acid is precipitated with calcium acetate after acidifying with acetic acid, and the precipitate is titrated with standard hydrochloric acid. Or the method frequently used was to acidify the filtrate strongly with sulfuric acid and titrate with potassium permanganate. The potassium permanganate used is calculated to oxalic acid.

Dr. Denis states that the above work[1] was confirmed repeatedly. She also made titrations of lactic acid by adding 0.1 *N* potassium permanganate to the alkalin solution. When no more potassium permanganate was reduced the solution was strongly acidified and the manganese dioxide reduced with 0.2 *N* oxalic acid. These experiments showed that 4.84 and 4.87 atoms of oxygen were consumed instead of 5 atoms per molecule, as demanded by the equation. The average difference of 3.4% was no doubt attributed to error. In experiment (*f*) above on zinc lactate the products accounted for 5.05 atoms of oxygen per molecule of lactic acid. This is an excess of 1%. The amount of potassium permanganate used was equivalent to 5.13 atoms of oxygen or 2.6% in excess. In experiment (*e*) the conditions used were much the same as those used by Ulzer and Seidel and the amount of acetic acid formed was larger.

Our own experiments have shown that the reaction is not as simple as was assumed by the former workers. The analytical error in working by Ulzer and Seidel's method may vary a great deal, depending on the temperature, the dilution, the concentration of the alkali, etc. The error in Denis' adaptation of it would never be so large because the potassium permanganate used in the oxidation is the basis of calculation.

Instead of having only the reaction

$$C_3H_6O_3 + 5O \longrightarrow C_2H_2O_4 + CO_2 + 2H_2O,$$

the following, also,

$$C_3H_6O_3 + 2O \longrightarrow CH_3COOH + CO_2 + H_2O$$

and

$$C_3H_6O_3 + 6O \longrightarrow 3CO_2 + 3H_2O$$

take place.

Further work along these lines on acetone, lactic acid and glycerol is now under way.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF COLUMBIA UNIVERSITY, No. 208.]

STUDIES ON AMYLASES: IV. A FURTHER INVESTIGATION OF THE PROPERTIES OF PANCREATIC AMYLASE.[2]

BY H. C. SHERMAN AND M. D. SCHLESINGER.

Received May 31, 1912.

In a previous paper,[3] we have described the preparation of several

[1] *Am. Chem. J.*, 38, 576.

[2] Read before the New York Section, May, 1912.

[3] THIS JOURNAL, 33, 1195.

samples of purified pancreatic amylase having in the air-dry state diastatic powers of over 3000 on the new scale developed and used in this laboratory,[1] corresponding to figures of about 5000 on Lintner's scale for purified diastases, or about eight times as powerful as the strongest amylase previously described. The preparations made during March and April, 1911, were quite uniform in activity but subsequently during the summer months some less active products were obtained, and on investigation it was found that in warm weather the enzyme deteriorates in solution in the course of the purification to such an extent as to call for a more definit control of conditions than had been found necessary during the winter and spring.

Method of Preparation.

The method as now used is as follows: Mix thoroughly 20 grams of pancreatin powder with 200 cc. of 50% alcohol at a temperature of 15° to 20°, allow to stand 5 to 10 minutes and filter through paper. During this filtration, which may occupy 1 to 2 hours, the temperature of the solution should not be allowed to exceed 20°. Pour the filtrate into 7 times its volume of a mixture of 1 part alcohol to 4 parts ether, shaking with a rotary motion. Within 10 to 15 minutes the precipitate will usually have collected as an oily layer at the bottom of the vessel. Decant the supernatant liquid, dissolve the precipitate in the smallest possible amount of pure water at a temperature of 10° to 15° and reprecipitate immediately by pouring into 5 volumes of absolute alcohol. Allow the flocculent precipitate to settle at a temperature not above 20°, filter, and dissolve in 200 to 250 cc. of 50% alcohol containing 5 grams of maltose. Pour this solution (which need not be perfectly clear at this point) into a 500 cc. collodion sac and dialyze against 2000 cc. of 50% alcohol at a temperature not above 20° and preferably not below 15°.[2] Replace the dialysate by an equal volume of fresh 50% alcohol after 15 hours and again after another 8 or 9 hours, continuing the dialysis for 40 to 42 hours.[3] Filter, and pour the clear filtrate into an equal volume of (1 : 1) alcohol-ether mixture, thus obtaining the final product. Filter in the cold and place immediately in a vacuum desiccator to dry.

Using the method with the precautions here given, we have obtained at different seasons of the year a number of preparations corresponding in activity as well as in other properties with those prepared in March

[1] THIS JOURNAL, **32**, 1082.

