AN EXAMINATION OF SOME BIOLOGICAL SYSTEMS FOR METHYLGLYOXAL

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Rene Evard

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AN ABSTRACT

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ABSTRACT

Several biological systems have been examined for the presence of methylglyoxal. The methods employed for this investigation offer several advantages over the previous techniques used for determing methylglyoxal: they are quite sensitive and specific, and they permit examination of biological systems under almost physiological conditions.

In most of these experiments, the material under investigation was lyophilized and the distillate collected. This distillate was either treated with chromotropic acid-sulfuric acid mixture and the concentration of methylglyoxal determined by fluorometry, or the carbonyl compounds present were converted to their oximes and the resulting mixture examined for methylglyoxime by paper chromatography.

No methylglyoxal could be found in baker's yeast, yeast fermentation mixtures, rat liver or rat blood. The addition of very low concentrations of methylglyoxal to these systems was also studied. No methylglyoxal could be recovered unless the system was previously inhibited with iodoacetic acid.

Several plants have also been examined. The older leaves were found to contain a substance which reacts with chromotropic acid to yield a fluorescent compound. Moreover, a substance was present which apparently forms an oxime. This "oxime" can be converted to a yellow nickelous complex, but the Rf value for the "oxime" is considerably greater than that for methylglyoxime.

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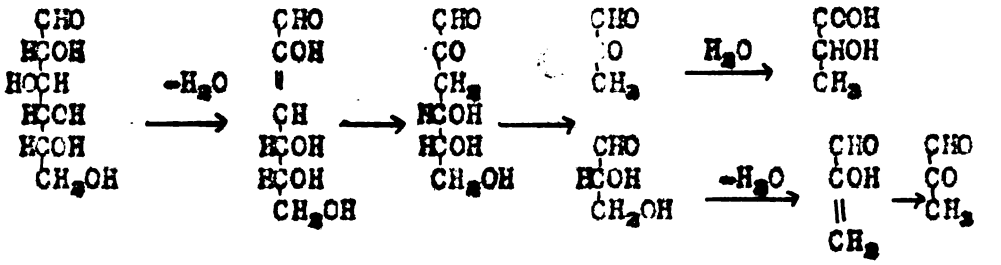
HISTORICAL INTRODUCTION

HISTORICAL INTRODUCTION

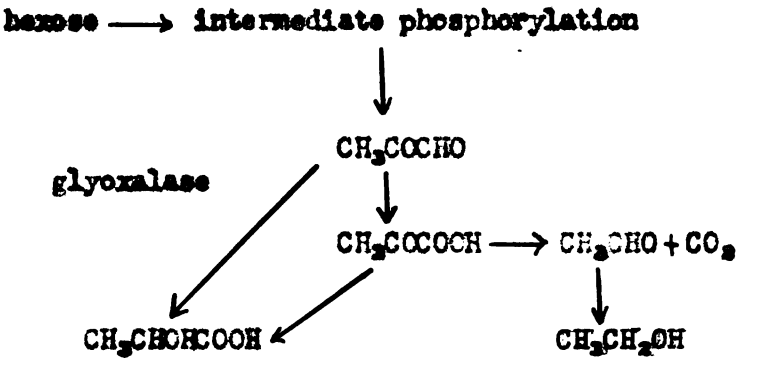
The now obsolete theory of methylglyoxal as an important intermediate in glycolysis first came into being around 1912. The close structural relationships between lactic and pyruvic acids and methylglyoxal contributed greatly to the attractiveness of the idea, and it was partially substantiated by identification of glyoxalase, which catalyzes the conversion of methylglyoxal to lactic acid, as well as by indications of the actual occurrence of methylglyoxal in a variety of biological systems. Thus, as early as 1913 Dakin and Dudley (1) reported the presence of glyoxalase in blood and in various tissues (liver, heart, muscle, brain, and kidney) of a number of animal species (man, dog, ox, sheep, rabbit, "fowl", and codfish). More recently, Hopkins and Morgan (2) found an exceedingly wide distribution of glyoxalase among invertebrates, algae, seaweed, fungi, and higher plants. An early claim to the identification of methylglyoxal in biological systems occurs in the report by Toennissen and Fisher (3) who isolated methylglyoxal p-nitrophenylosazone from an incubation mixture consisting of fructose-1,6-diphosphate and muscle and pancreas tissue. Ariyama (4) repeated this work and found evidence for the presence of methylglyoxal in the color formed on reaction with arsenophosphotungstic acid and cyanide ion. Neuberg and Kobel (5) reported isolation of methylglyoxal as its 2,4-dinitrophenylosazone from bacterial extracts

