Since the absorption region of monochloroacetic acid reaches to longer waves than that of formaldehyde, in these experiments, the formaldehyde solution was illuminated in the reaction vessel I. It was proved that in the short wave region both monochloroacetic acid and formaldehyde absorbed completely in our arrangement all the emitted light.

In some of the experiments in Table VIII the decomposition of the monochloroacetic acid was calculated from the decomposition observed in thirty or sixty minutes in order to avoid inaccuracy due to the formation and light absorption of larger amounts of glycolic acid. The quantum yield in column 5 of Table VIII is the number of aldehyde groups disappearing per absorbed quanta. It is not yet possible to tell the mechanism of the decomposition. Since the amount of aldehyde disappearing and the amount of acid formed are of the same order of magnitude, it seems that in this case the formation of acid is one of the main reactions. According to Pribram and Franke[23] formic acid is one of the reaction products, beside the formation of glycolaldehyde.[24,25]

Although the formation of glycolaldehyde would suggest that the primary process is also in this case the formation of formaldehyde molecules with free valences, still further investigation of the photochemical reactions in the formaldehyde solution seems necessary. The distinct shift of the absorption spectra to short wave lengths in aqueous solution and the formation of acid make equally probable that in formaldehyde solution the primary process and the secondary reactions are of another type than those which we discussed for acetaldehyde and propionaldehyde.

A detailed investigation of the photochemistry of formaldehyde in monochromatic light and different solutions is planned.

(23) Pribram and Franke, *Ber.*, **44**, 1035 (1911).
(24) *Cf.* C. Neuberg, *ibid.*, **33**, 3207 (1900).
(25) *Cf.* Fischer and Landsheimer, *ibid.*, **25**, 2552 (1892).

Summary

1. The photochemical decomposition of formaldehyde, acetaldehyde, acetaldol, crotonaldehyde and of propionaldehyde has been investigated in aqueous solutions in the unfiltered light of a mercury arc.

2. The quantum yield, *i. e.*, the number of aldehyde molecules disappearing per absorbed quantum, is 0.5 for formaldehyde, 2.5 for acetaldehyde, 1.8 for propionaldehyde and very much smaller than unity for crotonaldehyde.

3. In the photolysis of acetaldehyde acetaldol is formed according to $2CH_3CHO = CH_3CH(OH)CH_2O$. It is suggested that the formation of an aldehyde molecule with free valences is the photochemical primary process.

4. When acetaldol is illuminated the reaction products formed are soluble in water, whereas from crotonaldehyde insoluble products are formed. In the photolysis of propionaldehyde and formaldehyde some acid is formed.

THE DANIEL SIEFF RESEARCH INSTITUTE
REHOVOTH, PALESTINE RECEIVED JULY 28, 1937

[CONTRIBUTION FROM THE FLEISCHMANN LABORATORIES, STANDARD BRANDS INCORPORATED]

A Fermentation Test for Vitamin B. II

BY ALFRED S. SCHULTZ, LAWRENCE ATKIN AND CHARLES N. FREY

Introduction

It has long been known that certain naturally occurring substances cause a stimulation in the rate of alcoholic fermentation by yeast.[1] As recently reported,[2] we have discovered that crystalline vitamin B_1 and the synthetic product are powerful accelerators of fermentation. The importance of this accelerating action on the rate of fermentation was recognized immediately and an attempt made to devise a rapid method of vitamin assay. The short time required to carry out an assay makes the method a valuable tool to both research and industry.

We have accepted the terminology suggested at the Chapel Hill meeting (1937) and henceforth give the name thiamine to vitamin B_1.

(1) Euler and Swartz, *Z. physiol. Chem.*, **140**, 146 (1924).
(2) Schultz, Atkin and Frey, THIS JOURNAL, **59**, 948 (1937).

Apparatus and Methods

The rate of fermentation is measured by the amount of gas evolved in a given time interval. The thermostat, shaker and gasometer system has been described.[3] To make a run we line up our six reaction bottles and place in them all the ingredients of the final reaction mixture except the yeast suspension. When all is ready, the yeast suspension is added to each by a fast pipet, the stoppers fixed in place, the bottles placed in the shaking cradle and connection promptly made to the gasometers. After two or three minutes have elapsed for temperature equilibration we make our initial or "zero" reading. This whole starting procedure is so timed as to give the same time interval (approximately four minutes) from the addition of the yeast to the "zero" reading.

