[CONTRIBUTION FROM THE FLEISCHMANN LABORATORIES]

VEGETABLE AMYLASES. STUDY OF DIASTASE ACTION IN THE ABSENCE OF MALTOSE

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Foreword

Studies of the kinetics of enzyme action have been but rarely made in the absence of end products. Determinations of the course of the reaction in most previous studies of diastatic activity have been made either (1) by noting the rate of degradation of starch as in the method of Wohlgemuth[1] utilizing the change in iodine reaction or (2) by observing the rate of appearance of maltose, usually by copper reduction.[2] Nearly every investigator has attempted to show that his results follow the usual monomolecular reaction formula, with but varying success. Brown and Glendinning[3] as well as Luers and Wasmund[4] definitely state that the earlier portion of their curve would not fit a first order reaction and the monomolecular velocity constants calculated by other investigators[5] all tend to rise during the reaction. Others[6] who worked with relatively concentrated enzyme solutions are the only ones who report results which follow at all closely this law.

Kinetics in Absence of Maltose.—In the present investigation the determination of sugars formed in the reaction by the usual tedious procedure has been replaced by a method much more convenient. Yeast is added to the reacting solutions in sufficient quantity to ferment out the sugars as rapidly as they are formed and the carbon dioxide evolved is collected and measured at frequent intervals (Fig. 1). The results obtained in comparison with the more usual methods are shown in Fig. 2 and Fig. 3. The linear character of the action in this case is obvious and the inhibition caused by added maltose (reported also by Wohl and Glimm)[7] is quite apparent. As far as can be learned this is the first example of an enzyme reaction for which conditions have been found under which the velocity appears to be independent of the time over such a large portion of the reaction.[7a] If an explanation were to be offered for the apparent simplicity

[1] Wohlgemuth, *Biochem. Z.*, **9**, 1 (1910).

[2] Pollack, *Wochschr. Brau.*, **20**, 595 (1903).

[3] Brown and Glendinning, *J. Chem. Soc.*, **81**, 388 (1902).

[4] H. Luers and W. Wasmund, *Fermentforschung*, **5**, 169 (1922).

[5] Euler and Svanberg, *Z. physiol. Chem.*, **112**, 193 (1921).

[6] Luers in Carl Oppenheimer's "Die Fermente und ihre Wirkungen," **1928**, 5th ed. Vol. III, p. 883; Willstätter, Waldschmidt-Leitz and Hesse, *Z. physiol. Chem.*, **142**, 14 (1925).

[7] A. Wohl and E. Glimm, *Biochem. Z.*, **27**, 349 (1910).

[7a] P. Rona and R. Ammon [*ibid.*, 181 (1927)] report that lipase activity does not vary when the P_H is kept constant by titration.

of this action it would seem logical to conclude that all inhibiting factors, including reversible combination with end products, appear to be eliminated. The results also seem to substantiate the Michaelis[8] theory and the velocity of the reaction as measured is that of the decomposition of the enzyme–substrate complex, the concentration of which remains essentially constant as long as a sufficient excess of substrate is present.

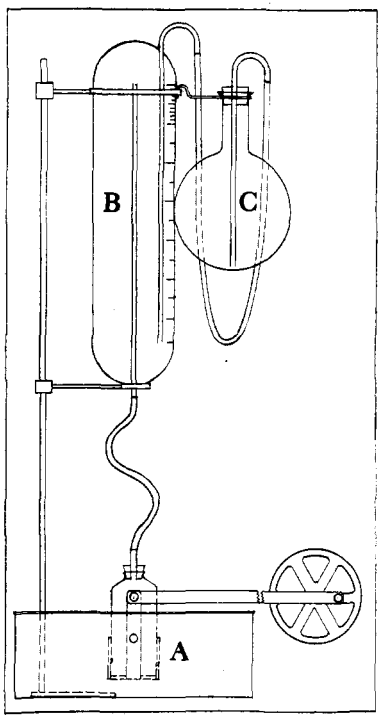

Fig. 1.—Apparatus: A, cradle in thermostat carrying six bottle clamps; B, 1-liter fermentometer; C, 1-liter flask as leveling bulb.

