X.—*The Influence of Certain Amphoteric Electrolytes on Amylolytic Action.*

By John Simpson Ford and John Monteath Guthrie.

In a recent publication on Lintner's soluble starch and the estimation of diastatic power (*J. Soc. Chem. Ind.*, 1904, **23**, 414), it was pointed out by one of us that under certain conditions the addition of asparagine to starch solutions undergoing hydrolysis by malt diastase gave rise to an increased production of maltose. From the experimental results obtained by working with starch preparations of varying degrees of purification, it was concluded that this augmentation of the hydrolysis was due, not to a specific action of the amide on the amylase, but to an indirect action in preventing or lessening the inhibitive influence of certain impurities in the starch solutions. It was established that the addition of asparagine to starches containing alkaline impurities increased the maltose production at temperatures above 40°, whereas addition to purer starches decreased the maltose production. It was also noted that asparagine was able to lessen the inhibitory influence of traces of copper, which were found to have a very destructive effect on amylolytic action. As these observations are of considerable interest and physiological import, we have further investigated this action of asparagine and also the influence of certain amino-acids on amylolytic hydrolysis.

Preparation of the Starch.

It was pointed out by one of us (*loc. cit.*) that soluble starch, prepared by Lintner's method or otherwise, is extremely difficult to purify; it obstinately retains traces of phosphorus compounds which

prolonged washing with water does not remove, and which are not readily eliminated by solution and precipitation of the starch.

Certain of the preparations of soluble starch used in this investigation were prepared by Lintner's process as usual, then further purified by solution in water and repeated precipitation by means of alcohol, first in the presence of hydrochloric acid and then without addition of acid. The method is tedious and from twenty to thirty precipitations may be necessary before a neutral product is obtained. We have now found that prolonged digestion and extraction of ordinary preparations from maize with dilute acid (HCl) removes the phosphorus compounds completely. After this treatment and washing with water, a few precipitations with alcohol yield an equally pure starch. The criteria of purity we employ are neutrality to rosolic acid and phenolphthalein, and absence of indications of phosphoric acid to molybdate solution in the ash of 5 grams ignited with sodium carbonate and nitrate. The latter is a severe test and it is not often that a preparation is obtained which does not show a faint coloration to this reagent. The specific conductivity of 2 per cent. solutions of such purified starches runs about 5×10^{-6} reciprocal ohms per c.c. at 25°, so that, although not pure, a close approximation to purity is evident. In connection with the drying of alcohol-precipitated preparations of starch, we have noticed on several occasions that starches which were neutral when tested immediately after filtration, showed faint acidity after drying. This acidity is probably due to slight oxidation of the alcohol; Duchemin and Dourlen (*Compt. rend.*, 1905, **140**, 1466) have recently shown that oxidation of alcohol takes place more readily than is generally supposed. Whatever the origin of the acidity may be, its formation renders the attainment of neutral preparations somewhat difficult if ordinary methods of drying in air be employed. We have found it convenient to dispense with the final drying in many cases, and also to supplement the method of alcohol precipitation by that of freezing out, the separated starch being sucked free from mother liquor on a Buchner funnel, then dissolved at once in boiling water. The strength of the solution is readily deduced from its specific gravity. Working in this manner we have obtained much purer preparations, the specific conductivity of 2 per cent. solutions being reduced to $1{\cdot}5 \times 10^{-6}$ at 25°. The dried alcohol-precipitated specimens, when moistened with water, form a jelly-like mass which, on heating, gives a solution of specific rotation $[\alpha]_{D4{\cdot}00} = 200—202°$. On hydrolysis with malt extract at 55°, the transformation products are the same as those yielded by ordinary starch paste, having the constants $[\alpha]_D$ 150°, R. 80. Therefore, if ordinary starch is a mixture of amylocellulose and amylopectin (Maquenne and Roux, *Compt. rend.*, 1905, **140**, 1303), our prepara-

tions must evidently have retained the same proportion of acid modified amylopectin, notwithstanding the prolonged purification and separation into fractions by alcohol precipitation and freezing out.

Preparation of the Amylase.

Ordinary preparations of malt diastase by Lintner's method (*J. pr. Chem.*, 1886, [ii], **34**, 378) are exceedingly impure, and are as a rule strongly alkaline in reaction. We have endeavoured to prepare purer specimens by modifying Lintner's alcohol method, the crude product being dissolved in water, containing potassium dihydrogen phosphate, and reprecipitated by addition of excess of ammonium sulphate. The precipitate so obtained was dialysed for some days and again precipitated, this time with alcohol. The diastase so obtained had only feeble amylolytic properties, and was still far from pure as regards freedom from mineral substances. As our object was to obtain an enzyme of considerable activity and relative freedom from saline impurity, we did not further pursue this method of preparation. Osborne and Campbell (*J. Amer. Chem. Soc.*, 1895, **17**, 503; 1896, **18**, 536) have made elaborate investigations on the chemical nature of amylase, and have prepared specimens of great activity and purity by the methods of "salting out" and dialysis. We therefore employed their methods, and from a quantity of highly active malt extract, kindly presented to us by the Distillers' Co., Ltd., Edinburgh, obtained a small yield of a preparation (F_2) suitable for our purpose. This had a diastatic power of fully 300° Lintner. The specific conductivity of a 2 per cent. solution was $7{\cdot}0 \times 10^{-4}$ at 25°. As only 1 c.c. of a solution of 5 to 15 milligrams per 100 c.c. was taken for the experiments to be described, the amount of impurity contributed by the enzyme preparation was less than that of the distilled water used.