to which fructose-1,6-diphosphate had been added. Similarly, Aubel and Simon (6) isolated methylglyoxal 2,4-dinitrophenylhydrazone from yeast and from dog muscle when these materials were incubated with hexose diphosphate. Needham and Lehmann (7) also claimed identification of methylglyoxal by the procedure of Neuberg and Kobel (5) in chicken embryo tissue. Case and Cook (8) reported that methylglyoxal occurs as an intermediate in lactic acid formation.

Neuberg and Kerb (9), Levene and Meyer (10), Dakin and Dudley (11), and Neuberg and Kobel (12) all proposed plausible schemes for glycolysis which involved methylglyoxal. Probably the most interesting was that of Levene and Meyer which is shown below.



The culmination of these schemes was reached in that suggested in 1929 by Neuberg and Kobel which is shown in the following outline:



The work of Meyerhof shattered these hypotheses, so that these ideas which were really never built upon firm experimental ground have since fallen into disrepute.

Notwithstanding this and the fact that its true role remains a mystery, the significance of methylglyoxal in biological systems cannot be disregarded.

Meyer (13) showed that methylglyoxal formation is possible from glyceraldehyde and other trioses phosphates. This formation is a chemical rather than an enzymatic reaction, because active muscle extract did not yield more methylglyoxal than boiled muscle extract.

Stohr (14) showed that methylglyoxal is toxic to rats. Kun (15) presented strong evidences showing the inhibitory effect of methylglyoxal on succinic dehydrogenase. Apparently methylglyoxal combines with -SH enzymes to form a stable condensation compound. Other -SH enzymes, -malic, -glutamic, triosedehydrogenases, hexokinases, adenosinetriphosphatases are also inhibited similarly to succinic dehydrogenase. Salem (16) showed that methylglyoxal is excreted in the urine of thiamine-deficient rats. This appears to be caused by a deficiency in glyoxalase activity in the liver. Therefore, the symptoms of thiamine deficiency can be attributed to methylglyoxal intoxication.

The physiological role of glyoxalase in tissue was suggested by Meyerhof (17) when he pointed out that this enzyme may eliminate the spontaneously formed methylglyoxal.

Lohmann (18) showed that reduced glutathione is the coenzyme of glyoxalase. Yamasoye (19), Jowett and Quastel (20), and Racker (21)

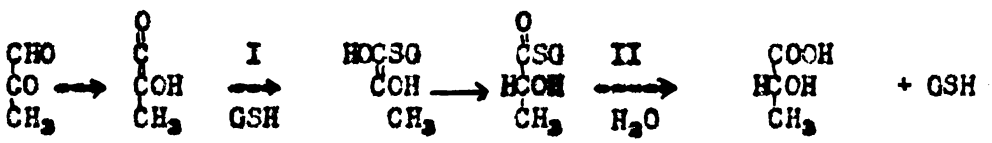
reported that methylglyoxal and glutathione form a compound, and suggested that this might be an intermediate in the glyoxalase reaction. Jowett and Quastel (20) observed that the -SH groups titrable by iodine decreased when methylglyoxal was added to a glutathione (GSH) solution. They proposed the following mechanism for glyoxalase action

$$\begin{matrix} CHO \\ CO \\ CH_3 \end{matrix} + GSH \longrightarrow \begin{matrix} CH \\ HCSG \\ CO \\ CH_3 \end{matrix} + H_2O \longrightarrow \begin{matrix} COOH \\ HCOH \\ CH_3 \end{matrix} + GSH$$

Platt and Schroeder (22) noticed that iodoacetic acid inhibits glyoxalase action by destroying glutathione, the enzyme itself not being harmed, which was shown by the fact that addition of glutathione would restaure the activity.