Fermentation of the products of the acid hydrolysis of the starch used gave 1250–1300 cc. of gas per 125 cc. of 5% solution. In our tests with the products of enzyme hydrolysis 950–1000 cc. was obtained. This corresponds to the usual 75% conversion and corroborates the residual dextrin theory.

Inactivation of Enzyme.—Among the factors which may be considered to explain any falling off in the initial rate[9] may be listed, (1) reversible combination of the products (mentioned above) with the enzyme, (2) insufficient saturation of enzyme by substrate, (3) irreversible destruction of the enzyme by heat, adsorption, etc. In the progress of this investigation it was found that shaking, particularly in the case of malt diastase, caused a serious diminution in activity as shown in Fig. 4, curves 1 and 2, quite similar to the effect of adsorbents such as diatomaceous earth or filter paper pulp (Fig. 4, curves 4 and 6, and Fig. 5, curves 3 and 6). The addition of small amounts of gelatin, saponin, albumin, casein and peptone, however, enabled each enzyme to act linearly (Fig. 4, curves 3, 5 and 7, and Fig. 5, curves 2 and 4), but if added after inactivation did not restore the activity. It should be noted, however, that soy diastase produces this type of reaction without the presence of any added protective agency. The inhibition is not oxidative for the presence of inert gases is without effect. Glycine and agar were found to be without action.

In explanation of the effect of shaking we may call attention to the work

[8] L. Michaelis and M. L. Menten, *Biochem. Z.*, **49**, 333 (1913).

[9] Haldane, "Enzymes," Biochemical Monographs, Longmans, Green and Co., London, **1930**, p. 92.

of Ramsden,[10] who found that shaking of protein solution caused a precipitation or "de-solution" of the solute by agglomeration of the viscous coatings formed on the free surfaces of the solution. It is highly probable that the enzyme is removed from the reaction in this way. Others[11] in studying the inactivation of enzymes by shaking found that the presence of small amounts of saponin and certain proteins prevented the inactivation and explained it on the basis of surface tension, *i. e.*, saponin, producing a lower surface energy than the enzyme, forced the latter away

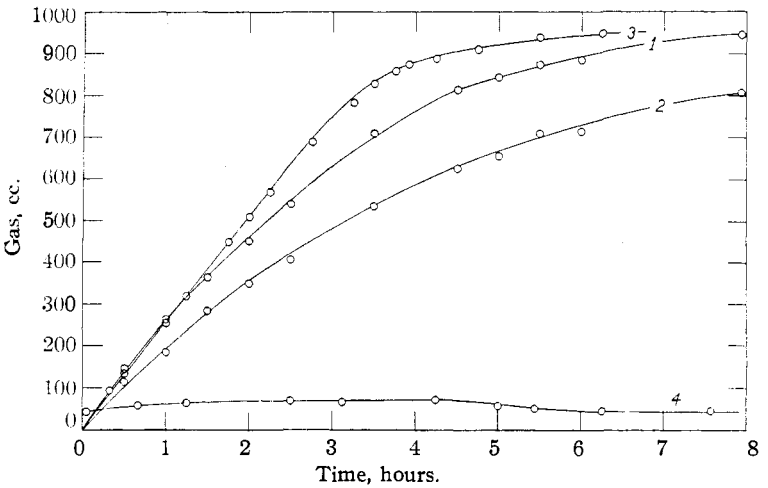

Fig. 2.—Malt diastase: effect of end-products on activity. Reacting solutions contained 125 cc. of 5% soluble starch solution, citrate buffer, 0.030 g. of malt sirup, 0.03 g. of saponin; total volume 146 cc., and (1) no added maltose, no yeast; sugars by copper reduction, calculated to equivalent gas. (2) Five per cent. added maltose, no yeast; sugars by copper reduction, calculated to equivalent gas. (3) No maltose, 5 g. of yeast, gas measured. (4) Residual reducing substances in (3) by copper reduction, calculated to equivalent gas.

from the surface, or competed more strongly for positions in the surface layer. We are led to consider the same explanation in the case of inactivation by adsorbents, *i. e.*, competition for positions on the adsorbing surface. In passing it might be mentioned that it does not seem that Przylecki's explanation of inactivation by adsorption[12] is substantiated, since the falling off in the velocity occurs even in the presence of excess

[10] W. Ramsden, *Proc. Roy. Soc.* (London), **72,** 156 (1904).