In our preliminary work the reaction mixture was simply composed of yeast, sugar, ammonium phosphate buffer and distilled water. We now prefer to add a mixture of inorganic salts to the above so that no question of salt deficiency may interfere. The following standard control

(3) Schultz and Landis, *ibid.*, **54**, 211 (1932).

reaction mixture has been adopted tentatively: 15 ml., solution A, an ammonium phosphate buffer made by dissolving $NH_4H_2PO_4$ and $(NH_4)_2HPO_4$ in the ratio of 5 to 3 in such concentration that these salts will not exceed 5% in the reaction mixture; 15 ml., solution B, a solution of dextrose, not over 40% in concentration, together with salts of potassium, calcium, magnesium, and traces of manganese and iron; 25 ml., yeast suspension, commercial baker's yeast; 45 ml. distilled water.

A solution or suspension of a suspected fermentation stimulator may replace a portion of the distilled water. Readings of the gasometers may be made at any convenient interval; we use fifteen-minute periods.

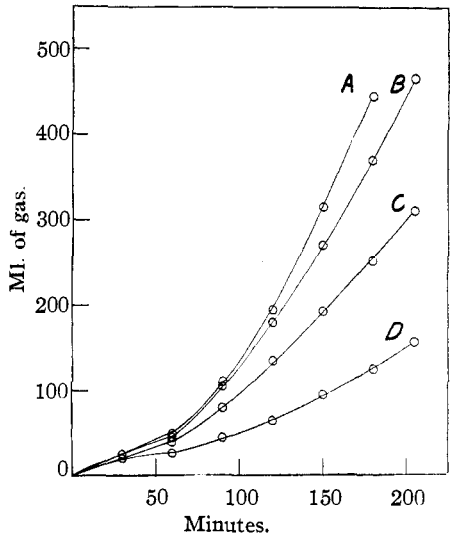

Fig. 1.—Maltose fermentation as affected by accelerators: A, yeast extract; B, soya meal; C, beet molasses; D, control.

Experimental Part

Figures 1 and 2 show the influence of certain substances on the rate of fermentation. These are older experiments and were made before the standard control reaction mixture was adopted, consequently solution B was not used. Furthermore, the measurement of the rate of fermentation of maltose was complicated by the presence of other sugars in the added substances. To overcome this difficulty we used a preliminary fermentation, *i. e.*, arranged the test so that all extraneous sugars were fermented out before the maltose was added. To compensate for the variation in sugar content between the different substances an additional half gram of dextrose was added to each at the beginning of this preliminary fermentation. We added the dextrose in order to eliminate any error due to a difference in the initial rate such as might easily occur if substance A contained an appreciable quantity of a fermentable sugar while substance B had none at all. These precautions were not considered necessary in the sucrose series, Fig. 2. Examination of these two figures shows that the relative stimulations produced by beet molasses, ground soy meal, and yeast extract are the same. The magnitude of the stimulation is shown likewise.

Thiamine gives a stimulation of equal magnitude and in Fig. 3 may be seen the effect of increasing quantities. Our standard reaction mixture is here used. When we apply this reaction to vitamin assay we prefer to use the three-hour gas total and to use quantities of material corresponding to 1–5 gammas of thiamine.

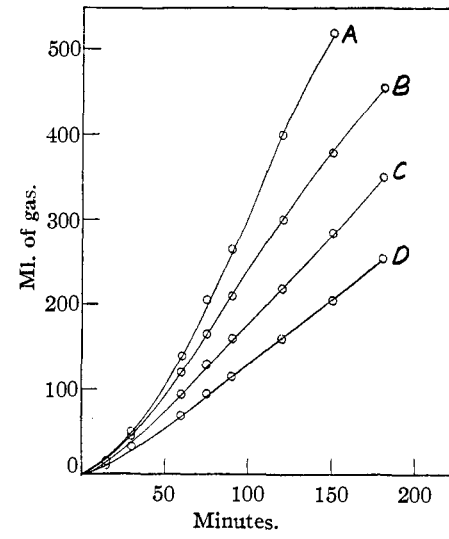

Fig. 2.—Sucrose fermentation as affected by accelerators: A, yeast extract; B, soya meal; C. beet molasses; D, control.