[2] If the temperature of dialysis is too low, the removal of the maltose is retarded with the result that the final precipitate may contain maltose.

[3] Too long a dialysis is apt to result in a less active product, doubtless because of deterioration of the enzyme in solution, especially after removal of the maltose, but an extension of the total time of dialysis up to 46 hours has not been observed to have any appreciable effect upon the activity of the product.

and April, 1911, and described in our last paper. Through study of the material thus obtained, as well as further observations upon the preparations described last year, we are enabled to present a somewhat fuller description of the properties of this pancreatic amylase.

Elementary Composition and Heat of Combustion.

Determinations of carbon, hydrogen and nitrogen were reported last year, calculated to the basis of dry, ash-free material. Subsequent observations made it probable that the apparent ash of these preparations is chiefly metaphosphoric acid derived from phosphorus present as an essential constituent. This is indicated both by the appearance of the residue obtained on ignition and by the fact that its weight only slightly exceeds that which would correspond to the phosphorus content as determined both in a recent preparation and in a composit sample made up of portions of the preparations of 1911.

Recalculating the carbon, hydrogen, and nitrogen in accordance with this view and including the data for phosphorus and sulfur recently determined, we find the following elementary composition:

C.	H.	N.	S.	P.	O (and undetermined).
51.9	6.6	15.3	1.0	0.8	24.4

As the limited amount of material prevented frequent repetition of determinations we have preferred to express percentages only to the first decimal place.

It will be noted that this material bears considerable resemblance to casein in its elementary composition; its resemblance to albumin in superficial appearance, solubility and coagulation on heating, and the fact that the filtrate from the coagulum contains proteose or peptone was pointed out in our previous paper.

Berthelot and André[1] reported the heat of combustion of casein as 5629 calories per gram; Stohmann and Langbein found for casein 5858 calories, for egg albumin 5735 calories, for peptone 5299 calories per gram.

One of our preparations (No. 37) burned by the same method in an Atwater-Berthelot bomb calorimeter,[2] showed 5568 calories per gram, calculated to dry, ash-free material in the usual way for comparison with the data just cited. Thus the heat of combustion is slightly lower than that of casein or albumin but considerably higher than that of peptone. This accords well with the elementary composition and the yielding of a coagulated protein and a proteose or peptone on heating in water, and favors the hypothesis of the formation of the enzyme from preexistent protein by a process involving incipient hydrolysis associated perhaps with very slight oxidation.

[1] Hawk and Gies, *Am. J. Physiol.*, **5**, 387.

[2] Atwater and Snell, THIS JOURNAL, **25**, 659.

Temperature of Coagulation.

Several of our preparations have been tested for coagulation temperature by heating in 1% water solution. Solutions of such concentration were usually not absolutely clear. Filtered solutions showed a very slight opalescence at 50° with no further change until the temperature reached 68–70°. At 68–70° there was a distinct cloudiness which separated at 70° into fine flocks. Held at 70°, the precipitate gathered into a few large flocks, leaving the remaining solution clear. After filtering out this coagulum the solution showed no further precipitation on heating to 100°.

Starch Splitting Power.

The method of determining diastatic power regularly used in connection with our studies of the purification of the amylase is in reality a measurement of the sugar forming rather than of the starch splitting power, the latter being more directly measured by methods in which one determines the disappearance of the starch-iodine reaction as in the Wohlgemuth method.

In our previous paper we reported a test of preparation 34 by a modification of the Wohlgemuth method in which digestion of the starch was carried to the point at which the products give no color reaction with iodine. The experience of this laboratory during the past year has led us to the belief that the end point recommended by Wohlgemuth—that at which iodine gives a color free from blue or violet—is more logical and serviceable than the so-called "colorless end point." When preparation 34 was examined by the original Wohlgemuth method, 0.0000025 gram of the enzyme[1] was found to digest 5 cc. of a one per cent solution of Kahlbaum's soluble starch to the point of giving a wine red reaction with iodine in 30 minutes at 40°, thus giving a Wohlgemuth figure of

$$D_{30'}^{40^\circ} = 2{,}000{,}000$$

Here the actual weight of starch digested was 20,000 times the weight of the enzyme and complete conversion into products giving only a wine-red color with iodine was accomplished in 30 minutes.

The same preparation (at a later time when it had deteriorated somewhat) was allowed to act upon 800,000 times its weight of starch. This it digested in 30 hours to products giving only a red color with iodine, while within 72 hours the digestion was carried completely beyond the erythrodextrin stage and the enzyme had formed reducing sugar equivalent to 455,000 times its weight of maltose.