[11] A. and S. Schmidt-Nielson, *Z. physiol. Chem.*, **68,** 317 (1910); E. Abderhalden and W. Guggenheim, *ibid.*, **54,** 331 (1907); M. M. Harlow and P. G. Stiles, *J. Biol. Chem.*, **6,** 359 (1909); A. O. Shaklee and S. J. Meltzer, *Am. J. Physiol.*, **225,** 81 (1909); H. C. Bradley, *J. Biol. Chem.*, **6,** 133 (1909).

[12] S. J. Przylecki and co-workers, *Biochem. J.*, **21,** 1025 (1927).

starch when the enzyme is adsorbed, although this difference may be due to P_H.

Concentration Effects.—Eadie[13] has shown that diastatic activity is practically proportional to enzyme concentration. In the case of soy diastase, Fig. 6, the enzyme concentration of curve 2 is two and one-half times that of curve 1 and the rate is within the limits of error two and one-half times as great. Variations of substrate concentration, Fig. 7, are more important as from them we may calculate the value of the Michaelis constant. This constant K_m for a flour diastase, calculated from the data

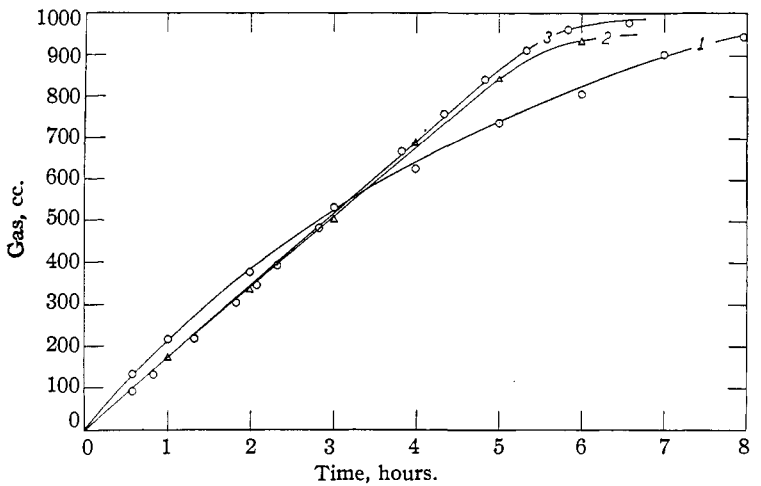

Fig. 3.—Soy diastase: effect of end-products on activity. Reacting solutions contained 125 cc. of 5% soluble starch solution, citrate buffer, 0.05 g. of ground soy bean, no saponin; total volume 135 cc., and (1) no added maltose, no yeast, drop in polarization calculated to equivalent gas. (2) Same, with 5 g. of yeast; drop in polarization calculated to equivalent gas. (3) Same, with 5 g. of yeast, gas measured.

presented in Fig. 7, is about 0.25 in units of per cent. starch. K. Sjoberg and E. Ericksson[14] have found K_ms 0.4 and 0.5 for malt amylase. It is believed that the results of this investigation substantiate further the Michaelis theory and the idea that the kinetics of enzyme action is fundamentally simple; the difficulty arises in sorting out the various inhibitions to which these labile substances are subject.

Experimental Procedure

Principle.—The basis of the method is the removal of the reaction product, maltose, as rapidly as it is formed in the reaction by fermentation

[13] G. S. Eadie, *Biochem. J.*, **20**, 1016 (1926).

[14] K. Sjöberg and E. Eriksson, *Z. physiol. Chem.*, **139**, 118 (1924).

with a sufficiently large quantity of yeast. A quantity of gas, carbon dioxide, is formed, collected and periodically measured, which is stoichiometrically related to the amount of sugar produced (under actual conditions 1 g. of maltose is equivalent to 220 cc. of carbon dioxide at 25° and 1 atm. pressure). Supersaturation of the reacting solution with carbon

