solution free from inorganic salts is relatively very inert has been frequently shown and is well emphasized in the interesting investigations of Sherman and his colleagues referred to some pages back. But especial importance attaches to the behavior of weak acids and alkalies, since, as already referred to, the question of the action of pepsin on the pancreatic enzymes is complicated by that of the action of the medium itself, that is, of the acidity or alkalinity.

That a neutral or faintly alkaline reaction favors the activity of the pancreatic diastase is usually assumed, but it is sometimes denied. There is also a wide divergence of view regarding the optimum alkalinity, where this is considered necessary. By some authors a slight degree of acidity is declared to favor the action of pancreatic diastase as well as that of the diastase from malt, where the effects of acid have been long known. According to Gruetzner[1] the harmful action of sodium carbonate is shown in a concentration of 0.05%, while Langley and Eves[2] find that the action of the salivary diastase is retarded with 0.0015% of the carbonate, and with a much smaller concentration of the hydroxide. The results of different observers appear to be out of agreement with each other largely because the effects of other electrolytes, always present but in varying proportions, have been generally left out of consideration. In most of these earlier observations the amount of electrolyte present was usually very small.

In our experiments we have worked with a salt content of 1 milligram of sodium chloride to the cubic centimeter of digesting mixture, as this amount appeared to be enough to practically activate the preparations used. We have carried out a number of observations on the digestion in presence of sodium bicarbonate and sodium glycocolate, as in their hydrolysis these salts yield very weak alkali.

[1] Gruetzner, *Pflueger's Archiv.*, **12**, 292.

[2] Langley and Eves, *J. Physiol.*, **4**, 18.

As enzymes we employed several commercial preparations and also a glycerol extract of hog pancreas. This was made by mincing the organs very fine and macerating through about three weeks with twice their weight of pure glycerol. The mass, thick at first, grew much thinner in time from the evident partial digestion. Finally the liquid was separated pretty thoroughly from the suspended particles by straining and by centrifugalization in a powerful machine capable of taking eight tubes of 100 cc. each at one operation. In this way we secured about 1600 cc. of a cloudy liquid which deposited but little on standing. This was extremely active in amylopsin and trypsin, although prepared with careful exclusion from contact with any portions of the intestinal mucosa. Even without the addition of salt the liquid was markedly active on starch, but the addition of the salt brought an increase in the activity. In all our experiments aqueous dilutions of this extract were used, in some cases a 1 to 10 dilution, and in the experiments to be explained first a 1 to 50 dilution, filtered.

In the first set of experiments an amount of this dilution was taken not quite sufficient to convert 50 cc. of 1% starch paste to the colorless end point in 6 minutes, and which would not carry it beyond the deep blue iodine stage in three minutes. This amount was found by experiment to be 3 cc. In the first experiments the alkaline enzyme solution, warm, and diluted to 50 cc., was added to the starch paste at the same temperature without previous incubation of the enzyme mixture. In later experiments the enzyme-alkali-salt mixtures were incubated 30 minutes and 60 minutes before adding to the starch. Two persons made the tests, working together so as to lose no time in removing portions of the digesting liquid for the iodine reaction. The sodium bicarbonate was added from a solution of the pure salt made up and kept in a stoppered bottle in the cold, so as to avoid any appreciable hydrolytic dissociation from elevated temperature.

Experiment A.—Action of enzyme and alkali on starch paste, without previous incubation. Taken, 50 cc. of 1% starch paste. Added 50 cc. of alkali-enzyme mixture containing 3 cc. of the I to 50 glycerol extract, 100 mg. of salt and sodium bicarbonate as shown below. Both solutions warmed to 40° before mixing.

Mg. $HNaCO_3$.	Time of contact, in minutes.				
	3.	6.	10.	12.	15.
0.....	Blue	Faint reddish	Colorless	Colorless	Colorless
25.....	Reddish purple	Yellowish	Colorless	Colorless	Colorless
75.....	Reddish purple	Reddish	Colorless	Colorless	Colorless
125.....	Blue	Reddish	Reddish	Colorless	Colorless
175.....	Blue	Purple	Reddish	Colorless	Colorless
200.....	Blue	Blue	Reddish	Yellowish	Colorless
300.....	Blue	Blue	Reddish purple	Yellowish	Colorless
400.....	Blue	Blue	Reddish purple	Yellowish	Colorless
500.....	Blue	Blue	Reddish purple	Yellowish	Colorless
600.....	Blue	Blue	Reddish purple	Yellowish	Colorless

It is interesting to note in this experiment that at the end of the 3-minute period the solution without alkali and those with the higher amounts of alkali had not changed enough to show an advanced iodine reaction. The mixtures with the smaller amounts of alkali were, however, somewhat advanced in digestion, showing the favorable influence of the slight dissociation of the bicarbonate at this temperature. It may be added that in other experiments with a narrower range in the alkali variations, 25, 50, 75, 100 and 125 milligrams, the stimulating effect was not found to extend beyond 100 mg.

