TRACER STUDIES OF PYRUVIC ACID METABOLISM IN YEAST

by

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TRACER STUDIES OF PYRUVIC ACID METABOLISM IN YEAST

SECTION I

INTRODUCTION

Very little is known at present regarding the biosynthesis of the amino acids, particularly the mode of formation of the carbon skeleton. However, with the advent of isotopes as tracers a new and versatile tool came into use. This allowed attacks on hitherto practically insolvable problems, among which are many biosynthetic reactions leading to the formation of the amino acids. Thus, a highly active field of research has developed in very recent years.

The work being presented, a study of pyruvic acid metabolism in yeast, has been carried out in preparation for and in conjuction with the investigation of the biosynthesis of amino acids. Yeast is a very advantageous organism to use for a problem of this type because (1) it is easy to grow in large, workable quantities; (2) the protein content is high, about 50 per cent; (3) the organism can easily be kept free of contamination; (4) yeast can synthesize all of the known amino acids from carbohydrate, or a similarly utilizable carbon source, and inorganic salts; (5) yeast has been shown to possess the same tricarboxylic acid cycle (18) as most animals have for the complete oxidation of carbohydrates.

Pyruvic acid was chosen as a compound worthy of investigation because of its key position in the metabolic scheme of reactions. Carbohydrate intermediates usually enter the tricarboxylic acid

cycle either as a form of pyruvate or acetate for oxidation to carbon dioxide and water. Alanine is derived from pyruvate simply by transamination, while two more amino acids arise from keto acid members of the tricarboxylic acid cycle, namely, aspartic acid from oxaloacetic acid and glutamic acid from ketoglutaric acid.

Pyruvic acid containing C^{14} in the carbonyl group has been employed in the present work. The carbonyl position was chosen so that all of the pyruvate which underwent simple decarboxylation would remain radioactive. Since such a two-carbon residue might be metabolically equivalent to acetate, the latter compound has also been included in a parallel experiment with pyruvate for purposes of comparison.

The formation of pyruvic acid by microorganisms was first noted in 1916 during a study of malic acid. A year later work appeared demonstrating the production of pyruvate from lactate by yeast. However, it was not until the early 1920's that a great volume of research began to be published on many phases of the metabolism of pyruvic acid in yeast. Leaders in this field at the time were Haag in France and Neuberg in Germany, both of whom contributed greatly. Neuberg is best known for his fermentation scheme of which pyruvate is a key member (10).

The conversion of various substrates into pyruvic acid and the effect of several factors on its formation have been investigated. Of special significance to this work are the data presented by Brechot and Haag (2). During a prolonged (thirteen day) fermentation,

the decrease in glucose and increase in alcohol was measured. Simultaneously there was noted an increasing pyruvic acid concentration which reached a maximum on the fourth day of the fermentation period, and then decreased nearly to zero at the end of the fermentation. The accumulation of pyruvic acid was apparently due to the absence of cocarboxylase since the acid tends to disappear when the concentration of this enzyme is above 3×10^{-9} per cent (6). It is stated that the low concentration of cocarboxylase rather than pH is the essential factor in pyruvic acid formation. As high as 0.44 per cent conversion of sucrose to pyruvic acid, bound as the semicarbazone, has been reported (4).

In addition to pyruvic acid formation by yeast, factors concerning the utilization of the compound have been investigated. Yeasts exhibit the phenomenon of diauxie towards the uptake of pyruvic acid in the presence of glucose. Diauxie is the situation whereby, in the presence of two carbohydrates, one is used virtually exclusively until exhausted, then the other is used. This phenomenon has been extensively investigated in microorganisms by Monod (9). Studying the competition for mixed substrates by yeast, Sperber and Runnström (16) found that glucose always displaced pyruvic acid and alcohol, even when its concentration was lower. Pyruvic acid and ethanol were metabolized simultaneously. Moreover, carbon dioxide production in the aerobic metabolism of glucose somewhat exceeded the oxygen consumption, the difference having been equivalent to the alcohol production. Inasmuch as pyruvic acid and

alcohol are both formed during glucose fermentation, it follows logically that neither would likely be utilized from an exogenous source in the presence of glucose.

The limiting factor in the utilization of exogenously supplied pyruvic acid has been postulated by Smythe as being the permeability of the cell membrane (14). This conclusion was founded on the following observations: (1) practically no pyruvic acid was utilized at pH 7 or above in phosphate buffer, however, it was metabolized readily in an acid medium; (2) urea increased the rate of pyruvic acid utilization three-fold, while various amino acids had a similar effect. These observations led Smythe to conclude that a physicochemical factor governs pyruvic acid utilization. His data were interpreted to indicate that the permeability of the cell membrane is increased under aerobic conditions and decreased under anaerobic conditions. The opposite is true for glucose and has been used to explain the Pasteur Effect.

In their investigation of the metabolism of exogenous pyruvic acid, Runnström, Sperber and Karlsson (12) compared the breakdown of pyruvate at the cell periphery and its fate in the cell interior. Peripherally, pyruvate was oxidized to carbon dioxide plus water and to carbon dioxide plus acetic acid. In the cell interior, decarboxylation occurred to produce carbon dioxide plus acetaldehyde. The proportion of the pyruvate that was oxidized to acetate increased with the initial concentration of pyruvate. Significantly, the addition of two per cent glucose per hour for three hours

prevented the anaerobic conversion of pyruvate. Apparently some extractable factor was essential for pyruvate utilization since yeast cells which had been impoverished by shaking in distilled water overnight, respired less and converted less pyruvate than normal. However, pyruvate conversion increased after one hour, indicating that some factor facilitating the reaction was being formed. The authors believed that this was von Euler's Z factor for facilitating glucose uptake. In the light of present day research this factor would also seem to bear a superficial resemblance to the pyruvate oxidation factor of O'Kane and Gunsalus (11).

