THE EFFECT OF TETRAMETHYLTHIURAM DISULFIDE ON THE ANAEROBIC BREAKDOWN OF GLUCOSE BY YEAST

By

Hugh D. Sisler

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 - 1346

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INTRODUCTION

In a previous study it was shown that the fungicidally active compound tetramethylthiuram disulfide (TMTD) inhibits respiration and fermentation (anaerobic CO_2 production) of Fusarium roseum Link (Sisler 1951). There appear to be no reports in the literature, however, which describe the effects of TMTD on specific enzyme systems involved in cellular metabolism. Manten, et al. (1950) found that the growth of Saccharomyces cerevisiae Meyen, a facultative anaerobe, was completely inhibited by 15 ppm. TMTD under aerobic conditions whereas under anaerobic conditions 50 ppm. were necessary. In an investigation of the fungicidal action of sulfur compounds, Klöpping (1951) concluded, on the basis of studies of respiration and of other studies, that TMTD does not disturb the common energy producing systems, but that it affects more specific processes in fungi, probably those belonging to the assimilatory phase of metabolism.

Sijpesteijn and van der Kerk (1952) showed that histidine may antagonize the toxic effect of TMTD on spore germination and suggested that the fungicide may interfere with the biosynthesis of histidine or with some essential reaction requiring histidine. They also reported that cysteine and methionine have slight TMTD-antagonizing activity.

The closely related compound tetraethylthiuram disulfide, TETD, would be expected to have a qualitative biological action almost identical with that of TMTD. Klöpping (1951) found that both compounds possessed essentially the same fungicidal activity. A review

of the results of studies of the effects of TETD on animal metabolism should contribute to an understanding of the action of TMTD on fungi.

Edwards (1949) and Nowinski, et al. (1950) have shown that TETD inhibits oxygen uptake by rat liver homogenates. The latter workers have reported reversal of inhibition by ascorbic acid and have concluded that TETD inhibits by acting as a competitive hydrogen acceptor.

A number of workers have reported inhibition of certain enzymes of animal origin by TETD. Keilin and Hartree (1940) found that aerobic oxidation of succinic acid by preparations of animal tissue was strongly inhibited following addition of sodium diethyldithiocarbamate, a reduction product of TETD. They found, however, that the inhibition of succinic dehydrogenase was caused by tetraethylthiuram disulfide, derived from the dithiocarbamate by an oxidation catalyzed by the cytochrome oxidase system, rather than by the dithiocarbamate itself. Alcohol treated enzyme preparations which lacked the property of reacting with cytochrome c, but which would catalyze the oxidation of succinate, as indicated by methylene blue reduction, were only slightly affected by TETD. The oxidation of para-phenylene diamine was not affected by TETD.

Hald and Jacobsen (1948) and Asmussen, et al. (1948) obtained evidence that the oxidation of ethanol was blocked at the acetaldehyde stage in human subjects when they were treated with TETD (antabuse, used clinically in the therapy of alcoholism) previous to ingestion of alcohol.

Kjeldgaard (1949) found that TETD inhibited aldehyde oxidase which was prepared from rabbit liver according to the method of Gordon, et al. (1940). Graham (1951) found that the Racker (1949) diphosphopyridine

nucleotide-linked aldehyde dehydrogenase from rat liver also was strongly inhibited by TETD. The drug apparently acted as a competitive inhibitor, competing with diphosphopyridine nucleotide (DPN) for active centers of the enzyme. A molar ratio of glutathione to TETD of 3 to 1 gave 50 percent restoration of activity while a molar ratio of 1700 to 1 of ascorbic acid to TETD was required for the same percentage of restoration. Graham further found that TETD also inhibited the breakdown of added citrate, succinate and ethanol by rat liver homogenates. When succinate was used, increasing the duration of contact between TETD and homogenate prior to the addition of substrate increased the degree of inhibition. Glutathione reversed, to a large extent, the inhibition by TETD of the oxidation of acetaldehyde and other substrates. In the case of ethanol or acetaldehyde, less inhibition occurred when DPN was added to the reaction mixture.

Richert, et al. (1950) found that TETD inhibited the oxidase portion of xanthine oxidase from rat liver. Xanthine oxidase from milk was not affected. They also found that succinoxidase was inhibited while d-amino acid oxidase was not affected.

Nygaard and Summer (1952) showed that triosephosphate dehydrogenase from animal tissue was strongly inhibited by TETD. Cysteine largely prevented this inhibition.

Relatively little has been reported on the effect of TETD on fungus metabolism. Ignazio (1951), however, has shown that it inhibits oxygen consumption, anaerobic glycolysis and dehydrogenase activity of bread yeast.

The present study was undertaken to determine which enzymatic processes in the anaerobic breakdown of glucose by yeast are affected

by TMTD, with the purpose of more clearly elucidating the mechanism by which this compound inhibits fungi.

MATERIALS AND METHODS

In order to study the effect of TMTD on the anaerobic breakdown of glucose, a preparation of dried brewers yeast[1], Saccharomyces cerevisiae Meyen, was made according to the method described by Meyerhof (1949). This dried yeast preparation would ferment glucose as well as various intermediates in the process of fermentation of glucose and it proved to be satisfactory for the purposes of this investigation. The fermentative activity of this preparation has remained constant for over a year while stored under refrigeration in a flask fitted with a paraffin treated cork.

For Warburg manometric studies of CO_2 production, 1.2 g. of the dried yeast preparation (intact dry cells), were suspended in 100 ml. of buffered solution[2]. One-ml. aliquots of this suspension, containing 12 mg. of air dried yeast, were added to each vessel.

Fresh (living) brewers yeast was also used in certain experiments. These cells were washed thoroughly with tap water, suspended in 0.04 M phosphate buffer (pH 6.3) and stored in the refrigerator. For use in fermentation experiments, aliquots of this stock suspension were diluted 1-100 with 0.04 M phosphate buffer and a one-ml. aliquot of this diluted suspension was added to each Warburg vessel.

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[1]Thanks are due Mr. Francis Omler of the Christian Heurich Brewing Co., Washington, D. C., for furnishing a liberal supply of fresh brewers yeast.

[2]This solution was composed of 0.02 M KH_2PO_4 and 0.01 M $MgCl_2$ adjusted to pH 6.5 with KOH. All materials added to the dried yeast preparation in the reaction vessels were likewise dissolved in or suspended in this buffered solution.

The dry weight of yeast in one ml. of the diluted suspension was 0.32 mg. after drying at 90° C. for 18 hours.

Manometric measurement of CO_2 released during fermentation was made at 30° C. The total fluid volume was two ml. The gas space of the vessels was filled either with N_2 or with 95 percent N_2 plus five percent CO_2 freed from oxygen by passing the gas over hot copper turnings. Where direct comparisons were made no differences in behavior attributable to differences in the gas phase were observed.

