TABLE II.—RESIDUAL HYDROCARBONS.

Boiling points at 1 mm.	Molec. wts. after 3rd recryst.	Corresponding to	Molec. wts. after 6th recryst.	Corresponding to	Iodine nos. after 3rd recryst.	Equal to atoms of iodine.	Iodine nos after 6th recryst.	Equal to atoms of iodine.
95–110°	Insufficient in quantity							
110–125°	284	Eicosylene $C_{20}H_{40}$, 280,	.....	.....	90	2.0	..	...
125–130°	305	Docosylene $C_{22}H_{44}$, 308,	.....	.....	85	2.1	..	...
130–135°	320	Tricosylene $C_{23}H_{46}$, 322,	.....	.....	80	2.1	..	...
130–140°	325	Tricosylene $C_{23}H_{46}$, 322,	332	$C_{24}H_{48}$, 336	83	2.1	66	1.7
145–150°	336	Tetracosylene $C_{24}H_{48}$, 336,	insufficient	.....	79	2.1	..	...
150–155°	360	Hexacosylene $C_{26}H_{52}$, 364,	"	.....	79	2.3	..	...
170–176°	366	Hexacosylene $C_{26}H_{52}$, 364,	395	.....	86	2.5	70	2.2
176–182°	382	Heptacosylene $C_{27}H_{54}$, 378,	392	.....	88	2.7	85	2.6
186–193°	402	Nonacosylene $C_{29}H_{58}$, 406,	409	.....	93	3.0	96	3.1

From a study of these tables it would seem that in these distilled wool grease oleins we had hydrocarbons of the olefin series, that is with but a single double bond, and of formula from $C_{20}H_{40}$ to $C_{30}H_{60}$.

These hydrocarbons are now under investigation, with the idea of determining some of their physical constants with greater accuracy, more particularly their boiling and melting points, their index of refraction and, if possible, the position of the double bond.

BOSTON, MASS.

[CONTRIBUTIONS FROM THE HAVEMEYER LABORATORIES OF COLUMBIA UNIVERSITY, NO. 177.]

STUDIES ON AMYLASES. I. AN EXAMINATION OF METHODS FOR THE DETERMINATION OF DIASTATIC POWER.

BY H. C. SHERMAN, E. C. KENDALL AND H. D. CLARK.

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The enzymes being known by their activities it is scarcely possible to make satisfactory progress in the isolation of any enzyme for investigation of its chemical nature until we have accurate knowledge of methods and conditions for determining quantitatively the increase of its activity or power with the progress of the purification. This may be illustrated by a reference to the papers of Osborne[1] and of Wroblewski[2] on the chemical nature of the amylase of malt.

In the case of the amylases there are also extremely important applications, both biochemical and economic, awaiting the development of reliable and comprehensive methods for the measurement of diastatic power. The fact that the salivary digestion of starch may continue

[1] Osborne, THIS JOURNAL, **17**, 587 (1895); Osborne and Campbell, *Ibid.*, **18**, 536 (1896); Osborne, *Ber.*, **31**, 254 (1898).

[2] Wroblewski, *Ber.*, **30**, 2289 (1897); **31**, 1127 (1898).

much longer in the stomach than was formerly supposed not only emphasizes the value of accurate measurements of the diastatic power of the saliva, but also adds interest to the use of both plant and animal amylases as aids to this part of the digestive process. The U. S. Pharmacopoeia standard for pancreatin has been changed so that the primary requirement now relates to amylolytic instead of to proteolytic power and recent food and drug legislation naturally suggests a more accurate standardization as well as a more comprehensive standard or standards which shall apply also to malt diastase and taka-diastase, both of which are likewise marketed as pharmaceutical preparations.

Malt extracts are now being purchased by bakers on the basis of diastatic power and with the economic completion in the production of tax-free industrial alcohol it is to be expected that the determination of diastatic power will soon become an important factor in the valuation of commercial American malts.

In consequence of the very different points of view from which investigators have approached the subject of diastatic power, many methods or modifications of methods have been employed for its measurement and the descriptions are widely scattered in chemical, physiological, and technical literature. In the interest of brevity anything like a comprehensive review of such methods must be omitted here and attention confined to those which appear to have been most used.

