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METABOLIC PATHWAYS IN ACID PRODUCTION
BY THE YEAST HANSENULA ANOMALA.

The Ohio State University, Ph.D., 1963
Botany

University Microfilms, Inc., Ann Arbor, Michigan

METABOLIC PATHWAYS IN
ACID PRODUCTION BY
THE YEAST HANSENULA ANOMALA

DISSERTATION

Presented in Partial Fulfillment of the Requirements
for the Degree Doctor of Philosophy in the
Graduate School of the Ohio State
University

By

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The Ohio State University
1963

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ACKNOWLEDGMENTS

I wish to express my appreciation for the continuing advice and encouragement given to me by Dr. William D. Gray throughout the course of this investigation. Dr. Gray, Dr. Bernard S. Meyer, and Dr. Clyde C. Allison were very helpful in their criticism of the manuscript.

I am grateful also for financial help received from the research fund of the Ohio Academy of Science and from the American Association of University Women, which granted me the Dorothy Bridgman Atkinson Fellowship in 1961-1962.

Wittenberg University was most generous in permitting me to use laboratory facilities there. Dr. W.C. Beaver of the biology department at Wittenberg kindly shared research space and contributed much helpful assistance.

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INTRODUCTION

The yeast cell has proved to be a useful organism for physiological studies. Saccharomyces cerevisiae, the yeast important to the baking and brewing industries, has been used most often; however, more recently some of the lesser known yeasts have been studied. One of these is Hansenula anomala Hansen (Sydow).

Some initial studies on the physiology of the genus Hansenula, and H. anomala in particular, indicated that an ester is produced. Finding a lack of definite information on the amount and identity of the metabolic products of H. anomala, Gray (1949) investigated conditions necessary for ester synthesis and isolated the volatile metabolic products ethyl acetate, ethyl alcohol, and acetaldehyde. He found that, after the ester had reached its maximum concentration, it disappeared from the medium at about the same rate it was produced. Apparently the ester is metabolized by the yeast.

Gray (1949) also reviewed the results of early physiological research on ester production by Hansenula

species, which dated back to 1894. Before discussing experimental work subsequent to that of Gray, it may be profitable to explore the cytological and physiological characteristics of the yeast Hansenula.

The fungus used in the present investigation is Hansenula anomala (Hansen) Sydow. The genus Hansenula is generally classified as an Ascomycete, family Saccharomycetaceae (ascosporogenous yeasts), subfamily Saccharomycetoideae, tribe Saccharomyceteae. Ascosporogenous yeasts are characterized by (1) naked asci resulting directly from conjugation of two vegetative cells, and (2) lack of a dicaryon state, a vegetative state in which each cell contains two nuclei which divide synchronically during cell divisions. (Lodder et al., 1958.)

The genus Hansenula includes all ascosporogenous yeasts which can assimilate nitrate. The genus was redefined by Wickerham (1951) as: "Yeasts which reproduce asexually by budding, or by budding accompanied by the formation of pseudohyphae or true hyphae, form 1-4 ascospores per ascus, and assimilate nitrate. The spores are mainly hat-shaped or Saturn-shaped. The ascus usually ruptures at maturity to liberate the spores."

The species Hansenula anomala is the prototype of the genus as described by early investigators. The ascospores are hat-shaped, usually with a thick brim. There is a wide range of cell shape and size. The cells occur singly, in pairs, or in chains.

The species exhibits pellicle formation, fermentation of glucose and sucrose, pseudomycelium formation, ester formation, and splitting of the B-glucoside arbutin. Maltose is almost always fermented, while the ability to ferment galactose and raffinose depends on the strain. No added vitamins are needed for growth.

H. anomala is the most widely distributed member of the genus. It has been isolated from many sources: fruits, brine, soil, saps and gums of trees, cocoa beans, and as a predominant organism in bin-stored rice. It is an occasional contaminant in industrial food and fermentation processes.

Candida pelliculosa is the imperfect stage of H. anomala. Before this was demonstrated by mating the two, C. pelliculosa was isolated from human sources, particularly cases of disease of the pulmonary tract. H. schneegii is considered by some workers to be another

sexually active mating type of H. anomala, although Wickerham (1951) considers it a separate species represented by a single strain.

The life cycle of H. anomala is similar to the pattern found in Saccharomyces ludwigii as discussed by Alexopoulos (1952). It exhibits a long diploid somatic stage and a very short haploid stage. The ascospores copulate, with conjugation occurring at the time the ascospores germinate. No conjugation takes place immediately preceding the formation of asci. Ascus rupture as the ascospores mature, except in some strains having cells large enough to accommodate the mature spores. Two ascospores fuse, with both plasmogamy and karyogamy taking place, and form a diploid cell. Meiosis converts these diploid somatic yeast cells into asci, each producing haploid ascospores, usually four in number. The ascospores represent the only haploid phase of the life cycle.

Wickerham (1951) and Wickerham and Burton (1954) have worked out phylogenetic lines in the genus Hansenula. They recognize lines of development from primitive species to more highly evolved species.

Wickerham suggests three lines of development in the genus, as shown by his phylogenetic diagram:

In the above diagram, the following symbols are used:

H, haploid; D, diploid; H+D, more haploid than diploid;

D+H, more diploid than haploid (based on Wickerham, 1951).

The lower line shows a loss in physiological activity as the nucleus became diploid. The three species in this line are found exclusively in coniferous trees. H. capsulata is entirely haploid and ferments glucose slowly; the other two are diploid but ferment no sugars. All three require an external source of vitamins.

