[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF COLUMBIA UNIVERSITY, NO. 326.]

INFLUENCE OF ASPARTIC ACID AND ASPARAGIN UPON THE ENZYMIC HYDROLYSIS OF STARCH.

BY H. C. SHERMAN AND FLORENCE WALKER.

Received August 29, 1919.

In 1893 Effront[1] stated that asparagin accelerates the hydrolysis of starch by malt and taka-diastase. A few years later[2] he obtained similar activation by addition of certain proteins and of a boiled cold water extract of barley. In 1904 he reported[3] that asparagin, aspartic acid, hippuric acid, creatin, creatinin, and the peptones increased the action of malt extract, while succinamide, the amines and their salts, and acid amides generally act unfavorably. This he found for several starches of different origin. He also found, however, that the more favorable the conditions for production of an optimum amount of sugar, the less marked is the effect of the amino acids.

Ford,[4] working with malt, found asparagin to be without effect on the activity of the enzyme. The apparent activation by amino acids and acid salts obtained by other investigators, he ascribes to the neutralization by these compounds of alkaline impurities in the starch.

According to Terroine and Weill[5] saccharification by pancreatic juice is greatly accelerated by α-alanine, glycine, leucine, valine, histidine, arginine, tyrosine, phenyl-alanine, aspartic acid, and glutamic acid. The activating power which they found the digestion products of protein to possess is, they think, most probably due to the amino-acids formed.

More recently Rockwood[6] has investigated the effects of several nitrogenous substances on the hydrolysis of corn starch by saliva and concludes that glycine, tyrosine, anthranilic acid and its *meta* and *para* isomers, aspartic acid, hippuric acid, proteins (serum albumin and gelatin) and amines of the methane series increase saccharification, whereas the amides (acetamide, propionamide and urea) sulfanilic acid, asparagin, succinimide and succinamide show no such effect. He also found that glycine and aspartic acid activate pancreatic extract.

Because of the lack of agreement among previous workers as to whether amino acids do or do not activate amylases, and in view of the fact that past investigations have seldom covered more than one enzyme, a systematic study of the influence of amino acids upon the action of several

[1] *Mon. sci.*, **41**, 266 (1893).

[2] *Compt. rend.*, **120**, 1281 (1895).

[3] *Bull. soc. chim.*, [3] **31**, 1230 (1904); *Mon. sci.*, [4] **18**, 561 (1904); *Compt. rend. Soc. Biol.*, **57**, 234 (1904).

[4] *J. soc. Chem. Ind.*, **23**, 414 (1904).

[5] *Compt. rend Soc. Biol.*, **72**, 542 (1912).

[6] THIS JOURNAL, **39**, 2745 (1917).

amylases, in purified as well as in their natural or commercial forms, seemed desirable.

Experimental.

Apparatus and Materials Used.—Description of apparatus, its selection and care, has been given in previous papers from this laboratory.

The starches used were potato, wheat, maize and rice starches purified in the laboratory by washing with cold, very dilute sodium hydroxide and water as described in a previous paper, and Merck's "soluble starch according to Lintner," washed 9 times with ordinary distilled and 6 times with triply distilled water. 100 cc. of a 1% dispersion, made neutral to rosolic acid with 0.01 *N* alkali or acid, were used for each digestion.

The enzyme preparations employed were those described in our recent paper upon rate of hydrolysis of starches of different origin.[1]

The water extract of potato was obtained by letting one grated potato of medium size stand overnight in 150 cc. of purified distilled water. This was filtered and the filtrate boiled. Its acidity was carefully determined, rosolic acid being used as indicator. Well purified specimens of aspartic acid and asparagin were used. The exact amount needed for each experiment was weighed out and dissolved in the least possible volume of triply distilled water plus the amount of alkali necessary for neutralization.

Method.—The procedure has been fully described in previous papers. When water-washed or alkali-washed starch was used as substrate, the amount of reducing sugar was determined by Fehling's volumetric method.[1] With Lintner's "soluble" starch the gravimetric method[2] which has been used in this laboratory since 1910 was usually employed. The potato extract and the aspartic acid and asparagin solutions were added to the starch dispersions in the cylinders after cooling and before adjusting to final volume.

Experiments with Potato Extract.

In recent experiments[1] on the action of enzymes upon starches of different origin, we found that, when similarly purified by washing with cold, very dilute alkali or with such alkali and then with ether (in either case followed by very thorough washing with pure water) all the starches tested were of practically equal digestibility. This was true whether the digestive agent employed were saliva, pancreatin, malt extract, taka-diastase, or a laboratory preparation of purified amylase of pancreas, malt or *Aspergillus oryzae*, except that in experiments in which purified pancreatic amylase acted upon purified potato starch the results obtained were abnormally low. In this particular case it appeared that the rigorous "purification" of both starch and enzyme had in some way diminished

[1] Sherman, Walker and Caldwell, THIS JOURNAL, 41, 1123 (1919).

