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Properties of compounds produced by the action of Acetobacter suboxydans upon i-inositol

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NOTE TO USERS

PROPERTIES OF COMPOUNDS PRODUCED BY THE ACTION OF *ACETOBACTER SUBOXYDANS* UPON i-INOSITOL

by

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INTRODUCTION

The harnessing of micro-organisms for commercial production of pure organic compounds was an achievement that, with the exception of alcohol production by yeast, belongs entirely to this century. Were it not for the fact that industrial organic chemistry has also made great strides in the last forty years, more substances would be prepared with the aid of these minute laboratories than are in fact produced at the present time. The organism with its enzymes and catalysts matches in efficiency the chemist with his high temperatures and pressures; and often some small economic change or the discovery of new nutrilites or procedures will turn the scales in favor of one or the other.

Future progress will depend upon an increased knowledge of the potentialities of micro-organisms through the elucidation of the principles underlying microbiological activity. The final aim is a better control of the activities of the organism and the ability to forecast the nature of its action under special conditions and on given raw materials.

Acetobacter suboxydans has been found to be a unique tool for the oxidation of polyhydric alcohols, of suitable configuration, to the corresponding ketose sugars, while other Acetobacter species may carry on a series of

dehydrogenations and decarboxylations to finally yield carbon dioxide and water. Bertrand (1904), in his classical investigations upon the comparison of the steric configuration of a polyhydric alcohol and the oxidizing action of Bacterium xylinum, came to the following conclusion, -- "The configuration favorable for dehydrogenation was that in which the hydroxyl of the third carbon was on the same side of the chain as the hydroxyl of the secondary alcohol group in the β- position." Generally, this rule has been found to hold true.

Dunning, Fulmer, Guymon and Underkofler (1938) tested the action of Acetobacter suboxydans upon i-inositol for several considerations. Since it contains five cis-secondary alcohol groups on the same side of the cyclic plane, one would expect it to be oxidized in accordance with Bertrand's rule (1904), but it would be interesting to ascertain the effect the closed ring exerted on the position and the number of secondary hydroxyl groups attacked. Such a reaction would permit the preparation of cyclic polyhydric ketoses not now available. These authors found that, under appropriate conditions, the i-inositol was oxidized to form a cyclic polyhydric ketose which preliminary studies indicated to be a diketo-i-inositol. Since the exact chemical nature of the product was not known they refer to the compound as "ketose", a term used throughout this thesis.

The complementary action of i-inositol in the Bios complex has been confirmed by several investigators since its identification by Eastcott (1928) as Bios I. Thus, the biochemical conversion of this compound into an oxidized product might throw additional light upon possible reversible oxidation-reduction systems and the configuration necessary for Bios I activity.

The purpose of this investigation was to study the chemical properties of the fermentation product and also to determine whether or not the Bios I activity of i-inositol had been affected by the limited oxidation involved. In the course of these studies it was planned, first, to examine the fermentation product in an effort to determine both the quantity and the position of the oxidized hydroxyl groups; and, second, to test the new cyclic "ketose" upon three different yeasts to determine its possible role as a yeast growth stimulant.

HISTORICAL

Inositol

Inositol and its derivatives possess considerable biological interest. Its wide occurrence in nature, its important physiological properties, and its close relationship in chemical constitution to the hexoses and some hydroaromatic compounds all combine to make it a potent chemical for investigation. The synthesis of inositol derivatives, when compared to analagous derivatives of straight chain hexoses, affords a comparison of the effect on the properties of a hexose in which the carbons are arranged in the form of a closed ring to those with an open chain. A study of its derivatives may also throw some light on the role played by i-inositol as Bios I.

Inositol first was discovered, as a constituent of meat extract, in 1850 by Scherer, and called "inosite" or "muscle sugar". It was first classed as a sugar because of its empirical formula, $C_6H_{12}O_6$ and its pronounced sweet taste. Vohl (1857), identified a substance isolated from the unripe fruit of green beans (Phaseolus vulgaris), and called it phaseo-mannitol; it is the same compound as inositol. He prepared the hexa- and tri-nitrate derivatives through the action of concentrated nitric and sulfuric acids on inositol.

These nitro-derivatives have recently found use as explosives and are now employed as detonators (Crater, 1934). Tanret and Villiers (1878) reported investigations in which they isolated Scherer's (1850) i-inositol from French beans, ash leaves, horse flesh, peas, green beans, acacia, cabbage, digitalis, potato plant, and asparagus. Later (1881), a procedure was given for its extraction from walnut leaves and it was found that its content varied with the season; one kilo of dried leaves gave one gram when collected in June and three grams when collected in August. Higler (1871) commented on the high concentration of i-inositol in grapes and stated that i-inositol is the precursor to the paralactic acid found in active muscle. As evidence, he reported the production of paralactic acid by a species of the genus Lactobaccillus organism using i-inositol as the substrate. Vohl (1876), contrary to Higler (1871), found that when i-inositol was fermented with putrid cheese or flesh, the product was ordinary lactic acid. Meilière (1908) showed that i-inositol was present in the animal body in many important organs, for example, the thyroid, nervous tissue and thymus. He was also able to isolate it from many groups and varieties of vegetables, plants, and fruits.

Maquenne (1887), in his classical investigations on i-inositol, showed that it was a unique secondary alcohol and not a sugar as assumed by many previous investigators. He described an improved method of isolation of i-inositol based

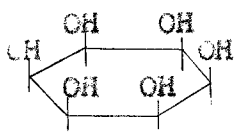
on the resistance of the compound to nitric acid. Further work was reported on its molecular weight, solubility and other physical properties, absence of reducing properties, resistance to further hydrogenation, and optical inactivity. Its hexagonal structure was established since it formed a hexacetate and a hexabenzoate which, upon treatment with hydriodic acid, gave tri-iodophenol. On oxidizing i-inositol with three to six times its weight of fuming nitric acid and evaporating to dryness, he obtained and identified oxalic acid, tetrahydroxyquinine, rhodizonic acid, croconic acid and triquinoyl. Wieland and Wishart (1914) further proved the cyclic nature of i-inositol by synthesizing it by the reduction of hexahydroxybenzene.

Since i-inositol occurs widely in nature as its methyl and ethyl ethers, it was necessary to identify isomeric i-inositols with their respective ethers. The ether was treated with hydriodic acid to give the free alcohol. Müller, (1912) showed that syllitol, cocositol and quercine were identical ethers and gave i-inositol on hydrolysis.

Pinite is the monomethyl ether of l-inositol. Additional derivatives of pinite and i-inositol were described by Griffin and Nelson (1915). The occurrence of methyl ethers of i-inositol in nature suggested the alkylation of i-inositol. Of the many agents tried, only dimethyl sulphate in alkaline solution proved successful. The alkyl i-inositols

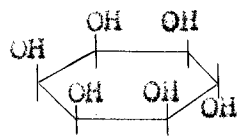
were optically inactive and hence probably not identical with those occurring in nature.

Maquenne (1887), Bouveault (1894) and Mohr (1903), showed that according to the arrangement of the hydroxy groups in i-inositol there were nine possible stereoisomers. Little was done to distinguish between the optical isomers until the work of Posternak and Posternak (1929). A schematic representation of the possible isomers is as follows:-



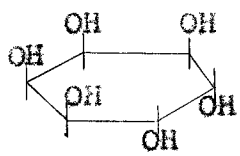
6 Planes of symmetry

Fig. 1



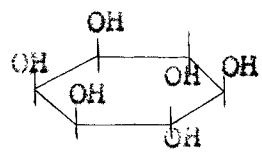
1 Plane of symmetry

Fig. 2



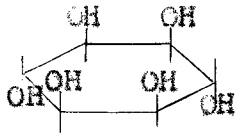
1 Plane of symmetry

Fig. 3



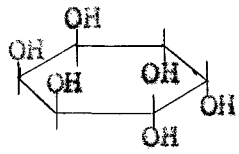
1 Plane of symmetry

Fig. 4



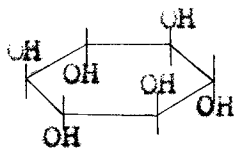
2 Planes of symmetry

Fig. 5



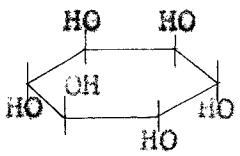
1 Plane of symmetry
Center of symmetry

Fig. 6

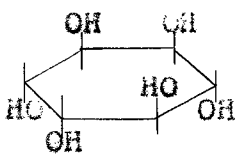


3 Planes of symmetry
Center of symmetry

Fig. 7



d



l

d, l - Inositol

Fig. 8

Only the last two formulas have no plane of symmetry and necessarily correspond to the d and l forms of i-inositol which are obtained from their respective ethers--pinite and quebrachitol. i-Inositol must be distinguished from the other seven formulas. Among other characterizing reactions were the oxidation of mono- or di-phosphate i-inositols with fumming nitric acid which gave meso- and d, l-tartaric acid, while oxidation with alkaline potassium permangante produced allo-mucic acid. Hence, i-inositol must have a configuration of five cis hydroxyls and one trans hydroxyl; that is, Fig. 2.