The vertical branch of the phylogenetic line is made up of species which also produce hat-shaped ascospores, but are not isolated from conifers. As the ratio of diploid to haploid cells increases from species to species, there is less dependence on an external source of vitamins. There is an increase in the number of sugars fermented and the intensity of fermentation. H. minuta produces asci as a result of the conjugation of a cell with its own small bud. This appears to be a characteristic which decreases with the change to a diploid somatic stage.

H. anomala occupies an intermediary position. It produces esters, a characteristic not shared by the three species at the bottom of this line, and is active in fermentation of sugars and utilization of alcohol and

organic acids. It exhibits a more advanced type of conjugation, that of germinating ascospores.

The third phylogenetic branch, shown diagonally on the diagram, consists of species having Saturn-shaped spores. Wickerham considers them to cover a relatively recent period of evolution, since none is completely haploid or completely diploid. They show the same general trends physiologically as the species with hat-shaped ascospores discussed above. Conjugation of adult vegetative cells occurs in all of these species and is generally isogamic. Only H. californica in this series requires the addition of vitamins to a synthetic medium.

Wickerham considers the most fundamental trend in these phylogenetic relationships to be the progressive development from a haploid nucleus, in the more primitive yeasts, to the diploid condition found in the more recently evolved species.

Other characteristics showing such trends are: from vitamin dependence to independence, from no fermentation to fermentation of a variety of sugars, from no pellicle formation on a liquid medium to pellicle

formation. The ability to form hyphae generally increases as the species become more diploid.

The most primitive type of conjugation in Hansenula appears to be that of a large cell and its small bud. Successively higher levels of development include heterogamy of independent cells, isogamy of independent cells, and conjugation of germinating ascospores or their immediate descendants. Another high form of conjugation is nuclear fusion within a germinating ascospore. The three methods of conjugation named first take place at the end of the vegetative stage of haploid cells; the remaining types occur at the beginning of the vegetative stage of the yeast cells which exist as diploid cells in the vegetative stage.

In developing the theory of phylogenetic lines in Hansenula, Wickerham is in opposition to the theory of Guilliermond (1937), who believed that haploid and diploid yeasts represent two separate lines of development, with the haploid yeasts arising from a form similar to Eremascus, and the diploid yeasts developing from Taphrina. According to Guilliermond (1940), the true yeasts have been derived from the mycelioid forms by reduction.

Many mycologists consider the yeasts and related forms to be primitive groups, perhaps showing evolution in their development from primitive to more recently evolved forms. Other workers consider them to be reduced, degenerate forms which represent the highest stage in the development of the Ascomycetes.

Physiological studies with H. anomala were continued in 1951 by a group including Davies and Peel, who identified acetic acid and detected a non-volatile acid, which was not identified, among the metabolic products of the organism. Tabachnick (1951), using chromatographic methods, detected acetic, malic, succinic, citric, and fumaric acids in the yeast cells. Hughes (1952) found only two acids produced by fermentation by H. anomala: volatile acetic acid and a non-volatile acid which was not identified. His investigation showed that this acid was not saturated, contained only one acidic group, and decomposed without melting at 212-217° C. No acids with these characteristics were listed in available tables of derivatives. Davies et al. (1951) reported that the acid did not prove to be pyruvic, lactic, or succinic.

Brock (1952) studied the effects of inhibitors on respiration in H. anomala. Observing that certain inhibitors of glycolysis and the tricarboxylic acid cycle did not appear to inhibit respiration, he suggested that an alternate pathway might exist. This pathway of carbohydrate metabolism was suggested to be similar to that demonstrated by Warburg, Lipmann, and Dickens, with others, and sometimes named for them. This is also called a pentose phosphate pathway or a hexose-monophosphate shunt. Together with glycolysis, it will be discussed more fully later.

We may illustrate the two possible pathways of carbohydrate metabolism thought to occur in yeast by the following diagram. The glycolytic pathway is often called an Embden-Meyerhof-Parnas scheme (EMP) after workers who demonstrated its existence. We may abbreviate the hexose-monophosphate shunt as HMP.

Whether or not the triose phosphate (glyceraldehyde-3-phosphate) produced from the two pathways forms a pool, as has been assumed, is not known. There is a possibility that there is intermixing between the two pathways (Horecker and Mehler, 1955).

The hexose-monophosphate pathway is well known to occur in yeast. Not only does it provide a route for the respiration of hexoses and pentoses, it also contributes to the synthesis of ribose, found in nucleic acids.

The results of Davis (1958) indicate that both glycolytic and hexose-monophosphate pathways are simultaneously active in H. anomala. Unpublished data by Gray indicate this also, as does the work of Sova (1960). Sova also substantiated the probable inter-mixing of both pathways.

The phosphorylation of glucose, fructose, glucosamine, and mannose all depends on the enzyme hexokinase. The rate of phosphorylation of these sugars can be determined for each of these alone by saturating yeast hexokinase with each. When these are added simultaneously to the enzyme system, the total activity is far less than would be expected if a summation of individual activities was taking place. This may be due to a competitive inhibition between the sugars for the active center of the enzyme (Axelrod, 1960).

Recent unpublished results by Gander with isolated enzyme systems indicate that fructose has an inhibitory effect on the enzyme systems of glycolysis. This inhibition may occur at one of the first steps in the glycolytic pathway, catalyzed by hexokinase.

Acetate formed in respiration by yeasts, either directly by the glycolytic pathway, or by the oxidation of ethanol formed by the hexose-monophosphate shunt, can be further catabolized. This would take place possibly by the tricarboxylic acid cycle; most of the intermediate compounds of the cycle have been found in yeast.