Extent of Fermentation

While investigating certain refinements of the sugar analysis method of Schultz and Kirby,[4] it was noted that the presence of certain substances caused an increase in the "yield" of gas, *i. e.*, ml. of gas per gram of sugar. This

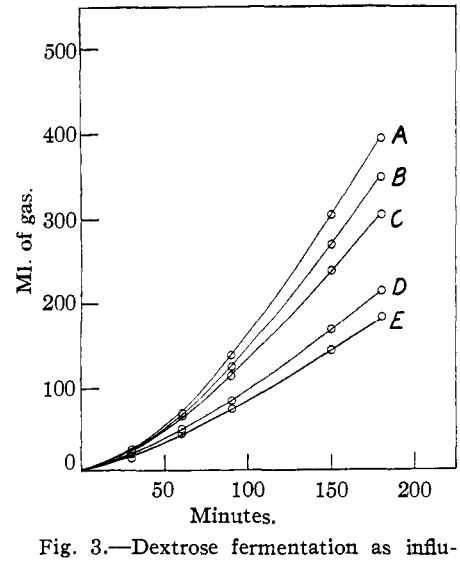

Fig. 3.—Dextrose fermentation as influenced by thiamine: A, 40 gamma of thiamine; B, 10 gamma; C, 5 gamma; D, 1 gamma; E, control.

increase in "yield" of gas is not surprising since it was known that the theoretical quantity of gas was not being

(4) Schultz and Kirby, *Cereal Chem.*, **10**, 149 (1933).

obtained. The substances which give the higher "yield" of gas are those substances known to contain the fermentation accelerator. It was therefore of interest to see whether thiamine has the same property. A normal control experiment, with no accelerator, gives about 210 ml. of gas per gram of sugar (dextrose). If a substance like ground soybean is added the yield of gas is increased by 10–15 ml. Thiamine gives a similar increase. The production of this additional gas is not a specific function of dextrose since it can also be obtained when sucrose or maltose replaces dextrose.

Another Accelerator

When we first started to use the gas test for thiamine assay we noted an anomalous situation. Autoclaved yeast shows no thiamine by rat growth but shows considerable gas stimulation. Furthermore, if a solution of the crystalline vitamin or thiamine is heated to boiling at an alkaline *p*H the vitamin is destroyed for rat growth but is still active according to the gas test. Therefore, when synthetic thiamine became available it occurred to us that the building blocks of the molecule might show activity. We obtained, therefore, from Merck and Company two substances used in the synthesis of thiamine, 4-methyl-5-β-hydroxyethylthiazole and 2-methyl-5-ethoxymethyl-6-aminopyrimidine.

Table I shows that the aminopyrimidine is effective in stimulating fermentation, whereas the thiazole is inactive. Thus we have another fermentation stimulator and, at the same time, a possible explanation for the anomalous effect of autoclaved yeast. A search was instituted among similar or related compounds for fermentation activators. None were found.

TABLE I

Addition, γ		Ml. gas 3/hrs.
None		185
100	thiazole	180
10	thiazole	185
100	2-methyl-5-ethoxymethyl-6-aminopyrimidine	350
10	2-methyl-5-ethoxymethyl-6-aminopyrimidine	363
4	thiamine	325
3	2-methyl-5-ethoxymethyl-6-aminopyrimidine	335

Thiamine Assay by the Gas Method

Since it is quite apparent that the fermentation stimulation is not a specific test for thiamine, caution must be observed in interpreting results. Like any indirect test the results must be correlated from time to time with the ultimate standard, animal growth, unless of course the crystalline compound is eventually taken as the standard. We find that when we analyze a series of yeasts or vitamin concentrates the accuracy with which we can check ourselves is as great as the accuracy of the gas measurement. Solutions for assay are prepared by making the unknown to volume and then sterilizing for twenty-five minutes in an Arnold sterilizer. Care is taken that the reaction of the solution before sterilization is acid to litmus. A quantity of the unknown is then used which will give a stimulation corresponding to some point between 2 and 5 gamma of the pure vitamin.

The use of our discovery for thiamine assay very largely depends upon the reproducibility of the stimulation. If we compare the total gas production, *i. e.*, control plus stimulation, in the 4 gamma test over any period of days we find an unsatisfactory variation. The same is true of the 2 gamma test. If, however, we take the daily difference between these two totals ($\Delta_{4\gamma-2\gamma}$) we find a very satisfactory constancy. Table II elucidates this effect. In this table we have also expressed the standard deviation, $\sigma\Delta_{4\gamma-2\gamma}$ in terms of International B_1 units. This is based on the conclusion that 4.0 gamma of pure thiamine is equivalent to 1 International unit. A gas test made with the Int. vitamin B_1 adsorbate showed this relation to be true within the limits of error.

TABLE II

AVERAGE OF 14 PAIRS OF MEASUREMENTS

	Average ml. gas	A. D. ml. gas	σ ml. gas	σ Int. B_1 Units
4 gammas	329.2	8.76	9.95	
2 gammas	264.7	6.34	8.07	
$\Delta_{4\gamma-2\gamma}$	64.5	3.3	4.23	0.032

Correlation with Rat Tests

In applying our method of assay we must regard 2 - methyl - 5 - ethoxymethyl - 6 - aminopyrimidine as an interfering substance. There is no qualitative test for this interfering substance and therefore recourse must be had to the rat test. If any significant difference should occur between the gas and the rat test for a particular type of product, the presence of the interfering substance may be suspected. It is fortunate that, with the exception of autoclaved yeast, we have found no natural source of the interfering pyrimidine or of its equivalent. Twenty-one parallel assays (gas and rat test) on yeasts and concentrates showed an average variation of 14.6% when the sign of the variation was disregarded.