Working on the mechanism of pyruvate catabolism, Chen and Tang attacked the problem using enzymatic inhibitors (3). Cyanide reduced the rates of respiration in the presence of pyruvate, the inhibitory effect being counteracted by methylene blue. The fact that both the rates of aerobic carbon dioxide production and of oxygen consumption were affected by cyanide and methylene blue simultaneously and to the same extent indicates that the oxidation of pyruvate is a direct oxidation to acetate and carbon dioxide instead of passing through the intermediate process of acetaldehyde formation, on which neither cyanide nor methylene blue has any effect. This conclusion was contradicted by recent work of Weinhouse, Millington and Lewis on the carbohydrate metabolism of yeast employing C^{14} (19). The latter's data supported the postulate that fermentation and oxidation of glucose, and therefore pyruvate, diverge at acetaldehyde.

In the research being reported, acetic acid has been tested in

a parallel experiment to pyruvic acid because of the possibility that pyruvate is metabolized via acetate, or a closely related two carbon unit. The importance of acetate as an intermediate in the oxidation of carbohydrates seems to have been established by Weinhouse and coworkers (18, 19). They determined that at least half of the glucose undergoing complete oxidation passed through acetate as an intermediate. In addition, it was demonstrated that the tricarboxylic acid cycle is a common pathway in yeast for the complete oxidation of both glucose and acetate.

The fat fraction of the yeast is especially important to observe when investigating acetate metabolism. White and Werkman (20) found the fat content of yeast to increase over 100 per cent on incubation with acetate; and the increased fat was derived almost entirely from the added acid. Here also, acetate was demonstrated to be readily incorporated into the carbohydrate metabolizing system of the cell.

The work being presented is thus a study of the formation and utilization of pyruvic acid under aerobic and anaerobic conditions. The results obtained aerobically are compared with acetic acid utilization in a parallel experiment. In order to compare the metabolic pathways, carbon and radioactivity balances were established by measurements on carbon dioxide expired, aldehyde evolved, alcohol formed, total uptake in yeast and residue in medium. In addition, a simple analysis of the yeast has been made with particular attention being given to the fat fraction.

SECTION II

EXPERIMENTAL

Apparatus. The apparatus for yeast growth and fermentations consisted of a culture flask equipped for the addition and removal of various substances, and suitable attachments for aeration and the collection of metabolic products swept out in the effluent gas. The culture flask was of 3-liters capacity, with 3 necks and a round bottom. A mercury-sealed stainless steel paddle stirrer was inserted through the center neck; adjacent to the stirrer was fastened a gas outlet tube and leads for a pair of electrodes to be used in checking the pH. The electrodes were fixed with the tips above the stirrer but beneath the surface of the medium. Inserted into each side neck was a 3/4 inch strip of stainless steel which served as a baffle to prevent swirling of the medium and promote turbulent motion. In addition, each side neck contained a small dropping funnel for adding acid or base as needed. A gas inlet tube, with a sintered tip for sparging purposes, entered through one side neck and extended to the bottom of the flask beneath the stirrer. The other side neck was equipped with a glass tube extending into the culture medium for the removal of samples as required. Removal was accomplished by applying a slight negative pressure to a filter flask containing a 15 ml centrifuge tube into which extended, through a rubber stopper, one end of the sampling tube.

The rate of sparging was determined by passing the gas through

a manometer type flow meter. The gas was then led into a soda-lime tower and finally through a sterilized cotton tube to remove suspended contaminants before entering the culture flask.

The exhaust gas was dispersed in 500 ml of saturated dimedon solution (dimethyl dihydro resorcinol) by means of a sintered disc. This served the purpose of trapping any aldehyde which might be swept out in the gas. Respiratory carbon dioxide was collected in two gas scrubbing bottles connected in series, each containing 225 ml of 3 N sodium hydroxide (CO_2-free). The apparatus was made air tight by sealing all connections with Tygon, a liquid plastic.

Growth of yeast. The organism was obtained by isolation from a cake of Fleischmann's Bakers' Yeast, and a stock culture was maintained on malt-agar slants. In order to obtain a workable quantity of cells, a culture was grown in a rich natural medium (Blue Ribbon Malt Extract 200 gm, potassium dihydrogen phosphate 2 gm, urea 1 gm, tap water to 1000 ml). The procedure was as follows: Fifty ml of this medium was inoculated with a loop of cells (about 5 mg) from the stock slant. This culture was allowed to incubate 16 hours, and then used as the inoculum for the remaining 950 ml of the medium. Growth was carried out in the culture flask described above, with stirring at 250 rpm. No aeration was used because it was deemed unnecessary and it also presented a serious foaming problem. During the 16 hours incubation period, 28 per cent ammonia was added to the culture medium, when necessary, to maintain the pH at 4.0-4.5. Sterile conditions were maintained to assure a pure culture for later use.

Since the pH electrodes could not be autoclaved, the whole unit including the stirrer was well washed with sterile water and 70 per cent ethanol before being placed into position in the culture flask. At the termination of the growth period, the culture was refrigerated overnight. The cells were then centrifuged and washed twice with cold distilled water. This procedure gave very viable cells for further experimentation.

Incubation with labeled pyruvic acid[1] or acetic acid[1]. Ten gm (wet weight) of fresh yeast were taken for the inoculation of a medium[2] similar to that of Snell, Eakin and Williams (15), and containing glucose as the only significant carbon source. The incubation period was 4 hours, an interval sufficient for the metabolism of all the glucose (Figure I). Fermentation conditions included stirring at 400 rpm, sparging with nitrogen or oxygen at 100 ml per minute, and maintaining the pH at 4.0-4.5 with 3 N sodium hydroxide. Ten ml samples were withdrawn at zero time and at each hour thereafter for glucose and pyruvic acid determinations. At the completion of the fermentation (4 hours) the cells were centrifuged and washed twice with cold distilled water. Incubation of the cells with glucose served to rid them of many extraneous materials which had been

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1 Kindly supplied by Dr. C. H. Wang (Wang, Labbe, Christensen, and Cheldelin; to be published).

2 Composition of the medium: glucose 18 gm, $(NH_4)_2SO_4$ 2.5 gm, NaCl 2 gm, KH_2PO_4 2 gm, $MgSO_4 \cdot 7H_2O$ 250 mg, $CaCl_2 \cdot 2H_2O$ 250 mg, H_3BO_3 1mg, $ZnSO_4$ 1 mg, $MnSO_4 \cdot 4H_2O$ 1 mg, $FeCl_3$ 1 mg, $TiCl_3$ 0.5 mg, $CuSO_4 \cdot 5H_2O$ 0.1 mg, KI 0.1 mg, Bacto-Yeast Extract 100 mg, and distilled water to 1000 ml.

carried over from the malt medium, as evidenced by the yeast changing from a light brown color to creamy white during this procedure. The incubation also assisted partially in adapting the yeast to the simpler medium. The quantity of yeast recovered at this stage was found in preliminary experiments to be approximately 4.0 gm (dry weight).