At the end of 6 minutes the mixture with no alkali gave in the iodine test a faint reaction, while with the 25 mg. of bicarbonate the color was just discernible. With the larger amounts of alkali the color passed through reddish, purple and blue for the 6-minute period, indicating clearly the effect of increasing amounts of the product of dissociation. At the 10- and 12-minute intervals it is seen how the reaction advances, even with large weights of the bicarbonates present, while at the end of 15 minutes the conversion is complete in all cases. These results point to a restraining, rather than to a destructive action on the enzyme. It is also plain that for weights of bicarbonate above 300 mg. the effect is about the same, no matter what the increase is. In other experiments, which need not be detailed here, it was found that in the restraining action 850 mg. seemed to be no more effective than 300.

Experiment B.—Action of enzyme and alkali on starch paste, after preliminary incubation of the alkali-enzyme solution. Taken, 50 cc. of 1% starch paste. Added 50 cc. of alkali-enzyme mixture containing 3 cc. of the I to 50 glycerol extract, 100 mg. of salt and sodium bicarbonate as shown below. The alkali-enzyme mixture was incubated 30 minutes at 40° and then added to the starch paste at the same temperature.

Mg. $HNaCO_3$.	Time of contact, in minutes.						
	3.	6.	10.	12.	15.	18.	22.
0	Blue	Reddish	Colorless	Colorless	Colorless	Colorless	Colorless
25	Reddish purple	Yellowish	Colorless	Colorless	Colorless	Colorless	Colorless
75	Purple	Reddish	Colorless	Colorless	Colorless	Colorless	Colorless
125	Blue	Reddish	Reddish	Colorless	Colorless	Colorless	Colorless
175	Blue	Blue	Reddish	Reddish	Colorless	Colorless	Colorless
200	Blue	Blue	Reddish	Reddish	Reddish	Colorless	Colorless
300	Blue	Blue	Reddish purple	Reddish	Reddish	Colorless	Colorless
400	Blue	Blue	Reddish purple	Reddish	Reddish	Reddish	Colorless
500	Blue	Blue	Reddish purple	Reddish	Reddish	Reddish	Colorless
600	Blue	Blue	Reddish purple	Reddish	Reddish	Reddish	Colorless

It will be noticed in Experiment B, as compared with A, that the various stages in digestion, as indicated by the iodine reaction, all appear, but

still more retarded in consequence of the half-hour preliminary incubation. The reaction time, for complete conversion, is increased five minutes or more. From this experiment, as well as from the last and the one to follow, it is evident that a small trace of alkali provides the best medium for the rapid conversion of the starch. With the ordinary carbonate the hydrolytic decomposition may readily furnish too much alkali for the purpose, and thus bring about destruction or marked retardation.

Experiment C.—The conditions here were the same as in *B*, except in the time of preliminary incubation of the alkali-enzyme mixture before mixing with the starch paste. In this case the incubation was 60 minutes. The effect of this longer incubation was to still further delay the stages in the conversion progression. The most rapid conversion was in the mixture with 25 mg. of bicarbonate. As the results in general were very much like those of B the details need not be given.

The Ferment is Not Destroyed.

It is well known that amylopsin is actually destroyed by caustic alkalies of even marked dilution, and that its action is much retarded by certain weaker dilutions. For the behavior of the hydroxide and normal carbonate of sodium there are numerous references in the literature.[1] But from a practical point of view, as regards especially the pancreatic diastase, it is desirable to know the behavior of an alkali coming from the hydrolysis of a compound like sodium bicarbonate, and to determin whether the diastase is permanently weakened or destroyed by the contact. The retardation of the rapidity of starch conversion may be due to some kind of a weakening of the whole mass of the ferment, or it may be due to the actual destruction of part of it.

To answer this question experiments like the above were repeated with certain amounts of bicarbonate, the incubation being carried on through one hour before adding the enzyme-alkali mixture to the starch, and enough weak acid being added at the end of the incubation to exactly or nearly neutralize the bicarbonate. In the first experiments six portions of starch of 50 cc. each were mixed with the enzyme solutions, made to 50 cc. as above, and previously incubated. Three of the enzyme solutions contained alkali and three did not. At the end of the incubation period the alkali was treated with hydrochloric acid in the proportions given in the table. For the complete neutralization of the 100 mg. of bicarbonate 10.8 cc. of 0.4% acid is necessary. To the other three enzyme solutions an amount of salt corresponding to that formed in the neutralization was added, the volumes made to 60 cc., and then mixed with the starch.