To distinguish between the d and l forms, l-inositol was oxidized with potassium permanganate and from the mucic and saccharic acids produced, formulas were assigned to these isomeric forms as was illustrated by Fig. 8.

An i-inositol compound frequently found in nature is phytin, a hexaphosphoric ester of i-inositol. Posternak (1900) was the first to successfully isolate phytin in pure form. In a series of papers (1903, 1905) he gave a comprehensive study of the physical and chemical properties of phytin, describing methods of preparation, results of analysis of pure products, and speculated on the constitution and biological functions of this product.

Pure phytin may be obtained either by isolation from vegetable matter or by direct esterification of i-inositol with phosphoric acid. Conversely, Bartow and Walker (1938), showed that calcium phytate was easily hydrolyzed to i-inositol and phosphoric acid in an autoclave at 5.6 - 6.3 kg. per sq. cm. pressure in 5 - 6 hours. The best yield of i-inositol obtained was 12 - 13 per cent based on phytin. Yields were not increased either by adding acid, increasing the pressure, or by lengthening the time of hydrolysis. The acid ester of phytin forms salts easily with the alkaline metals. Numerous investigators have prepared many salts, but mostly in an amorphous form and therefore of doubtful purity. Anderson (1912)(1914) has determined that no difference

exists in the salts prepared from commercial phytin and those prepared from phytin obtained from cottonseed meal, oats and corn. An excellent résumé of the literature on phytin has been prepared by Rose (1912).

Contardi (1921) carried out the oxidation of i-inositol in essentially the same manner as that of Maquenne (1887), using fuming nitric acid. However he was able to isolate only a material which he believed to be pure leuconic acid. Its identity was shown by the correct percentage composition and the preparation of an amorphous hydroxylamine derivative which gave the theoretical value for nitrogen when analyzed.

In view of the apparently contradictory claims of the above investigators, Gelormi and Artz (1930) repeated much of the previous work. Their results showed that in oxidizing i-inositol with nitric acid, a large number of products were obtained. All of the above mentioned products and hexahydroxybenzene were isolated and identified.

Bartow and Walker (1938) showed that oxalic acid was produced by the oxidation of i-inositol with concentrated nitric acid in the presence of iodine and bromine. In the absence of iodine or bromine, tetrahydroxy-quinone and rhodizonic acids were identified as products of the oxidation. Work was continued in 1939 by Hoglan and Bartow to obtain larger quantities of i-inositol, to improve the hydrolysis procedure by regulation of the pH of the precipitated phytin,

to find other cheaper sources of i-inositol and to prepare additional ester derivatives of i-inositol with members of the fatty acid series. The yield of i-inositol from wheat bran in five experiments was only 0.52 per cent. Bran was less satisfactory than steep water as a source of i-inositol.

Hoglan and Bartow (1940) in a further study on the properties of oxidized i-inositol, state that the sodium and potassium salts of tetrahydroxy-quinone were hydrolyzed upon treatment with hydrochloric acid. Aniline combined with tetrahydroxy quinones to give highly colored addition products. The color varied with the position of the substituents. Several yellow colored esters of fatty acids and tetrahydroxy-quinone were prepared. All the esters were crystalline and melted with decomposition.

Posternak (1936), by oxidizing i-inositol with nitric acid under certain conditions, was able to isolate a monoketo compound which he called inosose. This was the first ketone isolated belonging to the i-inositol series. Its properties were described and derivatives made, but no attempt was made to designate the particular hydroxyl group oxidized.

Since i-inositol occupies a unique position between the straight chain hexoses and the aromatic series, it would be very interesting to investigate the action of microorganisms upon it. Weiss and Rice (1917) found that i-inositol was

useful as a test medium for various species of bacteria. An investigation was made of the i-inositol fermentation of 144 strains of the paratyphoid enteriditis, typhoid, dysentery and colon groups.

Hewitt and Steabben (1921) tested i-inositol with Lactobacillus aerogenes but found no compounds produced which would form a phenylhydrazone. Succinic acid, acetic acid, formic acid, lactic acid, carbon dioxide and hydrogen were isolated and identified. Using the same organism and a medium composed of i-inositol, peptone, ammonium sulfate, calcium carbonate and calcium sulfite, Kumaguawa (1922) detected acetaldehyde in two days. Acids were also produced, and the fermentation was complete in twelve days. Bernhauer and Görlich (1935) used an i-inositol-yeast extract medium and found that Bact. gluconicum produced mainly acetic acid and probably some lactic acid. Aspergillus niger produced oxalic acid from i-inositol.

Because of the identification of i-inositol as Bios I by Eastcott (1928) and of their desire to form new cyclic ketones, Dunning, Fulmer, Guymon and Underkofler (1938) tested the action of Acetobacter suboxydans upon i-inositol in a yeast extract-sorbitol medium. They found that sorbitol (0.1 per cent) must be present for continued transfer in i-inositol medium. A compound was isolated from the fermentation which seemed to be a di-keto-inositol. Details were given for the development of the optimum cultural conditions

by Dunning, Fulmer and Underkofler (1940). The positive effect of the adjuvant materials as sorbitol, erythritol, glycerol, dextrose, and mannitol in the i-inositol fermentation was not associated with metabolic products thereof. They simply served as assimilable substrates permitting the growth of the organism and subsequent oxidation of the i-inositol.

Kluyver, Hof and Boezaardt (1939), in tracing the reason for a purple discoloration in a sample of salted beans, determined the causative organism to be Pseudomonas Beijerinckii Hof. The discoloration seemed to be associated with a substance present in the bean extract. This active compound was isolated and identified as i-inositol, which was biologically oxidized to form tetra-hydroxy quinone. Its calcium or barium salt was shown to be identical to the above purple pigment of discoloration.

Attempts to bring about an oxidation of i-inositol by a suspension of Pseudomonas Beijerinckii in manometric experiments of short duration, were unsuccessful; the oxygen uptake was too slow, since contamination occurred before the completion of the fermentation. Similar experiments using Acetobacter suboxydans--well known for its ability to carry out incomplete oxidations--gave better results. These bacteria suspended in a phosphate buffer of pH 6, to which 0.3 ml. of 0.1 mol i-inositol solution was added, gave a sharp oxygen uptake which levelled off in about one hour.

In several experiments it was found that at this time almost exactly one-half mol of oxygen per mol of i-inositol had been taken up. Hence, it seems that the i-inositol was converted to a monoketo-i-inositol. The purple coloration developed only after the fermented medium had stood in the air for some time and was probably due either to its autooxidation or to the gradual development of some contaminating organism. Other experiments were repeated in a similar medium buffered at pH 7.7. In more alkaline medium the A. suboxydans were suppressed by Pseudomonas species and the i-inositol was not oxidized. Such Pseudomonas species as aromatica var. quercto-pyrogallica and calco-acetica failed to oxidize i-inositol, while Pseudomonas fluorscens effected complete combustion of i-inositol to carbon dioxide and water.

It seems likely therefore, that Pseudomonas Beijerinckii specifically oxidized i-inositol to a triketo-i-inositol and that this colorless compound in contact with the air under went autooxidation to tetrahydroxy quinone which then reacted with calcium or magnesium ions, present in solution, to form the finely divided purple pigment.

Kluyver and Boezaardt (1939) described a biochemical method for the preparation of inosose, a monoketo-i-inositol, first prepared by Posternak (1936). They questioned the article of Dunning, Fulmer, Guymon, and Underkofler (1938) in which diketo-i-inositol was claimed to be the fermentation product from Acetobacter suboxydans acting upon i-inositol

under certain conditions. No mention was made of the addition of sorbitol to their buffered 3 per cent i-inositol -0.5 per cent yeast extract medium. Despite the fact that directions were given by Dunning et al (1938), Kluyver and Boezaardt (1939) disregarded the necessity of adding the sorbitol to the medium, and contrary to directions, they used buffered medium at pH 6 in their manometrical studies. Aeration was then introduced in the regular fermentation. A product was isolated from the fermentation, which, because of its slight solubility in water and a melting point of 200°, they identified it as being the same as Posternak's (1936) inosose. A yield of 90 per cent was reported, using this biochemical method of preparing inosose, as compared to a yield of 16 - 25 per cent obtained by Posternak (1936) and his nitric acid technique.

i-Inositol may now be classified as a member of the vitamin B complex as a result of work described by Woolley (1940). The dietary factor which was necessary for the normal maintenance of hair in mice was called the anti-alopecia factor. Experimental results with liver extracts and cereal grains indicated that this factor was i-inositol and its derivatives. Phytin caused restoration of hair and resumption of growth when added to the diet of depleted animals.

Bios

It has been seventy years since the "Bios" question first developed from a discrepancy in the experimental results obtained by Liebig (1871) and Pasteur (1860) on the growth of yeast. Pasteur (1860) stated that yeast could be grown on a medium containing sugar, salts, and ammonium tartrate as the source of nitrogen. Liebig (1871), although following Pasteur's (1860) procedure, was never able to confirm these results and a fierce controversy developed at that time. An explanation of this difference was not available until thirty years later when the investigations of Wildiers (1901) showed that a substance was present in boiled yeast extract which in addition to sugar, salts and nitrogen, was necessary for the normal growth of yeast. To this unidentified substance Wildiers (1901) gave the name "Bios", the Greek word for life. Although discovered contemporaneously with the analogous vitamins in human nutrition, the subject, notwithstanding numerous investigations, was much less developed and has engendered misconception and doubt even to the present time.