The mechanism of the Krebs tricarboxylic acid cycle, or citric acid cycle, has been clarified by the work of Lipmann, Ochoa, and others. Novelli and Lipmann (1947, 1950) observed that yeasts deficient in pantothenic acid do not utilize acetate as effectively as non-deficient yeasts. Coenzyme A, which contains pantothenic acid as part of its structure, was found to be concerned with the primary attack on acetate, presumably a condensation with oxalacetate to form citric acid. These investigators confirmed the catalytic function of coenzyme A in citric acid synthesis with cell-free extracts of yeast.

Activation of a two-carbon, or acetyl, fragment produced in respiration by coenzyme A is important in the composition of many major cell constituents: fats, phospholipids, proteins, and polysaccharides. The acetyl group, formed from pyruvic acid, a common intermediary in sugar oxidation, is a basic building block in biosynthesis.

The aims at the investigation, then, were two-fold:

(1) To block either the glycolytic pathway or the hexose-monophosphate shunt by using specific inhibitors of each, and (2) to determine whether an unknown non-volatile acid previously reported in H. anomala might be produced by the hexose-monophosphate shunt or by a glycolytic mechanism.

MATERIALS AND METHODS

The basal medium used by Kuehner (1951) and later by Hughes (1952) in their studies with Hansenula anomala was modified for use in the present investigation because it is a chemically definable medium.

Hansenula anomala (Hansen) Sydow, strain Number 20 of the Ohio State University stock culture collection, was used throughout this investigation.

In preparing inoculum for use in these studies, a 25 ml. portion of basal medium was autoclaved at 15 pounds pressure for 20 minutes, cooled, and inoculated with a loop needle from a slant of H. anomala. Slants of the yeast were cultured on the following medium: 0.7 per cent Difco Yeast Extract, 0.5 per cent KH_2PO_4 , 5.0 per cent glucose, and 3.0 per cent agar agar. The liquid culture was incubated without agitation for 24 hours at 30°C. At the end of the incubation period, 1 ml. portions of this inoculum were used to inoculate 100 ml. of basal medium in 250 ml. erlenmeyer flasks.

During the course of the investigation, various alternate methods for culturing were tested. One of these studies used successive transfers on liquid media instead of slants for growing inoculum; no demonstrable difference in results

was observed. Other studies using varying amounts of medium in different sizes of flasks were planned to test the effects of aeration. Additional tests showed that a pH of the basal medium of 6.2 before autoclaving seemed to promote best acid production, and that $30^{\circ}\text{C.} \pm 2^{\circ}$ was optimal for acid production. Length of culture period was studied; a high total acid production was obtained at **seven** and eight days. Eight days was the length of incubation in most of the investigation except where exceptions are noted.

The medium as used by Hughes (1952) contained 2 per cent glucose. In attempting to secure maximum total acid production this was raised to 4, 5, and 10 per cent glucose. The final amount is shown in Table 1, listing the composition of the medium.

When inhibitors were used, known molar solutions were made up and added 24 hours before harvest, except in the studies with fructose, where it was incorporated into the medium as will be indicated later.

Total acidity was determined by titration of 5 ml. aliquots of the fermentation medium (diluted with 25 ml. of carbon dioxide-free water) with 0.1000 N NaOH using phenolphthalein as indicator, and was expressed as milliliters of normal acid per 100 ml. of fermentation medium.

Table 1.--Composition of basal medium.

Constituent	Per liter of solution
Glucose	100.0 grams
Asparagine	2.0 grams
KH_2PO_4	1.5 grams
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.5 grams
CaCl_2 (Anhydrous)	0.28 grams
$(\text{NH}_4)_2\text{SO}_4$	2.0 grams
KI	0.1 milligrams
Trace element solution*	1.0 milliliters

*Composition of trace element solution:

Element	p.p.m./ml.
Mn as MnSO_4	0.01
Zn as ZnCl_2	0.07
B as H_3BO_4	0.01
Cu as CuCl_2	0.01
Mo as Mo_2O_3	0.01
Fe as FeSO_4	0.05

Volatile acid was determined in the following manner: 5 ml. of the fermentation medium and 0.5 ml. 10 per cent phosphoric acid were put into a Sellier tube in a modified Hortvet apparatus and 100 ml. of steam distillate was collected. This distillate was then titrated with 0.1000 N NaOH with phenolphthalein as indicator.

Non-volatile acid was determined as follows:
acidity due to non-volatile acid = total acidity minus volatile acidity.

For ethyl acetate determination a 50 ml. sample was removed from the culture. About 25 ml. of this sample were distilled into a 500 ml. erlenmeyer flask, and a few drops of phenolphthalein were added. The distillate was neutralized with 0.1 N NaOH until the color was just pink to phenolphthalein. Twenty-five ml. of 0.1000 N NaOH were added to the neutralized distillate and refluxed for one hour. The distillate was cooled and titrated with 0.1000 N **HCl**. Total ethyl acetate production per flask in milligrams was calculated as follows: (25 ml. - No. of ml. of HCl) x 8.8 x 2.

RESULTS

I. Preliminary investigations

A number of screening tests were conducted to determine how to obtain optimum total acid production. These were explained in detail in the preceding section and were concerned with length of culture period, temperature requirements, pH of the medium, area of the culture flask and other factors influencing aeration, and amount of glucose in the medium (Table 2). Studies were also made on the best method of preparing inoculum.

II. Inhibitors

The second area of investigation was concerned with the effect of various inhibitors on the production of volatile and non-volatile acids. Inhibitors were selected which were known to inhibit the metabolic pathways at specific points, as will be indicated. With all of these agents, a series of screening surveys were made to determine what concentrations would have the greatest effect.

TABLE 2.--Acid production by Hansenula anomala under different hydrogen ion concentrations and length of incubation period. PH of the medium before inoculation is given. Figures represent the average of six flasks in each trial.