Since small amounts of CO_2 are retained in solution within the range of pH at which these experiments were performed, the CO_2 values which are reported may differ somewhat from the absolute. However, in all cases the CO_2 values for various treatments are comparable since the pH values of the various treatments at the conclusion of the experiments were either identical or varied only slightly from each other (a discrepancy of approximately three percent between CO_2 values for the widest difference in final pH).

The potassium salt of 3-phosphoglyceric acid (PGA) was prepared from the barium salt[1] by dissolving the latter in 0.1$\frac{1}{4}$ N HCl, removing the barium as the sulfate by addition of the calculated equivalent weight of K_2SO_4 and centrifuging to remove the barium sulfate. Sufficient KH_2PO_4 and $MgCl_2$ were added so that in the final solution the molarity of inorganic phosphate and $MgCl_2$ would be the same as that in the regular buffered solution. The pH was adjusted

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[1]Nutritional Biochemicals Corporation, Cleveland, Ohio.

to 6.5 with KOH and the solution made to volume with distilled water.

Hexose diphosphate (HDP) was also used as the potassium salt prepared from the barium salt[1] in the same manner as the phosphoglycerate. The concentration of HDP was determined by the fructose method of Roe (1934). This preparation of HDP was used in studies in which the effect of TMTD on utilization of glucose, HDP, PGA and pyruvate were compared. In other experiments the unmodified magnesium salt (Schwarz Laboratories) was used as a source of HDP.

In order that the PGA, HDP and glucose substrates should be approximately equivalent with respect to inorganic ion concentration, solutions of glucose containing the same amount of HCl, KH_2PO_4, $MgCl_2$ and KOH as was used in preparing the HDP and PGA solutions were employed in the studies of fermentation by the dried yeast preparations. It was later determined that the TMTD effect on fermentation of glucose in solutions prepared in this fashion did not differ from its effect on fermentation of glucose in the regular buffered solution.

Adenosine triphosphate (ATP) as the tetrasodium salt and DPN (65 percent) were obtained from Schwarz Laboratories.

The TMTD[2] was purified by repeated recrystalization from chloroform solution. Uncorrected melting point of the purified preparation was 154-155° C. In making suspensions of this compound, a wetting

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[1]Schwarz Laboratories, Inc., New York, N. Y.

[2]The original sample of TMTD was obtained through the courtesy of E. I. du Pont de Nemours and Company, Inc., Wilmington, Del.

agent[1] was used to facilitate dispersal of the particles. An equivalent amount of wetting agent was added to all vessels. The final concentration was 0.00125 percent. At this concentration it has been shown previously, Sisler (1951), to have no measurable effect upon growth and respiration of F. roseum.

The yeast suspension was placed in the main compartment of the Warburg vessel while substrate and such factors, when used, as DPN, ATP, adenosine, cysteine, etc., were placed in one sidearm. TMTD (or buffer with wetting agent in case of control vessels) was placed in the other sidearm. The contents of the sidearms were added to the yeast suspension at the desired time during the experiment.

Acetaldehyde analysis was carried out in the following manner: At the conclusion of manometric determination of CO_2 production by dried yeast cells fermenting HDP, the manometers were removed from the water bath and the vessels, while still attached to the manometers, were immersed in ice water at 1° C. for approximately ten minutes. One-ml. aliquots of the contents of each of the vessels were pipetted into tubes containing one ml. of cold 10 percent sodium tungstate. One ml. of 0.67 N cold sulfuric acid was then added to each tube. Seven ml. of ice water were added and the tubes were plugged and centrifuged. The supernatant was removed and stored in a refrigerator. A three-ml. aliquot of the supernatant was placed in a distillation apparatus and neutralized to pH 7.0 with cold sodium

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[1] A commercial product designated as "WA" by the O. E. Linck Co., Inc., Montclair, N. J. The active ingredient in this product is Triton X-100 which is chemically an alkyl aryl polyether alcohol.

carbonate to prevent distillation of volatile acids such as pyruvic acid. Distillation and colorimetric determination of the acetaldehyde were then carried out according to the method of Stotz (1943).

The effect of TMTD on aldolase activity of yeast extracts was determined by a colorimetric method which was an adaptation of the method of Sibley and Lehninger as modified by Dounce, et al. (1950) for determining aldolase activity of animal tissue. Departures from the method described by Dounce, et al. were: (1) final concentration of HDP, 0.004 M instead of 0.005 M; (2) incubation temperature, 30° C. instead of 25° C.; (3) use of one ml. of 20 percent instead of two ml. of 10 percent trichloroacetic acid (this change was necessary so that following addition of trichloroacetic acid and preceding centrifugation all samples could be made equivalent with respect to added materials without causing a change in final volume and concentration of reactants); and (4) addition of hydrazine and buffer as a combined solution of one-ml. volume instead of separately in a total volume of 1.25 ml. (this permitted addition of TMTD, cysteine, etc., without exceeding the 2.5 ml. final incubation volume prescribed by the method).

As a source of aldolase, 0.5 g. of dry yeast cells was extracted for 30 minutes at 30° C. with 100 ml. of distilled water. One half ml. of the cell-free extract was used in each sample.

EXPERIMENTAL RESULTS

The effect of TMTD on fermentation of glucose was studied with fresh (living) yeast and with a preparation of dried yeast cells. The dried yeast preparation was used to investigate the effect of TMTD on the utilization of various intermediates in the process of fermentation because dried cells, unlike living cells are permeable to phosphate esters and coenzymes (Meyerhof, 1949). Fig. 1 shows an abbreviated scheme for fermentation of glucose by yeast. In all manometric determinations, CO_2 production was used as a measure of fermentative activity.

It was necessary to supply a phosphate acceptor (adenosine) in order to maintain active fermentation by the dried cells because adenosine triphosphatase (ATPase), which in living cells makes available a phosphate acceptor by splitting ATP, was apparently destroyed, at least in part, during the drying process to which the cells were subjected. In fig. 2 is shown an example of the adenosine effect on the course of fermentation of HDP by dried yeast cells. Also shown in this figure is the effect of an atmosphere of air and an atmosphere of 95 percent N_2 plus five percent CO_2 on fermentation of HDP. Even though there was no appreciable Pasteur effect as a result of the presence of oxygen, all subsequent experiments were carried out under anaerobic conditions. The necessity of added adenosine for a high rate of fermentation of HDP is apparent. For the sake of uniformity, one mg. of adenosine was added to each Warburg vessel in all subsequent manometric studies of fermentation

of all substrates even though its requirements for fermentation of substrates other than HDP was not established.

ATP is needed to initiate phosphorolation of glucose and was added as Na_4 ATP at the rate of one mg. per vessel when fermentation of glucose by the dried yeast preparation was studied. It was also used at the same rate in certain experiments with other substrates, but it had no effect upon their fermentation.