The Bios question then received little notice until 1919 when it was revived by Williams because of the obvious similarity between the action of Bios on yeast, and vitamins on higher animals. Attempts were made to correlate vitamin B potency of materials with their ability to stimulate yeast

growth by Williams (1919). These methods were based on the supposition that "Bios" and water-soluble B vitamins were identical; but they were proven unreliable and soon abandoned (Willaman and Olsen) (1923).

An adequate review of the existing literature on the Bios problem was made by Tanner (1925). Other reviews include those of Buchanan and Fulmer (1930), Miller (1930), Peskett (1932), Fulmer and Christensen (1934) and Fulmer (1939). Details concerning the literature during the above period may be obtained from these excellent reviews. Real progress was made mainly in the fractionation of Bios into a number of components. Lucas (1924) succeeded in fractionating Bios obtained from malt sprouts into what became known as Bios I and Bios II. Eastcott (1928) proved that Bios I was identical with i-inositol. It was not until 1932 that Miller, Eastcott, and Sparling succeeded in fractionating Bios II into components II-A and II-B through preferential adsorption of II-b on charcoal. Further purification of the Bios II-A concentrate by Miller and co-workers (1934) led to the isolation of hydroxy-amino-butyric acid as its copper salt. They also described experiments with thirty-two amino acids which showed that laevoleucine had the properties of Bios II-A; that is, when added to inositol and crude Bios II-B it added materially to the yeast crop.

Williams, Lyman, Goodyear, Truesdail and Holaday (1933) in applying their fractional electrolysis technique (1931)

were able to concentrate an acidic substance which seemed to be present in the extracts of diverse tissues representing many biological species and which exerted a stimulating effect on yeast growth. They concluded that the acid was of unusual biological importance and ascribe unto it the name Pantothenic acid which in Greek means "from everywhere". Its chemical properties were determined but its chemical structure was not elucidated.

The effects of inositol, crystalline vitamin B_1 and pantothenic acid on the growth of different strains of yeast were determined by Williams and Saunders (1934). Concentrated pantothenic acid was effective alone in doses of 0.008γ per ml. upwards on all the yeasts studied. The effect was enhanced by i-inositol and vitamin B_1 or both.

Farrell (1935) and Miller (1935) while studying the influence of i-inositol, Bios II-A, and Bios II-B on the reproduction of several kinds of yeast, noticed that Saccharomyces valbyensis gave no increased growth with the above addends; but gave a much higher yeast crop in the presence of filtered tomato juice. To this unknown constituent of tomato juice the name Bios V was given. Bios V was evidently destroyed by the chemical treatment during the isolation of Bios II, and therefore was separated and concentrated by precipitation with tannin (Miller) (1936). Miller (1936) stated that as a result of a note by Schultz, Atkin and Frey (1939), describing the effect of aneurin on

yeast, that he carried out experiments which showed Bios V and vitamin B_1 to be identical. They both had the same effect when tested with Saccharomyces valbyensis. At the same time he postulated the existance of Bios VII because neither crude Bios II-A nor Bios II-B could be replaced by purified preparations to give the maximum growth by Saccharomyces valbyensis.

Williams and Rohrman (1936) report the discovery that synthetic β-alanine was a highly potent agent for stimulating yeast growth. They observed that while pantothenic acid was active in the presence of asparagin, and relatively inactive in an asparagine-free medium; β-alanine was inactive in the presence of asparagin, and active only in the absence of asparagin. Miller (1936) added β-alanine to dextrose media containing salts, i-inositol and Bios II-B. Yeast growth was greatly increased, showing that β-alanine acted as Bios II-A. The further addition of l-leucine to β-alanine media caused a further increase in yeast count. However as was pointed out by Nielsen (1938) (1939) β-alanine exerted a toxic effect in the presence of asparagin and glutamic-acid, while when aneurin also was present, a stimulatory action was observed on yeast. The fact that very small amounts of β-alanine would stimulate growth was attributed to the probable presence of small amounts of aneurin in the yeast. Later Nielsen and Dagys (1940) showed that asparagin and glutamic acid could be replaced by citric, succinic, tartaric or malic acids.

Kögl and Tönnis (1936) described the preparation of a very active yeast stimulant from egg yolk. To this crystalline material they gave the name "biotin" and suggested a molecular weight of about two-hundred. A publication by Kögl (1937) states that biotin contains sulphur, basic units and a carboxyl group. Later (1938) he suggested the empirical formula $C_{11}H_{18}O_3N_2S$ with a molecular weight of two-hundred fifty-eight. Rainbow and Bishop (1939) identified Kögl's (1937) biotin with Bios II-B of Miller (1936). Its molecular formula was given as $C_7H_{12}N_2O_3$ with a molecular weight of one-hundred seventy-two. Only traces of sulphur of doubtful significance were reported. Other chemical findings suggested basic nitrogen (not in an amino form) and one carboxyl or pseudo-carboxyl group. The latter was suggested since the back-titration with acid followed a slightly different course from the titration with alkali. These facts together with the faint diazo reaction and the ultra-violet absorption maximum at 2630 A^o suggest that a pyrimidine ring may be present. The above authors (1939) agreed that biotin was extremely active either alone or in the presence of i-inositol. β-alanine was found to have some effect, but l-leucine was inactive. Kögl (1938) distinguished between the action of aneurin and biotin (a hormone-like potency) and that of β-alanine and i-inositol (specific nutritional factors). He regarded the former as definitely constituting an enzyme system.

Snell, Eakin and Williams (1940) described a quantitative test for biotin in which they measure the response of yeast to varying concentrations of biotin. Over a range of 0.00002 γ to about 0.0012 γ the test was reliable and quantitative. β-alanine and vitamin B_6 increased its effectiveness.

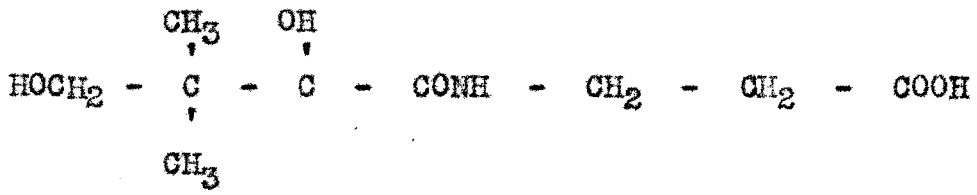
Williams and his co-workers continued their investigations on the concentration and identification of pantothenic acid and published a series of papers on their progress. In 1938 they reviewed the difficulties encountered in the concentration and purification of pantothenic acid from liver. A "unit" of pantothenic acid was that amount which when tested as indicated was equivalent to one gram of the dry rice bran extract. Working with sheep liver these investigators were able to obtain an amorphous preparation with a potency of 11,100 pantothenic units. Further fractionation of this material resulted in no increase in purity. Stimulation of yeast growth was determined quantitatively when only five parts of this concentrate was present in ten billion parts of culture medium (0.0005 γ per ml.). As a result of experiments on the analysis and determination of constituent groups, Williams et al gave $(C_8H_{14}O_5N)_2Ca$ as the formula for the calcium salt of pantothenic acid. The presence of specific active chemical groups was postulated on the assumption that any alternation of these chemical groups would also alter its physiological properties. Studies indicated the presence in the molecule of one carboxyl group, two

hydroxy groups and probably a substituted amide group.

Weinstock Jr., Mitchell, Pratt and Williams (1939) noticed that pantothenic acid was synthesized by yeast only when β-alanine was present in the medium. That -alanine was a cleavage product of pantothenic acid was shown by its isolation from such a mixture as β-napthalene-sulfo-β-alanine. While working with the chick anti-dermatitis filtrate factor obtained from liver, Woolley, Waisman and Elvehjem (1939) were also able to isolate β-alanine from alkali-inactivated concentrates. However activity returned upon recoupling the acidic fraction with synthetic β-alanine. Snell, Strong and Peterson (1939) were similarly able to identify the active ingredient in their concentrates for lactic acid bacteria, as pantothenic acid.

Further evidence for the structure of the lactone moiety was given by Mitchell, Weinstock, Snell, Stanberry and Williams (1940) and Stiller, Keresztesy, Finkelstein (1940). The synthetic β-alanine derivatives of several α-hydroxy γ-lactones showed slight physiological activity. A crystalline lactone was isolated from pantothenic acid hydrolysates and was characterized as α-γ dihydroxy-β, β-dimethylbutyric acid by degradative methods. Therefore the formula for pantothenic acid is (+) α,γ-dihydroxy-β,

β-dimethylbutyryl-β'-alanide.



The total synthesis of pure pantothenic acid and its resolution into d and l-enantiomorphs was immediately described by Williams and Major (1940), and Stiller, Harris, Finkelstein, Keresztesy and Folkers (1940). The (-) form of α-hydroxy-β,β, -dimethyl α-γ-butyrolactone was shown to be identical with the lactone obtained from the hydrolysis of pantothenic acid. (+) Pantothenic acid synthesized from this natural (-) lactone possessed the same physiological properties as pantothenic acid synthesized from synthetic (-) lactone.