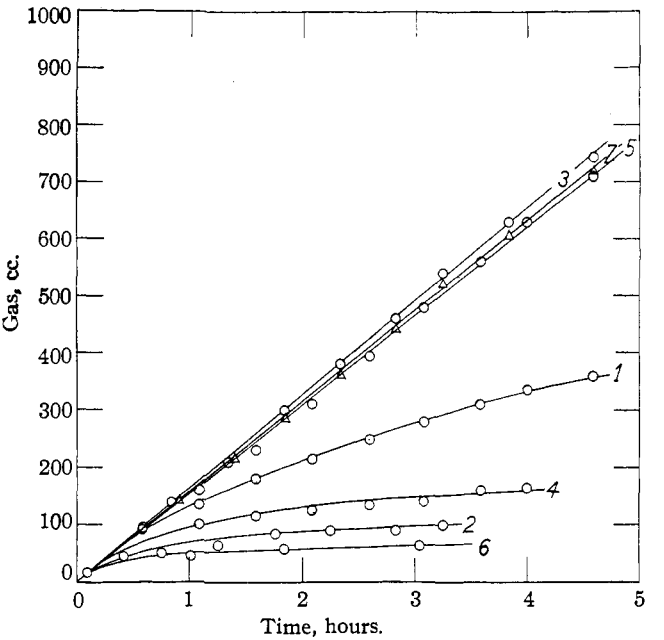

Fig. 4.—Malt diastase: effect of certain protective and destructive agencies on activity. Reacting solutions contained 125 cc. of 4.5% soluble starch solution, citrate buffer, 0.015 g. of malt sirup, 5 g. yeast; total volume 146 cc. and (1) nothing (or 0.5 g. of glycine or 0.5 g. agar). (2) Nothing (enzyme from a 1% solution of the malt sirup shaken two hours previous to test). (3) 0.5 g. of gelatin (or 0.03 g. of saponin, 0.5 g. of albumin, 0.5 g. of casein or 0.5 g. of peptone). (4) 1.0 g. of filter paper pulp. (5) 1.0 g. of filter paper pump plus 0.5 g. of gelatin. (6) 1.0 g. of diatomaceous earth. (7) 1.0 g. of diatomaceous earth plus 0.5 g. of gelatin.

dioxide is prevented by constantly shaking the mixture. It has been found necessary to "acclimatize" the yeast to the fermentation of maltose at large rates in order to prevent a short lag period at the beginning of enzyme action, and this as well as a preliminary saturation of the reacting solution with carbon dioxide is accomplished by an initial dosage of maltose before the addition of the enzyme. The changes due to the internal metabolic functions of the relatively small quantity of yeast used were found to be negligible in comparison with other experimental errors.

Apparatus.—Figure 1. Six 200-cc. wide-mouthed reaction bottles are attached by means of spring fastening clamps to a cradle of strap iron. The cradle is continuously rocked through an arc of about 20° in an electrically controlled thermostat by a small motor at a rate of about 120 oscillations per minute. Six 1-liter gasometers or gas burets are attached by flexible connections to their respective bottles. The water expelled by the evolved carbon dioxide is collected in liter wash bottles which also serve as leveling bulbs when taking readings. A layer of kerosene serves to minimize solution of carbon dioxide in the water. While such a layer is not completely impervious to the gas, it was found in a blank experiment that the rate of solution was negligible in comparison with that of the activity being measured.