In addition to the experiments with the purified starch and diastase, we have to record several made with ordinary preparations of Lintner's soluble starch and malt extract, the results of which we will consider first, as this is rendered necessary by a recent publication by J. Effront (*Moniteur Scientifique*, 1904, **61**, 561), in which he traverses the conclusions deduced by one of us (*loc. cit.*), and reiterates his opinion that the accelerating influence of asparagine and certain amino-acids on amylolytic action is a specific one, is independent of the temperature and alkalinity of the medium, and is exercised with all natural starches of whatever origin. The results given in his memoir do not, however, justify this conclusion, as the four starches which he employed are by his own showing obviously very impure. The titration values he records indicate that all the starches were alkaline, whilst the fact that different amounts of

maltose were yielded by each starch on treatment with equal amounts of malt extract is conclusive proof that the starches contained varying amounts of impurity. It may be pointed out that his purest preparation (D) is, as regards titration value to rosolic acid, ten times more impure than the most impure starch used as an example in the experiments recorded by one of us (*loc. cit.*), and, further, it is this starch which gives the smallest maltose production, which result is in our experience indicative of metallic contamination. Notwithstanding this, J. Effront, without making any effort to repeat his work on the lines suggested (*loc. cit.*) with purer preparations, or to verify or disprove our contention as to the significance of these titration values, or the influence of metallic impurity, seeks to extend his generalisations. We do not for a moment doubt the accuracy of his observations, but the mere repetition of experiments under the same conditions does not add additional value to the conclusions he has formed. In order to elucidate further the important and varying influence of the impurities in starches on amylolytic action, we have prepared several impure, as well as purified specimens, and, as will be seen from the results obtained with the more impure, it is possible to transcend the tenfold increase of maltose production mentioned by J. Effront and other workers. A normal preparation of Lintner's soluble starch was shaken with a natural water containing much calcium carbonate (0·4 gram per litre) and traces of iron salts. The starch (P*a*) was then filtered, washed with distilled water, and dried. It contained 0·006 per cent. of iron. With colour indicators, the values per 100 grams were as under:

Rosolic acid	7·2 c.c. $N/10H_2SO_4$.
Phenolphthalein	8·0 c.c. $N/10NaOH$.

One c.c. of malt extract, 1·2 grams of malt (d. p. 38° L.) to 100 c.c., was added to 70 c.c. of a 1·5 per cent. solution of this starch, one hour at 60·3°.

	Milligrams of maltose per 100 c.c.
Starch and malt extract without addition	3·0
,, ,, *plus* 15 milligrams of asparagine...	73·0
,, ,, ,, 30 ,, ,, ...	73·0
,, ,, ,, 50 ,, ,, ...	102·2
,, ,, ,, 70 ,, ,, ...	111·0
,, ,, ,, 100 ,, ,, ...	114·0

The original starch (P) under like conditions gave a decreased maltose formation in presence of asparagine.

We give below several experiments made with a number of starches of varying degrees of purification.

Influence of Asparagine on "Early" Maltose Production.

Series I.—One c.c. of malt extract, 0·6 gram of malt (d. p. 40°) per 100 c.c., to 70 c.c. of 1·5 per cent. solution of starch, one hour at 59·5°.

	Milligrams of maltose formed.			
	Pβ.	N.	M.	Mz.
Starch solution and malt extract without asparagine	29·2	64·2	55·5	49·6
,, ,, ,, *plus* 10 milligrams of asparagine	—	46·7	—	12·3
,, ,, ,, ,, 30 ,, ,,	64·2	46·7	67·1	12·3
,, ,, ,, ,, 50 ,, ,,	67·1	32·1	55·5	12·3
,, ,, ,, ,, 100 ,, ,,	55·5	—	29·2	—

The titration values of these starches per 100 grams were as under:

	Rosolic acid.	Phenolphthalein.
Pβ.	10·0 c.c. $N/10H_2SO_4$	8·0N/10NaOH
N.	0·2 ,, N/10NaOH	18·0N/10NaOH
M.	0·3 ,, $N/10H_2SO_4$	14·6N/10NaOH
Mz.	0·1 ,, N/10NaOH	0·2N/10NaOH

Series II.—One c.c. of malt extract, 0·4 gram of malt (d. p. 36°) per 100 c.c., to 70 c.c. of 1·5 per cent. starch solution, one hour at 59°.