These are of two main types: (1) the so-called "liquefaction" methods which aim to measure the power of the enzyme to completely convert a known amount of starch into products which no longer give the characteristic color reactions with iodine; (2) the saccharification methods which measure by means of alkaline copper solutions the amount of reducing sugar produced by the enzyme when acting under known conditions upon a sufficient excess of starch.

It will be convenient to refer to these two types as "iodine methods" and "copper methods" respectively.

Evidently the result obtained by either type of method will depend upon the conditions under which it is applied and further it may be expressed in a number of ways, for example, the amount of change produced by a given amount of enzyme in a given time, the amount of enzyme required to produce a given change in a given time, or the time required for a given amount of enzyme to produce a given change.

Kjeldahl[1] determined the reducing sugar formed by the action of a known amount of malt extract or saliva upon an excess of starch during 20 minutes at 57–59°, and considered that this was directly proportional to the amount of actual amylase present, or in other words, was

[1] Mittheilungen aus dem Carlsberger Laboratorium, *Dingler's Polytechn. J.*, **235**. 379, 452 (1880).

a true measure of the diastatic power, so long as the digestion was not carried beyond the point which corresponds to a conversion of about 40 per cent. of the original starch into maltose. This is frequently referred to by other writers as "Kjeldahl's law of proportionality" and has had considerable influence upon subsequent work.

Roberts[1] working with pancreatic extracts, saliva, and malt extract defined diastatic power as the number of cubic centimeters of a standard starch paste which could be converted by one cubic centimeter of the active solution during 5 minutes at 40° into products giving no color reaction with iodine.

Jungk[2] used a similar method for malt extract but determined the time required for 10 cc. of the extract to convert 10 grams of starch which he considered in the case of a good extract should not exceed 10 minutes at 40°.

Lintner[3] modified Kjeldahl's method in such a way as to ensure that the calculations of diastatic power should be based upon the production of a constant quantity of maltose by the action of the malt extract upon a definite amount of soluble starch. This was accomplished by measuring 10 cc. of a 2 per cent. starch solution into each of the several test tubes and adding to each tube a different amount of a malt extract, *e. g.*, 0.1, 0.2, 0.3, 0.4, 0.5 cc., etc. After standing for 1 hour at 21° the action was stopped by the addition of 5 cc. of Fehling solution to each tube, the tubes placed in boiling water for 10 minutes, and then examined to determine the first tube in which the copper was all reduced, *i. e.*, the smallest amount of extract which had produced sufficient maltose to reduce 5 cc. of Fehling solution. Lintner prepared a sample of diastase, of which 0.12 mg. produced under these conditions the maltose necessary to reduce the 5 cc. of Fehling solution. This preparation was rated as having a diastatic power of 100 and the diastatic powers of other preparations were calculated as inversely proportional to the amount of sample required to produce this fixed amount of reducing sugar. Thus, if 0.3 mg. were required $0.3 : 0.12 :: 100 : \times \ldots\ldots \times = 40$, the diastatic power of the preparation tested.

In applying this method to malt, however, 25 grams of the dried, ground sample are digested with 500 cc. of water and portions of the clear, filtered extract are employed as described for the diastatic solution. Then if V = the volume of extract required to give complete reduction of the Fehling solution

$$V : 0.1 :: 100 : \text{"diastatic power of malt."}$$

Since 0.2 gram starch are used in each test tube and the weight of

[1] *Proc. Royal Soc.*, **32**, 145 (1881); *J. Chem. Soc.*, **1881**, 1051.

[2] *Am. J. Pharm.*, **55**, 289 (1883), and *J. Chem. Soc.*, **46**, 529 (1884).