Mitchell, Snell and Williams (1940) conceived the idea that hydroxy-pantothenic acid may also possess considerable biological activity. This proved to be the case though results were variable with different organisms and test conditions.

Williams, Eakin and Snell (1940) studied the relationship of i-inositol, thiamin, biotin, pantothenic acid and vitamin B_6 to the growth of yeast. The effects of these substances were investigated alone and in combination when added to medium containing l-aspartic acid as the source of organic nitrogen. Three different strains of yeast were studied. i-Inositol and thiamin were not necessary for the

growth of two yeasts, while biotin alone was slow in exerting its stimulating effect, but in combination was very potent. Pantothenic acid or its percursor β-alanine was stimulating to all three yeasts. Of little importance was vitamin B_6 unless used with a variety of other stimulants.

A series was also run using a liver extract as the stimulant. Rapid growth was induced, thus showing that additional unknown substances were present in liver and yeast extracts. This fact was borne out by the claim of Alexander and Subbarow (1940) to have discovered yet a new yeast growth stimulant present in liver extract. The active material was stable to heat, acid and base hydrolysis, soluble in organic solvents, precipitated by phosphotungstic acid, adsorbed by charcoal, and not destroyed by nitrous acid at room temperature.

Inorganic salts in the presence of Bios may also have an effect on yeast growth as was shown by Lesh, Underkofler and Fulmer (1938). By studying the effect of Bios II, i-inositol and magnesium sulphate, alone and in combination on the growth of thirteen different strains of yeast they were able to divide their yeasts into the following three groups: (1) The addition of magnesium sulphate with Bios II did not give increased growth; (2) the addition of i-inositol to Bios II did not increase growth; (3) growth was increased under conditions (1) and (2). A similar investigation by Rainbow (1939) on the individual Bios requirements of seven

strains of Saccharomyces cerevisiae showed that the apparent differences in the results obtained by various workers on the Bios problem may be eliminated by consideration and specifications of (1) the yeast used, (2) the composition of the synthetic media, (3) the methods employed for sub-culture of the seeding yeast, the general technique-seeding rate, length of growth period, temperature of incubation, agitation conditions and criteria for growth.

This summary of the Bios problem shows that in spite of the many apparent difficulties, real progress has been made into the chemical nature of yeast growth stimulants. The discovery that Bios was not one pure chemical compound led to fractionation which resulted in the preparation of concentrates of more or less purity and finally, as in the case of pantothenic acid, to complete chemical synthesis. A number of yeast growth stimulants have been discovered and purified. The existence of still others is postulated.

It has been suggested by some authors that Bios may be of a co-enzymic nature. For example, a change in the source of nitrogen available to the yeast for assimilation would probably necessitate the bringing into action of a different enzyme or set of enzymes within the cell in order to bring about assimilation. In other words, the yeast would show different Bios requirements in the presence of different nutrients. Different workers have studied individual Bios components and not the whole system with the result that the

inter-relationship between the work in different laboratories has been obscured.

METHODS

Preparation and Determination of the Fermentation Product

Dunning, Fulmer and Underkofler (1940), described the optimum cultural conditions for the oxidation of i-inositol by Acetobacter suboxydans from the standpoint of, i-inositol concentration, sorbitol concentration, yeast extract concentration, surface volume ratio, hydrogen ion concentration, temperature of incubation and the effects of adding sodium chloride and potassium dihydrogen phosphate to the fermentation. Dunning (1938) prepared chemical derivatives which led him to believe "ketose" to be a diketo-i-inositol. Since the compound had not been completely characterized, the authors adopted the term "ketose" as a short term for the product of the oxidation of i-inositol obtained under the conditions used in their investigations.

The method employed in the present investigation to prepare, isolate, and purify the "ketose" used in the succeeding experiments was as follows: The medium contained 3 per cent i-inositol, 0.1 per cent sorbitol and 0.5 per cent yeast extract (Difco); 200 ml. of the medium were used in two-liter Erlenmeyer flasks. The flasks were plugged with cotton, capped with beakers, and sterilized at 15 - 17 pounds steam pressure for thirty minutes. A two or three day old culture of Acetobacter suboxydans growing on the

same medium was found to be best for use in inoculation. The inoculations amounted to 5 per cent of the medium. Incubation was at 28°C. for seven to ten days at which time the fermentation was completed. The flasks were not moved during this time to prevent rupturing the film which formed on the fermenting media. With the termination of the fermentation, the contents of each two-liter flask were poured into a large round bottomed flask. A sufficient amount of a saturated basic lead acetate solution was added to give a final concentration of 0.75 per cent. Before filtration the mixture was shaken with Fuller's earth and coarse norite. Excess lead was removed from the filtrate by treatment with hydrogen sulphide and the solution filtered with the aid of suction. The clear filtrate was concentrated in vacuum at 50°C. to one-half its original volume. Again a test for excess lead was made by passing in hydrogen sulphide and after filtering, the evaporation continued until crystallization began. Upon standing overnight in the cold, additional crystals formed which were filtered off the next day and washed with 50 per cent alcohol-water solution. The fermentation product thus obtained was slightly soluble in cold water but dissolved in hot water to give a brown colored solution. Ebullition with norite however, produced an almost colorless clear solution. Recrystallization was performed by concentration in vacuum to the point of crystallization and then alcohol was stirred in until a final

concentration of 60 - 70 per cent was reached. Purification consisted in crystallization at least once more from water and thrice by precipitation with alcohol. The resulting white crystals melted quite sharply, but the melting point varied from 184 - 195°C. with different preparations, or with the same preparation and different crystallizations. The product obtained after crystallization from water gave even higher melting points. Fifty per cent yields of "ketose" were obtained, based upon i-inositol.

The "ketose" was quantitatively determined in solution by a semi-micro method described by Guymon (1939). In order to do this, it was first necessary to standardize the procedure using known concentrations of the compound. Consequently, a standard solution was prepared which contained 10 mg. of "ketose" per 5 ml. of solution. Using this solution and following the procedure of Guymon (1939), it was found that a minimum heating time of twenty-five minutes was necessary for reproducible reduction values.

To calibrate the method for "ketose", reduction values must be obtained for varying concentrations of the compound using the standard heating time. The volumes of 0.0500 N sodium thiosulphate corresponding to given weights of sample per 5 ml. of solution are given in table 1.

From these data a curve was plotted giving the number of milligrams per 5 ml. of test solution corresponding to

Table 1

Reducing Values for Varying Concentrations of "Ketose" (ml. of 0.05N. $Na_2S_2O_3$ required for varying weights of sample in 5 ml. of solution).

Mg. of "Ketose" present in 5 ml. of solution						
1	2	3	4	5	6	7
0.697	1.529	2.450	3.244	4.357	5.464	6.554

varying volumes of 0.0500 N sodium thiosulphate required. The curve was nearly a straight line, but deviated slightly fron a linear relationship.

Biological Technique

Yeast Propagation in Synthetic Medium

Since various strains of yeast show wide differences in response to different stimulants, it is obvious that any particular stimulant must be tested by several representative yeasts before any conclusions can be drawn concerning its general behavior. Lesh, Underkofler and Fulmer (1938) studied the effect of magnesium sulphate upon the response of thirteen strains of Saccharomyces cerevisiae Bios I and Bios II. On the basis of their findings, they separated the yeasts into the following three groups for convenience of comparison. Group one included strains No. 5, 6, and 42,