	Mz_2.	P.	M.	N.
Starch solution and malt extract without asparagine	29·0	29·2	14·4	29·6
,, ,, ,, *plus* 35 milligrams of asparagine	17·6	20·4	29·0	23·8
,, ,, ,, ,, 50 ,, ,,	14·6	14·6	29·0	18·0

The titration values of these starches were, per 100 grams:

	Rosolic acid.	Phenolphthalein.
Mz_2.....	neutral	neutral
P.	2·0 c.c. N/10NaOH	19·0N/10NaOH
M.	0·3 ,, $N/10H_2SO_4$	14·6N/10NaOH
N.	0·2 ,, N/10NaOH	18·0N/10NaOH

The foregoing starches, with the exception of Pα, were free from metallic impurities such as iron or copper. We give below some results with starches containing metallic impurities.

L Starch.

Rosolic acid .....3·0 c.c. N/10NaOH per 100 grams.
Phenolphthalein...10·0 c.c. N/10NaOH ,, ,, ,,
Copper .....0·04 per cent.

One c.c. of malt extract, 4 grams of malt (d. p. 30°) per 100 c.c., to 70 c.c. of 1·5 per cent. solution, one hour at 40°.

	Milligrams of maltose formed.
Starch and malt extract without asparagine	79
,, ,, *plus* 15 milligrams of asparagine ...	324
,, ,, ,, 30 ,, ,, ...	333
,, ,, ,, 50 ,, ,, ...	330
,, ,, ,, 100 ,, ,, ...	324

Drosten's Starch.

Rosolic acid .....3·0 c.c. $N/10H_2SO_4$ per 100 grams.
Phenolphthalein... 12·0 c.c. $N/10NaOH$,, ,, ,,
Copper .....0·0075 per cent.

One c.c. of malt extract, 4 grams per 100 c.c., to 70 c.c. of 1·5 per cent. solution, thirty-five minutes at 40°.

	Milligrams of maltose formed.
Starch and malt extract without asparagine	84·6
,, ,, *plus* 75 milligrams of asparagine ...	128·4

Numerous other experiments have yielded similar results, and in conjunction with those already published (*loc. cit.*) confirm conclusively the opinion expressed there, that when augmentation of diastatic action of malt extract is obtained on the addition of asparagine, the augmentation is due to the influence of the asparagine in lessening the inhibitory effect of alkaline or other impurities present in the starch solutions, and not to a specific action in the amylase under such conditions. The conclusion we arrive at as to the influence of asparagine may be extended to the other substances which J. Effront (*loc. cit.* and *Bull. Soc. chim.*, 1904, [iii], **31**, 1230) states stimulate amylolytic action. He concludes that the amino-group, and not the amide, accelerates the action, because he obtained augmentation with aspartic acid, sarcosine, glycine, alanine, leucine (asparagine), glutaminic acid, and hippuric acid, whereas succinamide, acetamide and its homologues, benzamide, the amines, hydroxylamine, and hydrazine exhibit a retarding influence. It is to be observed that the substances he enumerates in the favouring group are either weak acids or amphoteric compounds.

In the paper already referred to, it was pointed out that asparagine, under the conditions of hydrolysis in question, was able to overcome or lessen the inhibitory effect of traces of copper on amylolytic action. We give in the following table some additional experiments as to its influence on metallic impurities.

Influence of Asparagine on Certain Metallic Impurities.

One c.c. malt extract, 4 grams per 100 c.c., to 70 c.c. of 3 per cent. starch, P solution, one hour at 40°.

	Grams of maltose formed.
Starch solution and malt extract without addition	0·32
,, *plus* 0·1 milligram of copper as sulphate	0·06
,, ,, 0·1 ,, ,, *plus* 0·1 gram of asparagine	0·29
,, ,, 0·1 ,, mercury as chloride	0·02
,, ,, 0·1 ,, ,, *plus* 0·1 gram of asparagine	0·01
,, ,, 0·1 ,, mercury as cyanide	0·08
,, ,, 0·1 ,, ,, *plus* 0·1 gram of asparagine	0·03
,, ,, 10·0 ,, copper as aminosuccinamate	0·08
,, ,, 10·0 ,, ,, *plus* 0·1 gram of asparagine	0·27
,, ,, 100·0 ,, asparagine	0·31

These results indicate that the protective influence of asparagine in the case of copper is due to the formation of copper aminosuccinamate and its lessened dissociation in presence of excess of asparagine and its salts, the free copper ions being so reduced in quantity as not to interfere greatly with normal amylolytic action. We have here an explanation of the fact already recorded by us (*J. Soc. Chem. Ind.*, 1905, **24**, 605), that traces of copper, which greatly inhibit the amylolytic action of precipitated malt diastase (Lintner's), have much less effect on the activity of malt extract.

It was pointed out (*loc. cit.*) that asparagine at temperatures above 40° reacted more strongly acid to colour indicators; we now supply evidence to show that this acidity is really exhibited by ordinary recrystallised specimens of the amide. This is clearly shown by their action on sucrose solutions at temperatures of 40° and 60°.

Acidic Function of Asparagine ($\mu = 0{\cdot}50$).