The yeast obtained from the glucose medium was used as the inoculum for one liter of similar medium, with the glucose replaced by 20 m mols of labeled acetic or pyruvic acid. Fresh medium was used in this phase of the experiment because the yeast was more active when transferred than when allowed to remain in the same medium. Incubation conditions were essentially the same as with glucose. As acetic acid or pyruvic acid was utilized, the medium became alkaline, and the pH was controlled by the addition of 3 N hydrochloric acid. Sparging of the medium was accomplished with oxygen when acetic acid was the substrate, whereas, with pyruvic acid oxygen or nitrogen were used in successive experiments. The oxygen experiments were incubated 4 hours; however, the nitrogen experiment was extended to 5 hours to allow for fuller utilization of the pyruvic acid.

On completion of the incubation, the yeast was centrifuged, washed twice with distilled water, and dried *in vacuo* over sodium hydroxide. The yeast crops were retained for degradation and analysis.

Measurement of radioactivity. The methods used to measure the radioactivity of the various types of samples were essentially the same as those described in detail by Wang, *et al.* (17). Solid

materials were converted to barium carbonate, which was then ground in ethanol and mounted in steel planchets. Counting data were corrected for self-absorption. Liquid samples were counted by depositing directly into planchets and evaporating to dryness under an infra-red lamp. It was unnecessary to apply a correction for self-absorption in these cases because less than 1 mg of sample was deposited as a thin film. Since radioactivity measurements were employed to follow the fermentation processes of acetate and pyruvate, the more rapid direct deposition method was used. A minimum aliquot of the medium which would give a reasonable number of counts (approximately 1000 per minute) was pipetted into a cupped planchet and neutralized with 0.1 N sodium hydroxide, then evaporated to dryness before counting. Although salt deposits reduced the accuracy, preliminary studies indicated the method to be suitable for control and comparative purposes.

Chemical determinations. Glucose was determined iodometrically by the method of Shaffer and Somogyi (13).

Pyruvic acid was assayed as its phenylhydrazone in the colorimetric method of Friedemann and Haugen (5).

The residual acetic acid in the culture medium at the end of the prescribed incubation period was determined by steam distilling an aliquot of the medium in the presence of excess magnesium sulfate. The distillate was titrated with standard alkali and the radioactivity determined after direct deposition.

Acetaldehyde was determined only by measurement of the radio-

activity in the dimedon solution in which it was trapped. Preliminary studies of radioactivity recovery indicated that the method was satisfactory. The mols of acetaldehyde present were calculated on the assumption that this compound had the same specific activity as the pyruvic acid. The procedure appeared justified, since the ethanol obtained had the same specific activity.

The iodometric method of Kozelka and Hine (8) was used in the determination of ethanol in the culture media. The specific activity of the ethanol was determined after its oxidation to acetic acid, steam distillation of the latter compound, titration and counting by the direct deposition method.

Analysis of yeast. For partial analysis, 3.00 gm of dried yeast were ground to a fine state with a mortar and pestle. The yeast was ether extracted in a Soxhlet Extractor for 24 hours to secure at least partial removal of the free fatty materials. The amount of extractables was governed by several factors such as culturing conditions, method of drying, and nature of the extraction solvent (1).

The extracted yeast was degraded in 30 ml of 20 per cent hydrochloric acid for 24 hours to insure complete hydrolysis of all proteins[3]. By extracting with ether in a liquid-liquid extractor for 24 hours, the hydrolysate was freed of remaining fatty materials.

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[3] It was essential that the hydrolysis of proteins be complete because further planned investigations involve the determination of the manner in which individual amino acids are labeled, with a view to elucidating the methods of their biosynthesis from pyruvic acid.

The extracted hydrolysate was filtered to remove humin and the total dry weight of hydrolysate determined by drying an aliquot *in vacuo* over phosphorus pentoxide.

The humin contained adsorbed fatty materials which were removed by extracting with ether in a Soxhlet Extractor for 8 hours. This extract was combined with that from the hydrolysate. The dry weight of humin was determined and its radioactivity measured by combustion and counting as barium carbonate.

Weights of the two ether extractable fractions, before and after hydrolysis, were determined by drying the ether solutions with sodium sulfate, transferring to a tared flask, and removing the solvent by heating followed by vacuum evaporation.

Radioactivities of the hydrolysates and fat fractions were determined by counting after direct deposition, the hydrolysate being used as the aqueous solution and the fat fractions being dissolved in absolute ethanol.

SECTION III

RESULTS AND DISCUSSION

Formation of pyruvic acid. With 100 millimols of glucose per liter as substrate and with oxygen for sparging, a maximum of 3.98 millimols of pyruvic acid accumulated in the culture medium. This is equivalent to 1.99 per cent conversion, assuming one mol of glucose to yield 2 mols of pyruvic acid. When pyruvic acid was bound as the semicarbazone during the course of a fermentation, sucrose has been reported as being converted to pyruvic acid to the extent of 0.44 per cent (4). These values (1.99 per cent and 0.44 per cent) differ considerably but deviations might be explained on the basis of experimental differences in procedure when factors such as oxygen tension have such a great effect (Figure I). Using air for sparging, the maximum concentration of pyruvic acid accumulation lies midway between that found with pure oxygen and with pure nitrogen aeration. It is noteworthy that only a trace of pyruvic acid is found under essentially anaerobic conditions. The relative amounts of pyruvic acid accumulated under three levels of oxygen concentration in the sparging gas (100 per cent, 20 per cent and 0 per cent) definitely show that oxygen plays a role in this process.