[3] *Ztschr. ges. Brauwesen*, **1885**, 281; *J. prakt. Chem.*, [2] **34**, 378 (1886).

maltose required to reduce 5 cc. of Fehling solution is only about 0.04 gram, it follows that the diastatic power is determined by this method for a point at which only about 20 per cent. of the starch has been converted into maltose. Hence the retarding effect of the maltose formed must act, if at all, to the same extent in all cases and so can not vitiate comparative determinations. On the other hand, if ten tubes containing 0.1 cc. to 1.0 cc. of the extract are used in testing a sample, there are only 10 points on Lintner's scale at which an accurate determination could be had in the first operation. If, as usually happens, the correct amount lies between the successive amounts taken, it is necessary to assume that the required volume is midway between the two, or to repeat the test with smaller increments of the extracts. Moreover, the probable error of the method increases rapidly with the diastatic power of the sample. If the end point falls between the last two tubes (0.9 cc. and 1.0 cc.) the diastatic power will be between 10 and 11.1, but if it falls between the first two tubes (0.1 cc. and 0.2 cc.) the diastatic power may be anywhere between 50 and 100.

Kjeldahl,[1] Chittenden and Ely,[2] and later Brown and Glendenning,[3] Ford[4] and other investigators have estimated gravimetrically by copper reduction the extent of the diastatic action.

Sykes and Mitchell[5] introduced a method which they held to be interchangeable with Lintner's while giving a closer result in a shorter time, especially when dealing with malt of high diastatic power. In this method 100 cc. of 2 per cent. starch solution are treated with 1 cc. of malt extract or diastase solution (of the concentration used by Lintner) for 1 hour at 21°, 50 cc. of Fehling solution added, and the whole heated rapidly to 98° and then placed in a boiling water bath for 7 minutes, after which the reduced copper is determined. The weight of copper found, divided by 0.438 (the quantity of copper in 50 cc. of Fehling solution) and multiplied by 100, gives the diastatic power in terms of the Lintner scale. The results obtained by this method on 10 samples of malt having diastatic power of 5 to 77 (Lintner scale) averaged 2 units (about 8 per cent.) higher than those by the Lintner method with extreme variations of —1.7 to +5.8 units and of —34 to +30 per cent. This was accepted by Sykes and Mitchell as a satisfactory agreement of results.

Francis[6] improved the iodine method by extending the time of diges-

[1] *Loc. cit.*
[2] *Am. Chem. J.*, **3**, 307 (1881–2).
[3] *J. Chem. Soc.*, **81**, 388 (1902).
[4] *J. Soc. Chem. Ind.*, **23**, 414 (1904).
[5] *Analyst*, **21**, 122 (1896).
[6] *Bulletin of Pharmacy*, **12**, 52 (1898).

tion to one-half hour, and specified exact conditions for the determination of the end point.

Takamine[1] determines diastatic power in comparison with a permanent standardized sample of taka-diastase by finding the relative amount of the standard sample and the sample under test which are required to accomplish the same conversion in the same time as judged by the iodine color reaction.

Vernon[2] in his study of pancreatic amylase used a method essentially like that of Roberts.

Johnson[3] has recently published a new form of color method in which different amounts of the sample under examination are added to a fixed amount of starch paste, the mixture held at 40° and portions withdrawn and tested with iodine at the conclusion of 8 minutes and each minute thereafter. The operation is repeated with smaller increments of sample, if necessary, until that amount is found which just suffices to digest the starch in 10 minutes to products which give no color with iodine. Working mainly with samples containing commercial pancreatic amylase, Johnson concluded that diastatic values which were equivalent to each other as indicated by this method were also equivalent as measured by the production of reducing sugar.

The first object of our experimental work has been to ascertain, if possible, the relative value of some of the methods in use for measuring diastatic power, the consideration which should determine the choice of a method, what modification, if any, is necessary to ensure reliability of results, and how generally applicable is any one method to the examination of amylases of different origins. Much work having already been done by others upon malt diastase, we have confined our experiments almost entirely to taka-diastase and pancreatin.

In reaching the results given below we have been very greatly aided by the extended and able preliminary work on this subject which was carried out by the late Arnold William Meyer in conjunction with one of us in this laboratory in 1907–'08. We have also profited by the experience of Mr. Robert Schwarz and Miss M. I. Alley, who during the course of the investigation have carried out in this laboratory a considerable number of experiments on certain phases of the work.

Experiments upon Taka-diastase.

Three samples of taka-diastase have been used: No. 1, a one-ounce sample purchased in 1907; No. 2, a mixture of 4 one-ounce samples purchased in 1908; No. 3, a specially prepared sample of much greater ac-

[1] *J. Soc. Chem. Ind.*, **17**, 118, 437 (1898).
[2] *J. Physiol.*, **27**, 174 (1901).
[3] THIS JOURNAL, **30**, 798 (1908).

tivity which was kindly furnished us directly from the laboratory of Dr. Takamine.