[2] Sherman, Kendall and Clark, *Ibid.*, 32, 1082 (1910).

the facility of interaction between them. Addition of a small amount of boiled, carefully neutralized, water extract of potato to the digestion mixture resulted in hydrolysis at a fully normal rate.

When such potato extract was added to the purified starch substrates which had shown normal digestibility, the rate of hydrolysis appeared to be increased slightly, not only in the case of purified pancreatic amylase, but also of commercial pancreatin and saliva. Upon the action of purified amylases of malt and *Aspergillus oryzae*, however, potato extract had much less, if any, effect. In all cases it appeared that, in the presence of a small amount of the water-soluble substances of the potato, potato starch was digested quite as readily as that of either wheat, maize or rice.

TABLE I.

Effect of 1 cc. Potato Extract upon Enzymic Hydrolysis of Starch.

	Per cent. of starch hydrolyzed in 30 min. by					
	Purified pancreatic amylase (No. 62).		Pancreatin.		Saliva.	
Description of starch.	Without potato extract.	With potato extract.	Without potato extract.	With potato extract.	Without potato extract.	With potato extract.
Wheat starch (alkali washed).....	..	42.2	36.0	38.8	40.2	44.3
Maize starch (alkali washed).....	37.5	42.0	..	..	40.6	45.8
Rice starch (alkali washed).....	37.0	40.5	..	..	41.5	46.3
Potato starch (alkali washed).....	25.5	47.7	..	..	43.5	49.6
Potato starch (water washed).....	38.4	47.3	37.3	40.3	..	..
Potato starch (Lintner "soluble")...	39.2	45.0	..	..	..	..

TABLE II.

Effect of 1 cc. Potato Extract upon Enzymic Hydrolysis of Starch.

	Per cent. of starch hydrolyzed in 30 min by			
	Purified malt amylase (No. 155).		Aspergillus amylase (prep. 22).	
Description of starch.	Without potato extract.	With potato extract.	Without potato extract.	With potato extract.
Wheat starch (alkali washed).	38.4	39.1	38.6	40.1
Potato starch (water washed).	41.0	40.8	39.9	41.1

Since the activating material in the potato extract was water-soluble and heat-stable and the optimum amounts of chloride and phosphate were added to the starch substrate in all cases whether potato extract was to be added or not, attention is naturally directed to the amino acids and acid amides as possibly responsible for the effect of the potato extract.

Experiments with Aspartic Acid and Asparagin.

The results of experiments on the influence of aspartic acid, carefully neutralized with sodium hydroxide, upon the rate of hydrolysis of different starches by different enzymes are shown in Tables III-V. The methods

employed were those described in our previous paper mentioned above, and, as in that case, only the data of experiments in the same table and by the same digestive agent are directly comparable with each other.

TABLE III.

Effect of Various Amounts of Aspartic Acid (Neutralized by Sodium Hydroxide) upon the Hydrolysis of Starch by Purified Pancreatic Amylase.

Description of starch.	Aspartic acid added (weight before neutralization). Mg.	Starch digested in 30 min. %.
Water-washed potato starch.....	None	38.8
	50	45.2
	100	44.2
Lintner soluble starch.....	None	39.1
	30	41.5
	50	44.9
	100	43.3

TABLE IV.

Effect of Neutralized Aspartic Acid upon the Hydrolysis of Starches by Saliva, Pancreatin and Purified Pancreatic Amylase.

	Per cent. of starch hydrolyzed in 30 min by					
	Saliva.		Pancreatin.		Pancreatic amylase prep. 62.	
Description of starch. Alkali washed.	Without aspartate.	With aspartate.	Without aspartate.	With aspartate.	Without aspartate.	With aspartate.
Wheat starch.....	41.1	47.4	40.1	44.6	36.5	40.3
Maize starch.....	40.7	48.0	40.5	44.8	36.7	43.2
Rice starch.....	41.6	49.8	41.1	43.8	37.4	40.3
Potato starch.....	..	..	41.2	47.9	25.7	43.6

TABLE V.

Effect of Neutralized Aspartic Acid upon the Hydrolysis of Starches by Malt Extract, Taka-Diastase and the Amylase Preparations Obtained from them.

	Per cent. of starch hydrolyzed in 30 min. by							
	Malt extract.		Malt amylase (prep. 155).		Taka-diastase.		*Aspergillus* amylase (prep. 22).	
Description of starch. Alkali washed.	Without aspartate.	With aspartate.	Without aspartate.	With aspartate.	Without aspartate.	With aspartate.	Without aspartate.	With aspartate.
Wheat starch...	54.7	54.5	40.2	45.3	50.0	51.0	42.4	42.8
Maize starch....	53.8	53.3	41.8	47.5	51.0	51.7	40.7	40.5
Rice starch.....	54.7	55.1	42.0	44.6	53.0	53.8	41.1	40.9

Tables VI and VII show the influence of aspartic acid and asparagin upon the hydrolysis of Lintner soluble starch by the various amylases. The amount of reducing sugar formed was determined gravimetrically and, for convenience of comparison with the results of previous workers, is indicated in the tables by the number of mg. of cuprous oxide found after correction by blank experiments with the starch and reagents.