The accumulation of pyruvic acid in the medium is seven times as great under aerobic conditions as it is under anaerobic conditions. Pyruvic acid may be formed to the same extent in each case but in the presence of oxygen, it is seemingly protected from the action of

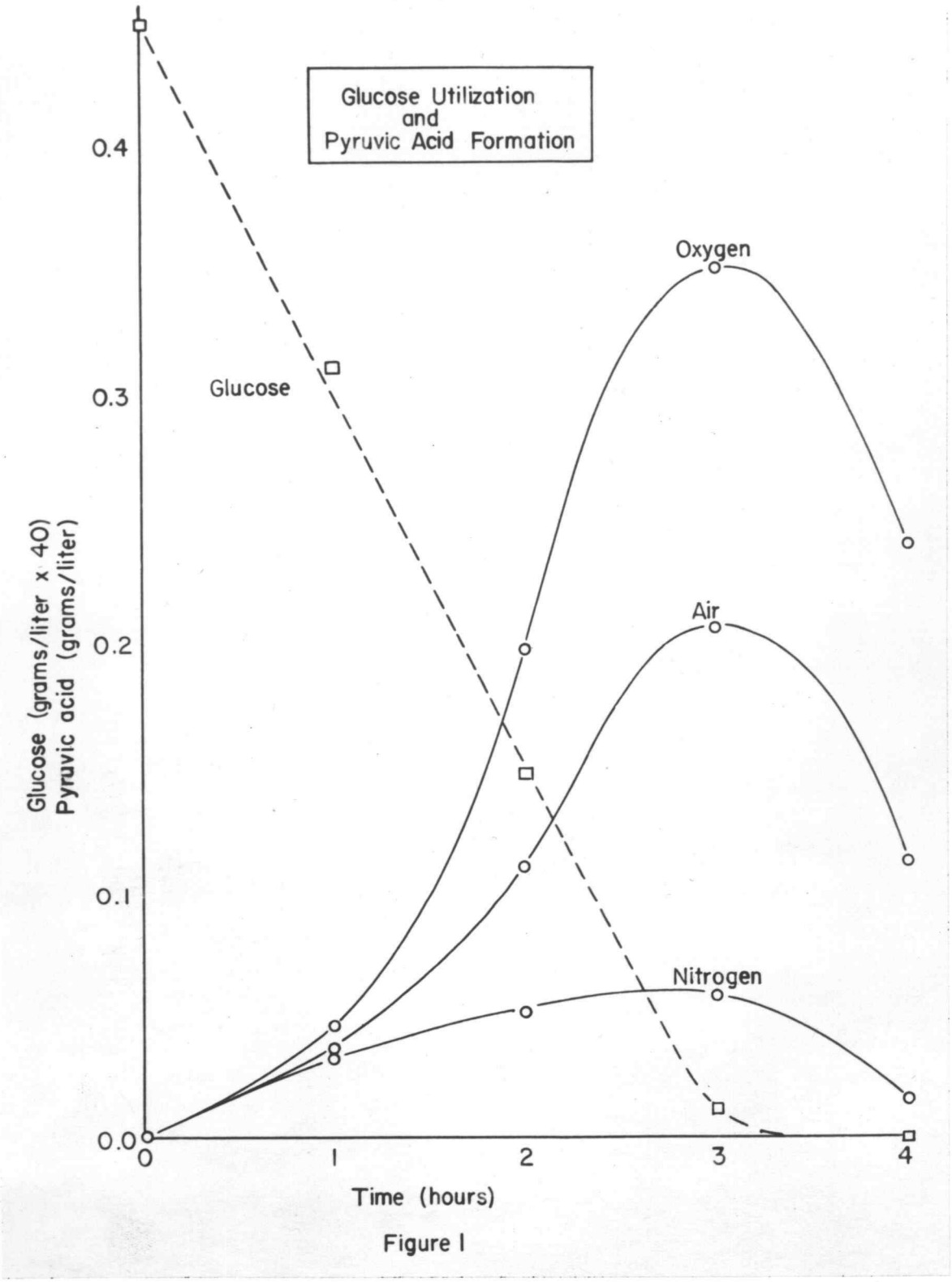
Glucose Utilization
and
Pyruvic Acid Formation
Glucose
Oxygen
Air
Nitrogen
Glucose (grams/liter x 40)
Pyruvic acid (grams/liter)
0.0
0.1
0.2
0.3
0.4
0
1
2
3
4
Time (hours)

Figure I

cocarboxylase. This coenzyme is rather easily oxidized to the disulfide form and may be so affected by molecular oxygen. The disulfide has no activity on pyruvic acid (7). Since the decarboxylation of pyruvate is an intracellular reaction (12), loss of cocarboxylase activity could be expected to allow some acid to escape into the medium in the presence of oxygen, without further conversion, so long as glucose is present to keep the forward reaction going and pyruvic acid forming.

Whereas the formation of pyruvic acid appears to be strikingly affected by oxygen, there is no significant difference in glucose utilization curves either aerobically or anaerobically. For this reason, only one curve illustrating the rate of uptake of glucose has been shown (Figure I). The exhaustion of glucose can be seen to coincide with the initiation of pyruvic acid uptake.

The data given differ with that of Brechot and Haag (2), especially regarding the observation here showing that no exogenous pyruvic acid was utilized before the glucose was almost completely exhausted. This was first noted in a preliminary experiment in which glucose and labeled pyruvic acid were added to the medium simultaneously. Only 5 per cent of the pyruvic acid had been metabolized at the end of 3 hours; and this may have taken place during the latter stages of the fermentation when the glucose level became very low. The finding also indicates that exogenous and endogenous pyruvate must not be in dynamic equilibrium, for if they were, considerably more radioactivity should have entered the cell

from the exogenous pyruvate, as occurs with acetate (19). In the preliminary experiment using pyruvic acid and glucose together, there was even then a slight increase in the total pyruvic acid present as found by chemical determination, indicating that the added pyruvic acid did not affect its accumulation in the medium as an end product of glucose metabolism.