Yamazoye (19) showed that the compound formed by methylglyoxal and glutathione in the presence of liver glyoxalase was different from that formed in its absence. He was able to isolate both. The compound formed in the absence of the enzyme was very labile and behaved like a mixture of glutathione and methylglyoxal. The "biological" compound formed in the presence of the enzyme above pH 7, slowly broke down to form glutathione and lactic acid. Hopkins and Morgan (2) demonstrated that ox heart glyoxalase was made up of two components. One of these is the "enzyme" which converts methylglyoxal and phenylglyoxal to the corresponding hydroxyacids at equal rates. The other component, which these authors called "factor", when added to the systems increased the rate of conversion of methylglyoxal, but had no effect on phenylglyoxal.

In the presence of the "enzyme", the "factor" was separated by adsorption of the letter on calcium phosphate gel at pH 5.6. Racker (21) demonstrated the presence of an intermediate by its absorption at 240 mμ, since neither glutathione, nor methylglyoxal show appreciable absorption at that wave length. Using enzyme preparations from yeast, Racker also studied the function and properties of glyoxalase I and II, which he claimed correspond to Morgan's "enzyme" and "factor", and he proposed the following mechanism for glyoxalase action.



Using an enzyme preparation from ox heart, and different techniques, Crook and Law (23) arrived essentially to the same conclusions as Racker.

Although methylglyoxal is no longer considered an intermediate in glycolysis, there still remain some possibility that it might be present in biological systems. This work presents an examination of several biological systems using methods which are specific for methylglyoxal and permit an investigation for this substance under almost physiological conditions.

EXPERIMENTAL METHODS

EXPERIMENTAL METHODS

Materials

Ethanol, nickelous sulfate, sodium acetate, anhydrous ethyl ether, disodium hydrogen phosphate and sodium chloride were all C.P. reagents. Matheson chromotropic acid was recrystallized from 50 per cent ethanol. Sulfuric acid was Merck reagent grade. Iodoacetic acid, n-heptanol, hydroxylamine hydrochloride and semicarbazide hydrochloride were Eastman Kodak Company best grade. Fructose-1,6-diphosphate was obtained from Nutritional Biochemicals Corporation. The yeast was a Fleischman product. DL-Glyceraldehyde was obtained through the courtesy of Dr. John C. Speck, Jr.

Apparatus

A Model 12-B Coleman photofluorometer, equipped with a B 2 primary filter and a PC-2 secondary filter, and the standard cuvettes supplied for this instrument were used in making all fluorescence measurements.

A lyophilizing apparatus was made from a U tube fitted with ground glass joints. At one end was attached a 50-ml flask in which was placed the material to be dried. The other end was connected to a tube which was placed into a dry ice-ethanol bath. The apparatus was evacuated with an oil pump.

Preparation and Standardization of Methylglyoxal Stock Solutions

These stock solutions were prepared and standardized according to the procedure described by Thornton and Speck (24).

One gram of DL-glyceraldehyde was dissolved in 25 ml. of 1 M sulfuric acid, and this solution was distilled. Fifteen milliliters of the distillate were collected and diluted to 100 ml. volume with distilled water. The resulting solutions were stored at 5°.

The methylglyoxal solutions were standardized by precipitation of the disemicarbazone. To a 10-ml. aliquot of the solution to be standardized was added a filtered solution of 1 g. of semicarbazide hydrochloride and 1.5 g. of sodium acetate in 15 ml. of distilled water. The mixture was allowed to stand for 24 hours. The precipitate was collected in a filter crucible and washed with a few milliliters of water. It was then dried in an oven at 105° and weighed.

Fluorometric Determination of Methylglyoxal

These determinations were carried out according to the procedure of Thornton and Speck (24) also.