Experiment D.—Effect of neutralization after incubation with alkali. Taken, 50 cc. of 1% starch paste. Added, after incubation, the enzyme solution, as above, containing salt as before. To the alkali-enzyme solutions, hydrochloric acid in amount

[1] See, for example, the discussion in Green, "The Soluble Ferments and Fermentation," or the work of Oppenheimer, already quoted.

shown was added after the incubation of 60 minutes at 40°, and then all solutions were mixed with the starch paste at the same temperature.

Mg. $HNaCO_3$.	Cc. 0.4 p. c. HCl.	Time of contact, in minutes. 3.	6.	10.	30.	60.	90.
0	0	Blue	Reddish	Colorless	Colorless	Colorless	Colorless
0	0	Blue	Reddish	Colorless	Colorless	Colorless	Colorless
0	0	Blue	Reddish	Colorless	Colorless	Colorless	Colorless
100	10.7	Blue	Blue	Blue	Blue	Blue	Blue
100	10.3	Blue	Blue	Blue	Blue	Blue	Blue
100	10.0	Blue	Blue	Blue	Blue	Blue	Blue

These results were somewhat surprising and suggested that neutralization failed to restore the activity of the ferment. But it was found that on standing at the same temperature the iodine reaction of the fourth mixture was purplish blue, of the fifth red, and of the last colorless, at the end of 12 hours. This points to retardation, but not destruction, and the peculiar effect is due to the sudden addition of the relatively large amount of hydrogen ions from the acid, which apparently react at once with the enzyme. Although finally decomposed by the acid, which is thereby neutralized, the bicarbonate behaves at the outset as a neutral salt, and the immediate effect is that of the addition of acid alone.

The experiment was then repeated with the same amount of bicarbonate and the same incubation, followed by the addition of a little acid, and then with more bicarbonate and a correspondingly larger acid addition. Very different results were now secured, as shown by the next table.

Experiments E and F.—Effect of partially neutralizing the bicarbonate. Taken, 50 cc. of 1% starch paste. Incubated salted enzyme solutions, with and without bicarbonate, as shown, and partially neutralized with acid, as shown. Added to the starch pastes and incubated again.

Mg $NHaCO_3$.	Cc 0.4 p. c. HCl.	Time of contact, in minutes. 3.	6.	10.	15.	20.	30.
0	0	Blue	Reddish	Colorless	Colorless	Colorless	Colorless
0	0	Blue	Reddish	Colorless	Colorless	Colorless	Colorless
0	0	Blue	Reddish	Colorless	Colorless	Colorless	Colorless
100	0.25	Blue	Reddish	Colorless	Colorless	Colorless	Colorless
100	0.50	Blue	Reddish	Colorless	Colorless	Colorless	Colorless
100	1.00	Purple	Yellowish	Colorless	Colorless	Colorless	Colorless
100	2.00	Blue	Reddish	Colorless	Colorless	Colorless	Colorless
500	2 00	Blue	Blue	Reddish	Colorless	Colorless	Colorless
500	3.00	Blue	Blue	Blue	Reddish	Yellowish	Colorless
500	4.00	Blue	Blue	Blue	Yellowish	Colorless	Colorless
500	5.00	Blue	Purple	Colorless	Colorless	Colorless	Colorless

These results are very interesting and show clearly that with the proper neutralization of the 100 mg. of bicarbonate there is complete restoration of the diastatic activity. With the addition of 1 cc. of the acid, which is about one-tenth of that required for complete neutralization, to form sodium chloride of all, the activity becomes very marked. With the larger

bicarbonate amount present a corresponding acid neutralization is necessary to secure the same effect. The action of the bicarbonate is, therefore, a temporary one.

The Effect of Dilution of Aqueous Solutions.

It has been shown that aqueous solutions of our glycerol extract were employed in most of the experiments recorded. The extract itself is apparently very stable and was used through a long period with evidently undiminished activity. Vernon[1] and others who have worked with such extracts state that they retain their activity through months. With water solutions, however, the case is quite different, and in a 1 to 50 dilution, or even in a 1 to 10 dilution there may be a very marked loss of activity in 10 to 15 hours, as when the solutions are allowed to stand over night. The loss of power is much increased by a slight elevation of temperature and at 40° may be very decided.

If, however, the dilutions are made with salt water in place of pure water the stability is found to be much greater. These facts are illustrated by a large number of experiments made with some commercial pancreatin preparations in an effort to find an explanation for the great variability in their relative strengths. With each pancreatin a solution of 500 mg. to 100 cc. was made, in one case with pure water and then with the addition of 50 mg. of salt. Of the fresh and salted solutions one portion was allowed to incubate 1 hour at 40°, while the second portion stood 1 hour at the room temperature, about 20°. Then small volumes of each solution were added to 50 cc. of starch paste, at 40°, and incubated at the same temperature, as shown below.