According to Weinhouse and co-workers (19), acetic acid is likewise excreted when glucose is oxidized. The amount was calculated by the isotopic dilution method and found to be 4.1 millimols during the metabolism of 17.9 millimols of glucose under aerobic conditions. Assuming that 2 molecules of acetic acid arise per molecule of glucose, there was a 11.5 per cent conversion into excreted acetic acid. The accumulation was lowered considerably under anaerobic conditions (to about 3.8 per cent). However, either value is much higher than that reported here for pyruvic acid accumulation (1.99 per cent). In any case, it can be concluded qualitatively that acetate and pyruvate are regularly produced from glucose during its metabolism, in line with our general concept of the glycolytic and oxidative patterns that exist in this organism. The question then arises as to the extent that pyruvic and acetic acids are metabolized similarly or differently.

Utilization rates of pyruvic acid and acetic acid. For determining any differentiation which may be present in the method of metabolism of acetic and pyruvic acids, a logical initial consideration might be the comparative rates of utilization of acetic and

pyruvic acids under similar conditions (Figure II). Starting with 20 millimols per liter of medium, pyruvic acid was found to disappear at a linear rate until it was virtually exhausted, as measured both by chemical assay and radioactivity of the medium. Total removal of the acid occurred within 4 hours in the presence of oxygen. During this same interval, 39 per cent of the acetic acid was metabolized, as measured by radioactivity of the medium. The radioactivity determinations were slightly greater than the chemical assay near the end of the fermentation of pyruvic acid, apparently because of some non-volatile radioactive metabolic products being excreted back into the medium. On the other hand, the specific activity of the pyruvic acid during the main course of the fermentation showed that it was not diluted by pyruvate from endogenous metabolism. The same was true for acetic acid, which accounted for all of the radioactivity in its medium, proving further that no significant amount of non-volatile radioactive metabolic products had been excreted into the medium.

Examination of the glucose and aerobic pyruvic acid utilization curves shows that they have almost identical slopes (Figures I and II), which are independent of the substrate concentration. The rates of uptake of glucose and pyruvic acid thus correspond to zero order reactions, and become first order only at very low substrate concentrations. Since, it is believed, glucose enters the cell by active enzymatic transfer, it would appear that pyruvic acid, likewise, enters the cell by a similar mechanism. On the other hand, the

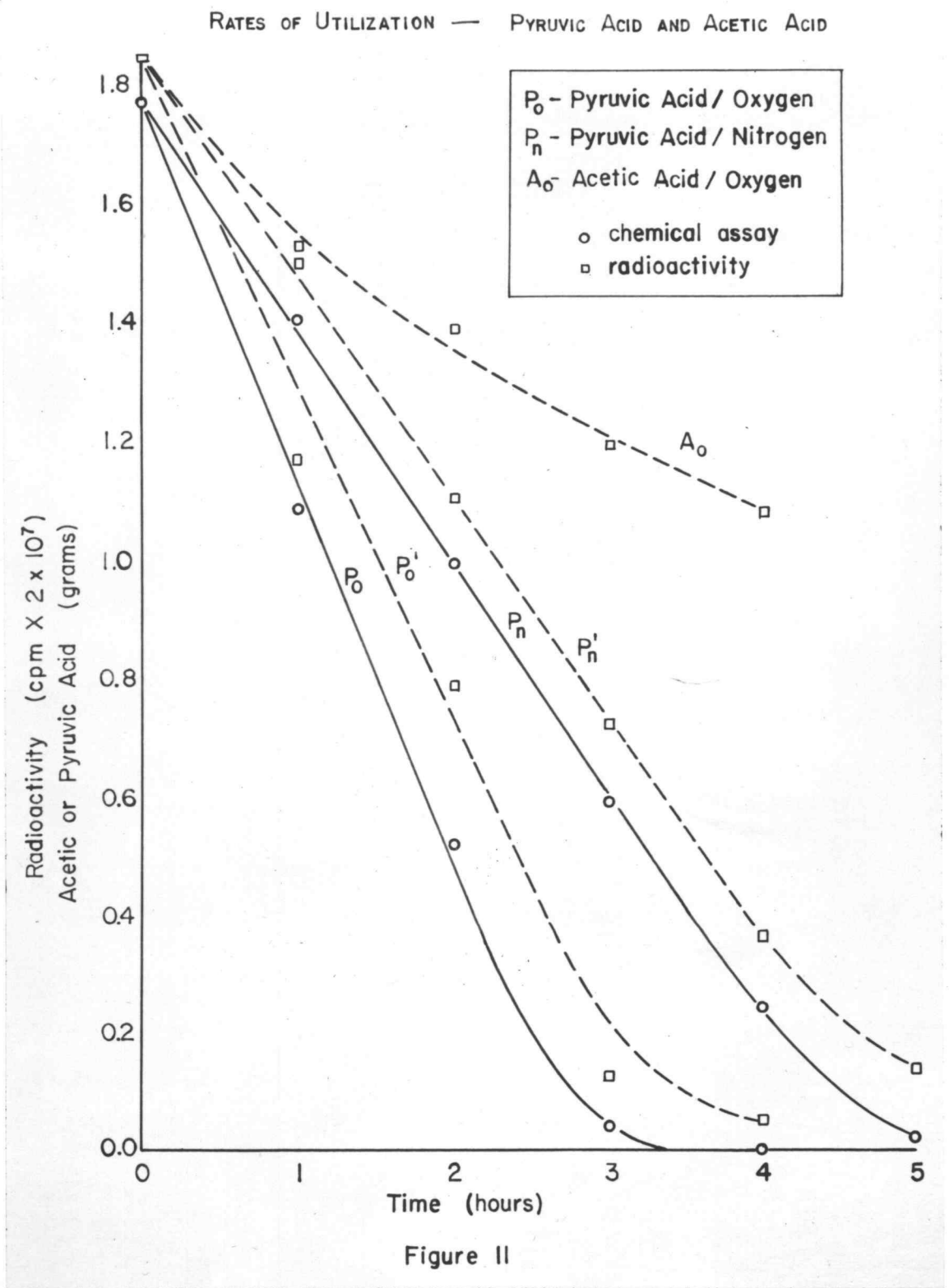
RATES OF UTILIZATION — PYRUVIC ACID AND ACETIC ACID
P_o - Pyruvic Acid / Oxygen
P_n - Pyruvic Acid / Nitrogen
A_o - Acetic Acid / Oxygen
o chemical assay
□ radioactivity
Radioactivity (cpm X 2 x 10⁷)
Acetic or Pyruvic Acid (grams)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Time (hours)
A_o
P_o
P_o'
P_n
P_n'

Figure II

utilization of acetic acid proceeds according to a first order reaction, which in turn suggests a different mode of utilization or transport across the cell membrane, perhaps simply through diffusion.