Acknowledgment.—We wish to express our thanks to Merck and Company for the samples of thiamine, the pyrimidine and the thiazole compounds. The thiamine assays (rat growth method) were made by R. F. Light, W. N. Benson and L. J. Cracas of Standard Brands.

Summary

Thiamine is a stimulator of fermentation. 2 - Methyl - 5 - ethoxymethyl - 6 - aminopyrimi-

dine is likewise a stimulator of fermentation. Increased "yield" of gas is caused by fermentation in the presence of these substances. The vitamin assay method described (gas method) is a rapid and reliable one for research or control purposes. Details are given for the performance of the above tests.

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NEW YORK, N. Y.

RECEIVED JUNE 14, 1937

[CONTRIBUTION FROM THE LABORATORIES OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

The Equilibrium of the Semiquinone of Phenanthrene-3-sulfonate with its Dimeric Compound

BY L. MICHAELIS AND E. S. FETCHER, JR.

Previous studies of the oxidation-reduction intermediates of various dyestuffs[1] have resulted in the conclusion that these intermediates are monomolecular radicals. By analogy to radicals of the triphenylmethyl and diphenylnitride type, which have long been known to exist in equilibrium with their dimeric, valence-saturated compounds, it is of interest to examine the possibilities of a similar dimerization of the radicals in question.

Dimerization, a bimolecular reaction, will depend upon concentration. Hitherto all studies have been made in the rather dilute solutions imposed by the limited solubility of most of the substances used. The concentration usually has varied from 10^{-4} to 3×10^{-3} mole/liter. Few dyestuffs of otherwise suitable properties are sufficiently water soluble to permit of any great extension of this concentration range. One such, however, has been found in potassium phenanthrenequinone-3-sulfonate, recently studied in dilute solution.[2] It is the purpose of this paper to extend the investigation of the course of the potentiometric titration of this dye to comparatively high concentrations.

Critical Review of the Experimental Technique.—In order to obtain comparable titration curves for widely varied concentrations of the dye and thus to examine the influence of the concentration upon these curves, it is necessary that two conditions be held constant, the ionic strength and the *p*H. The difficulty in maintaining these is increased by a high concentration of the dye, and it is virtually impossible to do this exactly.

In acid solution, the influence of the ionic strength is negligible within the range of its variation occurring in the experiments. This is not quite so in alkaline solutions, in which the dye is soluble enough to allow of a wider range in ionic strength. In two otherwise comparable experiments at *p*H 12.2, the index potential increased 5 to 7 mv. on increasing the ionic strength from $\mu = 0.1$ to 1.0. Hence, all titrations to be compared with each other should represent experiments at an approximately equal ionic strength. In order to overcome as much as possible the effect of the polyvalent ferricyanide ion added during the titration, the initial ionic strength of the dye solution was chosen as high as feasible. Two ranges of *p*H had to be selected for the experiments, one around *p*H 12.2, the other around 4.6. For *p*H 12.2, a 0.05 *M* solution of sodium hydroxide was made 1.0 molar with potassium chloride. At *p*H 4.6, we had to be satisfied with ionic strength $\mu = 0.2$. At a higher salt concentration the solubility of the dye is not great enough to reach the dye concentration desired. Acetate buffer of $\mu = 0.1$ was used, and μ was doubled by addition of potassium chloride. These salt concentrations were high enough to maintain the ionic strength sufficiently constant during any one titration, as well as over the range of dye concentrations used.

The second condition, that the *p*H should be the same in all experiments to be compared, need be fulfilled only approximately for the following reason. We wish to compare not the absolute values of the potentials, which of course depend on *p*H, but the shape of the curve, as expressed by the slope at comparable points. This shape does not depend at all on minor variations of *p*H in certain ranges. There is one such range from about *p*H 3 to 5, in which the shape of the titration curve is practically identical with that of a dye with no intermediate step. There is a second range at *p*H > 11.5, where the three normal potentials E_1, E_m, and E_2, plotted against *p*H,

(1) L. Michaelis, *Chem. Rev.*, **16**, 244 (1935).

(2) L. Michaelis and M. P. Schubert, *J. Biol. Chem.*, **119**, 133 (1937).