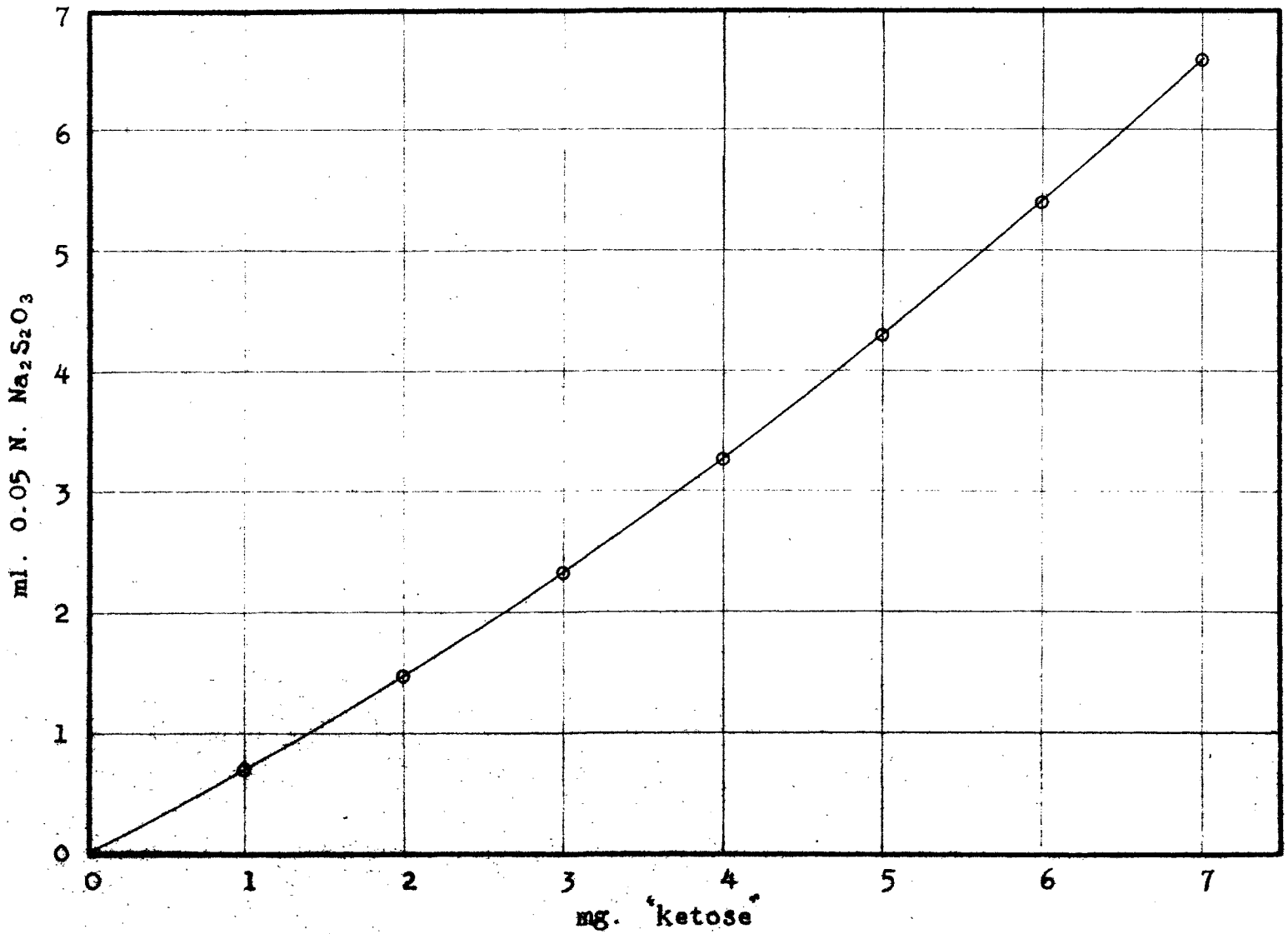


Fig. 9. Reduction equivalents for increasing concentrations of "ketose".

and were characterized by the fact that they did not give increased growth upon addition of magnesium sulphate and Bios II. The second group included strains No. 9, 11, 16, and 26, and were distinguished by decreased growth upon the addition of i-inositol and Bios II. The third group included strains giving increased growth when either i-inositol or magnesium sulphate was added with Bios II. Strains No. 7, 10, 18, 19, 22, and 41 belong in this last group.

The yeasts selected for investigation in this thesis were chosen as representative of each of the above three divisions, and comprise strains No. 42, 26, 16, 10, and 5. They are listed in the American Type Culture Collection as shown in table 2.

Table 2

Key to Strains of Saccharomyces cerevisiae

(A.T.C.C. = American Type Culture Collection)

Strain		
5	(Hansen)	A.T.C.C. No. 4360
10		A.T.C.C. No. 765
16		A.T.C.C. No. 764
26	(Hansen)	A.T.C.C. No. 4923
42	("Gebrude Mayer") Fleishman	No. 2.14-40

Each strain was transferred from a beerwort-agar slant into a flask containing medium composed of 5 grams sugrose, 0.188 grams ammonium chloride, 0.100 grams potassium

dihydrogen phosphate, 0.100 grams calcium chloride, 0.100 grams magnesium sulphate, 0.100 grams yeast extract (Difco) per 100 ml. of medium. After incubation at 28° for three to four days, they were inoculated into similar media. The yeast became very active and the third transfer was aseptically transferred to a sterilized centrifuge tube and centrifuged. The supernatant liquid was poured off and the yeast cells suspended in saline solution. After centrifuging and washing twice, the suspended cells were inoculated into the basal synthetic media containing only salts and sucrose.

The basal medium used in this investigation was one developed by Fulmer, Nelson and Sherwood (1921) and contained per 100 ml., 0.188 grams of ammonium chloride, 0.100 grams dipotassium phosphate, 0.100 grams of calcium chloride, 0.100 grams of magnesium sulphate, and 5 grams of sucrose. Stock solutions of the above salts were made up fifty times the concentration designated for the prepared medium. Reagent grade chemicals of known purity were employed exclusively. Pfanstiehl's C.P. grade sucrose was selected for use in the experiments.

In order to make direct comparisons of the yeast strains it was necessary to standardize the method of subculture and the preparation of the inoculum. The cultures were carried in 125 ml. Erlenmeyer flasks containing 25 ml. of basal synthetic medium. Transfer was made when 1 ml. of the

fermenting medium contained approximately the proper number of cells for the inoculation of each experimental flask. The yeast count in the active media was determined by means of a Thoma-Zeiss counting chamber. A count of one was equivalent to 250,000 cells per ml.. Inoculations were made such that the initial count was one.

Since yeast strain No. 42 was unable to acclimatize itself to the synthetic medium and only gave a count of five after a nine day growth period; its subculture was discontinued. Strain No. 5 grew very slowly but adapted itself rapidly enough to be tested in the experimental fermentations. Yeasts Nos. 26, 16, and 10 grew quite well in the basal medium and gave a count of 20 - 25 in forty-eight hours. During the entire acclimatization period it was important to guard against "dropping" the yeast by too rapid subculture (Fulmer and Nelson) (1923). Inoculations should be made only after a microscopic count and then always to at least a count of one. Also, due to the unsuitability of the medium, there was a marked tendency toward involution and care must be exercised or the culture will be lost. The involuted forms did not return to normal immediately when they were transferred into flasks containing biotin.

Photometer Standardization

Various methods have been developed for the determination of yeast growth. Wildiers (1901) used the loss of weight in

the flasks, due to the evolution of carbon dioxide, as a means of comparing growth. Other methods, such as weighing the moist or dried yeast, measuring the volume of yeast in a centrifuge tube, and determining the turbidity of yeast suspensions are also employed. Direct microscopic count is considered the most accurate and is therefore generally employed for the calibration of the other methods.

In the present investigation, yeast growth was determined by means of a K. W. S. Z. (Kraybill, Withrow, Shrewsbury, and Zscheile) photometer. This instrument measured the "per cent light transmitted" through the unknown solution compared to a suitable reference cell set to read 100 per cent transmission. The more turbid the solution, the lower the photometer reading or per cent light transmitted. Since uninoculated basal medium contained considerable precipitate, it was necessary to devise a procedure to eliminate this natural turbidity. The best found, was to use distilled water as the reference cell and to clarify the fermentation medium by the addition of one drop of concentrated hydrochloric acid from a medicine dropper to each 125 ml. Erlenmeyer flask. The medium was then agitated sufficiently to suspend all the yeast cells and poured into the "unknown" photometer tube. The light transmission was read directly by adjusting the resistance dials until the galvanometer deflection was zero. Care was taken that the same two reference tubes were used, and that they were inserted in

the same position each time. After use, the tubes were cleaned in potassium dichromate-sulphuric acid cleaning solution and rinsed well with distilled water.

The choice of suitable color filters for the photometer was necessary to obtain greater sensitivity and accuracy in determining yeast growth. The instrument was therefore calibrated for the red (No. 7) and purple (No. 1) filters by direct microscopic count. The correct filter should absorb enough light so that the fraction passing through the unknown solution can be accurately measured. The following experiment illustrates the choice of the proper filter, and the reliability and sensitivity of the photometer in yeast count studies.

Sufficient 125 ml. Erlenmeyer flasks containing 25 ml. of basal medium plus Bios II and i-inositol per flask were prepared so that counts could be made every two hours for the duration of the growth period. Direct microscopic counts and photometer readings, using the red and purple filters, were determined for each flask.