The rate of utilization of pyruvic acid in nitrogen is less than in oxygen, about 50 per cent more time being required for complete metabolism anaerobically, although uptake still occurs at a linear rate. The slower utilization may be due to reduced metabolic activity, or as Smythe has suggested (14), to decreased permeability of the cell membrane to pyruvate under anaerobic conditions. Recalling that the oxygen tension has no effect on glucose utilization, it would seem plausible to explain this difference in pyruvate uptake on a basis of permeability.

Metabolic products. A second factor to observe in a comparison of acetate and pyruvate metabolism should be the nature of some excretion products (Table I). Acids and nonvolatile substances accounted for 61 per cent of the radioactivity in the acetate medium while the oxygenated pyruvate medium contained only 3 per cent of the original radioactivity at the end of the fermentation period of 4 hours. The pyruvate medium incubated anaerobically contained 7 per cent of the radioactivity after 5 hours. The significance of these differences has been discussed above under utilization rates of acetic acid and pyruvic acid.

On examination of volatile products, it is seen that no detectable amount of ethanol appeared in the acetate medium (Table I). This is an expected result and confirms previous reports (19). In

TABLE I

ANALYSIS OF FERMENTATIONS OF PYRUVATE AND ACETATE BY YEAST

Fermentation Constituents	Acetate/Oxygen			Pyruvate/Oxygen			Pyruvate/Nitrogen		
	Quantity	Radioactivity cpm	Per cent	Quantity	Radioactivity cpm	Per cent	Quantity	Radioactivity cpm	Per cent
Substrate:									
Total gm & cpm	1.64	3.70×10^7	100	1.76	3.70×10^7	100	1.76	3.70×10^7	100
Millimols	20			20			20		
Sp.Act./m mol		1.85×10^6			1.85×10^6			1.85×10^6	
Sp.Act./m mol C		0.93×10^6			0.62×10^6			0.62×10^6	
Medium:									
Acids & Nonvol.		2.27×10^7	61		0.11×10^7	3		0.28×10^7	7
Ethanol gm & cpm	none	none	0	0.026	0.10×10^7	3	0.186	0.76×10^7	20
Millimols				0.565			4.05		
Sp.Act./m mol					1.77×10^6			1.87×10^6	
Acetaldehyde	none	none	0	(0.082) Calc.	0.35×10^7	9	none	none	0
Carbon Dioxide:									
Total gm & cpm	0.836	0.88×10^7	24	1.700	1.84×10^7	50	1.205	0.86×10^7	23
Millimols	19.0			39.6			27.4		
Sp.Act./m mol		4.64×10^5			4.77×10^5			3.14×10^5	
Yeast:									
Total gm & cpm	4.02	0.33×10^7	9	4.44	1.42×10^7	38	4.44	1.56×10^7	42
C assimilated gm (approx.)	0.0			0.2			0.2		
Radioactivity Recovery			94			103			93

the presence of oxygen, pyruvic acid, on the other hand, yielded 0.6 millimols of ethanol having a specific activity of 1.77×10^6 cpm/m mol. In a nitrogen atmosphere, 4.2 millimols of ethanol were formed, having a specific activity of 1.87×10^6 cpm/m mol. These specific activities both agreed, within experimental error, with that of the pyruvic acid serving as substrate (1.85×10^6 cpm/m mol). It can be concluded, therefore, that all of the ethanol arose directly from pyruvic acid, probably by means of decarboxylation to acetaldehyde and subsequent reduction to the alcohol. Seven times as much alcohol was formed anaerobically, most probably because its precursor (acetaldehyde) served as a major hydrogen acceptor in the absence of oxygen. Increased cocarboxylase activity may also be a contributing factor, as suggested for pyruvic acid accumulation during glucose fermentation.

In the aerobic pyruvate experiment, the dimedon trap contained nearly 10 per cent of the activity, presumably as acetaldehyde (Table I). The production of acetaldehyde is in accord with the postulate that pyruvate catabolism passes through acetaldehyde as an intermediate. As expected, none was found in the acetate experiment. Likewise, no aldehyde was liberated in the anaerobic pyruvate medium, probably because it was reduced as rapidly as formed. According to the principle of mass action, there would be a great affinity for any active hydrogen acceptor in the absence of oxygen. Since the cellular hydrogen transport enzymes under aerobic conditions must exist in a generally less reduced state, some of the

aldehyde formed could be expected to escape by way of the sparging gas. Since the aldehyde was not determined other than by radioactivity, it was impossible to calculate its specific activity.

Another fermentation product which gives an indication of metabolic activity is the carbon dioxide evolved (Table I). The pyruvic acid carbon has a specific activity equal to two-thirds that of acetic acid carbon. If these were oxidized to carbon dioxide by the same metabolic reactions, the specific activities should thus appear in the same ratio in the carbon dioxide as in the substrates. However, experimental results show the two substrates to yield carbon dioxide with equal specific activities. It may also be seen (Table I) that there was virtually no net growth of yeast with acetate, whereas about 0.4 gram of growth was recorded with pyruvate. In spite of this, 39 per cent of the radioactivity of the acetate disappeared; 9 per cent was incorporated into the yeast and an additional 24 per cent appeared as respiratory carbon dioxide. This suggests an equilibration between products of acetate metabolism and intermediary cellular metabolites; and the additional observation that the specific activity of the carbon in carbon dioxide is exactly half that of the carbon in acetate indicates dilution during oxidation and makes the foregoing equilibration picture appear certain.

A study of the aerobic pyruvate fermentation reveals that 39.6 millimols of carbon dioxide were produced from 20 millimols of pyruvic acid or 60 millimols of carbon metabolized. It can be concluded

from these data that 20 millimols of carbon, or one mol per mol of pyruvate was incorporated into the cells. This is reflected in the increased yield of yeast. If the carbon content of the yeast is taken as 50 per cent, an assimilation of 17.5 millimols of carbon occurred in the pyruvate experiments.