The effect of a range of concentrations of TMTD on fermentation of glucose by living yeast cells is shown in fig. 3. It is apparent that the higher concentrations of TMTD (1×10^{-3} and 5×10^{-4} moles/l.) inhibited fermentation of glucose during the entire course of the experiment. The lower concentrations (5×10^{-5} and 1×10^{-5} moles/l.) inhibited only in the latter part of the experiment and to a much lesser extent. Inhibition by the intermediate concentration (1×10^{-4} moles/l.) during the early part of the experiment was similar to that produced by the higher concentrations. In the latter part of the experiment, however, inhibition by this concentration, as indicated by the slope of the curve, was almost identical with that caused by the two lower concentrations.

In order to determine which portions of the process of fermentation are affected by TMTD, a comparison was made of its effect upon fermentation by dried yeast cells of glucose, of HDP, of PGA and of pyruvate at 0.01 M concentrations. Shown in fig. 4 is the effect of two concentrations of TMTD on fermentation of glucose when TMTD and substrate were added simultaneously. A concentration of TMTD of 1×10^{-4} moles/l. had a moderate inhibitory effect,

Fig. 1. An abbreviated scheme of fermentation of glucose by yeast.

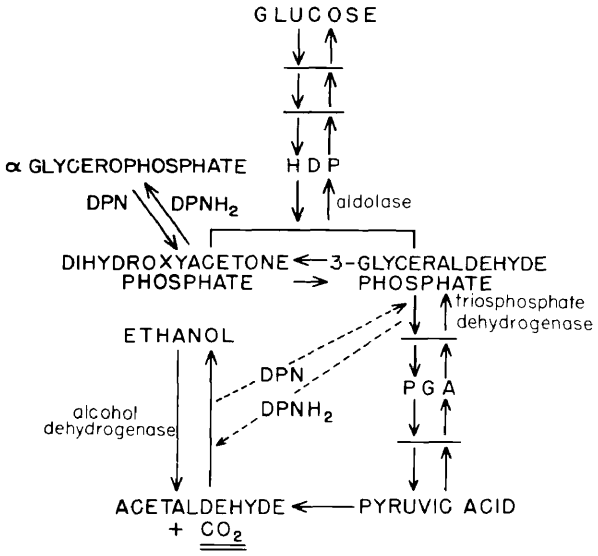

GLUCOSE
α GLYCEROPHOSPHATE
H D P
DPN
DPNH2
aldolase
DIHYDROXYACETONE PHOSPHATE
3-GLYCERALDEHYDE PHOSPHATE
triosphosphate dehydrogenase
ETHANOL
DPN
DPNH2
P G A
alcohol dehydrogenase
ACETALDEHYDE + CO2
PYRUVIC ACID

Fig. 2. Effect of adenosine and of composition of atmosphere upon fermentation of hexose diphosphate (HDP) by dried yeast cells as indicated by CO_2 production. HDP and adenosine were added at beginning of experiment. All vessels contained 0.01 M HDP and one mg. of adenosine. Initial pH, 6.5; final pH, 6.1.

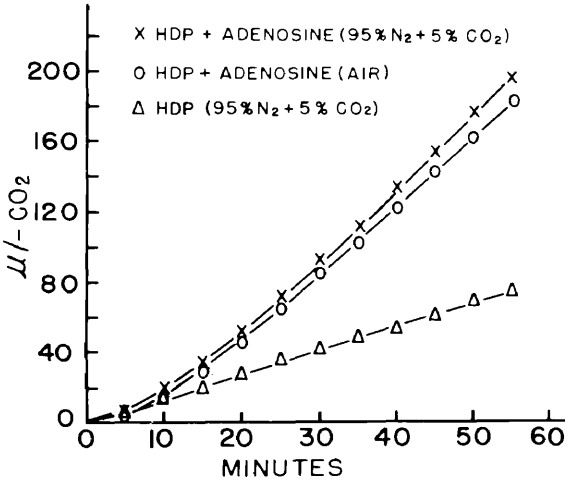
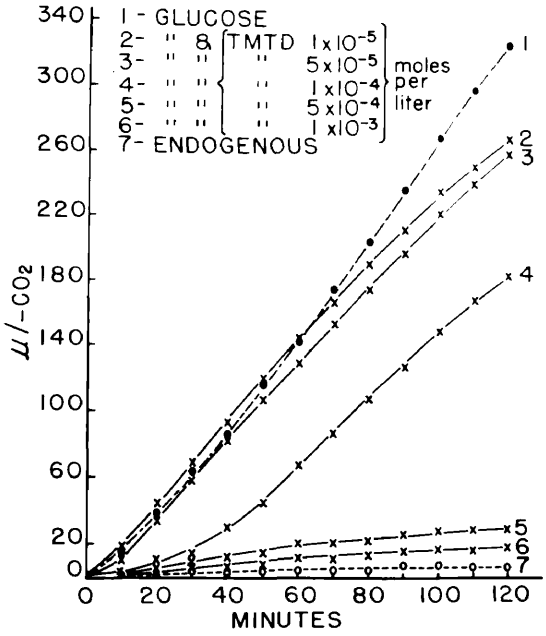

X HDP + ADENOSINE (95% N2 + 5% CO2)
O HDP + ADENOSINE (AIR)
Δ HDP (95% N2 + 5% CO2)
200
160
120
80
40
0
μl - CO2
0
10
20
30
40
50
60
MINUTES

Fig. 3. Effects of several concentrations of tetramethylthiuram disulfide (TMTD) on fermentation of glucose by living yeast cells as indicated by CO_2 production. Glucose and TMTD were added at the beginning of the experiment. All except seven contained one percent glucose. Gas phase was 95 percent N_2 plus five percent CO_2; initial pH, 6.3; final pH, 6.1-6.2.


1 - GLUCOSE
2- " & TMTD 1x10-5
3- " " " 5x10-5
4- " " " 1x10-4
5- " " " 5x10-4
6- " " " 1x10-3
moles per liter
7- ENDOGENOUS
μl/-CO2
340
300
260
220
180
140
100
60
20
0
0 20 40 60 80 100 120
MINUTES
1
2
3
4
5
6
7

whereas 5×10^{-4} moles/l. virtually eliminated fermentation of added glucose. It was determined in a number of experiments that TMTD had no effect on the rate of endogenous fermentation of the dried yeast cells.

The effect of the same two concentrations of TMTD on fermentation of HDP (fig. 5) was essentially the same as that on fermentation of glucose. When fermentation of HDP was allowed to become well established before addition of inhibitor, (fig. 6) 5×10^{-4} moles/l. quickly suppressed this established rate to a level characteristic of dried yeast suspensions to which HDP and TMTD, 5×10^{-4} moles/l. were added simultaneously (fig. 5).

The effect of TMTD, 1×10^{-3} moles/l. upon fermentation of PGA is shown in fig. 7. When TMTD and PGA were added to the dried yeast preparation simultaneously, CO_2 production was only slightly depressed. A concentration of TMTD of 5×10^{-4} moles/l. produced similar results. When TMTD at these concentrations was incubated with the yeast suspension for 45 minutes previous to the addition of PGA, inhibition of fermentation of this compound approximated 40 percent.