Standard solutions of methylglyoxal were made up freshly every day from the stock solution. One milliliter of the standard solution and 1 ml. of freshly prepared 2 per cent chromotropic acid were placed in a 25-ml. volumetric flask. Separate solutions containing the unknown and the blank were prepared in the same way. To each of these solutions were then gradually added 10 ml. of ice-cold concentrated sulfuric acid. The flasks were cooled during the addition by swirling in an ice-water

mixture. After addition of the sulfuric acid, each flask was stoppered and placed in a shallow water bath containing ice-water mixture. The flasks were then transferred together to a water bath maintained at 50° where they were swirled for five minutes. At the end of this time, the flasks were transferred together to a cool water bath where they were quickly brought to room temperature. Each flask was diluted to the mark with concentrated sulfuric acid, and the fluorescence of the unknown was compared with the standard, using a blue primary filter, and a yellow secondary filter for the measurements.

The experiments described below involve the examination of several biological systems which it was thought might contain methylglyoxal. The material examined was lyophilized and 1-ml aliquots of the distillate were treated with chromotropic acid, according to the procedure described above. The fluorescence was compared with that of a known standard.

Examination of Yeast

Five grams of baker's soft yeast were placed in the lyophilizing apparatus, and the distillate was collected.

Examination of Rat Blood

A male rat was sacrificed. Five milliliters of blood were immediately collected from the animal and placed in the lyophilizing apparatus.

Examination of Rat Liver

A female rat, eight months old, was sacrificed. The liver was removed immediately, placed in a flask, frozen and lyophilized.

Examination of Yeast Fermentation Mixtures

The fermentation mixture was prepared in the following manner: To 10 ml. of a 10 per cent solution of glucose and 10 ml. of a 0.1 M disodium hydrogen phosphate solution were added 5 g. of yeast. This mixture was allowed to stand for two hours at room temperature. Five milliliters of this suspension were lyophilized.

Examination of Liver Extract Incubated with Fructose-1,6-Diphosphate

Two male rats were sacrificed and their livers (total weight 18.3 g.) were removed. These were then ground in a mortar with clean sand and suspended in 50 ml. of borate buffer at pH 7.0. The borate buffer was prepared by titrating 200 ml. of 4 per cent boric acid solution with 0.1 N sodium hydroxide, using a pH meter to check the pH. Ten milliliters of this suspension were added to 25 ml. of 1.2 per cent fructose-1,6-diphosphate solution. This mixture was incubated for 24 hours at 37°. This was labelled mixture I. The remainder of the suspension was also incubated at 37° for 24 hours. Ten milliliters of this mixture were then added to 25 ml. of a 1.2 per cent solution of fructose-1,6-diphosphate and incubated further for a 24-hour period. This was labelled mixture II.

At the end of the incubation period, 5-ml. aliquots were removed from each mixture and lyophilized.

Examination of Yeast (Methylglyoxal Added)

To 3 g. of baker's soft yeast was added 1 ml. of 0.1 M iodoacetic acid solution. The mixture was allowed to stand for one hour at room temperature. One milliliter of a solution containing 0.0021 mg. per ml. of methylglyoxal was added to this. The mixture was immediately placed in the lyophilizing apparatus and the distillate collected (4 ml.).

Examination of Yeast Fermentation Mixture (Methylglyoxal Added)

A fermentation mixture was prepared in the following manner: Ten milliliters of a 0.1 M glucose solution, 10 ml. of 0.1 M disodium hydrogen phosphate solution and 5 g. of yeast were mixed. After standing for two hours at room temperature, 1 ml. of 0.1 M iodoacetic acid was added. The mixture was then allowed to stand for one half hour longer. Four milliliters of this suspension were removed and 1 ml. of methylglyoxal solution containing 0.0385 mg. of this substance was added. This portion was then lyophilized.

Examination of Rat Blood (Methylglyoxal Added)

A rat was sacrificed and the blood immediately collected. Five milliliters of the blood were placed in a flask to which was added 1 ml. of a solution of methylglyoxal containing 0.002l mg. per ml. The flask was connected to the lyophilizing apparatus and the distillate was collected.