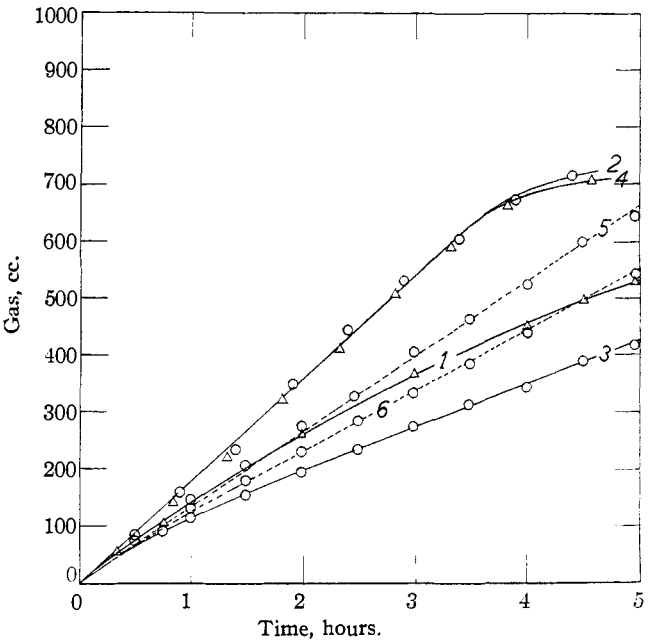

Fig. 5.—Wheat flour and ground soy bean diastase: effect of certain protective and destructive agencies on activity. Reacting solutions contained 125 cc. of 5% soluble starch solution, citrate buffer, 5 g. of yeast; total volume 146 cc., and (1) 0.035 g. of flour only. (2) Same, plus 0.5 g. of gelatin. (3) 0.035 g. of flour plus 1.0 g. of diatomaceous earth. (4) Same, plus 0.5 g. of gelatin. (5) 0.035 g. of ground soy bean only. (6) Same, plus 1.0 g. of diatomaceous earth.

Reagents.—A suitable unbuffered soluble starch solution of about 5 or 6% solids such as that prepared by the prolonged action of hydrochloric acid in the cold in commercial potato starch was used.[15] A buffer solution of 100 g. of potassium citrate, 20 g. of citric acid and 20 g. of primary ammonium phosphate per liter, sterilized by boiling, has been found satisfactory as it gives very closely the optimum P_H, 4.5 to 5.5. A quantity of fresh bakers' yeast, or any yeast of similar fermenting strength, and pure maltose complete the list of reagents.

[15] H. C. Gore, THIS JOURNAL, **47**, 281 (1925).

Procedure.—This general procedure was followed rather closely for most of the experiments reported herein. Five grams of fresh pressed yeast, 1 to 2 g. of maltose, 125 cc. of starch solution, 15 cc. of buffer solution and 3 cc. of 1% saponin solution or any other material, the action of which is to be tested, are placed in the reaction bottle in the cradle and the bottle is connected to the gasometer. Shaking is continued until the maltose is completely fermented (usually about two hours). The shaking is then stopped, the gasometers reset to zero, a definite volume of the aqueous solution or suspension of the diastatic material (1%) added and the time of addition noted. Shaking is resumed and continued until the test is finished, except for the short intervals during

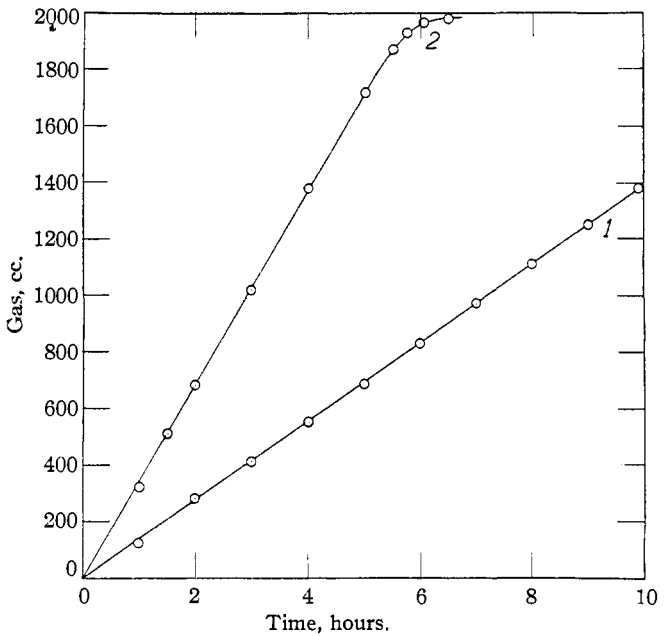

Fig. 6.—Soy bean diastase: effect of variations in enzyme concentration. Reacting solutions contained 270 cc. of soluble starch solution, citrate buffer, 10 g. of yeast; total volume 310 cc., and (1) 0.04 g. of ground soy bean. (2) 0.10 g. of ground soy bean.

which readings of the volume of gas are taken. These readings can be made conveniently at quarter or half hour intervals. It is desirable, although not absolutely necessary, to work in a constant temperature room; at any rate the fluctuations in room temperature should be kept to a minimum. For strictly comparable work barometer variations should also be taken into consideration. The thermostat was maintained at a temperature of 30 ± 0.2° in this investigation. A typical set of data is given in Table I, which shows the effect of gelatin in the preparation of the diastatic extract. The agreement with other methods is close, as is shown in Fig. 3, curves 2 and 3, in which the rate of degradation of starch was followed polariscopically in a parallel experiment. The experimental points are calculated from the ratio of cc. of gas to degrees Ventzke, equal to 17.4 cc. per degree; for curve 1 (maltose present) the ratio is 61.4.