Lintner's method was carried out with all precautions which we had found suggested by any previous workers, using carefully prepared[1] and neutralized[2] soluble starch and neutralized[2] distilled water in specially cleansed Jena glass apparatus and working in a room kept free from laboratory fumes.

Taka-diastase No. 1 showed a diastatic power of 11.4 on Lintner's scale at 21° and 24.5 at 40°. No. 2 showed 7.8 at 21° and 17.5 at 40°. No. 3 showed 38.9 at 21° and 95.1 at 40°.

The gravimetric method showed with taka-diastase No. 1 (when 2.5 mg. of enzyme acted for 1 hour at 21° upon 100 cc. of 2 per cent. soluble starch) an amount of reducing sugar corresponding to a Lintner figure of 11.1. This result is in substantial agreement with that of the Lintner method. The difference between maximum and minimum in 9 successive determinations by this method was about 4 per cent. while in Lintner's method it was about 15 per cent., probably because of the errors involved in measuring out the very small amounts of enzyme solution required.

Johnson's method, while a great improvement over the Pharmacopoeia test, did not in our hands yield definite or satisfactory results because of the difficulty experienced, when following Johnson's directions, in securing a homogeneous starch paste and in determining the end point with sufficient accuracy.

A new iodine method seemed desirable which should differ from those of Roberts, Jungk, and Johnson in combining the use of a more dilute and homogeneous starch paste, a large volume of digestive mixture, and a fairly long time of digestion (as recommended by Francis) in order to secure a better mixture and more uniform action and diminish the proportional error involved in the determination of the end point.

The starch paste is prepared as follows: Enough clean air-dry potato starch to contain 10 grams of water-free substance is suspended in about 100 cc. of cold distilled water; enough more distilled water to make one liter was poured into a 2 to 3 liter flask immersed in a brine bath and connected with a reflux condenser. The bath is then heated and if the water boils the heating is stopped so that the water may cool somewhat, then the suspension of starch is poured *very carefully* into the hot water, the heating resumed and the paste boiled 2 hours under the reflux condenser. This long boiling renders the starch paste less viscous, more homogeneous and transparent, and more easy of digestion by the amylase.

[1] The small reducing power which the starch still retained after 65 careful washings on a Buchner funnel was determined and the proper correction applied.

[2] Rosolic acid was used as the indicator of neutrality in both cases.

In order to determine how long the boiling of the starch paste should be continued, experiments were made in which 250 cc. portions of the paste were treated with 40 mg. of taka-diastase No. 2 dissolved in 20 cc. of water and the time required for digestion to products giving no color with iodine was determined as follows: Boiled ½ hour, required 50 min.; 1 hour, required 46 min.; 1½ hours, 36 min.; 3 hours, 33 min.; 6 hours, 30 min. Hence boiling beyond 1½ hours had little effect upon the digestibility of the starch as thus determined. Moreover, it was noted that up to 2–3 hours the solutions remained colorless while on 3–6 hours' boiling they became slightly yellowish. Two hours was therefore decided upon as the best length of time for boiling the starch paste.

The paste at a temperature not above 40° is weighed out in 250-gram portions (equivalent to 2.5 grams anhydrous starch) into Erlenmeyer flasks of 350–400 cc. capacity and immersed in a water bath kept at 40°. The desired amount of enzyme is then introduced along with 20 cc. of water (in which the enzyme may be dissolved, or which may be used to wash it into the flask), the contents of the flask well mixed and the temperature of 40° carefully maintained. The digestion is considered completed when 0.25 cc. of the contents of the flask removed and mixed with 5 cc. of the dilute iodine test solution[1] in a test tube shows, when viewed against a white background, no color which can be distinguished from that of the untreated iodine test solution. The experiment is repeated with different amounts of enzyme, if necessary, until that amount is found which completes the digestion in 30 min. (± 1 min.). The result may then be conveniently expressed by dividing the weight of starch (2.5 grams) by the weight of enzyme required to digest it under these fixed conditions.