Days	pH	ml 0.1 N acid	
6	5.0	0	total acid
6	5.0	0	total acid
6	6.5	6	total acid
		2	non-volatile acid
10	6.1	0	total acid
6	6.1	6	total acid
		2	non-volatile acid
12	6.5	0-4	total acid
10	6.5	11	total acid
		2	non-volatile acid
10	6.1	10	total acid
		6	non-volatile acid
10	6.1	12	total acid
		6	non-volatile acid

Medium in above trials is basal medium with two per cent glucose.

Generally a relatively weak concentration was used to begin with, and successively stronger solutions were then used. In some cases, there were indications in the literature as to what concentration might be used as a starting point.

A list of the inhibitors used and their probable place of action in the metabolic pathways may be helpful in visualizing the attack on the problem.

The inhibitors known to affect glycolysis include fluoride, iodoacetic acid, 2,4-dinitrophenol, and sodium bisulfite. The hexose-monophosphate shunt is inhibited by cyanide and atabrine.

III. Results of investigations with inhibitors

(1) Screening tests as to concentration were begun with a concentration of 10^{-5} M and with some inhibitors were gradually reduced to a concentration of $10^{-0.25}$ M. These will be indicated specifically in the next section. It must be kept in mind that at the stronger concentrations there may be side effects of any poison which would have an overall effect on the metabolism of the cell.

(2) As indicated above, the inhibitors affecting

glycolysis are fluoride, 2,4-dinitrophenol, sodium bisulfite, and iodoacetic acid. If these inhibit glycolysis at any point, we might expect an increase in non-volatile acid coming down through the alternate metabolic pathway, the hexose-monophosphate shunt.

With both sodium fluoride and 2,4-dinitrophenol, results with concentrations of 10^{-4} M in each case showed an increase in non-volatile acid over that of the control. In no case was there a complete blocking of glycolysis; some volatile acid was always produced. This was true of all the glycolytic inhibitors (Table 3).

In the case of sodium bisulfite, results were somewhat inconclusive, with amounts of total acid obtained varying widely, and with no definite ratios of volatile and non-volatile acids being observed.

Some of the most promising results came with the use of the sodium salt of iodoacetic acid. This poison is believed to inhibit triosephosphate dehydrogenase, an enzyme in the glycolytic pathway.

With sodium iodoacetate in concentrations as small as 10^{-2} M, an increase in the amount of non-volatile acid over that of the control was noted. Further tests used sodium

TABLE 3.--Effect of glycolytic inhibitors on production of acid. TA = total acidity; NVA = non-volatile acidity; these are expressed as ml 0.1 N acid/ 100 ml. The basal medium contained two per cent glucose. Figures represent an average of three flasks per trial.

NaF (10^{-4}) as inhibitor

control		with inhibitor	
TA	NVA	TA	NVA
trial 1 12.5ml	6ml	18ml	7.5ml
2 14.0	6	20	9.0

2,4-dinitrophenol (10^{-4}) as inhibitor

control		with inhibitor	
TA	NVA	TA	NVA
trial 1 14ml	6ml	26ml	13ml
2 11	6	21	11

iodoacetate in successively increasing concentrations up to $10^{-0.25}$ M. The best results in increasing the non-volatile acid/volatile acid ratio over that of the control were obtained at this concentration (Tables 4 and 5).

(3) Inhibitors thought to affect the hexose-monophosphate pathway through the cytochrome system are cyanide and atabrine.

If these are effective inhibitors, we might expect a low amount of non-volatile acid produced with a corresponding increase in the amounts of volatile acid produced.

Potassium cyanide was used in a gradient of concentrations from 10^{-5} M to $10^{-0.25}$ M. Some indications of a low non-volatile acid/volatile acid ratio were observed at the weaker concentrations. This effect became pronounced at a concentration of 10^{-1} M and continued to appear at stronger concentrations (Table 6).

Atabrine is another inhibitor of the monophosphate shunt mechanism, although it blocks the pathway at an earlier point than cyanide does.

Atabrine was observed to lower the non-volatile acid/volatile acid ratio at concentrations of 10^{-3} M and 10^{-2} M.

TABLE 4.--Effect of the glycolytic inhibitor sodium iodoacetate on acid production. Acidity is expressed as ml 0.1 N acid/ 100 ml. TA = total acidity; NVA = non-volatile acidity. Figures represent the average of three flasks in each trial. Per cent glucose in the basal medium is given.

2% glucose		control		10 ⁻² M iodoacetate as inhibitor	
		<u>TA</u>	<u>NVA</u>	<u>TA</u>	<u>NVA</u>
trial	1	20ml	11ml	31ml	8ml
	2	32	10	35	22

2% glucose		control		10 ⁻¹ M iodoacetate as inhibitor	
		<u>TA</u>	<u>NVA</u>	<u>TA</u>	<u>NVA</u>
	1	9	7	15	7
	2	13	7	15	11
	3	22	12	20	13
	4	20	8	27	11

4% glucose					
	1	24	13	43	31
	2	45	36	46	33

5% glucose					
	1	36	26	19	17
	2	44	36	38	30

10% glucose					
	1	41	25	45	17
	2	26	12	46	40
	3	40	36	14	10

TABLE 5.--Effect of the glycolytic inhibitor sodium iodoacetate on acid production at different glucose concentrations. Acidity is expressed as ml 0.1 N acid/ 100 ml. TA = total acidity; NVA = non-volatile acidity; VA = volatile acidity. Figures represent the average of three flasks in each trial.