Examination of Plants for Methylglyoxal

The leaves of the following plants were investigated for methylglyoxal: Maple (Acer saccharum), mulberry (Morus alba), white oak (Quercus alba), white fir (Abies concolor), blue grass, ivy (Parthenocissus tricuspidata), and tobacco (Nicotiana rustica). In addition, the roots and stems of tobacco plants were also examined.

Preparation of Leaf Extract

Fresh leaves were collected, without the stems, washed and placed in a deep freeze overnight. When ready for use, they were thawed, placed in a muslin cloth, and pressed in the hydraulic press. The juice was immediately frozen with dry-ice and lyophilized.

Paper Chromatography

Attempts were made to identify methylglyoxal in maple leaves by formation of the methylglyoxime and separation and identification of this substance by paper chromatography according to the procedure of Speck (25).

Twenty grams of fresh leaves were collected, washed and ground in a Waring blender. To this mixture were added 100 ml. of distilled water, 1 g. of hydroxylamine hydrochloride and 1.5 g. of sodium acetate (trihydrate). This suspension was saturated with sodium chloride, and extracted with five 50-ml. portions of anhydrous ethyl ether. The combined extracts were dried overnight over anhydrous sodium sulfate. The ether was distilled off, the last portion being removed under vacuum at the water pump in order to avoid overheating of the residue.

The residue was then dissolved in 0.5 ml. of water and a few drops of the solution placed on a sheet of Whatman No. 1 filter paper. The solvent used for paper chromatography was water-saturated n-heptanol. After allowing the solvent to rise for 20 hours, the sheets were dried in the air and sprayed with a 3 per cent solution of nickelous sulfate to which had been added 0.1 ml. of 28 per cent ammonia per 100 ml. The chromatograms indicated several brown spots and considerable "tailing".

In another attempt to identify methylglyoxal in leaves, 35 g. of freshly collected maple leaves were ground in a mortar with acid-washed sand and pressed with 15 ml. of distilled water. To this solution was added 1 g. of hydroxylamine hydrochloride and 1 g. of sodium acetate. The solution was filtered and treated for paper chromatography according to the procedure described above. The chromatograms obtained in this manner also showed considerable "tailing" and several brown spots.

In order to definitively prove the presence or absence of methylglyoxal in plants, maple leaves were collected, washed and placed in the deep freeze overnight. They were then thawed, placed in a muslin cloth and pressed in the hydraulic press. The extract was immediately frozen and lyophillized. To the distillate (approximately 50 ml.) was added 1 g. of hydroxylamine hydrochloride and 2 g. of sodium acetate. This solution was treated for paper chromatography according to the procedure previously described. The results are indicated in Table IV.