In the anaerobic pyruvate fermentation, 27.4 millimols of carbon dioxide and 4.5 millimols of ethanol (9 millimols of carbon) were produced from 20 millimols of pyruvic acid (60 millimols of carbon). Therefore, 23.6 millimols of carbon should have been retained. The material balance was less satisfactory here, since only 17.5 millimols of carbon were incorporated into the cells. However, the carbon dioxide was considerably diluted, so that there seems to be about twice the turnover with cellular constituents when pyruvate is metabolized anaerobically. It can be concluded that either the pyruvic acid is diluted by cellular constituents as suggested for acetic acid, or that the unlabeled groups are more susceptible to oxidation. In both types of pyruvate experiment, there was a net assimilation of substrate carbon giving an increased recovery of yeast over that obtained with acetic acid as substrate. Conclusions based on carbon dioxide data definitely indicate quantitative differences in exchange metabolism (aerobic or anaerobic) between pyruvic acid and cellular constituents, compared with the corresponding exchanges with acetate.

Analysis of yeast. The last method employed to study the metabolism of acetic and pyruvic acids has been the analysis of the yeast

cells after incubation with each of these substrates in parallel experiments (Table II). Pyruvic acid was also investigated anaerobically. Since the acetic acid was not completely utilized, as was the pyruvic acid in each case, the specific activity data for acetate has been corrected to 100 per cent utilization for comparative purposes. The whole yeast from the acetate fermentation is then seen to have taken up an average of 63 per cent of the radioactivity based on that found (by average) in the pyruvate fermentations. Thus, it appears that acetate is less readily available for syntheses than is pyruvate. Although anaerobically, the yeast incorporated 10 per cent more radioactivity than was found aerobically, it does not necessarily indicate a difference in metabolism because this deviation falls within the limits of experimental procedures.

The ether-extracted hydrolysates of yeast contain mainly amino acids and carbohydrates. From the acetate experiment, this hydrolysate fraction accounted for 58 per cent of the total radioactivity incorporated into the cells. In contrast, yeast incubated with pyruvate under oxygenation had 77 per cent of the radioactivity in this fraction. Anaerobically, there was only 69 per cent radioactivity in the extracted hydrolysate. However, the specific activity (cpm/mg) in the latter case is equal to that obtained under aerobic conditions. Therefore, the same types of reactions must be involved in amino acid syntheses[4] from pyruvic acid under either condition.

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[4] Recent experiments have shown that the neutral fraction of extracted hydrolysate, consisting mainly of carbohydrates, contains but a few per cent of the radioactivity in the yeast; and practically the total radioactivity can be accounted for in the amino acids.

TABLE II

ANALYSIS OF YEAST AFTER PYRUVATE AND ACETATE FERMENTATIONS

Yeast Fraction	Acetate/Oxygen			Pyruvate/Oxygen			Pyruvate/Nitrogen		
	Total cpm	Weight (mg)	cpm/mg	Total cpm	Weight (mg)	cpm/mg	Total cpm	Weight (mg)	cpm/mg
Whole Yeast	2.46×10^6 100%*	3000	820 (2100)**	9.57×10^6 100%*	3000	3190	10.54×10^6 100%*	3000	3510
Extracted Hydrolysate	1.42×10^6 57.7%	2790	509 (1300)	7.32×10^6 76.5%	2950	2480	7.22×10^6 68.5%	2750	2620
Humin	0.09×10^6 3.7%	245	367 (940)	0.35×10^6 3.7%	243	1440	0.40×10^6 3.8%	255	1570
Free Fat Fraction***	0.05×10^6 2.0%	22.7	2200 (5650)	0.11×10^6 1.1%	34.2	3220	0.12×10^6 1.1%	31.4	3820
Bound Fat Fraction***	0.70×10^6 28.4%	406	1720 (4410)	1.64×10^6 17.1%	614	2670	1.81×10^6 17.2%	529	3420
Recovery	91.8%			98.4%			90.6%		

*Percentages indicate the amount of radioactivity in the various fractions, based on whole yeast as 100%
**Specific activities converted to 100% utilization of acetate
***Fat fractions are mixtures of ether extractable substances.

Acetic acid must play a smaller role in this respect since the hydrolysate in this case has about one-half of the specific activity of that with pyruvic acid.

The data on humin show it to contain the same percentage of the radioactivity in each of the three fermentations. Likewise, the quantity of humin recovered can be considered as equal in every case. It follows that the specific activity of the humin from the acetate yeast again amounts to 63 per cent of that of the average values from pyruvate yeasts. The observations that the quantity of humin recovered is the same in each case and also contains the same percentage of radioactivity seem to imply a constant composition for humin. Furthermore, the rate of turnover of the constituents of humin is equal to the turnover in whole yeast.

Since fat and sterol metabolism involve the functioning of C_2 units, an investigation was made of the radioactivities and amounts of lipid material obtained. The free fat fractions which were extracted from the whole yeast contained an average of 5.7 per cent (by weight) of the total fat in the yeast. The value (5.7 per cent) was constant for the free fat in all of the yeast samples. The specific radioactivity in this fraction was over twice as large as in the whole yeast when grown in acetate, but in the two pyruvate experiments there was no difference between the specific activities of the free fat and the whole yeast. It thus appears that pyruvate becomes equilibrated fairly uniformly throughout the cell, whereas acetate, in line with general experience, exhibits a preferential

fat-forming activity. Free fat, although low in quantity in the acetate experiment, nevertheless, had a specific activity about twice as high as with pyruvate, thus supporting the present concept that C_2 units from acetate are readily converted into fat and contribute additional radioactivity over that obtained from pyruvate.

Results obtained from the bound fat fraction were generally parallel to those from the free fat fraction. Since this latter fraction contained all ether extractables, it was found in much greater quantities, about 14 to 17 times. Furthermore, the specific activities were reduced about 20 per cent, indicating a difference in the rate of turnover of the free and bound fat fractions. Acetate caused 28 per cent of the radioactivity of the yeast to be deposited in the bound fat. Again there was agreement between the two pyruvate experiments with 17 per cent of the radioactivity occurring in these fractions.