control					10 ^{-0.5} M iodoacetate as inhibitor			
	TA ml	NVA ml	VA ml	NVA/VA ratio	TA ml	NVA ml	VA ml	NVA/VA ratio
4% glucose								
trial 1	24	13	11	1.0:1	63	24	39	0.60:1
2	45	36	9	4.0:1	45	35	10	3.50:1
3	56	26	30	0.8:1	54	31	23	1.25:1
5% glucose								
	35	29	6	5:1	20	12	8	1.50:1
10% glucose								
1	29	13	16	1:1	32	16	16	1:1
2	64	56	8	7:1	36	32	4	8:1

control					10 ^{-0.25} M iodoacetate as inhibitor			
10% glucose								
1	44	32	8	4:1	28	20	8	2.5:1
2	36	32	4	8:1	28	26	2	13.0:1
3	38	28	10	2.8:1	24	20	4	5.0:1

TABLE 6.--Effect of potassium cyanide on acid production at different glucose concentrations. Acidity is expressed as ml 0.1 N acid/ 100 ml. TA = total acidity; NVA = non-volatile acidity; VA = volatile acidity. Figures represent the average of three flasks in each trial.

		control		KCN 10^{-4} M as inhibitor	
2% glucose	trial	<u>TA</u> ml	<u>NVA</u> ml	<u>TA</u> ml	<u>NVA</u> ml
	1	14	6	18	0
	2	11	6	18	0
		control		KCN 10^{-3} M	
	1	4	1	4	0.7
	2	19	12	20	3.3
		control		KCN 10^{-2} M	
	1	14	10	8	6
	2	21	9	30	20
		control		KCN 10^{-1} M	
	1	19	12	20	3.3
	2	8	6	9	6
4% glucose		control		KCN 10^{-1} M	
	1	100	92	63	60
	2	44	34	30	20
	3	40	36	45	26
10% glucose		42	32	32	22
4% glucose		control		KCN $10^{-0.5}$ M	
	1	41	35	21	13
	2	49	42	30	20
	3	100	92	76	62
	4	44	34	42	28
	5	40	36	37	26

TABLE 6.--Continued

trial	control				KCN $10^{-0.5}$ M			
	<u>TA</u> ml	<u>NVA</u> ml	<u>VA</u> ml	<u>NVA</u> / <u>VA</u>	<u>TA</u> ml	<u>NVA</u> ml	<u>VA</u> ml	<u>NVA</u> / <u>VA</u>
1	74	66	8	9:1	24	14	10	2.0:1
2	40	36	4	9:1	14	10	4	2.5:1
3	64	56	8	7:1	40	34	6	5.7:1
trial	control				KCN $10^{-0.25}$ M			
	<u>TA</u> ml	<u>NVA</u> ml	<u>VA</u> ml	<u>NVA</u> / <u>VA</u>	<u>TA</u> ml	<u>NVA</u> ml	<u>VA</u> ml	<u>NVA</u> / <u>VA</u>
1	44	32	8	4.0:1	32	16	16	1.00:1
2	48	38	10	3.8:1	14	6	8	0.75:1
3	38	28	10	2.8:1	12	4	8	0.50:1
4	38	34	4	8.5:1	20	16	4	4.00:1

Increasing the concentration to $10^{-0.5}$ M did not have any effect on the activity of the inhibitor. In no case was atabrine as effective as cyanide (Table 7).

Atabrine and cyanide were also used in combination. Here the effect of inhibition of the hexose-monophosphate shunt was about the same as with atabrine alone (Table 7).

With neither cyanide nor atabrine, nor with the combination of the two, was a complete blocking of the hexose-monophosphate shunt mechanism observed.

IV Results of investigations with fructose as an inhibitor

During the course of the investigation, reports were released from the Department of Agricultural Biochemistry at the Ohio State University by Dr. John Gander indicating that D-fructose might act as an inhibitor of some of the enzymes in the glycolytic pathway. These experiments were based on the use of isolated enzymes, and not with living organisms.

This suggested that fructose might also act as an inhibitor of glycolysis in the yeast cell, and investigations were begun on this phase of the project. In theory, fructose might interfere with one of the first steps in

TABLE 7.--Effect of atabrine and potassium cyanide on acid production. Acidity is expressed as ml 0.1 N acid/ 100 ml. TA = total acidity; NVA = non-volatile acidity; VA = volatile acidity. Figures represent the average of three flasks in each trial. All cultures are on a ten per cent glucose basal medium.

TA	NVA	TA	NVA	TA	NVA	TA	NVA				
control		control with foil		with atabrine 10 ⁻³ M		atabrine 10 ⁻³ M with foil					
70	64	52	46	54	42	56	46				

control		control with foil		with atabrine 10 ⁻² M		atabrine 10 ⁻² M with foil					
58	46	44	40	66	52	52	42				

control		with atabrine 10 ⁻³ M		KCN 10 ⁻¹ M + atabrine 10 ⁻³ M		KCN 10 ⁻¹ M					
42	32	38	26	32	24	32	22				

TA	NVA	NVA/ VA	TA	NVA	NVA/ VA	TA	NVA	NVA/ VA	TA	NVA	NVA/ VA
control			atabrine 10 ⁻² M			KCN 10 ^{-0.5} M +atabrine 10 ⁻² M			KCN 10 ^{-0.5} M		
trial 1											
74	66	9:1	40	34	7:1	24	14	2:1	24	14	2:1
trial 2											
40	36	9:1	32	24	3:1	24	18	3:1	22	10	1:1
trial 3											
64	56	7:1	48	38	4:1	34	24	2.5:1	40	34	6:1

control			atabrine 10 ^{-0.5} M			KCN 10 ^{-0.25} M +atabrine 10 ^{-0.5} M			KCN 10 ^{-0.25} M		
48	38	4:1	34	26	3:1	44	38	6:1	14	6	0.75:1

glycolysis, catalyzed by hexokinase; respiration would then occur through the hexose-monophosphate shunt mechanism, and an increase in non-volatile acid might be expected if this acid is formed through this pathway.