20 per cent. sucrose solution.	Grams of invert sugar per 100 c.c. 20 hours at 40°.	60°.
50 c.c. of sucrose solution, water to 100 c.c.	0·013	0·019
,, ,, *plus* 0·5 gram of asparagine, *plus* water to 100 c.c.	0·019	0·285
,, ,, ,, 1·0 ,, ,, ,, ,,	0·020	0·343
,, ,, ,, 1·2 milligrams of hydrochloric acid, *plus* water to 100 c.c.	0·146	—
,, ,, ,, 0·6 ,, ,, ,, ,,	—	0·971

The asparagine used in the preceding experiments was purified by recrystallisation from alcohol. The molecular conductivity at 25° for $v = 16$ was 0·50, a value in close agreement with that given by Walden (*Zeit. physikal. Chem.*, 1891, **8**, 483). As we now know that the active acidity of such recrystallised asparagine is mainly due to the presence of impurity, we will return to this subject subsequently.

We can infer that ordinary specimens of asparagine in virtue of their acid function overcome the inhibitive effect of hydroxyl ions present in our alkaline starches. We have not, however, so far offered any proof that starches with titration values indicating alkalinity, that is, requir-

ing the addition of acid to bring about neutrality to rosolic acid, are really alkaline. The following experiment shows that such starches at least possess a potential, if not an actual, alkalinity.

To 2 grams of each starch (in 70 c.c. water), 20 c.c. of 10 per cent. sucrose were added and 10 c.c. of dilute hydrochloric acid, equal to 1·2 milligrams of hydrogen chloride. Twenty c.c. of sucrose solution *plus* 10 c.c. of the acid were also made to a similar volume. The solutions (in Jena flasks) were kept for seventeen hours at 60°.

The invert sugar produced was as under:

Starch.	Grams of invert sugar.
Mz2	0·293
Pβ	0·147
M	0·250
Aqueous sucrose *plus* 1·2 milligrams of hydrogen chloride	0·350

The starch Mz2 was neutral to rosolic acid and phenolphthalein; the values of the other starches have already been given. As the viscosity effect would be the same in each case, the reduction of sugar inversion may be regarded as due to alkalinity or to a lessened dissociation of the acid caused by the salts present. The starches were free from chlorides, the salts being phosphates; whether at such dilutions double decomposition may be looked for is doubtful. In any case this reduction of the number of free hydrogen ions is for our purpose tantamount to alkalinity. Experiments were made to see if these starches increased the rate of mutarotation of freshly dissolved glucose. The results were negative, the rate of change of rotation being the same with each starch. Possibly this indicates that no free hydroxyl ions are present in the so-called alkaline starches; something, however, is present which is capable of reducing the amount of free hydrogen ions of the added acid.

We have already shown that ordinary preparations of asparagine exhibit distinctly acid properties at 60°, whereas, allowing for reduced velocity of reaction, at 40° it has practically no acidic function. As amylolytic action is admittedly greatly influenced by the degree of alkalinity or acidity of the medium, J. Effront's contention that the favouring influence of asparagine on amylolytic action is independent of the temperature and degree of alkalinity of the starch becomes untenable. Apart from the question of the active acidity of ordinary asparagine at higher temperatures, this amide, glycine and other amino-acids are amphoteric electrolytes, having potential acid and basic functions, capable of neutralising acid or alkali to an extent dependent on the relative proportions of acting substances and the temperature. This is shown by their influence on amylolytic action, to be described later, and also by the following results obtained by

methods used by Walker (*Zeit. physikal. Chem.*, 1889, **4**, 389), Winkelblech (*ibid.*, 1901, **36**, 546), and others. The diminution in concentration of $H^{\cdot}$ and OH' ions in solutions of hydrochloric acid and caustic soda (due to salt formation) was observed by measurements of electrical conductivity.

Asparagine and Hydrochloric Acid at 25°.

Concentration.	Molecular conductivity.
N/10 hydrochloric acid	381·6
plus 0·012 mol. of asparagine	345·1
,, 0·025 ,, ,,	313·4
,, 0·05 ,, ,,	248·4
,, 0·10 ,, ,,	150·8
,, 0·20 ,, ,,	100·6
,, 0·30 ,, ,,	86·3

Asparagine and Caustic Soda at 25°.

Concentration.	Molecular conductivity.
N/10 caustic soda	204·6
plus 0·025 mol. of asparagine	166·6
,, 0·05 ,, ,,	133·3
,, 0·10 ,, ,,	62·7
,, 0·20 ,, ,,	56·7
,, 0·40 ,, ,,	53·6

Asparagine and Potassium Chloride at 25°.

Concentration.	Molecular conductivity.
N/10 potassium chloride	128·5
plus 0·05 mol. of asparagine	127·5
,, 0·10 ,, ,,	127·0
,, 0·20 ,, ,,	125·4
,, 0·40 ,, ,,	120·1

Glycine and Hydrochloric Acid at 25°.

Concentration.	Molecular conductivity.
N/10 hydrochloric acid	381·7
plus 0·025 mol. of glycine.	303·4
,, 0·05 ,, ,,	259·5
,, 0·10 ,, ,,	153·3
,, 0·20 ,, ,,	102·7
,, 0·40 ,, ,,	93·6
,, 0·80 ,, ,,	87·6

Glycine and Caustic Soda at 25°.