With pyruvate as the substrate, CO_2 production by dried yeast suspensions with TMTD, 1×10^{-3} or 5×10^{-4} moles/l., was in some experiments slightly above and in others slightly below that of untreated suspensions. Results of such an experiment are illustrated in fig. 8.

Although pre-incubation of dried yeast suspension with the inhibitor resulted in appreciable inhibition of fermentation of PGA, in none of the experiments was fermentation of either PGA or

Fig. 4. The effect of two concentrations of tetramethylthiuram disulfide (TMTD) on fermentation of glucose by dried yeast cells as indicated by CO_2 production. Glucose, adenosine, ATP and TMTD were added at time indicated by arrow. Gas phase, 95 percent N_2 plus five percent CO_2; initial pH, 6.5; final pH, glucose 5.9, glucose and TMTD (1×10^{-4} moles/l.) 5.9, glucose and TMTD (5×10^{-4} moles/l.) 6.0, endogenous 6.1.

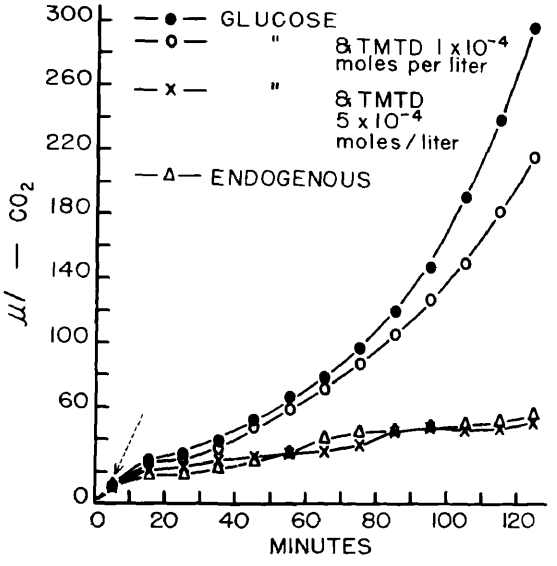

300
260
220
180
140
100
60
0
μl — CO2
—●— GLUCOSE
—o— "
—x— "
—Δ— ENDOGENOUS
& TMTD 1 x 10-4 moles per liter
& TMTD 5 x 10-4 moles / liter
0
20
40
60
80
100
120
MINUTES

Fig. 5. The effect of two concentrations of tetramethylthiuram disulfide (TMTD), 1 and 5 X 10^{-4} moles/l., on fermentation of hexose diphosphate (HDP) by dried yeast cells as indicated by CO_2 production. HDP, ATP, adenosine and TMTD added at time indicated by arrow; gas phase, 95 percent N_2 plus five percent CO_2; initial pH, 6.5; final pH, 6.2 except endogenous which was 6.1.

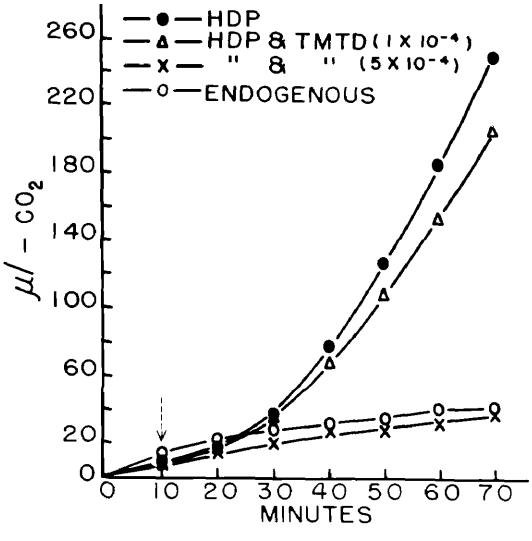

—●—HDP
—△—HDP & TMTD (1 X 10⁻⁴)
—x— " & " (5 X 10⁻⁴)
—o—ENDOGENOUS
260
220
180
140
100
60
20
0
μl – CO₂
0 10 20 30 40 50 60 70
MINUTES

Fig. 6. Effect of tetramethylthiuram disulfide (TMTD), 5×10^{-4} moles/l., upon fermentation of hexose diphosphate (HDP) by dried yeast cells as indicated by CO_2 production when TMTD was added 25 minutes after addition of HDP. Adenosine and HDP were added at beginning of experiment; TMTD at time indicated by arrow. Gas phase was 95 percent N_2 plus five percent CO_2.

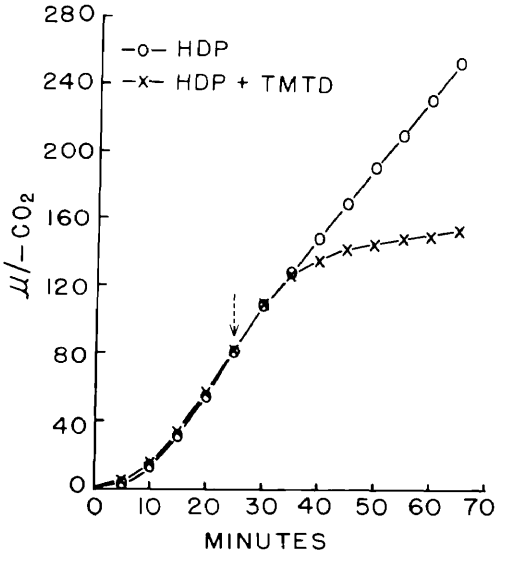

280
240
200
160
120
80
40
0
μl – CO2
–o– HDP
–x– HDP + TMTD
0 10 20 30 40 50 60 70
MINUTES

Fig. 7. The effect of tetramethylthiuram disulfide (TMTD), 1×10^{-3} moles/l., on fermentation of 3-phosphoglyceric acid (PGA) by dried yeast cells as indicated by CO_2 production. Adenosine, PGA, TMTD and ATP were added to the yeast suspension at the time indicated by arrow. Gas phase was N_2.