Experiments G and H.—A weak pancreatin, S, 500 mg. to 100 cc. Taken, 50 cc. of 1% starch paste in each case. In *G* added to the starch one-half of the pancreatin solution which had been previously incubated 1 hour at 40°, and in H added to the starch the same volume of pancreatin solution which had stood 1 hour at room temperature.

	Time of contact, in minutes.									
	G.						H.			
Cc. of ferment.	5.	10.	15.	20.	50.	70.	5.	10.	15.	20.
4	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Red	Reddish	Colorless
6	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Reddish	Colorless	Colorless
8	Blue	Blue	Blue	Blue	Blue	Blue	Purple	Colorless	Colorless	Colorless
10	Blue	Blue	Blue	Blue	Blue	Blue	Purple	Colorless	Colorless	Colorless

These tests illustrate very clearly the effect of a short period of incubation at 40° on the pancreatin solution. Even after 70 minutes with the largest volume of ferment solution taken there is apparently no action on the starch paste. With the same ferment without incubation the reaction is fairly rapid. With salt added, however, as now to be shown, the reactions are rapid under both conditions.

[1] Vernon, *J. Physiol.*, **27**, 269.

Experiments I and J.—The same weak pancreatin, S as before, and same strength, but 50 mg. of salt added. Taken, 50 cc. of 1% starch paste. In I added to the starch one-half of the pancreatin solution which had been previously incubated 1 hour at 40°, and in *J* added to the starch the same volume of pancreatin which had stood 1 hour at the room temperature.

	Time of contact, in minutes.					
	I.			J.		
Cc. of ferment.	5.	10.	15.	5.	10.	15.
4	Purple	Reddish	Colorless	Reddish	Colorless	Colorless
6	Red	Colorless	Colorless	Colorless	Colorless	Colorless
8	Reddish	Colorless	Colorless	Colorless	Colorless	Colorless
10	Yellowish	Colorless	Colorless	Colorless	Colorless	Colorless

We see here the remarkable effect of the salt, even in the small amount used. This trace of salt protects the ferment in the incubated solution so that the action on the paste becomes evident in the shortest time of contact and with the lowest concentration. The amounts of ferment added to the starch paste are 20, 30, 40 and 50 mg. in each test, and in the experiments with salt, 2, 3, 4 and 5 mg. of the latter. A weak ferment may be made to appear strong in this way, and in making tests it should be known whether or not the ferment mixture contains much of the salt. This particular pancreatin, which is one of the common brands on the market, is extremely weak, since in the incubated solution no conversion takes place in 70 minutes, with any ferment concentration employed, and in the unincubated sample a conversion in 20 minutes with the lowest concentration. Tests showed this product to be free from chlorides.

Experiment K.—To show the behavior with a really active pancreatin, sample P, solutions were made as above, without salt and with it. The incubation periods were the same as before, but in every case the conversion was carried to the colorless end point in less than 5 minutes. This sample contained a little chloride, and with more added the conversion of unincubated portion was almost instantaneous.

It is interesting to note now the behavior of a pancreatin of medium activity treated in the same manner.

Experiments L and M.—Pancreatin C, medium strength, 500 mg. to 100 cc. Taken, 50 cc. of 1% starch paste. In *L* used part of the pancreatin solution which had been incubated 1 hour at 40°. In *M* used the same pancreatin solution, but not incubated. Added to starch paste at 40° and incubated further as shown.

	Time of contact, in minutes.							
	L.						M.	
Cc. of ferment.	5.	10.	15.	30.	60.	85.	5.	10.
4	Blue	Blue	Blue	Purple	Reddish	Yellow	Reddish	Colorless
6	Blue	Purple	Purple	Red	Colorless	Colorless	Colorless	Colorless
8	Blue	Red	Reddish	Reddish	Colorless	Colorless	Colorless	Colorless
10	Blue	Red	Reddish	Colorless	Colorless	Colorless	Colorless	Colorless

Experiments N and O.—The same pancreatin, C, but salted, same strength. Taken, 50 cc. of 1% starch paste. In N used part of the pancreatin after the usual incubation

of 1 hour. In O used ferment not incubated. The amount of salt added was 50 mg. to the 100 cc. of ferment solution.

	Time of contact, in minutes.				
	N.			O.	
Cc. of ferment.	5.	10.	15.	5.	10.
4	Red	Reddish	Colorless	Colorless	Colorless
6	Red	Colorless	Colorless	Colorless	Colorless
8	Reddish	Colorless	Colorless	Colorless	Colorless
10	Colorless	Colorless	Colorless	Colorless	Colorless

According to this general scheme we have examined practically all the products made in this country, which, however, come from a limited number of factories, although sold under different brand names. The behavior of the whole group is represented by these tables from the weakest to the strongest, with the exception of a few samples which were found to be inert under all conditions, and one product which was remarkably strong. The results suggest the necessity of somewhat more uniform methods of examination of all such products where comparative values are desired, since the activity may depend on many factors, of which salt addition is one. In the intestines the presence of sufficient salt for acceleration is always assured from the diet.