Concentration.	Molecular conductivity.
N/10 caustic soda	204·6
plus 0·025 mol. of glycine.	168·7
,, 0·05 ,, ,,	136·0
,, 0·10 ,, ,,	66·0
,, 0·20 ,, ,,	65·0
,, 0·40 ,, ,,	63·5
,, 0·80 ,, ,,	62·4

Glycine and Potassium Chloride at 25°.

Concentration.	Molecular conductivity.
N/10 potassium chloride	128·5
plus 0·1 mol. of glycine ...	127·2

α-Alanine and Hydrochloric Acid at 25°.

N/10 hydrochloric acid	381·7
plus 0·025 mol. of α-alanine	303·4
,, 0·05 ,, ,,	236·5
,, 0·10 ,, ,,	138·8
,, 0·20 ,, ,,	96·7
,, 0·40 ,, ,,	86·1

α-Alanine and Caustic Soda at 25°.

Concentration.	Molecular conductivity.
N/10 caustic soda	204·4
plus 0·025 mol. of α-alanine	161·3
,, 0·05 ,, ,,	124·4
,, 0·10 ,, ,,	62·2
,, 0·20 ,, ,,	61·4
,, 0·40 ,, ,,	59·2

α-Alanine and Potassium Chloride at 25°.

Concentration.	Molecular conductivity.
N/10 potassium chloride	128·5
plus 0·1 mol. of α-alanine...	127·2

It is not necessary to enter into any general discussion of the above results, which we record simply to show in a qualitative manner the amphoteric nature of these substances. The theoretical and quantitative aspect of the subject is fully developed by Winkelblech (*loc. cit.*) and Walker (*Proc. Roy. Soc.*, 1904, **73**, 155, and **74**, 271).

Purification of Asparagine.

We stated previously that the active acidity exhibited by ordinary specimens of recrystallised asparagine is due to the presence of impurity. This impurity we find is present in all preparations we have examined which have been "purified" by the customary methods of recrystallisation. Walker has recently shown (*loc. cit.*) from theoretical calculations that pure asparagine should have a molecular conductivity of 0·087 at $v = 16$. By crystallisation from water twenty-four times he has prepared a specimen with $\mu = 0{\cdot}096$, using water of $k = 0{\cdot}7 \times 10^{-6}$ at 18°. We have prepared asparagine of a similar degree of purity, and find by reducing the duration and temperature of dissolution that it is possible to obtain this purity after about twelve recrystallisations. We dissolve the finely-ground amide in a minimum amount of "conductivity" water at 60—65°, cool rapidly, stirring vigorously so as to obtain a crop of very small crystals. These are freed from the mother liquor and washed with a small quantity of ice-cold water, the treatment being repeated until a product of constant conductivity is obtained. The yield, from the nature of the process, is very small. Asparagine of this purity exhibits practically no active acidity; this is evident from its slight inversion of sucrose in the experiments recorded below, the small fall of angle being due to the production of aspartic acid. We find, under like conditions of heating, an obvious increase in the conductivity of aqueous solutions of asparagine; heating on a water-bath for a short time is also sufficient to cause slight decomposition. This provides an explanation of the difficulty of obtaining pure asparagine by simple recrystallisation; when the substance is dissolved in hot or boiling

water, aspartic acid is produced, and probably some of the ammonia also formed is driven off, as ammonium aspartate solutions lose ammonia on boiling. On cooling to crystallise, or on addition of alcohol, it is probable that the aspartic acid forms a salt with the amide, the presence of which, or of the ammonium salt, in small quantity is competent to account for the apparent increase of acidity observed on heating solutions of such preparations of asparagine. Even in the presence of an excess of asparagine, the salt undergoes hydrolytic dissociation when the solutions are heated, giving rise to the presence of free hydrogen ions. Apart from this, if we regard asparagine as an internal ammonium salt, it is possible that its hydrolytic dissociation gives rise to the presence of the traces of free $H^{\cdot}$ ions observed in the solutions of our pure asparagine. At the same time we consider that the marked acid function observed by ourselves and by Degener (*Chem. Centr.*, 1897, **2**, 936) is due mainly to the presence of saline impurity in our preparations.

These observations, whilst they force us to modify somewhat our views as to the influence of pure asparagine on pure amylase and starch, do not invalidate our deductions from the foregoing experiments with more or less impure starches. It is perfectly certain that no one has hitherto worked with such pure asparagine, and opinions as to the influence of this amide on amylolytic action have been deduced from experiments made with ordinary preparations which contain an approximately constant amount of impurity. Further, the potential acid function of the pure substance is capable of neutralising impurities, so we need only modify our views to the extent that pure asparagine added to pure amylase and starch will have little influence, whereas ordinary specimens inhibit the action. The inhibition of action brought about by the addition of asparagine to the transformations with the purer starches recorded in the preceding part of this paper is due to the acid-forming impurity in the amide. Pure asparagine does not retard the hydrolysis, nor does it augment this reaction unless the starch contains certain impurities.