TABLE VI.

Effect of Neutralized Aspartic Acid and Asparagin upon Hydrolysis of Lintner Soluble Starch by Saliva, Pancreatin and Purified Pancreatic Amylase.

		Mg. cuprous oxide.		
Aspartic acid, Mg.	Asparagin, Mg.	Saliva.	Pancreatin.	Pancreatic amylase No. 59.
None	None	342	322	277
50	None	371	340	306
100	None	381	336	305
None	50	380	341	306
None	100	391	343	312
50	50	384	342	305
25	25	379	341	310

TABLE VII.

Effect of Neutralized Aspartic Acid and Asparagin upon Hydrolysis of Lintner Soluble Starch by Malt Extract, Taka-Diastase and the Amylase Preparations Obtained from Them.

			Mg. cuprous oxide.		
Aspartic acid, Mg.	Asparagin, Mg.	Malt extract.	Malt amylase No. 155.	Taka-diastase.	*Aspergillus* amylase 22.
None	None	191	209	291	258
50	None	196	212	291	260
100	None	...	220	294	261
None	50	195	223	295	260
None	100	...	218	299	265
50	50	196	220	295	264
25	25	196	222	295	263

It will be seen from Tables I to VII that the addition of a small amount of neutralized aspartic acid not only corrects the tendency to abnormally low results in the case above noted, but also appreciably increases the rate of action of saliva, pancreatin, purified pancreatic amylase and purified malt amylase upon all of the starches here tested. Under the conditions of our experiments it had no appreciable effect upon the action of malt extract, taka-diastase or the laboratory preparation of *Aspergillus* amylase.

Asparagin behaves in like manner except for what appears to be a very slight activation of taka-diastase and purified *Aspergillus* amylase, which may be due to some unrecognized experimental error.

The above tables also show that the addition of both aspartic acid and asparagin to the same digestion mixture does not result in greater activation than one of the compounds alone would produce, when used in optimum concentration.

A comparison of the results obtained with potato extract on the one hand, and with aspartic acid or asparagin on the other, brings out the fact that the former did not activate malt amylase, while the pure substances appeared to do so. A possible explanation of this is that the

direct cause of the increased amylolytic activity is in all such cases amino acids rather than protein. Potato extract in the small quantity used might furnish too little preformed amino acid to produce a distinct activation. The pancreatic enzymes and saliva being proteolytic as well as amylolytic could form activating amino acid from the protein of the potato extract, while purified malt amylase, being protease free, derived no such benefit.

As noted above, activating effects of amino acids upon the digestion of starch have been reported by some previous experimenters while others have held that such observations do not indicate a true activation, but only a favorable influence of the added amino acid upon the hydrogen ion concentration of the digestion mixture. In order to test this point the hydrogen ion concentrations of our digestion mixtures with and without the added (neutralized) aspartic acid and asparagin were determined electrometrically. Typical results are shown in Tables VIII and IX.

TABLE VIII.

Rate of Digestion and Hydrogen Ion Concentration with and without Neutralized Aspartic Acid.

	Pancreatic amylase prep. 62.	
Expt. 1.	Without aspartate.	With aspartate.
Starch hydrolyzed in 30 min., %	35.8	42.3
Hydrogen ion concentration as P_H	6.63	6.68
Expt. 2.		
Starch hydrolyzed in 30 min., %	40.5	47.7
Hydrogen ion concentration as P_H	6.63	6.62

TABLE IX.

Effect of Neutralized Asparagin on Hydrogen Ion Concentration.

Solutions activated as for digestion by malt amylase.				
Asparagin (before neutralization) Mg	None	50	100	100
Hydrogen ion concentration as P_H	4.95	4.94	4.95	4.96[a]
Solutions activated as for digestion by taka-diastase.				
Asparagin (before neutralization) Mg	None	50	100	100
Hydrogen ion concentration as P_H	5.31	5.31	5.39	5.33[a]

[a] Determined before digestion. Other determinations made at end of digestion.

In Expt. 1, Table VIII, neutralization of aspartic acid was governed simply by titration of solution with rosolic acid as indicator. In Expt. 2, guided by electrometric measurements made in Expt. 1, a little less alkali was used to "neutralize" the added aspartic acid. These results (Tables VIII and IX) show plainly that, in our experiments at least, the activating effect of sodium aspartate or of asparagin is not attributable to change in the hydrogen ion concentration of the digestion mixture.