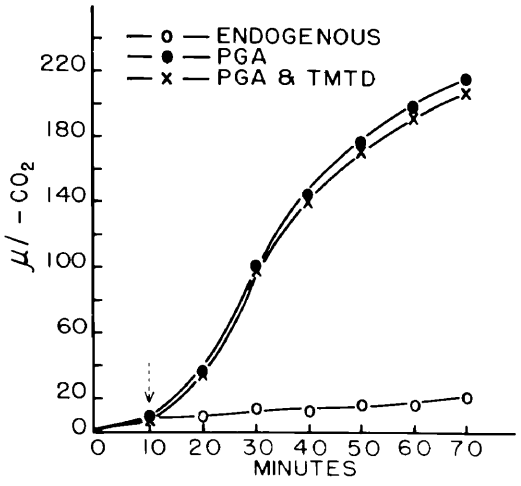
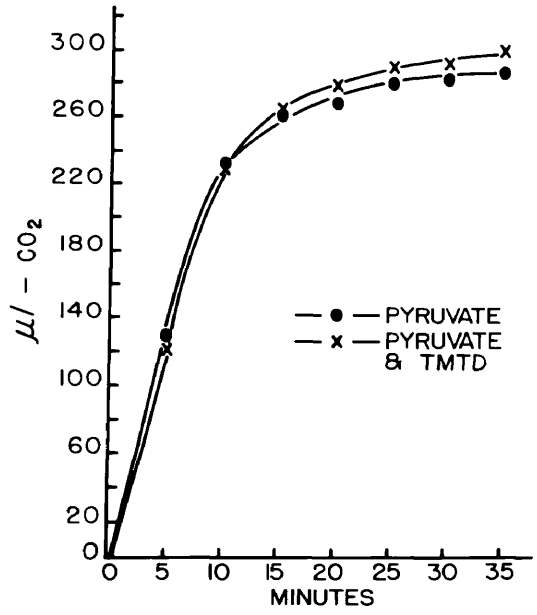

— o — ENDOGENOUS
— ● — PGA
— x — PGA & TMTD
220
180
140
100
60
20
0
μl - CO2
0 10 20 30 40 50 60 70
MINUTES

Fig. 8. Effect of tetramethylthiuram disulfide (TMTD), 1 X 10^{-3} moles/l., upon fermentation of pyruvate by dried yeast cells as indicated by CO_2 production. TMTD, pyruvate, ATP and adenosine were added to the yeast suspension at the beginning of the experiment. Initial pH 6.5; final pH, TMTD plus pyruvate 6.6, pyruvate 6.7; gas phase N_2.


300
260
220
180
140
100
60
20
0
μl - CO2
0 5 10 15 20 25 30 35
MINUTES
— ● — PYRUVATE
— x — PYRUVATE & TMTD

of pyruvate so profoundly affected as was that of glucose or of HDP.

Results of the experiments which have been described indicate that the main effect of TMTD on fermentation of glucose is inhibition of conversion of HDP to PGA. The first step in this conversion, which is catalyzed by the enzyme aldolase, involves splitting the 6-carbon compound, HDP to form two triosephosphates, dihydroxyacetone phosphate and glyceraldehyde-3-phosphate.

The effect of a range of concentrations of TMTD on the aldolase activity of a cell-free extract of dried yeast was determined. Fig. 9 shows that aldolase activity is inhibited by concentrations of TMTD of 5, 10 and 50 X 10^{-5} moles/l., whereas a concentration of 1 X 10^{-5} moles/l. stimulates aldolase activity. This stimulation occurred consistently in a number of experiments and might possibly be attributed to binding of traces of inhibitory heavy metals which may be present in the preparations by this low concentration of TMTD which is not inhibitory. Stimulation also occurred in the presence of 4 X 10^{-4} M iodoacetate, a triosephosphate dehydrogenase inhibitor. Concentrations of TMTD of 0.1 X 10^{-5} and 0.5 X 10^{-5} moles/l. had no effect upon aldolase activity.

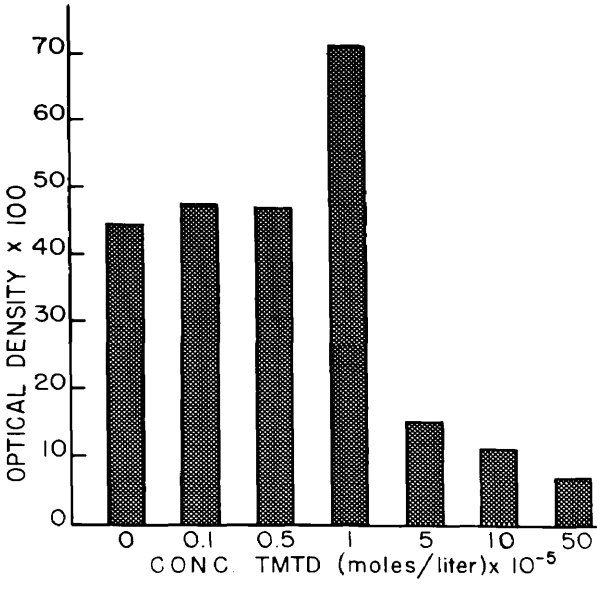
Glutathione or cysteine effectively prevents inhibition of aldolase activity by TMTD. A concentration of 2 X 10^{-3} M cysteine completely eliminated the inhibitory effect of TMTD, 5 X 10^{-4} moles/l. (fig. 10).

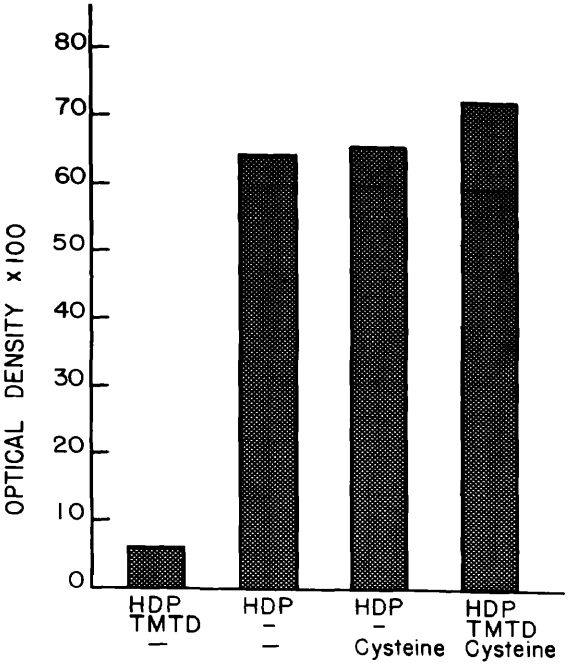
With dried yeast cells and HDP, both at the same concentrations as were used in the manometric determinations of CO_2 production, the effect of TMTD at 5 X 10^{-4} and 1 X 10^{-4} moles/l. on aldolase activity were similar to that of the same concentrations of TMTD on aldolase

Fig. 9-10. Aldolase activity of a cell-free extract of yeast as indicated by the optical density of the 2,4-dinitrophenylhydrazine derivative of the trioses.

Fig. 9. The relative effect of a range of concentrations of tetramethylthiuram disulfide (TMTD) on aldolase activity. Concentration of hexose diphosphate (HDP) was 0.004 M. The data represent the mean of four experiments.

Fig. 10. Prevention of TMTD-inhibition of aldolase activity by cysteine. TMTD, 5×10^{-4} moles/l.; cysteine, 2×10^{-4} M; HDP was 0.004 M. The data represent the mean of four experiments.