Action of Asparagine, Glycine, and α-Alanine on Sucrose.

Rotation of solutions in a 2-dcm. tube at 16°.

	After 20 hours at	
	40°.	60°.
50 c.c. of 10 per cent. sucrose solution, *plus* 0·375 gram of glycine, diluted to 100 c.c.	6·62	6·61
50 c.c. of 10 per cent. sucrose solution, *plus* 0·445 gram of α-alanine, diluted to 100 c.c.	6·60	6·58
50 c.c. of 10 per cent. sucrose solution, *plus* 0·375 gram of asparagine,* $\mu=0{\cdot}10$, diluted to 100 c.c.	6·60	6·50

Action of Asparagine, Glycine, and α-Alanine on Sucrose (continued).

Rotation of solutions in a 2-dcm. tube at 16°.

	After 20 hours at 40°.	After 20 hours at 60°.
50 c.c. of 10 per cent. sucrose solution, *plus* 0·375 gram of asparagine,* $\mu=0·20$, diluted to 100 c.c.	6·57	6·47
50 c.c. of 10 per cent. sucrose solution, *plus* 0·375 gram of asparagine,* $\mu=0·50$, diluted to 100 c.c.	6·50	5·83
50 c.c. of 10 per cent. sucrose solution, *plus* 1·2 milligrams of hydrochloric acid, diluted to 100 c.c.	6·45	4·80
50 c.c. of 10 per cent. sucrose solution, *plus* 0·6 milligram of hydrochloric acid, diluted to 100 c.c.	—	5·09
50 c.c. of 10 per cent. sucrose solution, *plus* water only, diluted to 100 c.c.	6·62	6·60

Rotation of each solution before heating = 6·63±0·03°.

* At $v=16$.

The potential basic function of the compounds is well illustrated by the manner in which they decrease the inversion of sucrose by acid. The salts formed undergo very considerable hydrolytic dissociation in dilute aqueous solution, hence a large excess of the base must be added to reduce this. As for our purpose we have only to consider the influence of the substances on minute traces of acid, the experiments recorded below were carried out with acid (HCl) in presence of a distinct excess of the base.

Basic Function of Asparagine, Glycine, and α-Alanine.

Rotation of solutions in a 2-dcm. tube at 16°.

	After 20 hours at 40°.	After 20 hours at 60°.
50 c.c. of 10 per cent. sucrose solution, *plus* 1·2 milligrams of hydrochloric acid, diluted to 100 c.c.	6·50	4·80
50 c.c. of 10 per cent. sucrose solution, *plus* 1·2 milligrams of hydrochloric acid, *plus* 150 milligrams of asparagine, $\mu=0·10$, diluted to 100 c.c.	6·58	5·85
50 c.c. of 10 per cent. sucrose solution, *plus* 1·2 milligrams of hydrochloric acid, *plus* 150 milligrams of asparagine, $\mu=0·20$, diluted to 100 c.c.	6·55	5·82
50 c.c. of 10 per cent. sucrose solution, *plus* 1·2 milligrams of hydrochloric acid, *plus* 150 milligrams of asparagine, $\mu=0·50$, diluted to 100 c.c.	6·53	5·60
50 c.c. of 10 per cent. sucrose solution, *plus* 1·2 milligrams of hydrochloric acid, *plus* 5 milligrams of asparagine, $\mu=0·10$, diluted to 100 c.c.	—	4·95
50 c.c. of 10 per cent. sucrose solution, *plus* 1·2 milligrams of hydrochloric acid, *plus* 75 milligrams of glycine, diluted to 100 c.c.	6·62	6·06
50 c.c. of 10 per cent. sucrose solution, *plus* 1·2 milligrams of hydrochloric acid, *plus* 89 milligrams of α-alanine, diluted to 100 c.c.	6·62	6·04

Rotation of each solution before heating = 6·68±0·03°.

Influence of Asparagine, Glycine, and a Alanine on the Hydrolysis of Purified Amylase and Starch.

As these compounds are practically neutral substances, it might be presumed that they would have little influence on amylolytic action when added to the purified starch and amylase described at the beginning of this communication. As a matter of fact, however, it was found that they slightly augmented the action, more maltose being formed in their presence than in the aqueous solution without such addition. The first interpretation we made of this slight augmentation was that the substances in virtue of their amphoteric properties neutralised such minute traces of acidity or alkalinity as were accidentally present in our solutions.