Investigations were set up using the standard 10 per cent glucose medium as a control, replacing 2 per cent of the glucose with D-fructose, and using a complete replacement of the glucose with 10 per cent D-fructose.

On cultures grown on 10 per cent fructose, and also on those grown on a medium where 2 per cent fructose replaced the same amount of glucose, an increase in the number of cells was observed; there was an increase in total acid production; more non-volatile acid was produced compared to the control; and production of volatile acid also increased.

Effect of varying concentrations of fructose.--A more intensive study was conducted using further gradations in relative concentration of glucose and fructose. As indicated in Table 8, the results of these studies substantiated those of the preliminary tests. There was increased total acid production when fructose

replaced part of the glucose in the basal medium. This increase in total acid production did not become greater as more fructose was used. An increase in volatile acidity on fructose was noted in most cases; non-volatile acid production was higher on fructose in each case. Production of ethyl acetate decreased on media containing fructose as compared to the 10 per cent glucose control.

Observation of acid and acetate over a ten day period.--

The next phase of the project was the determination of total acid, volatile and non-volatile acids, and ethyl acetate produced each day over a ten day period on 10 per cent glucose and 10 per cent fructose media. This was repeated fourteen times. These results are recorded in Table 9, and in Figures 1 - 6.

Total acid production was higher on the fructose medium than on glucose. On fructose, total acidity reached a peak at seven days; on glucose, total acid continued to rise until the tenth day. Volatile acid was considerably higher on fructose, where it continued to rise until the tenth day. On glucose, volatile acid reached a peak at nine days.

TABLE 8.--Effect of graded concentrations of glucose and fructose on acid and acetate production. TA = total acid; NVA = non-volatile acid; VA = volatile acid.

percentage of sugars in basal medium		TA ml (acidity expressed as ml 0.1 N acid per 100 ml)	VA ml	NVA ml	acetate milligrams
10% glucose a		35.3	2.6	32.6	
8% glucose + 2% fructose		45.6	7.3	38.3	
10% glucose b		33.0	6.0	27.0	316.8
6% glucose + 4% fructose		41.0	7.0	34.0	156.6
10% glucose b		33.0	6.0	27.0	316.8
5% glucose + 5% fructose		50.0	10.0	40.0	126.7
10% glucose c		42.0	8.0	34.0	332.6
4% glucose + 6% fructose		54.0	8.0	46.0	119.7
10% glucose b		33.0	6.0	27.0	332.6
3% glucose + 7% fructose		46.5	6.0	40.5	147.8
10% glucose d		33.0	3.5	29.5	420.6
10% fructose		49.3	10.7	38.5	98.6

- a. average of three series; no acetate was determined
b. average of two series
c. an eight-day series; all others were seven-day
d. average of four series

TABLE 9.--Acid and acetate production over a ten day period. Figures represent the average of fourteen series. TA = total acid; VA = volatile acid; NVA = non-volatile acid.

	10% glucose				10% fructose			
	TA	VA	NVA	acetate	TA	VA	NVA	acetate
days after inoculation	ml	ml	ml	mg	ml	ml	ml	mg
1	3.85	--	--	53.20	1.66	--	--	45.18
2	6.71	--	--	61.83	6.89	--	--	73.66
3	8.82	1.52	8.07	82.81	8.67	2.07	6.46	79.35
4	10.95	1.95	9.00	99.14	15.75	3.42	12.14	100.30
5	12.27	2.64	9.92	109.88	15.53	4.96	10.57	130.64
6	15.56	2.70	12.86	114.92	20.67	5.89	14.78	144.60
7	16.12	2.92	13.27	130.46	22.57	6.54	15.92	147.22
8	16.70	3.22	13.48	118.76	19.78	7.00	12.78	149.44
9	17.72	3.60	14.06	94.18	18.01	6.91	9.99	137.57
10	19.70	3.50	16.00	95.97	21.64	9.86	13.21	141.93

Figure 1. -- Acid production on 10% glucose over a ten day period. Figures represent the average of fourteen series.

Figure 2. -- Acid production on 10% fructose over a ten day period. Figures represent the average of fourteen series.

Figure 3. -- Production of total acid over a ten day period. Figures represent the average of fourteen series.

Figure 4. -- Production of volatile acid over a ten day period. Figures represent the average of fourteen series.

Figure 5. -- Production of non-volatile acid over a ten day period. Figures represent the average of fourteen series.

Figure 6. -- Ethyl acetate synthesis over a ten day period. Figures represent the average of fourteen series.

Non-volatile acid production on the fructose medium reached a peak at seven days. This peak was approximately the same as the highest value for glucose, which was recorded at ten days.

Ethyl acetate production was higher on the medium containing fructose. On glucose, highest acetate production was at seven days; on fructose, at eight days. After seven days, acetate production on glucose fell off sharply. On fructose, acetate levels remained approximately the same until the end of the ten day period.

Levels of acid and acetate after twenty days.--A further experiment was conducted to investigate the levels of acid and ethyl acetate after a longer period of time. Ten flasks on each of the two media, 10 per cent glucose and 10 per cent fructose, were harvested after twenty days. The results of this experiment (Table 10) showed that the amounts of total acid reached similar levels after this period of time; non-volatile acid was slightly higher on the glucose medium, while volatile acid was slightly higher on the fructose medium. Acetate production was also higher on the fructose medium.

Table 10.--Acid and acetate levels after twenty days. Figures represent the average of ten flasks for each medium. Initial pH was 6.3. TA = total acid; VA = volatile acid; NVA = non-volatile acid.