OPTICAL DENSITY x 100
0
10
20
30
40
50
60
70
0
0.1
0.5
1
5
10
50
CONC. TMTD (moles/liter)x 10^{-5}

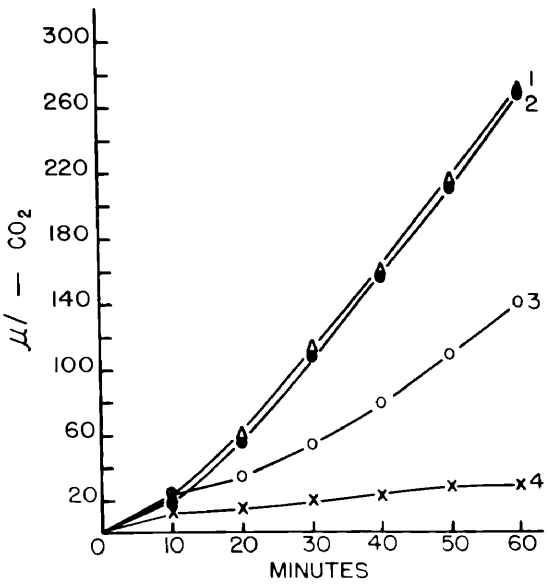

OPTICAL DENSITY x100
0
10
20
30
40
50
60
70
80
HDP
TMTD
—
HDP
—
—
HDP
—
Cysteine
HDP
TMTD
Cysteine

activity of the cell-free extracts. Glutathione or cysteine also offsets the TMTD effect under these conditions. Similar results were obtained by the method of Sibley and Lehninger (1949) when 0.62 mg. of dried yeast per sample in suspension were used as a source of aldolase.

TMTD-inhibition of fermentation of HDP by dried yeast preparations may likewise be prevented with cysteine or glutathione. The effect of 2×10^{-3} M cysteine in offsetting inhibition of fermentation of HDP by TMTD, 5×10^{-4} moles/l., is shown in fig. 11. When cysteine was added to the yeast suspension simultaneously with HDP and TMTD, there was no suppression of the rate of CO_2 production. However, if the inhibitor was incubated with the yeast suspension for 30 minutes previous to the addition of HDP and cysteine, only partial protection was brought about by the cysteine. Keilin and Hartree (1940) obtained similar results with glutathione on inhibition of aerobic oxidation of succinic acid by TETD. In experiments not illustrated a concentration of 1×10^{-3} M cysteine offset approximately 96 percent of the TMTD-inhibition and a concentration of 5×10^{-4} M offset approximately 40 percent of the inhibition when added to the dried yeast preparation simultaneously with HDP and TMTD at a concentration of 5×10^{-4} moles/l.

Cysteine or glutathione also protects fermentation of glucose by fresh yeast cells from the effect of TMTD. The effect of glutathione is shown in fig. 12. It might be noted that in the latter part of the experiment, some inhibition occurs even in the presence of glutathione. This inhibition might be attributed to an insufficient amount of glutathione to afford complete protection or to

Fig. 11. The effect of cysteine on inhibition by tetramethylthiuram disulfide (TMTD) of fermentation of hexose diphosphate (HDP) by dried yeast cells as indicated by CO_2 production. Treatment 1 contained TMTD, 5×10^{-4} moles/l. and 2×10^{-3} M cysteine added simultaneously with HDP at zero time; Treatment 2 contained 2×10^{-3} M cysteine added simultaneously with HDP at zero time; and Treatment 3 contained TMTD, 5×10^{-4} moles/l. incubated with the yeast suspension for 30 minutes before addition of HDP and 2×10^{-3} M cysteine which were added at zero time. Treatment 4, endogenous; initial pH, 6.5; final pH, Treatment 1, 6.1; Treatment 2, 6.2; Treatment 3, 6.1; Treatment 4, 6.2; gas phase, 95 percent N_2 plus five percent CO_2.


300
260
220
180
140
100
60
20
μl — CO2
0
10
20
30
40
50
60
MINUTES
1
2
3
4

by-products of breakdown of TMTD which inhibit even in the presence of glutathione.

Adding ATP or increasing the amount of adenosine normally used did not affect the rate of fermentation of HDP by dried yeast with or without TMTD. However, the addition of DPN (one or two mg. of 65 percent DPN per vessel) simultaneously with TMTD largely prevented TMTD inhibition of fermentation of HDP (table 1; curve 3, fig. 13 and curve 4, fig. 14). The rate of fermentation in the absence of TMTD was slightly increased by DPN.

TABLE 1. The effect of DPN on inhibition by tetramethylthiuram disulfide (TMTD) of fermentation of hexose diphosphate by dried yeast cells as indicated by CO_2 production.[a]

Concentration		Microliters CO_2 produced in one hour				
TMTD	DPN (65%)	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Percent Inhibition (Mean)
None	1 mg./ vessel	257.0	252.4	252.5	--	19.7
5×10^{-4} moles/l.	1 mg./ vessel	207.9	202.3	200.3	--	
None	2 mg./ vessel	276.6	--	--	247.9	13.9
5×10^{-4} moles/l.	2 mg./ vessel	233.0	--	--	218.7	
None	None	225.8	--	206.6	--	91.5
5×10^{-4} moles/l.	None	18.4	--	18.6	--	

[a]Gas phase was 95 percent N_2 plus five percent CO_2. DPN, HDP and TMTD were added to the yeast suspension simultaneously at the beginning of the experiments.

Fig. 12. Fermentation of glucose, as indicated by CO_2 production, by living yeast as affected by tetramethylthiuram disulfide (TMTD), TMTD plus glutathione and by glutathione alone. Glucose, glutathione and TMTD were added at time indicated by arrow. TMTD, 5×10^{-4} moles/l.; glutathione, 2×10^{-3} M; gas phase, 95 percent N_2 plus five percent CO_2.

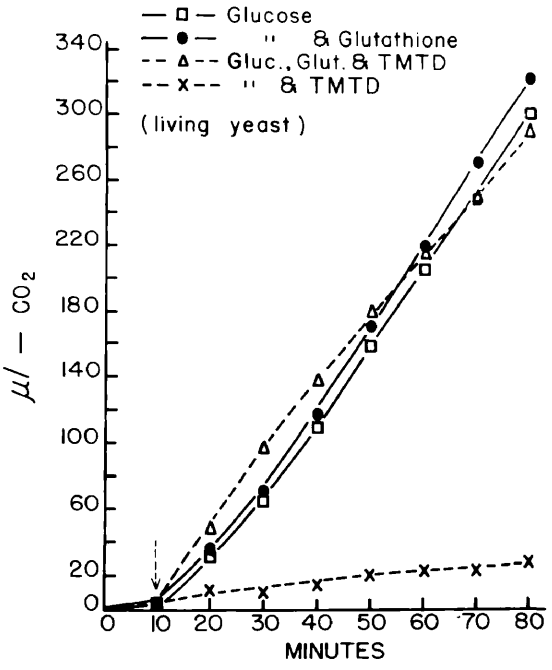
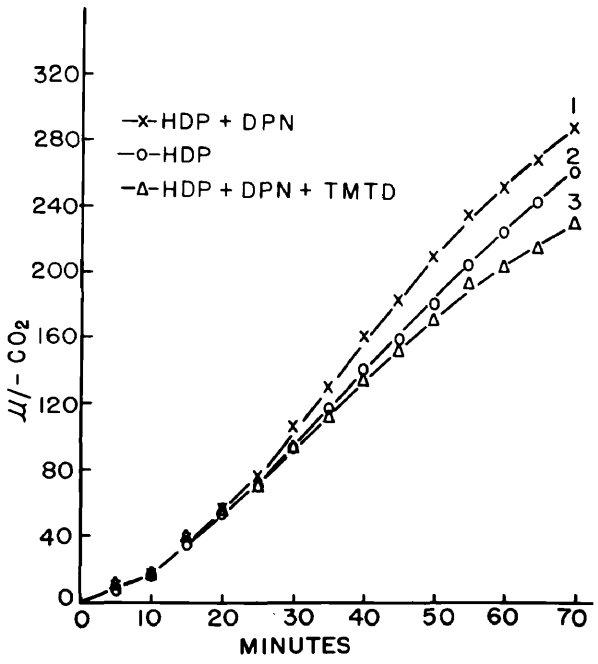