The Action of Weak Acids on Amylopsin.

Like the other actions, that of acids on the starch-digesting ferment of the pancreas has been much discussed. It seems desirable to bring out certain conditions here which have not been clearly recognized, although they have been in part pointed out by previous writers.

Amylopsin is much more sensitive to the action of traces of acids than to alkalies, and the final effect is more or less dependent on the presence of salts, which may not only serve to stimulate the catalytic action of the ferment, but may also act to depress the activity of the acid hydrogen in its paralyzing behavior. Attempts have been made to show that diastase absorbs gaseous hydrochloric acid through some other than a basic property and that a large part of the gas may be removed by suction without having fully destroyed the ferment. Panzer[1] has published a lengthy investigation on this subject but the weight of acid added to the ferment preparations was in all cases so large as to permit no conclusion concerning the behavior of the small acidities which really come into consideration where the physiological action is concerned. The same is true of the experiments where aqueous acid was used with gram or half-gram weights of the ferment. In one case a gram of a certain diastase preparation and 0.06775 gram of hydrochloric acid, made up to about 80 cc., stood an hour and was then neutralized with sodium hydroxide and diluted to 100 cc. Portions of this dilution were then allowed to act on starch paste, the iodine and sugar tests being made from time to time.

[1] Panzer, *Z. physiol. Chem.*, **82**, 276.

The figures given show a greatly diminished diastatic conversion, as one might expect from the rather high acid concentration. In discussing this and other experiments, the author states that the small amount of salt formed in the neutralization can not be in any way responsible for the modification of the diastatic action, since such small quantities are of no significance. This conclusion does not appear to be warranted by facts already referred to and which will be brought out below. The salt here is undoubtedly of very considerable importance.

We have made some experiments on the action of acid, employing the same general scheme used in following up the effect of alkaline and neutral incubation. Our glycerol extract was used first and then a number of commercial preparations as before.

Experiment P.—Ten cc. of 1 to 50 glycerol extract, plus 100 mg. of salt and quantities of acid as shown below made up to 50 cc. for each test. Incubated in each case through 30 minutes at 40° and added to 50 cc. of 1% starch paste at the same temperature.

Mg. of HCl.	Time of contact, in minutes. 3.	6.	10.	15.	30.	60.
0	Colorless	Colorless	Colorless	Colorless	Colorless	Colorless
1	Blue	Blue	Blue	Blue	Blue	Blue
3	Blue	Blue	Blue	Blue	Blue	Blue
5	Blue	Blue	Blue	Blue	Blue	Blue
7	Blue	Blue	Blue	Blue	Blue	Blue
9	Blue	Blue	Blue	Blue	Blue	Blue

In neutral solution the extract is seen to be very active, but in acid even of the lowest concentration the modification of the ferment amounts to practical destruction, since no perceptible starch conversion takes place, even after the longest incubation period of one hour. In the next experiments stronger solutions of the ferment were used, with somewhat different results.

Experiments P′ and P″.—In P′ 10 cc. of a 1 to 10 glycerol extract were diluted to 50 cc. with the addition of 100 mg. of salt, and in P″ 10 cc. of the glycerol extract made up 1 to 5 were diluted in the same way. In the dilutions volumes of weak acid as shown below were added, and after incubation of 30 minutes the mixtures were added to starch paste as before. Each dilution in P′ contains, therefore, 1 cc. of the original glycerol extract, and in P″ 2 cc. of the original extract.

Mg. of HCl.	Time of contact, in minutes. P′. 3.	6.	10.	60.	P″. 3.	6.	10.	60.
0	Colorless	Colorless	Colorless	Colorless	Colorless	Colorless	Colorless	Colorless
1	Blue	Purple	Reddish	Colorless	Blue	Purple	Reddish	Colorless
3	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Purple
5	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Purple
7	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Purple
9	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Purple

The action of the acid is not quite as marked here as in the case of the very weak glycerol extract dilution, since with the addition of 1 mg. of

acid there is a distinct color change in 6 minutes and complete conversion in an hour, but in the higher acidities the action of the acid is still very sharp. In the next test a still greater concentration of ferment was used.

Experiment Q.—Five cc. portions of the original extract were mixed with 100 mg. of salt and weights of acid, as given below, and diluted to 50 cc. These dilutions were incubated 30 minutes at 40° and then mixed with 50 cc. of starch paste of 1% strength and again incubated.