Within the limits of experimental error smooth curves were obtained when microscopic count was plotted against photometer readings. In an effort to obtain a straight line relationship, various other functions of count and transmission were plotted. Of these, the most successful were count vs. log photometer reading, and count vs. log I_o/I, where I_o was 100 per cent light transmitted and I was the

Table 3

Photometer Standardization with Yeast Count

Time in Hours	Yeast Count	Photometer Reading	
		red filter	purple filter
0	1	91.8	90.0
2	1.4	91.5	89.0
4	2.4	91.3	88.0
6	4.6	90.3	87.0
8	5.6	89.9	86.0
10	9.1	87.8	84.0
12	11.0	85.8	82.0
14	16.1	84.0	78.0
16	19.0	80.8	76.0
18	22.8	79.0	74.0
20	36.0	73.7	66.0
22	57.0	66.0	57.0
24	55.0	66.2	58.0
26	72.0	60.5	51.0
28	91.0	55.0	45.0
30	87.0	54.1	44.0
32	103.0	50.5	39.0
34	122.0	45.7	34.0
36	153.0	38.6	29.0
38	132.0	40.3	30.0
40	180.0	37.0	27.0
42	210.0	31.6	23.0
44	240.0	25.5	19.0
46	236.0	24.6	19.0
48	230.0	22.0	18.0
50	275.0	20.3	15.0
52	310.0	20.8	14.5
54	367.0	13.4	9.5
56	387.0	12.4	8.0
58	334.0	12.4	9.0
60	450.0	10.7	8.0
62	486.0	9.5	7.0
64	425.0	10.5	8.0

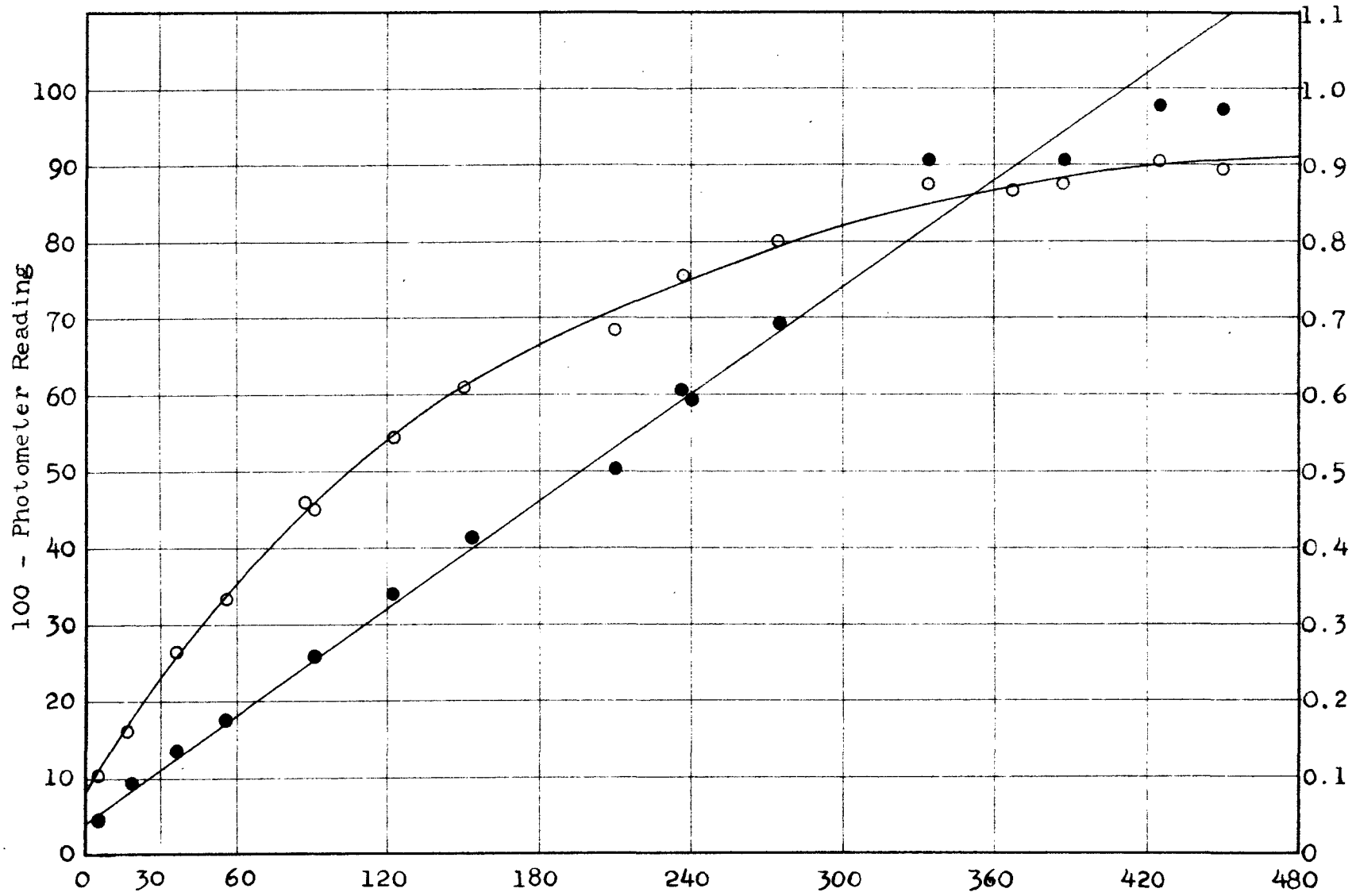


Fig. 10. Photometer standardization by direct count.
(red filter)

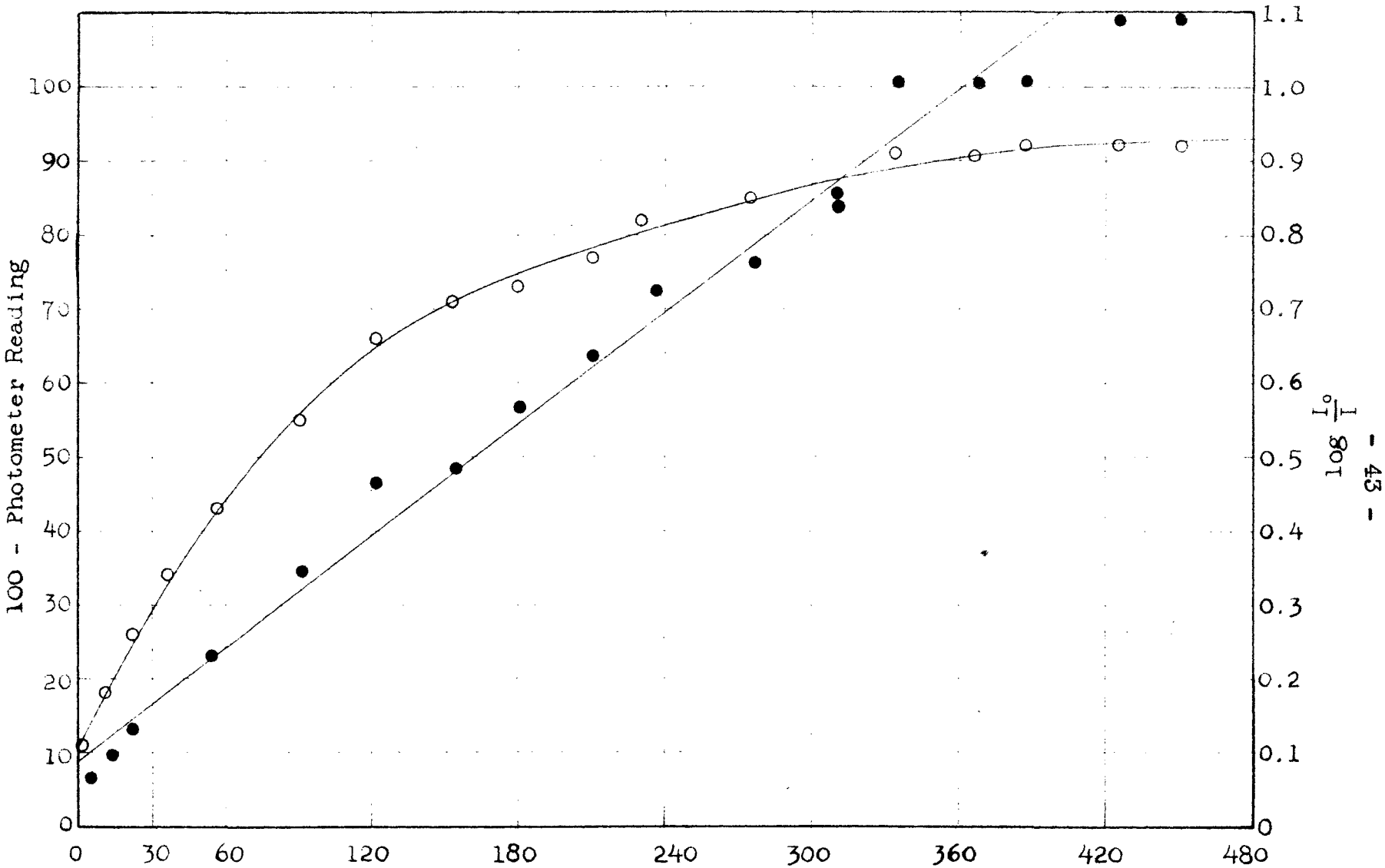


Fig. 11. Photometer standardization by direct yeast count. (purple filter)

reading of the unknown. Thus it appeared that yeast cells in suspension obeyed Beer's law which is expressed in the mathematical expression,

$$C = \frac{2.3}{kl} \log \frac{I_o}{I}$$

where C = concentration of solute or yeast count

k = constant

l = length of adsorbing column.

Since the same comparison tubes were employed for each reading, l became a constant and the equation resolved to,

$$C = k' \log \frac{I_o}{I}$$

where k' = new constant.

In the region of very low photometer readings the experimental values digress from the theoretical. Hence the sensitivity of the photometer decreases with high yeast counts. The remedy is to dilute the unknown to the region in which the linear relationship holds true. Curves were fitted to the experimental data by the method of least squares. Equations calculated for the red and purple filters were respectively:

$$C = 454.6 \log \frac{I_o}{I} - 23.9$$

and

$$C = 394.4 \log \frac{I_o}{I} - 35.2$$

When tested for the goodness of fit it was found that the correlation coefficient r was 0.994 for the red filter and 0.9881 for the purple filter. It was therefore evident that the red filter was better than the purple filter for yeast growth measurements.