The results obtained with the three samples of taka-diastase were as follows, the times required with different amounts of sample being given to show the probable error of the method.

No. 1 — 37 mg. required 34 min.; 40 mg. 27 min.; 43 mg. 22 min.; 38 mg. 31 min.

No. 2 — 40 mg. required 40 min.; 43 mg. 37 min.; 46 mg. 33 min.; 49 mg. 31 min.

No. 3 — 7 mg. required 34 min.; 9 mg. 30 min.; 11 mg. 27 min.

If the weight of starch, 2.5 grams, be divided in each case by the weight of taka-diastase which digested it in 30 or 31 min. the following values for diastatic power are obtained: No. 1, 66; No. 2, 51; No. 3, 278.

If these values be compared with the Lintner figures given above for

[1] Two grams of iodine and 4 grams of potassium iodide are dissolved in 250 cc. water. For use, 2 cc. of this solution are diluted to 1 liter and 5 cc. of this dilute solution are employed for each test.

the same samples at the same temperature we find the ratio of the Lintner figure to the value as found by the new iodine method to be for No. 1, 1 : 2.7; for No. 2, 1 : 2.9; for No. 3, 1 : 2.9.

Hence as expressing the comparative diastatic powers of the three samples of taka-diastase, the results by the new iodine method ran approximately parallel to the results obtained by the Lintner method at the same temperature.

Experiments upon Pancreatin.

Five commercial preparations of pancreatic amylase representing the products of the principal American makers were used in the experiments here described.

The Lintner method was applied to these as to the samples of taka-diastase, but with much less satisfactory results. Duplicate determinations often showed wide divergence and lower results were obtained at 40° than at 21°.

The gravimetric copper method also gave unsatisfactory results. The amount of reducing sugar formed by a given amount of enzyme was less here than in Lintner's method and there was not a fixed relation between the amount of enzyme used and the weight of cuprous oxide formed.

Thus the 46 mg. of cuprous oxide in five cc. of Fehling solution were obtained in a Lintner test from 1.7 mg. of pancreatin. The same weight of pancreatin under the conditions of the gravimetric copper method gave only 14.4 mg. cuprous oxide, and increasing amounts of enzyme gave the following results:

Pancreatin, milligrams.....	2.5	5.0	10.0	12.0	15.0
Cuprous oxide, milligrams....	27.0	62.8	202.6	270.0	379.0

In these experiments where only a small proportion of the starch was converted into maltose in any case, a given weight of pancreatin had a greater effect in 10 cc. than in 100 cc. of starch solution, while with different weights of pancreatin in the same volume (100 cc.) of starch solution the effect increased in greater proportion than the weight of pancreatin added. In both cases dilution appears to have injured the activity of the pancreatin.

The influence of dilution was studied directly by conducting experiments in pairs so arranged that the amounts of starch and enzyme and the conditions of time, temperature, etc., were exactly the same while the amount of water in which the action took place differed. At the end of the digestion enough water was added to the more concentrated solution to make the volumes the same for the determination of the reducing sugars which had been formed.

MILLIGRAMS CUPROUS OXIDE FROM REDUCING SUGARS FORMED BY 5 MILLIGRAMS ENZYME PREPARATION.

Enzyme and starch.	In 55 cc	In 110 cc.
Taka-diastase No. 1, 2 grams starch	328	332
Taka-diastase No. 1, 4 grams starch	361	361
Pancreatin No. 1, 0.5 grams starch	170	115
Pancreatin No. 2, 3.0 grams starch	273	185
Pancreatin No. 4, 0.5 gram starch	110	68
Pancreatin No. 5, 0.5 gram starch	94	66

Here the taka-diastase produced the same effect when working in different amounts of water, but the pancreatins produced much less in 110 cc. than in 55 cc.—the activity of the pancreatic amylase was evidently diminished by its dilution with neutralized distilled water.

From these results and those above given it is evident that the diastatic power of pancreatic amylase was largely influenced by the ratio of amylase to water. Under these conditions, therefore, the testing of fixed amounts of commercial preparations containing varying amounts of actual amylase cannot be expected to give reliable results. This is because of the influence of electrolytes in activating the amylase.