Amylase in this relatively pure state is extremely sensitive to minute traces of impurity (compare Osborne and Campbell, *loc. cit.*), so much so that we have found it somewhat difficult to obtain concordant results in duplicate determinations. Normal amylolytic action does not take place under such conditions of laboratory experiment. In the plant or natural product in which the enzyme works, the media contain mixed phosphates, other salts, amides, and amino-acids, which ensure the degree of neutrality most suitable for amylolytic action. This point has as yet received insufficient attention from biologists, mainly through the misleading values obtained by ordinary titration methods when applied to the examination of animal and vegetable fluids or extracts. Foa (*Compt. rend. Soc. Biol.*, 1905, 58, 865), by measurements of electromotive force with hydrogen electrodes, has lately given examples of this in the case of various animal fluids. In continuing our work and by using greater precautions to exclude accidental contamination, we came to the conclusion that such traces of acidity as might be incidental to our methods of working were insufficient to provide an explanation for certain apparently anomalous results obtained. It occurred to us that starch itself might possibly possess some feeble acid properties, and that if so it might be possible to obtain some evidence of salt formation by the observation of the conductivity of caustic soda solutions in presence of starch. A substance of such a feebly acid nature would form salts readily hydrolysable in aqueous solution. But, in accordance with the law of mass action, if sufficient starch were added the hydrolytic decomposition would be prevented. Such experiments cannot be carried out fully under the conditions available to us owing to the comparatively slight solubility of soluble starch. We have, however, made the determinations tabulated below, which are sufficient to prove that soluble starch of very great purity has feebly acid properties, which, feeble although

they are, are adequate to explain many of the apparently peculiar results we obtained in our experiments with the purified soluble starch and amylase.

Soluble Starch and Caustic Soda at 25°.

Concentration.	Molecular conductivity.
N/25 caustic soda	209·5
,, ,, *plus* 0·42 gram of starch per 100 c.c.	191·6
,, ,, ,, 0·85 ,, ,, ,,	179·6
,, ,, ,, 1·70 ,, ,, ,,	151·2
,, ,, ,, 3·40 ,, ,, ,,	114·5

Soluble Starch and Hydrochloric Acid at 25°.

Concentration.	Molecular conductivity.
N/25 hydrochloric acid	390·3
,, ,, *plus* 0·42 gram of starch per 100 c.c.	388·7
,, ,, ,, 0·84 ,, ,, ,,	385·6
,, ,, ,, 1·70 ,, ,, ,,	381·3
,, ,, ,, 3·40 ,, ,, ,,	372·0

Soluble Starch and Potassium Chloride at 25°.

Concentration.	Molecular conductivity.
N/25 potassium chloride	132·5
,, ,, *plus* 0·42 gram of starch per 100 c.c.	132·2
,, ,, ,, 0·84 ,, ,, ,,	131·7
,, ,, ,, 1·70 ,, ,, ,,	129·6
,, ,, ,, 3·40 ,, ,, ,,	126·0

These experiments were made with a starch preparation which, in 2 per cent. solution, had a specific conductivity of $2{\cdot}5 \times 10^{-6}$ at 25°, and so could not contain sufficient impurity to influence greatly the results tabulated. Observations made with other preparations yielded similar values. It is obvious from the results with caustic soda that starch forms compounds with the alkali, the conductivity being reduced fully 45 per cent. by the addition of 3·4 grams of starch per 100 c.c., whereas with hydrochloric acid and potassium chloride the reductions are 4·7 and 5·0 per cent. respectively. The further bearing of these and other results on the nature and constitution of starch we reserve for a subsequent communication.

Experiments with Purified Amylase and Starch.

Influence of Glycine, α-Alanine, and Asparagine.

Conditions of Experiment.—Starch: 70 c.c. of a 1·5 per cent. solution taken. Amylase: 5 milligrams of the preparation F_2 already described were dissolved in 100 c.c. of water; 1 c.c. of this added to each starch solution. The action was allowed to proceed for one hour, when it was stopped by the addition of caustic soda.

I. 59·5°.

	Milligrams of maltose formed.
Starch solution and amylase without addition	95
,, ,, ,, *plus* 75 milligrams of glycine	100
,, ,, ,, ,, 89 ,, α-alanine	103
,, ,, ,, ,, 150 ,, asparagine	99

In this experiment, a dried alcohol-precipitated specimen of soluble starch was used. A 2 per cent. solution had $k^* = 5 \times 10^{-6}$ at 25°.

II. 45 minutes at 54·5°.

	Milligrams of maltose formed.
Starch and amylase without addition	50
,, ,, *plus* 0·01 milligram of caustic soda	42
,, ,, ,, 0·01 ,, hydrogen chloride	42
,, ,, ,, 50 milligrams of potassium chloride	64
,, ,, ,, 75 ,, glycine	45
,, ,, ,, 89 ,, α-alanine	64
,, ,, ,, 150 ,, asparagine "A"	55
,, ,, ,, 150 ,, asparagine "B"	8
,, ,, ,, 1·0 milligram of caustic soda	nil
,, ,, ,, 1·0 ,, ,, ,, *plus* 75 milligrams of glycine	64
,, ,, ,, 1·0 ,, ,, ,, *plus* 89 milligrams of α-alanine	64
,, ,, ,, 1·0 ,, ,, ,, *plus* 150 milligrams of asparagine "A"	60

The starch used was purified by "freezing out" in the manner described. A 2 per cent. solution gave $k = 2·0 \times 10^{-6}$ at 25°.