The accelerated transformation of the starch to reducing sugar in the presence of the sodium aspartate or of asparagin is, therefore, not refer-

able to hydrogen ion concentration. Neither is it referable to the sodium ion nor to the mere concentration of electrolyte since, in all of the experiments with pancreatic amylase, optimum concentrations of sodium chloride and sodium phosphate were present.

While sodium aspartate results in higher activity of pancreatic amylase than can be induced by sodium chloride and sodium phosphate alone, it was also found by further experiments that the aspartate cannot replace the chloride in the activation of this enzyme. The percentages of starch digested in 30 minutes by purified pancreatic amylase in the presence of different combinations of these 3 salts were as follows:

With phosphate and chloride	41.2%
With phosphate, chloride and aspartate	48.3%
With phosphate and aspartate only	25.8%

In this connection we may recall the fact shown in previous papers that our purified pancreatic amylase (the most active enzyme of which we have knowledge when tested in the presence of proper concentrations of chloride and phosphate) shows no activity in the absence of added electrolytes.

The experiments described in the present paper show, therefore, a rather specific effect of the amino acids tested in that they induced still further activity in the enzyme already "fully activated" by the addition of optimum amounts of chloride and phosphate.

Summary.

The action of saliva, pancreatin, and purified pancreatic amylase on alkali-washed potato, wheat, maize and rice starches and on Lintner "soluble" starch was accelerated by the addition of small amounts of boiled, neutralized water extract of potato, while the action of the vegetable amylases tested was not influenced by the addition of the potato extract.

The addition of neutralized aspartic acid or asparagin accelerated the action of saliva, pancreatin, and purified pancreatic and malt amylases. Clear evidence of activation was not obtained in the case of malt extract, or the preparations made from *Aspergillus oryzae*.

The addition of both sodium aspartate and asparagin to the same digestion mixture produces practically the same activation as does one of these substances alone. Thus the activating effects of these substances are interchangeable rather than additive.

In what sense is this activation specific? It was not due, in these experiments, to change in hydrogen ion concentration, nor merely to a more favorable concentration of electrolyte. The amino compounds to be tested were added to a substrate which already contained the optimum concentrations of sodium chloride and phosphate, the reported activation being thus additional to the activity induced by chloride and

phosphate. Moreover, sodium aspartate is not interchangeable with sodium chloride in the activation of purified pancreatic amylase.

The work is being extended to a similar study of the effects of other amino acids.

We are greatly indebted to the Carnegie Institution of Washington for grants in aid of this investigation.

NEW YORK, N. Y

[CONTRIBUTION FROM THE LABORATORIES OF PHARMACEUTICAL CHEMISTRY, UNIVERSITY OF WISCONSIN.]

REDUCTION OF DIHYDROXY-THYMOQUINONE BY MEANS OF PALLADIUM-HYDROGEN.

BY NELLIE A. WAKEMAN.

Received September 5, 1919.

Thymoquinone and its oxidation and reduction products form an interesting series of compounds which have, from time to time, received considerable attention at the hands of organic chemists. The writer's interest in these compounds was aroused by the seemingly important role which they appear to play in the pigmentation of several species of *Monarda*, and it has been held no less by the behavior of the substances themselves than by their biochemical significance.

Two or three years ago, in reviewing the work of Pall[1] and Skita[2] upon the hydrogenation of organic compounds by means of palladium as a catalyst, it was learned that one of them[3] had in this manner reduced quinone to hydroquinone. The question of the action of thymoquinone toward this reagent therefore naturally presented itself, and accordingly the action was tried in alcoholic solution. Thymoquinone reduced very readily. Upon evaporation of the solvent, it yielded hydrothymoquinone which was identified by its melting point, its solubility, and its crystalline form.

The reduction of thymoquinone to hydrothymoquinone ordinarily presents little difficulty. A more interesting reaction promised to be the reduction of dihydroxy-thymoquinone to tetrahydroxy-cymene by the same reagent.

Zincke,[4] in 1881, reduced the mother liquors from which dimethyl-amino-thymoquinone had crystallized, by the use of sulfurous acid, thus obtaining a solution which, upon oxidation, yielded a mixture of mono- and dihydroxy-thymoquinone. Zincke believed that the reduced liquid

[1] *Ber.*, **38**, 1401, 2414 (1905); **40**, 1392, 2201 (1907); **41**, 805, 818, 2272, 2287 (1908); **42**, 1572, 2239, 3930 (1909).

[2] *Ibid.*, **41**, 2938 (1908); **42**, 1627 (1909); **43**, 3393 (1910); **44**, 2862 (1911); **45**, 1948 (1912).

[3] *Ibid.*, **43**, 3393 (1910).

[4] *Ibid.*, **14**, 92 (1881).