Preparation of Bios II-A and Bios II-B Concentrates

Bios II concentrate was prepared from malt sprouts according to the method of Lucas (1924). Bios I or i-inositol was precipitated with barium hydroxide and alcohol. Miller, Eastcott and Maconachie (1933) described a method for the fractionation of Bios II into Bios II-A and Bios II-B by the preferential adsorption of Bios II-B on charcoal in an acid solution. Our procedure, based on the Bios II concentrate obtained from 500 grams of malt sprouts was as follows:

Twenty grams of norite A charcoal were shaken for fifteen minutes with 200 ml. of a solution containing enough 2 N sulphuric acid and the crude Bios II solution to make the total solution 0.665 N sulphuric acid. The solution was filtered and washed with 50 ml. of water; the filtrate was shaken with another 10 grams of charcoal, which was filtered off and washed as before. This process was repeated the third time and the charcoal washed well with 500 ml. water. The combined filtrates and washings were freed from sulphuric acid by baryta, and evaporated under vacuum to a volume 200 ml. The charcoal from the above operations was combined

and shaken for 30 minutes with 200 ml. of freshly prepared "acetone-ammonia reagent" (5 ml. of concentrated ammonia, 35 ml. of water, acetone to 200 ml.), filtered off, and washed with 75 ml. of the reagent. This operation was repeated twice more. The united extracts and washings evaporated under vacuum, and the residue dissolved in water to a volume of 200 ml.

To test the completeness of fractionation, several fermentation series were prepared in the following manner:

0 - - control (basal medium, salts and sugar)
1 - - basal medium + i-inositol
2 - - " " + Bios II-A + i-inositol
3 - - " " + Bios II-A
4 - - " " + Bios II-B
5 - - " " + Bios II-B + i-inositol
6 - - " " + Bios II-A and Bios II-B
7 - - " " + Bios II-A and II-B + i-inositol

Results of yeast growth with time are shown in table 4.

Table 4

The Effect of Charcoal Adsorption on Bios II Fractionation (Blue filter, yeast strain, No. 26)

Series	Photometer Reading Time in hours				
	0	12	24	48	72
0	91	91	90	84	72
1	91	91	90	81	67
2	91	90	89	76	63
3	91	90	90	80	67
4	91	88	82	28	9
5	92	88	76	21	7
6	92	89	79	25	8
7	92	89	78	20	6

It is evident that no significant fractionation had occurred since there was practically the same growth for series 5 containing Bios II-B and i-inositol as there was in series 7 containing Bios II-B, i-inositol and Bios II-A.

In another experiment twice the quantity of Bios II-A concentrate was added to the series, but the results were similarly negative.

A new Bios II concentrate was prepared from malt sprouts according to the directions of Lucas (1924), and fractionated as directed above by charcoal adsorption. Again it appeared that no separation had occurred although the charcoal was well washed to remove the last traces of Bios II-A. The Bios II-B fraction contained all the activity thus showing that Bios II-A must be adsorbed also.

The next fractionation procedure tried was one developed by Rainbow and Bishop (1939) for dried egg yolk. After the crude Bios II concentrate had been prepared their directions were followed with the exception that our final dilution was made in water instead of alcohol.

The following series were prepared to test whether or not this procedure had resulted in the fractionation of Bios II concentrate into Bios II-A and Bios II-B.

0 - Basal Medium (control)
1 - Basal Medium + Bios II-A (2 ml./100) + i-inositol
2 - Basal Medium + Bios II-B (2 ml./100) + i-inositol
3 - Basal Medium + Bios II-A + Bios II-B (2 ml./100 each) + i-inositol
4 - Basal Medium + Bios II-A (1 ml./100) + Bios II-B (2 ml./100) + i-inositol

5 - Basal Medium + Bios II-A (2 ml./100)
6 - Basal Medium + Bios II-B (2 ml./100)
7 - Basal Medium + Bios II-A + Bios II-B.

Table 5

The Effect of Mercuric Chloride in the Fractionation of Bios II. (Yeast Strain No. 10)

Series	Photometer Reading Time in Hours		
	16	44	64
0	93.5	88.2	73.3
1	92.4	66.1	55.3
2	90.3	62.5	47.0
3	89.0	55.5	42.5
4	89.2	58.0	44.9
5	90.4	70.3	54.6
6	91.3	65.4	47.6
7	92.2	63.3	48.4

A greater difference was exhibited between series 2 and 3 than had been obtained in previous similar experiments. At least a certain amount of fractionation had occurred. It was also noticeable that series 3 containing 2 ml. of Bios II-A per 100 ml. of medium gave a lower photometer reading than series 4 which contained only 1 ml. of Bios II-A per 100 ml. solution. Consequently, the concentration of Bios II-A was increased to 3 ml. of concentrate per 100 ml. of medium for the subsequent experimental fermentations.

Test Materials

Stock solutions of the stimulants were prepared in appropriate concentrations and stored in the refrigerator under

toluene.

In all the succeeding experiments, unless otherwise stated, the following fixed quantities of materials were employed for tests per 100 ml. of synthetic medium:

i-Inositol- - - - - - 0.002 grams (Pfanstiehl Chemical Co., Waukegan, Ill.)

"Ketose"- - - - - - - 0.002 grams (Purified i-inositol-Acetobacter suboxydans fermentation product. M.P. 185°)

Bios II-A - - - - - - 3 ml. of the concentrate prepared by the author and described above.

Bios II-B - - - - - - 2 ml. of a concentrate prepared as described above.

β -Alanine- - - - - - 0.000,1 gram (S.M.A. Corporation, Chagrin Falls, Ohio).

Calcium Pantothenate- 0.000,03 grams (Merk & Co., Inc., Rahway, N. J.)

Vitamin B_1- - - - - - 0.000,02 grams crystalline thiamine hydrochloride. (S.M.A. Corporation)

Vitamin B_6- - - - - - 0.000,02 grams crystalline hydrochloride. (S.M.A. Corporation)

l-Leucine - - - - - - 0.000,02 grams (University of Illinois)

Biotin- - - - - - - - 0.000,000,01 grams prepared from biotin concentrate no. 1000, which is an aqueous solution of a concentrate prepared from natural sources, standardized to contain

0.0001 grams of biotin per ml. of solution, as determined by comparative assay against crystalline biotin using the assay method described by Snell, Eakin and Williams (1940). (S.M.A. Corporation)

Preparation of the Experimental Medium

The required volume of basal medium was prepared in a round bottomed flask. Into each 300 ml. Erlenmeyer flask, representing the number of series to be run were poured 175 ml. of medium. Inverted beakers were placed over them to avoid contamination. Sufficient quantities of the stimulant stock solutions were added to the proper flask to give the correct concentration per 100 ml. of medium. A homogenous suspension was obtained by thoroughly shaking the contents and 25 ml. pipetted into each of seven 125 ml. Erlenmeyer flasks for each series in the fermentation. Each flask was then plugged, capped with 50 ml. beakers to keep the cotton dry, and sterilized at 15 pounds pressure (121°) for 20 minutes. When taken from the autoclave the caps were removed, and after cooling to room temperature the flasks were put in the incubator for temperature equalization. Inoculations were made from a 48 hour culture and always to a count of one with photometer readings performed at definite intervals of zero, twelve, twenty-four, forty-

eight, seventy-two, ninety-six, and one-hundred-twenty hours.

EXPERIMENTAL

Chemical Properties of the Keto Compound ("Ketose") Produced by the Action of *Acetobacter suboxydans* upon i-Inositol

In a preliminary communication Dunning, Fulmer, Guymon and Underkofler (1938) dealt with the action of *Acetobacter suboxydans* upon i-inositol to produce a compound which results indicated to be a diketo-i-inositol. Ensuing investigations reported by Dunning (1938) gave further evidence in support of this assumption. The optically inactive compound did not absorb bromine or iodine in neutral water solution, but did absorb approximately two equivalents of iodine in alkaline solution, thus denoting the presence of two keto groups. A dinitro-phenylhydrazone was prepared which, after repeated precipitation from alcohol, melted at 193 - 5°C. and upon analysis contained the per cent nitrogen corresponding to two ketone groups. No mention was made of the possibility of osazone formation. The tetra-acetyl derivative of "ketose" was prepared by refluxing acetic anhydride with the fermentation product in the presence of a little zinc chloride. Attempted oxidation of "ketose" with alkaline potassium permanganate yielded so many products that the identification of significant fragments was impractical.

Upon elementary quantitative analysis the experimental

carbon and hydrogen values did not check those calculated for a diketo-i-inositol.

	Calculated	Found	
Per cent carbon	40.91	- 40.7 ,	40.6
Per cent hydrogen	4.55	6.17 ,	6.12

Purification of "ketose" by the preparation of a phenylhydrazone followed by the recovery of the original compound, gave similar values upon analysis.

The high hydrogen values could not be reconciled to a diketo-i-inositol by any assumption regarding water of crystallization or association.