	TA	VA	NVA	acetate
	ml	ml	ml	mg
TRIAL 1				
10% glucose	24.0	8.0	16.0	122.20
10% fructose	21.5	9.0	12.5	136.72
TRIAL 2				
10% glucose	23.0	5.0	18.0	116.16
10% fructose	23.0	7.2	16.0	128.48

Comparing the levels at twenty days with those reached at the end of the ten day series, it may be observed that levels of acid production had in general risen slightly, except for volatile acid production on fructose, which fell slightly from the ten day amount. Acetate production on glucose increased while acetate production on fructose was not as great at twenty days as at ten days.

DISCUSSION

A scheme of glycolysis (Nord and Weiss, 1958) with the points of inhibition by the specific inhibitors indicated might be represented as:

Fluoride inhibits the enzyme enolase (number 8 in the diagram) by forming a complex with the magnesium ion attached to the enzyme and with phosphate, forming a protein-magnesium fluorophosphate.

2,4-Dinitrophenol is called an "uncoupling agent." It reduces the formation of ATP without affecting oxidation, or uncouples phosphorylation from oxidation. This would occur at step 6, where ADP is changed to ATP, or at steps involving the conversion of 1,3-diphosphoglyceric acid to 3-phosphoglyceric acid, and of 2-phosphoenolpyruvic acid to enolpyruvic acid.

Use of both fluoride and 2,4-dinitrophenol at concentrations of 10^{-4} M resulted in an increase in non-volatile acid. This would indicate that glycolysis was blocked, with the increased non-volatile acid being produced by an alternate mechanism, possibly the hexose-monophosphate shunt.

Sodium bisulfite combines readily with aldehyde and ketone groups, and could thus combine with 3-phosphoglycer-aldehyde produced in glycolysis. It also forms an addition compound with a molecule containing a carbonyl group and a

double bond, as enolpyruvic acid contains. The possibility of its forming such a compound with enolpyruvic acid in glycolysis might account for the results obtained. No definite ratios of volatile and non-volatile acids were observed. It is possible that some of the intermediate products in glycolysis were tied up with bisulfite at some point. This might account for the varying amounts of total acid observed.

Some of the most promising results came with the use of the sodium salt of iodoacetic acid. This poison is believed to inhibit triosephosphate dehydrogenase, an enzyme in the glycolytic pathway (number 5), by combining with free sulfhydryl groups on the enzyme.

Results with sodium iodoacetate would indicate that glycolysis is being blocked and that increased non-volatile acid is being produced by an alternate pathway, possibly the hexose-monophosphate shunt. This proved to be the most effective of the compounds used to inhibit glycolysis.

Inhibitors thought to affect the hexose-monophosphate pathway through the cytochrome system are cyanide and atabrine.

If these are effective inhibitors, we might expect a low amount of non-volatile acid produced, with a corresponding increase in the amounts of volatile acid produced.

The hexose-monophosphate pathway and its relation to the cytochrome system (Lamanna and Mallette, 1953, and Axelrod, 1960) might be indicated as follows:

- enzymes: 1 glucose-6-phosphate dehydrogenase
or Zwischenferment
2 6-phosphogluconate dehydrogenase
3 phosphoriboisomerase
4 transketolase

Cyanide inhibits the enzyme cytochrome oxidase, also known as cytochrome a_3 , probably by combining with certain atoms in the prosthetic group of the enzyme and thus preventing the enzyme from functioning normally. It will be noted that this inhibition occurs at the very end of the cytochrome system.

Some indications of a low non-volatile acid/volatile acid ratio were observed at concentrations of potassium cyanide from 10^{-5} M to $10^{-0.25}$ M. This effect became pronounced at the greater concentrations. This might be interpreted as evidence that the hexose-mono-phosphate shunt is being blocked and that acid is being produced primarily through glycolysis.

Atabrine poisons the cytochrome system near the beginning, by inhibiting an enzyme made up of a flavo-protein and a prosthetic group called alloxazine mononucleotide. Atabrine competes with this prosthetic group for a place on the free protein, thus preventing formation of the complete enzyme system.

Atabrine was observed to lower the non-volatile acid/volatile acid ratio at concentrations of 10^{-3} M

and 10^{-2} M. Increasing the concentration to $10^{-0.5}$ M did not have any observable effect on the activity of the inhibitor. Thus atabrine appeared to block the hexose-monophosphate shunt mechanism; however, in no case was atabrine as effective as cyanide.

Atabrine and cyanide were also used in combination. Here the effect of inhibition of the hexose-monophosphate shunt was about the same as with atabrine alone. We may interpret this as demonstrating that the effects of cyanide and atabrine are not additive.

With neither cyanide nor atabrine, nor with the combination of the two, was a complete blocking of the hexose-monophosphate mechanism observed.

Possible interpretation of results with fructose.--In general, production of total acid, volatile acid, and ethyl acetate were greater on 10 per cent fructose than on 10 per cent glucose at seven days and at ten days. Volatile acidity and ethyl acetate production continued to be greater at twenty days, with total acidity being about equal on the two media.

Non-volatile acidity was greater on 10 per cent

fructose from four to seven days; from eight to ten days and at twenty days non-volatile acid production on glucose became greater than that on fructose.

The increase in non-volatile acid on a medium with 10 per cent fructose at seven days appears to confirm the theory that fructose may interfere with glycolysis at some point, and that the alternate pathway, the hexose-monophosphate shunt, might then produce increased non-volatile acid.

An increase in volatile acid on the 10 per cent fructose medium, contributing to a greater total acid production than in the 10 per cent glucose control, might be due to the fact that some fructose is phosphorylated and subsequently acted on by glycolytic enzymes, finally producing volatile acetic acid.