The Lintner value readily can be calculated from the data thus obtained by substituting in the formula °L. = fv/tw. In this expression v is the

TABLE I

QUANTITIES OF MATERIALS USED IN EACH EXPERIMENT

Maltose, 1 g.; buffer, 15, cc.; starch soln., 125 cc.; yeast, 5 g.; gelatin, 0.5 g.

Expt.	After sugar was fermented enzymes added as follows: cc.	G.	
1	3.5	0.035	1 g. Flour + 0.5 g. gelatin in 100 cc.
2	3.5	.035	1 g. Flour, no gelatin in 100 cc.
3	6.0	.06	1 g. Ground malt + 0.5 g. gelatin in 100 cc.
4	6.0	.06	1 g. Ground malt, no gelatin in 100 cc.
5	1.5	.015	5 g. Malt sirup + 0.5 g. gelatin in 500 cc.
6	1.5	.015	5 g. Malt sirup, no gelatin in 500 cc.

GAS EVOLVED AT TIMES INDICATED, CC.

Start..... Time	11:20 1	11:20 2	11:20 3	11:21 4	11:21 5	11:21 6
12:15	135	100	135	130	135	120
12:45	225	165	230	220	220	200
1:15	300	230	310	305	290	280
1:45	395	300	405	400	370	365
2:15	480	370	500	490	450	450
3:35	700	550	740	730	660	670
3:50	745	600	790	780	705	715
4:00	760	610	820	815	730	740
4:30	825	680	890	885	810	815
5:25	940	815	1000	995	940	940

volume of gas in cc. produced in t hours by w g. of diastatic material during the linear portion of the curve and f a conversion factor. For °L. equivalent to that determined polariscopically at 20°,[15] $f = 0.038$, the gas being determined by exact adherence to the procedure described above. If the factor weight of 0.038 g. of enzyme is used, the cc. per hour will be directly the °L. of the material. The temperature coefficient for the soy, wheat flour and malt diastases has been found to be about 1.9 per 10° between 20 and 30°, in agreement with the results of previous investigations. As reported also by Ford and Guthrie,[16] it has been found that in the preparation of the aqueous suspension of flour a small amount of protective substance such as 0.5 g. of gelatin per 100 cc. of 1% suspension of diastatic material should be present in order to obtain the maximum value of diastatic activity.

Precautions.—The necessity of having the yeast in an acclimatized state and the solution saturated with carbon dioxide before beginning an experiment has been mentioned. In addition it is necessary to adjust the enzyme concentration or the amount of yeast added so that the latter is never fermenting at its maximum rate, *i. e.*, the yeast must keep ahead of the enzyme. The "threshold value" of sugar or reducing substances which apparently is required for active fermentation is indicated in Fig. 2, curve 4. In actual determinations of diastatic power the addition of 0.03 g. of saponin or 0.5 g. of gelatin to the reacting mixture is necessary

[16] Ford and Guthrie, *J. Inst. Brew.*, **14**, 61 (1908).

to prevent adsorption at the surface. It should be noted that the filtration of any diastatic solution[17] is extremely likely to decrease its activity (Fig. 4, curve 4).

Limitations.—Experiments are limited to amylases which are active in the P_H range in which active fermentation occurs, *viz.*, 4.0 to 6.0. The fermentation rate also limits the ratio of enzyme to substrate concentrations which can be studied. When small concentrations of starch are involved (Fig. 7, 0.168% starch) it becomes necessary to substitute a 100-cc.