In these experiments no electrolyte was added other than the small amounts of salts accidentally present in the soluble starch and the neutralized distilled water, hence there was insufficient electrolyte present either to fully activate the enzyme or to protect it from deterioration on standing in water solution. Hence the greater the amount of water present the less favorable the condition for enzyme activity. The optimum conditions as worked out for pancreatin and which permit of the accurate determination of its diastatic power by the grav metric method were not worked out until after our experiments with the iodine methods and will, therefore, be described later.

Johnson's iodine method gave us even less satisfactory results with pancreatin than with taka-diastase for the reason that as the digestion proceeds the test portions from the digestion mixture, when they no longer give a blue color with iodine, do give a red color which on continuing the digestion persists for a comparatively long time and disappears by almost imperceptible degrees so that it is extremely difficult to decide when the digestion should be considered finished.

The new iodine method showed in our experiments upon pancreatin the same advantages over Johnson's method as were noted in the experiments with taka-diastase, but still did not give a very satisfactory end point because of the rather long duration and gradual fading of the red color reaction. That the activity of the commercial pancreatin may be greatly increased by addition of a small amount of salt was shown almost as prominently by this iodine method as by the determination of the reducing sugar formed. Each of the four samples of commercial

pancreatin tested digested 2.5 to 7 times as much starch under standard conditions in a solution containing 0.1 per cent. of added sodium chloride as in one to which no electrolyte was added. The stronger two preparations were tested in 0.3 per cent. salt solution and showed the same apparent activity as in the 0.1 per cent. solution.

By these and many other analytical data which must here be omitted for the sake of brevity, we became convinced that it is illogical and misleading to attempt to determine the diastatic power of pancreatin by its action upon pure starch in pure water because such a medium is too poor in electrolytes to permit the amylase to function normally, and that the conditions necessary for the amylase to function normally should be worked out and incorporated in the method to be used in the future for the determination of diastatic power. This led to a somewhat extended study of the action of pancreatic amylase the main results of which will be given in the next paper. The analytical procedure employed in that study and the optimum conditions for pancreatin as there found are included in the following description. Under these optimum conditions we obtain in testing commercial pancreatins results about 20 times as high as when no electrolyte is added.

PROPOSED GRAVIMETRIC METHOD.

Materials and Conditions.

Starch.—The starch for careful gravimetric work should be Lintner soluble starch, which gives a perfectly clear solution with boiling water. Ford[1] discusses very fully the preparation of such a starch. It should have a reaction with rosolic acid equivalent to less than 0.5 cc. of 0.01 normal acid or alkali per gram of anhydrous starch. In the anhydrous state it is quite hygroscopic; air-dry it contains about 15 per cent. of moisture. The lower the reducing power the better, but a reduction of 30 mg. of cuprous oxide per gram of anhydrous starch is not too large for accurate work.

Water.—The water used must be as nearly pure and as constantly uniform as is possible; redistilled and practicaly neutral to rosolic acid.

Temperature.—We prefer a temperature of 40° for all diastatic power determinations. This is obviously most suitable for the amylases of animal origin and there are important advantages and no important disadvantages in the use of the same temperature for the vegetable amylases. The solutions and flasks should be as near 40° as possible at the time of actually mixing the enzyme and the starch. The temperature should remain constant during the entire time of reaction, as a variation of one degree causes about 10 per cent. error in the result.

Time.—Since the time must in any case be somewhat arbitrarily chosen,

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

we have decided upon 30 min. as best meeting the requirements of accuracy and convenience of working.

Enzyme.—The enzyme may be dissolved in pure water if its power is to be tested immediately. If it is to stand, it should be dissolved in water containing 4 cc. of fiftieth-molar disodium phosphate per 100 cc. The test should be made within an hour in any case. The amount of enzyme to be weighed out will depend entirely on its strength.

One may dissolve the enzyme in a small amount of water and use a very small volume, or use a larger volume for the solution of the enzyme and measure larger amounts for the determination. We employed the former method, using a special 1 cc. pipette which was very accurately graduated. The actual weight of enzyme added to the starch solution varied from 0.1 to 1.0 mg.

Activating Agents.—These will doubtless differ with the different amylases. For pancreatic amylase acting on two per cent. starch, add 300 mg. sodium chloride and 7 cc. of fiftieth-molar disodium phosphate per 100 cc. (final volume) of reaction mixture.