Mg. of HCl.	Time of contact, in minutes.					
	3.	6.	10.	15.	30.	45.
0	Colorless	Colorless	Colorless	Colorless	Colorless	Colorless
1	Colorless	Colorless	Colorless	Colorless	Colorless	Colorless
3	Colorless	Colorless	Colorless	Colorless	Colorless	Colorless
5	Colorless	Colorless	Colorless	Colorless	Colorless	Colorless
7	Reddish	Colorless	Colorless	Colorless	Colorless	Colorless
9	Reddish	Reddish	Colorless	Colorless	Colorless	Colorless
11	Red	Red	Red	Red	Reddish	Reddish

With this stronger concentration of the enzyme, its destruction appears to be very much delayed. The results seem to suggest that the effect depends not only on the acid concentration but on the enzyme concentration also. In other words, a given weight of acid will destroy or inhibit the action of a given amount of enzyme only. A weight combination between the two is suggested in this way, but there is another possibility which must be noticed, and that is that the increasing weight of inert protein substance added with the largest weights of ferment binds to some extent the acid and weakens its action.

In this connection it may be asked what is the effect of the addition of alkali soon after the addition of acid to the ferment. In the experiments of Panzer, referred to, an attempt was made to answer this question, but the somewhat unfavorable conditions of the experiments made the answer uncertain. Some of our experiments on this point suggested a partial recovery of activity, by speedy addition of alkali. In mixtures incubated for some time the addition of alkali had but little effect, but when sodium bicarbonate was added soon after the acid, with no incubation, the result was somewhat different, as shown by these experiments.

Experiment R.—Ten cc. of the 1 to 10 glycerol extract as in P′ made up with 100 mg. of salt and acid as below to 50 cc. No incubation. Added to 50 cc. of 1% starch paste and incubated as below, for each acid concentration.

Mg. of HCl.	Time of contact, in minutes.					
	3.	6.	10.	15.	30.	90.
0	Colorless	Colorless	Colorless	Colorless	Colorless	Colorless
1	Colorless	Colorless	Colorless	Colorless	Colorless	Colorless
3	Blue	Blue	Blue	Blue	Blue	Blue
5	Blue	Blue	Blue	Blue	Blue	Blue
7	Blue	Blue	Blue	Blue	Blue	Blue
9	Blue	Blue	Blue	Blue	Blue	Blue

This experiment differs from P′ only in this that the incubation was omitted, before adding to the starch paste.

Experiment S.—Same conditions as in R, but 1 minute after adding the acid to the glycerol extract dilution, and shaking, the equivalent amount of sodium bicarbonate was added and then the mixture with the starch paste was made.

Mg. of HCl.	Time of contact, in minutes.					
	3.	6.	10.	15.	30.	60.
0	Colorless	Colorless	Colorless	Colorless	Colorless	Colorless
1	Colorless	Colorless	Colorless	Colorless	Colorless	Colorless
3	Yellowish	Colorless	Colorless	Colorless	Colorless	Colorless
5	Blue	Blue	Purple	Purple	Purple	Purple
7	Blue	Blue	Blue	Blue	Blue	Blue
9	Blue	Blue	Blue	Blue	Blue	Blue

The effect is clearly shown with the 3-milligram concentration of the acid but with the stronger concentrations of the acid even the very quick addition of the sodium bicarbonate does not restore the converting power of the ferment. Experiment R shows that three milligrams of the acid, not neutralized at once, destroys the diastase activity to such an extent that there is practically no starch conversion in 90 minutes. This is a strength of 0.006% in the ferment solution, before adding the starch. That is, 1.6 cc. of 0.1 normal HCl to 100 cc. destroys even the beginning of the starch conversion. Kendall and Sherman[1] have shown that about one-fourth of this stops the conversion to the sugar stage in a diastase-starch mixture. Our results are not very different from those found by Kjeldahl for the effects of dilute sulfuric acid on malt infusions. He found that 10 mg. of the acid for 100 cc. of paste and infusion practically prevented the starch conversion.[2]

Langley and Eves[3] state that 0.005% of hydrochloric acid is destructive to the action of ptyalin, while one-third of this slows the reaction perceptibly, which accords pretty well with our experience.

But it must be recognized that the amount of the ferment, or the accompanying proteins, plays a very important part here, as shown by Experiment Q where a dilution of the same glycerol extract, but five times as strong as the last, was employed. In that experiment, even after incubation, a marked diastatic activity was found in a solution with 18 milligrans of HCl to 100 cc. of ferment solution. This fact may reconcile the somewhat conflicting statements given by Langley and Eves[4] and by Chittenden and Ely[5] for the destructive effect of the acid. Small amounts of acid are undoubtedly stimulative under certain conditions, and often

[1] *Loc. cit.*, p. 1091.

[2] See Effront, "Die Diastasen," p. 121 (1900).

[3] Langley and Eves, *Jour. Physiology*, 4, 18.