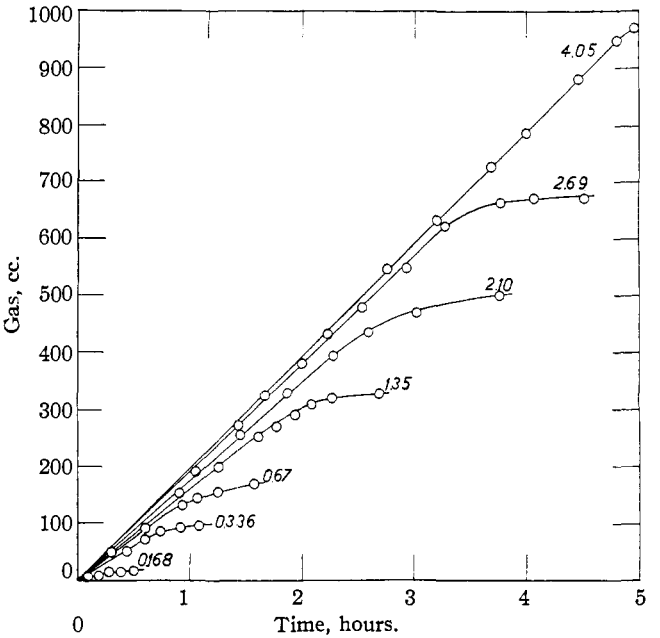

Fig. 7.—Wheat flour diastase: effect of variations in substrate concentration. Reacting solutions contained citrate buffer, 0.01 g. of saponin, 0.038 g. of flour, 5 g. of yeast; total volume 140 cc. and the percentages of soluble starch (dry basis) indicated by the figures.

gas buret for the one-liter fermentometer. In addition it is necessary to add the enzyme by a Victor Meyer capsule or similar device after the initial maltose has been fermented out to guard against change in concentration of carbon dioxide in the vapor phase. Smaller amounts of carbon dioxide formed may be measured by weighing in soda lime tubes. The principle of this method would lend itself to the investigation of invertase or maltase activity by the use of the proper invertase or maltase free yeast.

[17] D. J. Levy, Abstract 3091, Walton, "Comprehensive Survey of Starch Chemistry."

Summary

In the description of this investigation the following points have been presented.

1. Diastatic action of certain vegetable amylases in the absence of inhibitive agencies such as maltose, irreversible adsorption on interfaces due to shaking or on diatomaceous earth or filter paper pulp, is found to be a linear function of time and enzyme concentration throughout large variations in substrate concentration.

2. Adsorptive agents such as extended liquid–vapor surface films caused by continued shaking, diatomaceous earth or filter paper pulp are found to have marked inhibitive effects. The initial presence of small amounts of saponin, gelatin, albumin, casein or peptone, however, prevents this inhibition. Glycine and agar are found to be without effect.

3. Unpurified, ground soy bean diastase does not appear to be subject to adsorption at extended liquid–vapor surface films caused by shaking. Wheat flour and malt sirup enzymes, however, are quite subject to this effect.

4. The Michaelis constant for a flour diastase has been found to be about 0.25 in terms of percentage substrate concentration.

5. A convenient method for the study or determination of diastatic activity is described in which the sugars are fermented out by yeast as rapidly as formed, and the resulting carbon dioxide collected and measured at frequent intervals.

NEW YORK, N. Y.

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF THE JOHNS HOPKINS UNIVERSITY]

THE CHEMICAL COMPOSITION OF OIL OF RUVETTUS PRETIOSUS, THE "CASTOR OIL FISH"[1,2]

BY WARREN M. COX, JR., AND E. EMMET REID

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Ruvettus pretiosus was described ichthyologically by Cantraine[3] in 1837 and by Lowe[4] in 1841. The latter author reports, "it is affirmed that the bones abound in an oil or marrow, which, when they are sucked incautiously, produces diarrhea." Since this initial observation various in-

[1] The material in this article is extracted from a thesis submitted by Warren M. Cox, Jr., in partial fulfilment of the requirements for the degree of Doctor of Philosophy at the Johns Hopkins University.

[2] Presented to the American Chemical Society at its meeting in Atlanta, Georgia, April, 1930.

[3] F. J. Cantraine, "Memoire sur un Poisson nouveau (*Ruvettus temminckii*) trouvé dans le Canal de Messine, etc." Nouveaux Memoires Academie Royale Sciences et Belles Lettres, Bruxelles **1837**, Vol. 10, 1–2.

[4] Richard T. Lowe, "A Synopsis of the Fishes of Madeira, etc.," *Trans. Zoölogical Society*, London, 2, 180–181 (1841).