Procedure.—Prepare 400 cc. of 2 per cent. soluble starch solution and the enzyme solution of such a strength that 1 cc. will contain from 0.4 to 1.0 mg. of enzyme. By means of a 1 cc. Mohr's pipette, accurately calibrated in hundredths, measure into four 200 cc. Erlenmeyer flasks such volumes of the solution as will contain 0.2, 0.5, 0.8, and 1.0 mg. of enzyme, respectively.[1] Now 100 cc. of the starch solution previously warmed to 40° is poured into each flask and the digestion allowed to proceed for 30 minutes, the temperature being accurately maintained at 40°. At the expiration of the 30 minutes, stop the reaction quickly by mixing at once with 50 cc. of Fehling solution and immerse the flask in a large bath of boiling water for 15 minutes. See that the water of the bath is kept boiling and that it stands above the level of the contents of any of the flasks. At the end of this heating filter quickly and determine the reduced copper by any accurate method. We prefer to filter through Gooch crucibles having thick felts of specially prepared asbestos, wash the cuprous oxide thoroughly with hot water, then twice each with alcohol and ether and weigh it as such after drying for at least 20 min. in a boiling water oven. The weight of cuprous oxide must not exceed 300 mg.

Correct the weight of reduced copper or cuprous oxide found for the reducing power of the soluble starch by subtracting from it the weight obtained in a "blank" test in which the starch solution is treated directly with the Fehling reagent.

Under the conditions here described the reducing power of maltose

[1] The quantities suggested are such as were suitable for the pancreatins which we have tested. In testing other substances larger or smaller quantities may be necessary.

was found to be approximately the same as in Defren's method, so that the weight of cuprous oxide may be calculated to cupric oxide and the corresponding weight of maltose taken from Defren's table.

To express the results in terms somewhat comparable with those of Lintner's scale, multiply the weight of cuprous oxide by 0.2415, divide by the time in hours, and by the weight of enzyme preparation in milligrams. Results so obtained cannot, however, be considered as interchangeable with those found by Lintner's method because of the conditions of the determination and the form of the velocity curve of the reaction.

As will be more fully shown in the next paper, the rate of the amylolytic action is not constant up to a conversion of a considerable percentage of the original starch as Kjeldahl and others have held, but decreases as the conversion proceeds from the start, so that whatever the method of procedure the differing weights of cuprous oxide cannot be taken as standing in simple proportion to the actual diastatic power. However, if the velocity curve of the amylolytic action be plotted with time as abscissas and yield of reducing sugar as ordinates a scale may be established which will permit of an expression of true diastatic power based upon the weight of cuprous oxide obtained as above described. If 300 mg. of cuprous oxide be taken as 100 on such a scale the value on this scale of any lesser weight of cuprous oxide is obtained by expressing the abscissa corresponding to such weight as percentage of the abscissa for 300 mg. cuprous oxide. Calling the series thus obtained values of K, the diastatic power of the sample under examination is determined by dividing the value of K corresponding to the weight of cuprous oxide found by the number of milligrams of sample used to effect this conversion.

In the next paper in the consideration of the velocity of the reaction the mathematical relation between time and extent of conversion will be shown. From this relation an integral equation is deduced making it possible to establish this series by a mathematical means. The use of this equation verifies the experimental results and gives a convenient method for establishing values of K.

The accompanying table was constructed by finding the value for K from the equation for the action of pancreatic amylase upon 2 per cent. starch for every 10 mg. of cuprous oxide up to 300 mg.

VALUES FOR K FROM CUPROUS OXIDE FOUND.

Cuprous oxide. Mg.	K.	Cuprous oxide. Mg.	K.	Cuprous oxide. Mg.	K.	Cuprous oxide. Mg.	K.
30	9.1	100	31.2	170	54.1	240	78.3
40	12.2	110	34.4	180	57.5	250	81.8
50	15.3	120	37.6	190	60.9	260	85.4
60	18.4	130	40.9	200	64.3	270	89.0
70	21.6	140	44.2	210	67.8	280	92.6
80	24.8	150	47.5	220	71.3	290	96.3
90	28.0	160	50.8	230	74.8	300	100.0

In the equation, amounts of enzyme and time are reciprocal quantities. It is therefore evident that diastatic powers may still be compared when determined under different conditions of time as well as of amounts of enzyme employed.