In general, ethyl acetate production does not appear to be affected to any great extent by fructose. Early experiments indicated that acetate production was inhibited by fructose in the medium, but subsequent, more intensive experimental work did not substantiate this.

Probably both pathways are operating simultaneously; when fructose is incorporated into the medium, the

principal part of respiration may be occurring through the monophosphate shunt, but some may be taking place through glycolysis. Results would indicate that fructose does not completely block glycolysis, but may inhibit it to some degree.

The exact mechanism of this inhibition is unknown, but may be due to some interference with one of the first steps in glycolysis, where glucose would ordinarily be phosphorylated and converted to phosphorylated fructose. Fructose may inactivate the enzyme necessary for some of these conversions. The enzyme most likely to be involved is hexokinase.

The enzyme system responsible for the conversion of fructose-1,6- diphosphate has been found to be extremely sensitive towards oxidation and might be inhibited under aerobic conditions. This suggests that yeasts follow a pathway other than glycolysis in breaking down carbohydrates aerobically.

It is possible that some internal changes in the aeration of the medium during the experimental period may influence the extent to which each pathway is used by the yeast.

Fructose-6- phosphate can be catabolized via the hexose-monophosphate mechanism during aerobic oxidation. However, most evidence indicates that in living cells, glycolysis and not the hexose-monophosphate shunt is the prevailing mechanism for the utilization of glucose (Nord and Weiss, 1958).

The study of a purified enzyme system may give much information about specificity of substrates and rates of reaction. However, the activity of the system in the living cell may differ from the results obtained with purified enzymes.

Results obtained with a particular species may also differ from those of another species; it is difficult to draw general conclusions based on results with a particular species. Genetic and environmental differences between species may contribute to apparent variations in the predominance of one pathway or another.

The results from the investigations with fructose would indicate that non-volatile acid may be produced by both a glycolytic mechanism and a hexose-monophosphate pathway. It is not possible to state that one pathway or

the other is the predominant one by which non-volatile acid is produced.

It seems likely that two or more metabolic pathways may be operating simultaneously in H. anomala, with some interaction between the pathways being possible.

SUMMARY

Results of the present investigation appear to indicate that in the yeast Hansenula anomala there are two pathways by which hexose may be utilized by the yeast cell.

It was possible to block the glycolytic pathway by using the inhibitors sodium iodoacetate, sodium fluoride, and 2,4-dinitrophenol, and thus increase the amount of non-volatile acid produced over that produced by the control without inhibitor. This would indicate that hexose metabolism was occurring by an alternate pathway, possibly a hexose-monophosphate shunt mechanism. These three inhibitors are known to inhibit glycolysis at three specific steps in the pathway; it may be significant that the best results were obtained by using sodium iodoacetate, which affects one of the earlier steps in the pathway, the conversion of 1,3-diphosphoglyceraldehyde to 1,3-diphosphoglyceric acid. None of the inhibitors

effected a complete blocking of glycolysis, although the production of volatile acid was reduced.

Conversely, experiments were designed to reduce the amount of non-volatile acid produced by the hexose-mono-phosphate shunt. In this case the inhibitors used were potassium cyanide and atabrine. Both of these appeared to inhibit the shunt mechanism to some extent, with potassium cyanide proving more effective. Here, increased volatile acid, presumably produced by glycolysis, resulted; again, it was not possible to block the pathway completely. These two inhibitors are known to affect the shunt mechanism at two separate points, with cyanide inhibiting a later reaction in the pathway than atabrine.

These results would indicate that two alternate pathways exist in the metabolism of H. anomala. Under certain conditions it is possible that one or the other pathway may operate alone; results from the present study would indicate, however, that the pathways more often are involved simultaneously. Perhaps the best evidence for this was obtained from the results using fructose as an inhibitor.

Fructose differs markedly from the other inhibitors

used in that it is a sugar very similar to glucose, rather than a metabolic poison like the inhibitors used earlier. Previous research indicates, however, that fructose may act in some way as an inhibitor of glycolysis.

The present results indicate that fructose may interfere with glycolysis, since an early increase in non-volatile acid is evident. Apparently when glycolysis is inhibited, respiration occurs through the alternate shunt mechanism. At the same time, an increase in volatile acid was also noted. This might be due to a phosphorylation of the fructose and its incorporation into the glycolytic pathway. The greater total acid production on fructose over that on glucose might then be due to the fact that both pathways are operating simultaneously. It is not possible to state that the hexose-monophosphate shunt is the predominant avenue for respiration in the yeast cell on a medium containing fructose.

The studies with fructose also point to some interaction between the two pathways, with fructose being converted to glucose and then being metabolized through

the shunt mechanism, after one of the first steps in glycolysis is impeded by the presence of fructose. The reverse reaction, the conversion of phosphorylated glucose to fructose-6-phosphate, is an early step in glycolysis. It is also possible that further interaction between the two pathways occurs.

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AUTOBIOGRAPHY

I, Janet Posey Toy, was born in Follansbee, West Virginia, on June 7, 1930, the daughter of Clyde D. and Marie Posey. I attended public schools in that community, and received the degrees Bachelor of Arts and Master of Science from West Virginia University, at Morgantown, in 1951 and 1952 respectively. While working toward the Master's degree, I was a teaching assistant in biology and a research assistant to Dr. V. G. Lilly and Dr. H. L. Barnett in the field of physiology of fungi. From 1952 until 1959 I taught in public high schools in Weirton and Follansbee, West Virginia, and Germantown, Ohio. In 1957 I began to work toward the degree Doctor of Philosophy at the Ohio State University under an Academic Year Institute grant from the National Science Foundation. In 1958 I was married to William Minor Toy. From 1959 until 1961 I was a member of the biology faculty at Wittenberg University, Springfield, Ohio, in which city I now reside.