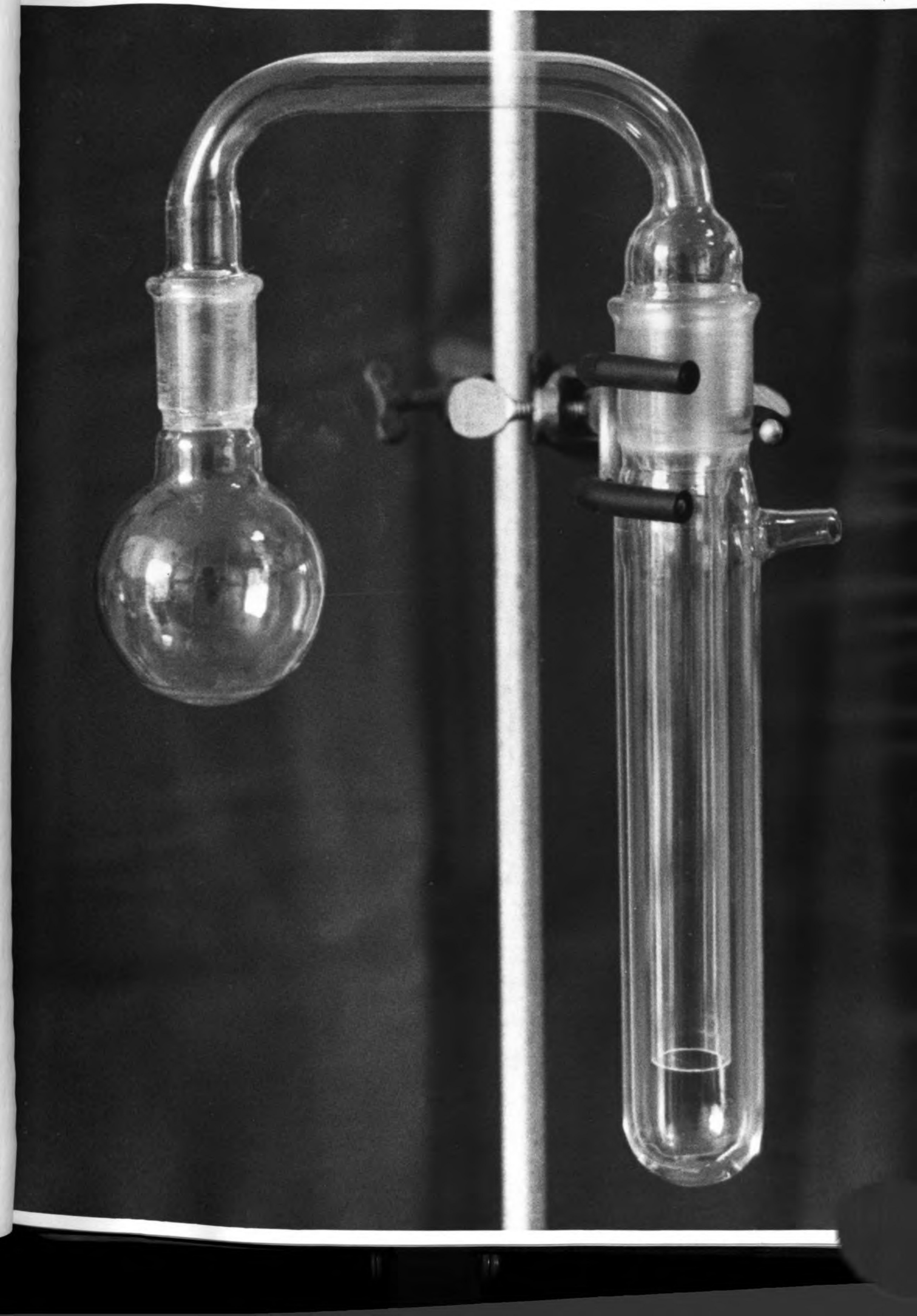
Attempt to Identify Methylglyoxal by Paper Chromatography in Rat Liver Extracts

A male rat was sacrificed and the liver immediately removed.

It was placed in a Waring blender with a solution of 1 g. of hydroxylamine hydrochloride and 1.5 g. of sodium acetate in 50 ml. of distilled water. This suspension was treated for paper chromatography according to the procedure previously described.

Attempt to Identify Methylglyoxal in Yeast by Paper Chromatography

Twenty grams of baker's soft yeast were placed in a Waring blender with 50 ml. of distilled water containing 1 g. of hydroxylamine hydrochloride and 1.5 g. of sodium acetate. This suspension was treated for paper chromatography according to the procedure previously described.



RESULTS AND DISCUSSION

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No methylglyoxal could be found in yeast, yeast fermentation mixtures, rat blood or rat liver. The methods employed for this investigation offer several advantages over the previous techniques used for determining methylglyoxal. They are quite sensitive and specific and they permit an examination of biological systems under almost physiological conditions.

A study was made in order to determine whether it is possible to recover any methylglyoxal when it is added at low concentration to these systems. It was found that when methylglyoxal was added at concentrations of 0.0013 mg. per ml. to yeast, yeast fermentation mixtures or rat blood none could be recovered. These results can be attributed to the immediate formation of a complex between methylglyoxal and glutathione. (Several investigators (18,19,20) have shown that glutathione acts as a coenzyme in the transformation of methylglyoxal to lactic acid.) Since this reaction can be inhibited by adding iodoacetate (22) these experiments were repeated with iodoacetate present. The results, which were not always reproducible, are reported in Table I.