The following example will illustrate how much more closely the results thus found correspond with actual amylolytic power than do the mere weights of cuprous oxide. In a set of determinations under uniform conditions 0.15, 0.30, 0.45, 0.60 mg. of pancreatin yielded respectively 76, 147, 217, 286, mg. of cuprous oxide.

From the table we find the corresponding values of K to be 23.5, 46.5, 70.2, 94.8. Dividing these values by the respective weights of enzyme we obtain 156, 155, 156, 158. If we did not use the table, but divided the weights of cuprous oxide by three times the number of milligrams of enzyme, we would obtain 169, 163, 160, 159.

Furthermore, by application of this table, results obtained with different times of conversion may still be expressed on the same scale. With the proper activating agents present, any time of conversion from 15 minutes to two hours may be employed; and, if the amount of cuprous oxide does not exceed 300 mg., the results may be calculated as follows: Divide 30 (the standard time for the method described above) by the actual time of conversion in minutes, and multiply by the value of K as found in the above table. The product will equal the value which would have been obtained for K under standard conditions.

The procedure of the Lintner method may likewise be used and from the known weight of cuprous oxide in the tube in which the 5 cc. of Fehling solution is just reduced one may (after correcting for the reducing powers of the starch used) make use of the above table and express the results on the new scale of diastatic values.

Summary.

1. There seems to be no good reason for the diversity of conditions of temperature and time which have been given by different writers in the past, and it seems desirable that in all determinations of diastatic power the amylase be allowed to act at 40° and that 30 minutes be adopted as the standard time.

2. The Lintner method is sound in principle but does not prescribe optimum conditions, is not very delicate, and does not give a satisfactory degree of accuracy when applied to preparations of high diastatic power.

3. The gravimetric method as here described is capable of much greater accuracy and gives results which can either be stated in terms of cuprous oxide actually weighed, or of maltose, or in terms comparable with those of the Lintner scale, or on a new scale based upon the velocity curve of the reaction.

4. The new iodine method is believed to increase considerably the practicable accuracy of this type of determination and while it probably cannot be made as delicate as the improved gravimetric method, yet for those cases in which a satisfactory end point can be obtained, it has the practical advantage of simplicity and the theoretical advantage of marking the completion of a fairly definite step in the digestive process, whereas the copper reduction method measures the amount of a substance or substances produced by successive steps through intermediate products which are but imperfectly known.

5. With the three samples of taka-diastase examined, the end point in the iodine method was fairly satisfactory, consisting in a change from light blue to nearly colorless, and the results by the method were consistent with those by the Lintner method. It remains to be determined by examination of a larger number of samples under a variety of conditions whether the two types of method will give parallel results with taka-diastase throughout, or whether each method is capable of giving information which the other does not.

6. In the case of pancreatin, the iodine method does not seem capable of great accuracy because of the uncertainty in judging the point of change from a blue to a red reaction with iodine, and the comparatively long duration of the stage which gives the red color and which fades almost imperceptibly into the faint straw color of the iodine test solution itself.

7. With the copper reduction methods also, much more difficulty was experienced with pancreatin than with taka-diastase. In spite of all efforts to eliminate interfering impurities, irregular results were persistently obtained. This fact indicated the need of a detailed study of the action of pancreatic amylase, the results of which study are described in the paper which follows.

8. In the absence of added electrolytes the pancreatic amylase was so incompletely activated that it could not behave normally and was extremely susceptible to slight variations in the amounts of salts, acids, or alkalies accidentally present, so that the results were both misleadingly low and irregular. It is evident that in testing amylolytic power it is not the presence of electrolytes but their absence or insufficiency that constitutes the disturbing condition, and that reliable results are obtained only when the enzyme is properly activated so that it can function normally.

9. A gravimetric method is proposed which utilizes the results of a quantitative study of the action of pancreatic amylase, which study is described in the following paper. By this method, employing optimum amounts of salt and alkali for activation, we obtain in testing commercial pancreatins, results about 20 times as high as when no electrolyte is added.