Preparation 39 digested 1,000,000 times its weight of starch beyond the erythrodextrin stage in 48 hours.

[1] To obtain this quantity for the test, 25 mg. of the preparation were dissolved in a volume of 50 cc., 1 cc. of this solution was diluted to 100 cc. of which 0.5 cc. was used for the test.

Preparation 50 digested 1,000,000 times its weight of starch completely to the erythrodextrin stage in 30 hours and to products giving no reaction with iodine in from 48 to 96 hours, at the expiration of which time it had produced reducing sugar equivalent to 516,000 times its weight of maltose.

In these experiments, since a one per cent solution of starch was used, the concentration of enzyme in the solution was 1 : 100,000,000.

Reducing Sugars Formed.

In earlier work in this laboratory it was found that under conditions similar to those which obtain in the determination of diastatic power, the yield of reducing sugar does not exceed that which would correspond to a conversion of about 85% of the starch originally present.

When, however, relatively large amounts of our strong preparations are allowed to act upon limited amounts of starch, larger yields of reducing sugar are obtained, and in some cases the digestion products have shown greater reducing power than would correspond to a complete conversion of starch to maltose. Thus when 20 milligrams of preparation 39 acted upon 100 cc. of 1% soluble starch for 72 hours the resulting solution showed greater reducing power than would have resulted from a quantitative conversion of the starch into maltose. This result naturally suggested that glucose as well as maltose had been formed by the action of the purified amylase upon the soluble starch. In order to test this in another way a portion of the digestion product of a similar experiment was hydrolyzed to glucose and the increase in reducing power determined. This increase was less than it should have been had the digestion products consisted of dextrin and maltose, or maltose alone, indicating again that some of the maltose had already been split to glucose by the action of the amylase.

The question of the formation of glucose by the action of our pancreatic amylase was further studied in a series of experiments carried out with the collaboration of Mr. P. W. Punnett, in which the digestion products were evaporated on sand, extracted with a small amount of strong alcohol, the alcohol evaporated, the extracted sugars weighed, dissolved in 20 parts of water and submitted to the osazone test. By means of test experiments, which will be more fully described in a later paper, it was found that the presence of glucose to the extent of about 1 part to 30 parts of maltose and dextrin could be demonstrated by the appearance of an osazone in the hot solution, while the time required for the appearance of the osazone, under the fixed conditions of the test, gives some indication of the amount of glucose present. The delicacy of this method is doubtless due in large measure to the extraction with a limited quantity of strong alcohol, which is designed to eliminate along with the dextrin a considerable part of the maltose present and thus minimize the retard-

ing action of maltose upon the formation and precipitation of the glucosazone.[1]

By means of this method it was found that both commercial pancreatin and the purified pancreatic amylase formed glucose. Our method of purification appears to result in concentration of glucose forming, as well as of maltose forming power. It was also found that both the commercial pancreatin and the purified pancreatic amylase reduced the rotatory power and increased the reducing power of a solution of pure maltose, thus affording additional evidence of the possession of a maltose splitting power.

Deterioration.

Not only is the activity of pancreatic amylase manifested only in the presence of electrolytes but the enzyme rapidly undergoes deterioration when kept in solution in water alone. The more completely the amylase is separated from the substances which naturally occur with it, the more rapidly it deteriorates in solution.

Without taking space to present here the full data of our experiments upon the rate of deterioration the following typical cases may be cited. Preparation 34 having a power of 3480 lost 45.5% of its activity on standing in pure water at room temperature for 20 minutes; a less highly purified preparation with a power of 2000 lost 32.5%; a high grade commercial pancreatin with a power of 425 lost 19%; a pancreatin of lower quality with a power of 150 lost only 11%. In all cases the diastatic power was completely lost within 24 hours.

In an aqueous solution containing the mixture of salt and phosphate used in determining diastatic power, or in 50% alcohol, the amylase was much more stable, purified preparations losing one-third to one-half of their activity in 24 hours, while the loss in the commercial pancreatins varied from about one-third down to a loss too small to measure.

While Hudson was able to explain the deterioration of invertase in hot water, as due simply to the hydrolytic action of the water upon the enzyme, the case of the deterioration of pancreatic amylase appears to demand consideration of other factors. One question which at once presented itself was whether, in view of the protein nature of our amylase preparation, its destruction in water solution might be due in part at least to its digestion by trypsin (or other protease) accompanying the amylase in the pancreas and not completely removed by our method of purification.

Proteolytic Action.