Although these recoveries are rather low, they nevertheless can be considered significant--particularly in view of the instability of methylglyoxal either at low concentrations or in the presence of biological systems. These results indicate that if methylglyoxal

TABLE I

RECOVERY OF METHYLGLYOXAL ADDED TO SYSTEMS CONTAINING IODOACETATE

System	Methylglyoxal Added, Mg.	Methylglyoxal Recovered, Mg.	Per Cent Recovery
Yeast	0.0026	0.0008	32.3
Yeast Fermentation Mixtures	0.038	0.0019	5.0
Blood (Rat)	0.0021	0	0

accumulated appreciably at any stage of glycolysis one should be able to detect it by these methods.

Ariyama (4) and several other workers reported that liver extracts, when incubated with hexose diphosphate yield methylglyoxal. It was decided to repeat this work in order to determine whether methylglyoxal could be detected under these circumstances by reaction with chromotropic acid and fluorometry. The results of this experiment, which are reported in Table II and which indicate the presence of methylglyoxal, support Ariyama's work.

The original liver homogenate-borate mixture was also tested for methylglyoxal. No methylglyoxal could be detected in this suspension when no fructose-1,6-diphosphate had been added.

These results shown in Table III indicate the presence of significant amounts of methylglyoxal in plants, especially in the older green leaves. In order to determine definitely its presence or absence,

TABLE II

EXAMINATION OF LIVER HOMOGENATES INCUBATED WITH FRUCTOSE-1,6-DIPHOSPHATE

	Blank	Standard*	Solution I	Solution II
Fluorometer Readings	0	74	100	37

* The standard contained 0.00096 mg. of methylglyoxal

TABLE III

RESULTS FROM THE EXAMINATION OF PLANTS

Plant Examined	Fluorometer Readings	
	Unknown	Standard*
Maple Leaves	46	85
Grass	0	84
Ivy Leaves	4	95
Mulberry Leaves	0	92
Oak Leaves	10	92
White Fir Leaves	16	92
Tobacco Leaves	14	69
Tobacco Stems	27	100
Tobacco Roots	0	73

* The standard solution contained 0.00096 mg. of methylglyoxal

50-ml. portions of distillate from lyophilization of maple leaf juice were examined by the procedure involving paper chromatography of the oximes. The results of these experiments are shown in Table IV.

TABLE IV

RESULTS OF PAPER CHROMATOGRAPHY OF LEAF EXTRACT (DISTILLED)

	Rf Value	Quality of Spot
Methylglyoxime	0.68	Bright pink and yellow
Unknown	0.96	Yellow, with "tailing"

These results indicate that methylglyoxal does not occur in the leaves of this plant. Instead some other volatile compound having the property of forming an oxime which gives a yellow complex with nickelous ion seems to be present. This substance appears to react with chromotropic acid under the conditions of the procedure for determination of methylglyoxal to yield a fluorescent compound. The compound undoubtedly merits further investigation because of its probable significance in photosynthesis.

SUMMARY

1. Yeast, yeast fermentation mixtures, rat blood, rat liver and plants were examined for methylglyoxal. The methods involved:

   a) Lyophilization and fluorometric determination of methylglyoxal in the distillate after reaction with chromotropic acid-sulfuric acid mixture.

   b) Lyophilization of the mixture followed by conversion of carbonyl compounds in the distillate to their oximes and separation of the oximes by paper chromatography.

2. No methylglyoxal could be detected in yeast, yeast fermentation mixtures, rat blood or rat liver. Methylglyoxal could be recovered after being added to these systems, providing iodoacetate was added also.

3. Evidence for methylglyoxal production was obtained with rat liver homogenates incubated with fructose-1,6-diphosphate.

4. A volatile substance was found in plants which gives a fluorescent compound on reaction with chromotropic acid under the conditions of the procedure for methylglyoxal.

5. This substance cannot be methylglyoxal since it could not be converted to an oxime having an Rf value identical with that of methylglyoxime.

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Date Due