[4] Langley and Eves, *Loc. cit.*

[5] Chittenden and Ely, *Am. Chem. J.*, 4, 107.

by the formation of an alkali salt. Where relatively large amounts appear to have but slight action, it may be on account of the presence of much protein, which combines with the acid, or on account of salts like bicarbonates or phosphates, which in a sense neutralize it through liberation of a weaker acid of lower dissociation. It is certain that the ferment, not protected in some such manner, is extremely sensitive.

The above experiments have shown that the action of weak alkalies of the hydroxyl strength of sodium bicarbonate solutions and that of weak hydrochloric acid on amylopsin are essentially different as far as the recovery after neutralization is concerned. But in all such comparisons it is important that some salt, preferably sodium chloride, be present. This point is well brought out in some experiments with the three commercial pancreatins already used to show the action of water. Solutions of these were made with 500 mg. to 100 cc. along with certain weights of acid. Some of the solutions were incubated and some not.

Experiments T and U.—Employed pancreatin S, before used. Took 500 mg. plus 6 mg. of HCl to 100 cc. In experiment T half of this solution was incubated 30 minutes at 40°. The other half, U, was not incubated, but stood at the room temperature through the same time. Then portions were added to 50 cc. of 1% starch paste, and incubated again, as shown.

	Time of contact, in minutes.								
	T.				U.				
Cc. of ferment.	5.	10.	15.	35.	5.	10.	15.	25.	35.
4	Blue	Blue	Blue	Blue	Blue	Purple	Red	Reddish	Reddish
6	Blue	Blue	Blue	Blue	Blue	Purple	Red	Reddish	Reddish
8	Blue	Blue	Blue	Blue	Blue	Purple	Red	Yellowish	Colorless
10	Blue	Blue	Blue	Blue	Blue	Purple	Red	Colorless	Colorless

Comparing these experiments with G and H, it will be seen that here, as there, the solution becomes inert after incubation. The presence of acid, in the last experiments, delays the reaction markedly. In repeating the experiments with just half the acid present it was found that with the incubated portion the starch conversion never got beyond the blue test stage, but in the portion not incubated the colorless end point was reached in the starch volumes with 8 and 10 cc. of ferment in 5 minutes and in all of them in less than 15 minutes.

When salt was added in the amount of 50 mg. to the 100 cc., with 6 mg. of acid and with 3 mg. of acid, no protecting effect was noticed in incubated portions, but in portions not incubated the reactions were all completed in about half the time. In the process of manufacture this sample was left very free from salt, and had possibly been purified by some method of dialysis. It is known that some manufacturers, in the effort to produce a more active ferment, have undertaken a concentration of this kind, but the effect appears to be just the opposit of that desired.

In the next experiments a pancreatin was used which contained relatively much salt, left from the manufacturing process, but more probably added to increase activity. This is the sample of Experiment K.

Experiments V and W.—Pancreatin P, 500 mg. to 100 cc. with 12 mg. of HCl added. Incubated part of the solution through 30 minutes at 40°, V, and allowed the other portion, W, to stand the same time at room temperature. Mixed small volumes with 1% starch paste, 50 cc., and incubated again, as shown.

	Time of contact, in minutes.							
	V.					W.		
Cc. of ferment.	5.	10.	15.	25.	35.	5.	10.	15.
4	Blue	Blue	Blue	Blue	Blue	Reddish	Colorless	Colorless
6	Blue	Blue	Blue	Blue	Blue	Colorless	Colorless	Colorless
8	Blue	Blue	Blue	Blue	Blue	Colorless	Colorless	Colorless
10	Blue	Blue	Blue	Blue	Blue	Colorless	Colorless	Colorless

In a solution of the same ferment made up with 6 mg. of acid to the 100 cc. the conversion times were essentially the same in the not-incubated portion, while in the incubated portion the reaction was not carried to the point of perceptible change in the iodine test. With this acid concentration the salt is not able to protect the ferment or hasten the conversion, when incubated. With a still smaller acid concentration, however, some effect is seen.

Experiments X and Y.—Pancreatin P, 500 mg. to 100 cc. and 3 mg. of HCl added. Part of the solution was incubated 30 minutes at 40°, X, and part stood at the room temperature the same time, Y. Then portions of each were added to 50 cc. portions of 1% starch paste and incubated again, as shown.

	Time of contact, in minutes.						
	X.					Y.	
Cc. of ferment.	5.	10.	20.	40.	60.	5.	10.
4	Blue	Blue	Purple	Red	Colorless	Colorless	Colorless
6	Blue	Red	Reddish	Colorless	Colorless	Colorless	Colorless
8	Blue	Colorless	Colorless	Colorless	Colorless	Colorless	Colorless
10	Blue	Colorless	Colorless	Colorless	Colorless	Colorless	Colorless

Some experiments were made with pancreatin C, also. In the acid treatment, as well as in the incubation with water alone, it stands between pancreatins S and P, and examination shows it to contain a little salt. With more added it acts very much as does P.