With the collaboration of Miss Una G. Ruth a number of the preparations were tested for proteolytic power and in all cases our purified amyl-

[1] Sherman and Williams, THIS JOURNAL, 28, 629.

ases showed greater proteolytic activity than the commercial pancreatins from which they had been prepared.

The method used in testing proteolytic power was essentially Loehlein's modification of the Thomas and Weber method. Fifty grams of casein were dissolved in a mixture of about 400 cc. water with 40 cc. *N* sodium hydroxide and the volume made up to 1 liter; 100 cc. portions of this solution were treated with different amounts of the substance to be tested and kept at 40° for 60 minutes, then mixed with 100 cc. of 20% solution of sodium sulfate, and 22 cc. of 0.5 *N* HCl added slowly with thorough stirring. After allowing ½ to 1 hour for the precipitate to settle the solution was filtered and 200 cc. of filtrate were titrated with 0.1 *N* sodium hydroxide solution, using alizarin as indicator. The excess of acid found in the solution after precipitation of the undigested casein, minus that found in a blank experiment in which no enzyme was added, affords a measure of the amount of proteolysis which has taken place through enzyme action and thus indicates the proteolytic power of the enzyme. For the present this is expressed only in relative terms as follows: Preparations, 33, 34, 43, 52 and 56 each of which had approximately 8 times the amylolytic (saccharifying) power of the pancreatin from which they were prepared, showed respectively by the method just described, approximately 7, 7, 5, 6 and 6 times the proteolytic power of the original pancreatin. These measurements of proteolytic power are probably subject to errors of 10 to 15% in the manipulation of the method, in addition to which there may be other discrepancies due to possible incomplete activation of the proteolytic power under the experimental conditions. Considering the nature of the method of purification by which these preparations were obtained, it is evident that the proteolytic and amylolytic activities must be due either to the same substance or to substances remarkably alike in their behavior in extraction and precipitation. It may be noted that a by product obtained near the end of our process of purification (the material which gradually precipitates from the solution of amylase in 50% alcohol during dialysis and which, when freed from mother liquor, had little, if any, amylolytic action) showed even higher proteolytic power.

Two specially purified dry extracts of pancreas made in another laboratory by a different method showed higher proteolytic powers than our purified preparations, although the amylolytic powers were but one-fifth to one-third as high. A high grade commercial trypsin of similar proteolytic power showed only one-fifteenth the amylolytic power of our preparations.

A comparison of the results obtained upon both our own and other preparations raises the question whether one of the enzymes of the pancreas is both a protease and an amylase and whether such a substance

may lose its amylolytic power with no decrease, or even with an increase, of proteolytic activity.

We are again indebted to Messrs. Fairchild Brothers and Foster and to the Parke, Davis Company for material.

Summary (of this and the preceding paper).

A method is described for the purification of pancreatic amylase which on frequent repetition at different times has yielded a product of fairly uniform properties and activity.

This product showed a heat of combustion of 5568 calories per gram, and the following approximate composition: C 51.9, H 6.6, N 15.3, S 1.0, P 0.8, O (and undetermined), 24.4.

It gave a pronounced Hopkins-Cole tryptophane reaction and also typical protein reactions in the xanthoproteic and biuret tests and with Millon's reagent.

It is soluble in pure water and coagulates at about 70°, the coagulum showing a violet, and the filtrate a rose red, biuret reaction. This filtrate also gives a white, flocculent precipitate when poured into strong alcohol. This preparation shows the properties of an active protease as well as amylase and some activity as a maltase. The amylolytic property has been more fully investigated than the other two.

The amylolytic power undergoes rapid deterioration in pure water solution. In water containing small quantities of sodium chloride and disodium phosphate, or in 50% solution of alcohol or acetone, it deteriorates much less rapidly.

Tested under proper conditions at 40°, this material shows an amylolytic power of about 3500 on the new scale of diastatic power, about 5000 on the Lintner's scale, about 2,000,000 on Wohlgemuth's scale.

Acting at a concentration of 1 : 100,000,000 in a 1% starch solution, it converted 1,000,000 times its weight of starch to the erythrodextrin stage in 30 hours and within 96 hours had completely digested the starch and intermediate dextrins to products giving no reaction with iodine and had formed over 500,000 times its weight of reducing sugar. calculated as maltose.

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THE HYDROLYTIC ACTION OF GLYCINE ON ETHYL BUTYRATE.

BY S. LIEBOWITZ

Received May 24, 1912.

If the hydrolysis of fats and esters by lipases is caused by a protein-like body, the assumption may be made that certain specific groupings