From analytical data of the yeast, it can be concluded that acetic acid is very active in fat synthesis and concentrates in this fraction. Pyruvic acid, on the other hand, is almost equally distributed (on a cpm/mg basis) in all yeast fractions examined. The latter observations strongly indicate that pyruvic acid is metabolized in a manner similar to that of a "normal" carbohydrate (glucose). Furthermore, with respect to incorporation into the yeast, pyruvic acid, whether under aerobic or anaerobic conditions, is metabolized basically in the same manner.

SUMMARY

1. During fermentation of glucose by yeast, pyruvic acid accumulates in the growth medium. The amount accumulated increases with increase in oxygen tension. The phenomenon is postulated as being due to the inactivation of cocarboxylase by oxygen.

2. Pyruvic acid is not utilized by yeast when glucose is present, even with glucose in very low concentrations.

3. When pyruvic acid is the sole carbon source, it is utilized at a linear rate, about twenty per cent as rapidly as glucose. Whereas the glucose utilization rate is unaltered by either aerobic or anaerobic fermentation conditions, pyruvic acid utilization rate is reduced fifty per cent anaerobically. In both cases, the uptake of pyruvic acid behaves as a zero order reaction.

4. Aerobically, acetate is utilized at only forty per cent of the rate of pyruvate. Furthermore, the uptake of acetate is non-linear and appears to behave as a first order reaction.

5. While no ethanol arises from acetate, there is ethanol found in the pyruvate media. Specific activities indicate that all of the ethanol arises directly from the pyruvic acid.

6. Aldehyde is liberated from pyruvic acid under aerobic conditions. Its formation indicates that the aldehyde (presumably acetaldehyde) is an intermediate in pyruvate dissimilation.

7. The amount of carbon dioxide evolved during fermentation indicates that one-third of the pyruvate carbon is assimilated into the yeast cell with concomitant growth. Carbon dioxide from acetate

is diluted fifty per cent indicating equilibration with cellular carbohydrates. Growth is insignificant in the presence of acetate.

8. Analyses of the yeast indicate that pyruvate is about twice as effective as acetate in synthesizing protein. Pyruvate is about half as active as acetate in the synthesis of fatty constituents.

9. The products of pyruvic acid metabolism are almost uniformly distributed in all cellular constituents examined. In distinction, acetic acid metabolites concentrate in the fat fraction.

10. Whether aerobically or anaerobically, pyruvic acid seems to be metabolized, in general, by similar metabolic pathways.

BIBLIOGRAPHY

1. Bills, Charles E., O. N. Massengale, and Paul S. Prickett. Factors determining the ergosterol content of yeast. Journal of biological chemistry 87:259-264. 1930.

2. Brechot, Paul and Erwin Haag. Production of pyruvic acid in the course of alcoholic fermentation. Comptes rendus des seances de l'academie des sciences 208:1847-1848. 1939.

3. Chen, H. K. and P. S. Tang. The kinetics of cell respiration. Oxidation of pyruvic acid by Escherichia coli, Pseudomonas pyocyaneus, Staphylococcus aureus, and Saccharomyces cerevisiae. Journal of cellular and comparative physiology 16:293-299. 1940.

4. Eliasberg, P. Demonstration of pyruvic acid in alcoholic fermentation by means of the semicarbazide method. Zeitschrift für physiologische Chemie 189:254-260. 1930.

5. Friedemann, Theodore E. and Gladys E. Haugen. Pyruvic acid. II The determination of keto acids in blood and urine. Journal of biological chemistry 147:415-442. 1943.

6. Haag, Erwin and Charlotte Dolphin. Mechanism of accumulation of pyruvic acid during alcoholic fermentation in a synthetic medium. Compte rendu des seances de la societe de physique et d'histoire naturelle de Geneve 57:73-75 (in Archives des sciences physiques et naturelles 22 July-August. 1940).

7. Karrer, P. and Max Visconti. The mechanism of vitamin B_1 activity and the knowledge of cocarboxylase. Helvetica Chimica Acta 29:711-718. 1946.

8. Kozelka, F. L. and C. H. Hine. Method for determination of ethyl alcohol for medicolegal purposes. Industrial and engineering chemistry, analytical edition 13:905-907. 1941.

9. Monod, Jacques. The phenomenon of enzymatic adaptation. Growth 11:249-256. 1947.

10. Neuberg, Carl and Ernst Simon. The nature of pyruvic acid fermentation. Biochemische Zeitschrift 187:220-253. 1927.

11. O'Kane, D. J. and I. C. Gunsalus. Pyruvic acid metabolism, a factor required for oxidation by Streptococcus faecalis. Journal of bacteriology 56:499-506. 1948.

12. Runnström, J., E. Sperber, and Eva Karlsson. The permeability of the cells of bakers' yeast for pyruvic acid under various physiological conditions. Arkiv för Kemi, Mineralogi och Geologi 13B:No. 10 5pp. 1939.

13. Shaffer, Philip A. and Michael Somogyi. Copper-iodometric reagents for sugar determination. Journal of biological chemistry 100:695-713. 1933.

14. Smythe, C. V. The utilization of pyruvic acid by bakers' yeast. Journal of biological chemistry 125:635-651. 1938.

15. Snell, Esmond E., Robert E. Eakin, and Roger J. Williams. A quantitative test for biotin and observations regarding its occurrence and properties. Journal of the American chemical society 62:175-178. 1940.

16. Sperber E. and J. Runnström. Competition of certain substrates for the enzymes of the living cell of baker's yeast. Biochemische Zeitschrift 300:373-380. 1939.

17. Wang, C. H., Tsoo E. King, Vernon H. Cheldelin, and Bert E. Christensen. Methionine reversal of 2-chloro-4-aminobenzoic acid inhibition in Escherichia coli. Journal of biological chemistry 188:753-758. 1951.

18. Weinhouse, Sidney and Ruth H. Millington. Acetate metabolism in yeast, studied with isotopic carbon. Journal of the American chemical society 69:3089-3093. 1947.

19. Weinhouse, Sidney, Ruth H. Millington, and Katherine F. Lewis. Oxidation of glucose by yeast, studied with isotopic carbon. Journal of the American chemical society 70: 3680-3683. 1948.

20. White, A. G. C. and C. H. Werkman. Assimilation of acetate by yeast. Archives of biochemistry 13:27-32. 1947.