In all these experiments it is seen that incubation, also with salt present, is destructive of the enzymic activity. With the amounts of acid used here, as in the experiments with the glycerol extract of pancreas, the subsequent addition of sodium bicarbonate does not appear to restore the normal strength, which suggests destruction of the enzyme rather than neutralization.

In Experiments X and Y the amount of acid used was small, 3 mg. to 100 cc. of the ferment solution, or 0.003%. With this concentration of

acid it is seen that after incubation there is hydrolysis of the starch, but somewhat delayed, which may be interpreted as showing that so small an amount of acid is not sufficient to combine with and destroy all the groups on which the ferment activity depends. It will be recalled that the similar relation noticed with one of the glycerol extracts suggested a weight relation between acid and ferment. This relation cannot be that of combination between the acid and a basic group, because in such case the neutralization with a weak alkali of the type of sodium bicarbonate should restore the activity. With an acid concentration sufficient to permanently inhibit the diastatic activity it is likely that the combination is of a different order.

In interpreting the behavior of these small amounts of acid on the commercial pancreatins, as well as on the glycerol extracts, another fact must be kept in mind. We have referred to the possibility of combination of acid and protein as accounting for the protection of the enzyme, but there is another possible factor in the case. All these preparations possess a certain slight potential alkalinity due to phosphates and other weak inorganic and organic salts present in the original pancreas tissue, and the addition of hydrochloric acid would result in the displacement of some of the acid of these salts.

Most of the pancreatin preparations on the market seem to be essentially finely divided powders of the dried and fat-free gland, and not evaporated extracts, as the descriptions in the literature would lead one to expect. It is true that some products are apparently made by concentration of an extract, but most of them are probably pancreas powders. This being the case we should expect the presence of the salts of the original tissue, and ash determinations on commercial products seem to bear out this supposition. We find in a considerable number of these products an ash content of about 6%, which is consistent with the assumption just made. In products which seem to contain added salt or bicarbonate the ash is more. Some manufacturers apparently recognize the value of salt addition in securing a greater stability and a higher starch-converting power.

It will be seen at a glance that the question of the action of hydrochloric acid is much complicated by the presence of varying amounts of salts, and especially the salts with weak acids. The results obtained above by the addition of small amounts of acid become difficult of interpretation when we recognize that these amounts of acid are small when compared with the potential base present. It would appear probable that in the absence of these natural salts in the pancreas preparations the amylolytic activity would be inhibited by mere traces of hydrochloric acid, and some experiments now in progress seem to point in that direction.

These pancreas powders contain phosphates in very different proportions, as well as organic phosphorus, apparently. Henderson[1] has pointed out the great importance of mixtures of mono- and disodium phosphate in the preservation of the neutrality of certain fluids. It is likely that a somewhat similar function is exerted here, but further study is necessary to make clear all the relations involved.

In this investigation we have been assisted by Mr. George W. Muhleman, to whom our thanks are due.

Summary.

It is suggested in this paper that starch paste for diastatic comparisons should be made from laboratory-prepared potato starch, sound and fully ripe potatoes being employed. Long washing and boiling of commercial starch will not always be sufficient to secure a suitable product.

The amylolytic activity of pancreas preparations is best exhibited in a mixture containing about 25 milligrams of sodium bicarbonate to 100 cc. of paste and ferment solution. Larger amounts of bicarbonate retard the reaction somewhat but do not appear to destroy the ferment, since the full rapidity of conversion may be recovered by the partial neutralization of the soda by weak hydrochloric acid. The addition of enough acid to convert all the soda to salt, at once, destroys the ferment, however.

Glycerol extracts of the pancreas retain their amylolytic power through many months, but by dilution with water this activity is speedily lost. The loss of digesting power is very marked after a short incubation of the diluted extract at 40°, but if salt is present the destructive effect of incubation is much diminished. The effect of incubation of commercial preparations is much the same as with the glycerol extracts, and the presence of salt lessens the disturbing action of alkalies here, also.

The pancreatic diastase is extremely sensitive to the action of traces of strong acids, which was shown by experiments with glycerol extracts and hydrochloric acid. Salt is a protection here, as before. The action of the acid is much more marked than is that of weak alkali, and neutralization with soda does not bring about recovery. Destruction of the enzyme probably follows the contact with acid. The weak inorganic and organic salts present in all pancreas preparations are important factors in modifying the action of added acids, and doubtless, also, of added alkali. The behavior of phosphates may be of the first importance in this regard, especially in the commercial pancreatins, which are largely pancreas powders. Without the presence of these salts the addition of the slightest trace of acid would be doubtless much more marked, and possibly destructive.

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[1] L. J. Henderson, *Ergebnisse der Physiologie*, **8**, 254.