— □ — Glucose
— ● — " & Glutathione
-- △ -- Gluc., Glut. & TMTD
-- x -- " & TMTD
(living yeast)
340
300
260
220
180
140
100
60
20
0
μl — CO2
0 10 20 30 40 50 60 70 80
MINUTES

Fig. 13. The effect of DPN on inhibition by tetramethylthiuram disulfide (TMTD) of fermentation of hexose diphosphate by dried yeast cells as indicated by CO_2 production. HDP, adenosine, DPN and TMTD were added simultaneously at the beginning of the experiment. Treatment 1 contained one mg. of DPN (65 percent) while treatment 3 contained one mg. DPN (65 percent) plus TMTD, 5×10^{-4} moles/l. Gas phase was 95 percent N_2 plus five percent CO_2.


320
280
240
200
160
120
80
40
0
μl - CO2
—x—HDP + DPN
—o—HDP
—Δ—HDP + DPN + TMTD
1
2
3
0
10
20
30
40
50
60
70
MINUTES

Although DPN largely prevented the TMTD-inhibition of fermentation of HDP, neither DPN nor reduced DPN were effective in preventing inhibition of aldolase activity by TMTD. The fact that DPN largely prevents TMTD-inhibition of fermentation of HDP without preventing aldolase inhibition would indicate that aldolase is in sufficient excess in the yeast preparation, at least in the absence of added DPN, to supply enough triosephosphates for near maximal activity even in the presence of TMTD at a concentration of 5×10^{-4} moles/l. (table 1). Apparently aldolase withstood the drying process better than certain other enzymes of the fermentative system or else it was not a limiting enzyme in the living cells.

The same fact strongly suggests that TMTD-effect is mainly upon the triosephosphate dehydrogenase system. Slight inhibition of fermentation of HDP in the presence of DPN (two mg. per vessel) may result from a TMTD-effect on aldolase.

An effect on triosephosphate dehydrogenase could be the result of a TMTD-inhibition of alcohol dehydrogenase. In this reaction, reduced DPN is oxidized when acetaldehyde is reduced to ethanol. Interference with this reaction would result in a lack of the coenzyme, DPN to serve as a hydrogen acceptor in the oxidation of glyceraldehyde-3-phosphate unless the reduced DPN were reoxidized in the reduction of dihydroxyacetone phosphate to glycerol phosphate. It is apparent that in the presence of TMTD and added DPN, the systems which oxidize reduced DPN (alcohol dehydrogenase and alpha glycerol phosphate dehydrogenase) were active since the amount of DPN required to offset the TMTD-effect could account for only a small percentage of the CO_2 that was produced unless the reduced DPN were reoxidized and

recycled. For example, it can be calculated that the one mg. of DPN (65 percent) per vessel which was added could have accounted for less than 22 microliters of CO_2 if it were not recycled. However, in two experiments (table 1) approximately 185 "extra" microliters of CO_2 were produced in the presence of TMTD as a result of addition of one mg. of DPN.

Evidence that alcohol dehydrogenase is active is the fact that acetaldehyde does not accumulate in excess of that in untreated suspensions when HDP is fermented in the presence of TMTD and added DPN (table 2). However, the excess acetaldehyde may have condensed with dihydroxyacetone phosphate in the presence of aldolase to form methyltetrose-1-phosphate as was shown by Meyerhof, et al. (1936).

The ineffectiveness of DPN when added after addition of inhibitor and substrate (fig. 14) would indicate that the inhibitory effect on the triosephosphate dehydrogenase system is not a result of a direct reaction of TMTD with DPN to remove it from the fermentative system, but rather an effect upon triosephosphate dehydrogenase or enzymes involved in the oxidation of reduced DPN.

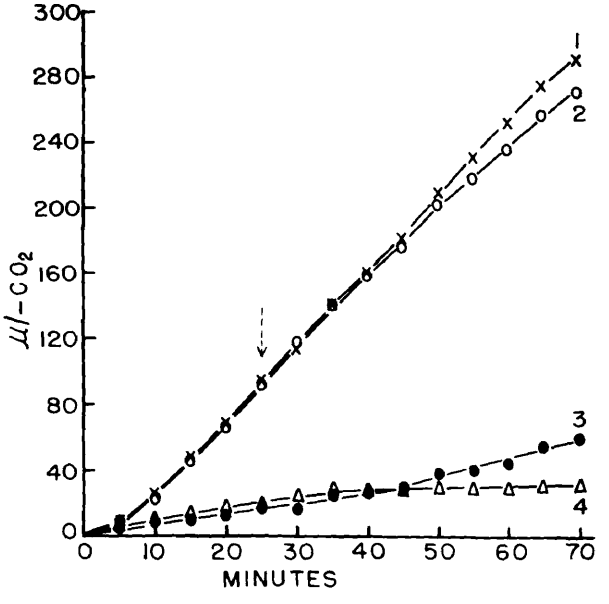
TABLE 2. Acetaldehyde accumulation and CO_2 production in one hour by 12 mg. air dried yeast fermenting hexose diphosphate (HDP) in the presence of added DPN and of added DPN plus tetramethylthiuram disulfide (TMTD) at 30° C.

Added to yeast suspension at beginning of experiment	Acetaldehyde	
	Micrograms per vessel[a]	Microliters CO_2
0.01 M HDP plus 2 mg. DPN (65%)	71.3[b]	247.9
TMTD (5×10^{-4} moles/l.) plus 0.01 M HDP plus 2 mg. DPN (65%)	64.3	218.7

[a]Total fluid volume per vessel two ml.

[b]All figures represent the mean of triplicate determinations.