III. 1 hour at 52·2°.

	Milligrams of maltose formed.
Starch and amylase without addition	224
,, ,, *plus* 0·01 milligram of caustic soda	235
,, ,, ,, 0·01 ,, hydrogen chloride	207
,, ,, ,, 10·0 milligrams of potassium chloride	280
,, ,, ,, 37·5 ,, glycine	257
,, ,, ,, 44·5 ,, α-alanine	269
,, ,, ,, 75·0 ,, asparagine "A"	232
,, ,, ,, 75·0 ,, asparagine "B"	190

A two per cent. solution of the starch gave $k = 2·8 \times 10^{-6}$ at 25°. Amylase, F_2, 15 milligrams to 250 c.c., 5 c.c. to each solution. The asparagine marked "A" in series II and III was a highly purified specimen of $\mu = 0·094$. "B" was an ordinary laboratory "pure"

* No correction has been made, in any of these values of k, for the conductivity of the solvent water for which $k = 1$ to $1·5 \times 10^{-6}$.

specimen recrystallised five times from water, $\mu = 0{\cdot}50$, $v = 16$. The glycine was a specially purified specimen, $\mu = 0{\cdot}05$. The α-alanine was crystallised several times, but was not of the same degree of purity as the "A" asparagine or glycine, $\mu = 0{\cdot}25$, $v = 16$. The potassium chloride was a specially purified preparation used for conductivity values. The water used in the experiments detailed here had $k = 1$ to $1{\cdot}5 \times 10^{-6}$ at 25°. We may mention incidentally that with the still and spray traps described by us (*J. Fed. Inst. Brew.*, 1905, **11**, 3, 218) water of this purity is easily obtained in quantity by a second distillation, a little calcium hydroxide being added to the contents of the still.

Other experiments which we need not detail gave similar results. We, however, record a series of experiments where the amylase used was slightly acid. The acidity of 100 milligrams being equivalent to 0·86 c.c. of N/100NaOH with rosolic acid, 1·17 milligrams of this amylase was added in each case. The starch was the same as in No. I. The asparagine was the impure "B" specimen.

IV. 1 hour at 58·5°.

					Milligrams of maltose formed.
Starch and amylase without addition					210
,,	,,	*plus* 75 milligrams of glycine			215
,,	,,	,, 89	,,	α-alanine	236
,,	,,	,, 150	,,	asparagine "B"	45
,,	,,	,, 0·01	,,	caustic soda	252
,,	,,	,, 0·01	,,	,, *plus* 75 milligrams of glycine	254
,,	,,	,, 0·01	,,	,, *plus* 89 milligrams of α-alanine	276
,,	,,	,, 0·01	,,	,, *plus* 150 milligrams of asparagine "B"	50
,,	,,	,, 5·0	,,	,,	nil
,,	,,	,, 5·0	,,	,, *plus* 500 milligrams of glycine	160

The maltose formed was determined by gravimetric copper reduction, the only method in our opinion which yields sufficiently accurate results for such work. In connection with the values for maltose formed it must be noted that glycine and α-alanine are not without slight influence on the copper reduction. We have applied experimentally determined corrections in obtaining the above maltose values. Further, owing to the extremely sensitive nature of amylase, even when taking the greatest precautions to exclude atmospheric and other impurities, there is considerable risk of discrepant results through adventitious contamination. Although it is possible that in all the highly purified starches with which we worked some unrecognised impurity may have been present, we feel nevertheless justified in

concluding that isolated amylase cannot bring about a normal hydrolysis in starch solutions which are free from saline substances. Even the addition of a neutral salt such as potassium chloride increases the velocity of the reaction (compare Osborne and Campbell, *loc. cit.*). The addition of certain other salts has a like effect; for example, addition of a few milligrams of a mixture of $10KH_2PO_4 + 1Na_2HPO_4$ greatly increases the speed of hydrolysis. In this case, we can conceive the increased action as being due, at least partly, to the attainment of the degree of neutrality most suitable for amylolytic action. The increase with the mixed phosphates is greater than that with potassium chloride, hence we may assume that the influence of the neutral salt depends only on the change it produces in the osmotic pressure of the starch and enzyme solution.

As we are unable to continue this line of investigation, we now record our results, which, although in themselves possibly not conclusive, are at least suggestive, and may be of some assistance to other workers who are in a position to prosecute such investigations under more favourable conditions than obtain in an industrial laboratory. We consider that our results are sufficient to establish that:

(1) Asparagine and the amino-acids mentioned have no specific influence in augmenting the action of amylase: the apparent augmentation of action sometimes obtained by the addition of these amphoteric compounds (or of feeble acids) is due to their neutralising alkaline (or other) impurity in the starch or enzyme solution.

(2) Normal amylolytic action takes place in neutral solution. In the plant substance, this neutrality is brought about by equilibrium between the basic and acid compounds present.

(3) Until the conditions influencing the action of enzymes are more fully established, it is inadvisable to formulate mathematical laws as to the kinetics of enzymic hydrolyses.

(4) Purified soluble starch has the properties of an extremely feeble acid; it is capable of yielding negative ions under the influence of strongly positive ones.