Fig. 14. The effect of DPN on inhibition by tetramethylthiuram disulfide (TMTD) of fermentation of hexose diphosphate by dried yeast cells as indicated by CO_2 production. HDP and adenosine were added to all treatments at beginning of experiment. TMTD, 5×10^{-4} moles/l., was added to treatments 3 and 4 at beginning of experiment. DPN was added to treatments 1 and 3 at time indicated by arrow. DPN (65 percent), 1 mg. per vessel; gas phase, 95 percent N_2 plus five percent CO_2.


300
280
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μl-CO2
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MINUTES
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DISCUSSION AND CONCLUSIONS

While it has been shown that TMTD inhibits aldolase activity, this effect apparently is not of primary importance in the inhibition of the overall fermentation process in the dried yeast preparation used in this study. It might, however, assume greater importance in living cells or systems in which aldolase were limiting.

The evidence obtained in this study indicates that the major portion of the inhibitory effect of TMTD is upon the triosephosphate dehydrogenase system. Nygaard and Sumner (1952) showed that triosephosphate dehydrogenase from rabbit muscle was strongly inhibited by TETD. Their work indicated that TETD competes with substrate, but not with DPN for the enzyme.

Since the data from the present study practically eliminate the possibility of a lack of DPN as a result of a direct reaction of TMTD with DPN to remove it from the fermentative system, it was concluded that the TMTD-effect is directly upon triosephosphate dehydrogenase, or upon enzymes which oxidize reduced DPN.

The fact that DPN was much more effective in offsetting inhibition when added simultaneously with TMTD than when added later in the experiment would indicate that this coenzyme protects the apoenzymes from TMTD.

This interpretation is supported by the work of Rapkine, Rapkine and Trpinac (1939) who showed that DPN protects SH groups of triosephosphate dehydrogenase from oxidation by oxidized

glutathione, GSSG, (oxidized glutathione contains the S-S linkage as does TMTD). They also showed that DPN afforded greater protection when added to preparations containing the enzyme before addition of the inhibitor (GSSG), than when it was added after the inhibitor.

The effect of DPN and of glutathione (reduced) in offsetting TMTD-inhibition of fermentation by yeast is similar to that obtained by Graham (1951) in preventing liver aldehyde oxidase inhibition by TETD with the same two compounds. Kjeldgaard (1949) reported that when the S-S linkage of TETD is reduced to SH, the compound is no longer inhibitory to aldehyde oxidase. Kelin and Hartree (1940) showed that the oxidized form of TETD, with the S-S linkage, is responsible for inhibition of succinic dehydrogenase. Inhibition of succinic dehydrogenase and of the Racker aldehyde oxidase from liver by TETD is prevented by glutathione. Keilin and Hartree, 1940; Graham, 1951 and Nygaard and Sumner, 1952, have suggested that the action of cysteine or glutathione in preventing TETD inhibition may be that of preventing interference with essential SH groups in the structure of certain enzymes. It is possible that the action of cysteine or glutathione in preventing TMTD-inhibition of the fermentative process in yeast may be that of reducing the S-S linkage of this inhibitor to non-inhibitory SH groups.

The role of cysteine or glutatione in protecting yeast aldolase may be that of keeping the ferrous iron, which is necessary for the activity of this enzyme, in a reduced state in the presence of TMTD which would tend to oxidize the iron.

Either the oxidized compounds TMTD and TETD or their reduced forms appear to be capable of causing fungus-inhibition. The oxidized forms of these inhibitors are apparently responsible for inhibitions aleviated by glutathione, cysteine and ascorbic acid. The reduction product of TETD, diethyldithiocarbamate, reacts readily with copper ions and is generally recognized as an inhibitor of copper containing enzymes. Dimethyldithiocarbamate also reacts readily with copper ions and would be expected to behave similarly toward copper containing enzymes.

The thiuram disulfides inhibit a number of enzymatic processes generally regarded as common to living cells. The effectiveness of TMTD in controlling fungi when used as a fungicide, while not causing serious injury to higher plants may not necessarily result from qualitative differences in enzymatic processes in the two groups of organisms, but to various other factors. Among the latter may be: differences in content of cysteine, glutathione, histidine and DPN; relative amounts of susceptible enzymes present; differences in permeability to the toxicant; relative surface area of cells exposed to the toxicant; and relative capacity of the cells to reduce or to decompose the inhibitor.

SUMMARY

It was shown that TMTD at several concentrations inhibits fermentation of glucose by living brewers yeast cells under anaerobic conditions. Fermentation of glucose and of HDP by dried yeast preparations was likewise inhibited. The degree of inhibition of fermentation of these two substrates was approximately equal. Under the same conditions fermentation of PGA and of pyruvate was affected only slightly or not at all by TMTD. Incubation of TMTD with suspensions of dried yeast previous to addition of substrate produced inhibition of fermentation of PGA approximating 40 percent. However, in all cases, fermentation of glucose and of HDP was much more profoundly affected by TMTD than was fermentation of PGA or of pyruvate.

On the basis of these observations it was concluded that the main effect of TMTD is inhibition of some process or processes involved in conversion of HDP to PGA. Further study revealed that TMTD inhibits aldolase activity and that cysteine or glutathione prevents this inhibition. Cysteine or glutathione also offsets TMTD-inhibition of fermentation of glucose by living yeast cells and of fermentation of HDP by dried yeast.

DPN, when added simultaneously with substrate and TMTD, offsets a large portion of the TMTD-inhibition of fermentation of HDP, but it has no effect on TMTD-inhibition of aldolase activity. This indicates that inhibition of the triosephosphate dehydrogenase system, rather than of aldolase, is primarily responsible

for the TMTD-effect upon fermentation of HDP by dried yeast preparations.

The data indicate that in the presence of TMTD and DPN the systems involved in the oxidation of reduced DPN are active since, (1) the amount of DPN required to offset the TMTD-effect is not sufficient to account for the extra CO_2 produced as a result of adding DPN to dried yeast suspensions containing HDP and TMTD unless DPN is reoxidized and recycled; and (2) acetaldehyde does not accumulate when HDP is fermented in presence of TMTD and added DPN.

The fact that DPN has only a slight effect in preventing TMTD-inhibition when added following addition of inhibitor and HDP, lead to the conclusion that DPN protects the enzyme, triosephosphate dehydrogenase, or enzymes involved in the oxidation of reduced DPN. It was concluded that the main effect of TMTD upon fermentation of glucose by yeast is upon one or more of these enzymes.

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VITA

Name in full: Hugh Delane Sisler

Permanent address: Friendsville, Maryland

Degree to be conferred; date: Doctor of Philosophy, 1953

Date of birth: November 4, 1922

Place of birth: Friendsville, Maryland

Secondary Education: Friendsville High School

Collegiate Institutions attended:	Dates	Degree	Date of degree
University of Maryland	Sept. 1946 Aug. 1949	B.S.	August 1949
University of Maryland	Sept. 1949 June 1951	M.S.	June 1951

Publications:

Cox, C. E., and H. D. Sisler. 1951. Some metabolic and morphologic changes in germinating conidia of Fusarium. (Abs.) Phytopathology 41: 8.

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