Quantitative Hydrogenation of "Ketose"

The discrepancy between the calculated and experimental carbon and hydrogen per cent values suggested the possible presence of monoketo-i-inositol in the fermentation compound. A quantitative hydrogenation in the presence of a suitable catalyst should reveal the degree of oxidation that had occurred and give an insight into the purity of the compound. Consequently, some "ketose" was purified by reaction with phenylhydrazine and subsequently recovered from the solid derivative. A determination was also made on inosose, prepared and purified as described by Posternak (1936). Preliminary experiments showed that either platinum-oxide or sodium-amalgam catalyzed their reduction to i-inositol. The reduction was performed in a semi-micro apparatus with

constant rocking. The weighed sample was dissolved in water, some weighed catalyst added and the system closed. The volume of hydrogen absorbed was read from a micro-buret after pressure equalization by a mercury-filled leveling bulb. Data are presented in table 6 where all volumes were calculated at 26°C. and 740 mm. pressure.

Table 6

Quantitative Hydrogenation of Inosose and "Ketose"

Compound	Wt. of sample mg.	Wt. of catalyst mg.	H_2 absorbed by PtO_2	H_2 absorbed by sample	Theoretical H_2 absorbed
Inosose	37.5	8.6	1.98	6.19 ml.	5.49 ml.
"Ketose"	21.4	9.4	2.15	4.97 ml.	6.32 ml.

It is evident that inosose absorbed more hydrogen than the amount theoretically calculated. This was understood since oxidation of i-inositol by concentrated nitric acid could be expected to form other ketone products besides inosose, which were not entirely eliminated in the phenylhydrazine treatment.

Conversely, "ketose" did not take up the theoretical amount of hydrogen as calculated for a diketo-i-inositol. In other words only 78.64 per cent of the theoretical volume of hydrogen as calculated for diketo-i-inositol was absorbed by "ketose". Thus it appeared that purified "ketose" was not pure diketo-i-inositol, but a mixture of mono-,

di- and possibly higher keto-i-inositols.

Oxidation Equivalent Analysis

A method has been described by Williams, Rohrman, and Christensen (1937) and Williams, and Woods (1937) on the selectivity of iodic acid in the oxidation of organic compounds. The procedure was based upon the oxidative action of iodic acid according to

$$6KIO_3 + 3H_2SO_4 = 3K_2SO_4 + 3H_2O + 3I_2 + 150.$$

After the prescribed heating time, and with subsequent cooling, the excess KIO_3 was titrated with standard sodium thiosulfate solution.

$$KIO_3 + 5KI + 3H_2SO_4 = 3K_2SO_4 + 3H_2O + 3I_2$$

$$3I_2 + 6Na_2S_2O_3 = 3Na_2S_4O_6 + 6NaI$$

Since the mono- and diketo-i-inositols are in different states of oxidation the amount of oxygen necessary for complete oxidation would vary. Thus one might determine whether the sample was predominantly a mono- or diketo-i-inositol by the equivalent oxidation values obtained.

The micro procedure of Williams, Rohrman, and Christensen (1937) was followed explicitly and the results shown in table 7.

The "milligrams of potassium iodate used up" was obtained by subtracting the milligrams of potassium iodate equivalent to the sodium thiosulfate needed for titration from the total weight of potassium iodate added, and had

Table 7

Equivalent Oxidation Values for "Ketose"

($Na_2S_2O_3$= 0.090 N)

Sample	Wt. in mg.	Wt. KIO_3 mg.	Time of Heating (min.)	$Na_2S_2O_3$ ml.	mg. KIO_3 used up	mg. O_2 equiv.	mg. O_2 Theor.
Blank		82.336	20	24.5	5.844	0.719	
Blank		63.464	20	19.3	1.627	0.304	
Blank		100.302	40	30.0	4.18	0.782	
Blank		69.640	40	21.2	1.715	0.321	
Sucrose	5.00	105.30	20	22.69	32.6	5.58	5.60
"Ketose"	5.273	95.682	20	20.78	29.103	4.69	4.79
"Ketose"	3.178	75.98	20	18.38	17.10	2.85	2.89
"Ketose"	3.601	97.45	40	23.45	22.316	3.45	3.28
"Ketose"	3.262	76.12	40	17.60	19.730	3.13	2.97

not been corrected for the blank. The theoretical weight of oxygen required for the combination of "ketose" was calculated on the assumption that "ketose" was pure diketo-i-inositol. When the heating time was twenty minutes, the experimentally determined oxygen equivalent values were considerably closer to the theoretical than when the heating time was forty minutes; and was therefore considered the best heating time. It was at once apparent that "ketose" was predominantly a diketo-compound, due to the close check in equivalent oxygen values obtained in this experiment.

Methylation Studies

Direct oxidation of "ketose" resulted in too many oxidative fragments for significant identification. It

was thought that if the hydroxyl groups could in some way be protected by a methoxy group, then oxidation would break the ring adjacent to a carbonyl group. Subsequent identification of the oxidation products should then help to ascertain the position of the carbonyl group or groups.

Previous attempts to methylate "ketose" had failed for various reasons. The methyl-iodide-methyl alcohol-silver oxide method failed because of the oxidative action of silver oxide on "ketose". Diazomethane gave no reaction with the fermentation product. Finally, 10 grams of "ketose" were put in a glass stoppered bottle filled with 250 ml. absolute methyl alcohol. The mixture was saturated with dry hydrogen chloride and the stopper sealed with paraffin. The bottle was then put away for approximately one and one-half years with intermittent shaking. At the end of this time the wax seal was broken and some of the brownish liquid taken out for analysis. The unreacted "ketose" was removed by filtration. Evaporation in vacuum yielded a thick syrup which could not be induced to crystallize. This residue was extracted with chloroform, distilled to dryness and the remaining syrup dried in a dessicator. Solution was made in hot alcohol, which, upon cooling yielded flaky, brown crystals which melted at 59°C. These were crystallized three times from alcohol and yielded a tan colored compound. After drying in vacuum a methoxyl determination was made. The negative results showed that

the sample was not methylated.

Methylation procedures using alkaline dimethyl-sulphate at -5°C. were also tried, but pertinent results could not be obtained. Griffin and Nelson (1915) described a procedure for the alkylation of i-inositol with dimethyl-sulphate. The method consisted of refluxing the compound with an excess of dimethyl-sulphate in 25 per cent sodium hydroxide solution for twenty-four hours. After neutralization with sulphuric acid, an excess of barium hydroxide solution was added and the solution filtered. Carbonation served to remove the excess barium hydroxide as the carbonate. The solution was made alkaline with ammonium hydroxide and any unchanged "ketose" removed by precipitation with basic lead acetate. Excess lead in the filtrate was precipitated by hydrogen sulphide treatment and filtered. After boiling off the hydrogen sulphide the filtrate was concentrated under vacuum to a syrupy consistency. The syrupy mass was dried at 100°C. in a vacuum to form a hard hygroscopic compound, which was pulverized and emptied into acetic anhydride containing a few drops of concentrated sulphuric acid. The mixture was heated for one hour, cooled, filtered, and the dark red solution evaporated to a syrup. This residue was extracted with chloroform, filtered, and the chloroform distilled off. The dark colored syrup thus obtained was dissolved in alcohol, boiled with norite, and filtered. The solution was evaporated to a pale yellow syrup, extracted

with ether, and again distilled to a syrup. After solution in alcohol, followed by evaporation to a syrup, the residue was poured into chopped ice; solution was complete. The water was evaporated to a small volume which was extracted with ether. When the ether was distilled off a heavy viscid material resulted. Since this syrup could not be crystallized, a methoxyl determination was made upon it. It was found that the liquid contained 27.4 per cent methoxyl. Assuming "ketose" to be all diketo-i-inositol there was a possible 44.9 per cent methoxyl, while if one hydroxyl group was acetylated and three methylated, the per cent methoxyl would be 30.6 per cent. Acetyl groups do not interfere with the methoxyl determination.

From these results it was plain that some methylation had occurred. Operations using the same method but larger quantities should give methylated products in sufficient yields to permit significant oxidation experiments.

Factors Influencing the i-Inositol Fermentation

The Effect of pH upon the Fermentation

Posternak (1936) described the preparation of a monoketo-i-inositol by the oxidation of i-inositol with concentrated nitric acid and gave the name inosose to the compound. Recently Kluyver and co-workers (1939), claimed the production of inosose by the action of Acetobacter

suboxydans upon i-inositol in a phosphate buffered medium at pH 6. It was possible that the difference in results between this work and that of Kluyver might be connected with the effect of pH upon the Acetobacter fermentation.

To determine if the reducibility of the fermentation product was a function of the pH of the fermenting media, an experiment was devised consisting of the following four series.

0 - Control (unbuffered basal medium)
1 - basal medium plus phosphate buffer to pH 6.4
2 - basal medium plus phosphate buffer to pH 6.0
3 - basal medium plus phosphate buffer to pH 5.8

The basal medium contained 3 per cent i-inositol, 0.5 per cent yeast extract and 0.2 per cent sorbitol.

After sterilization and inoculation from an actively growing culture of Acetobacter suboxydans, the fermentation was followed by sugar analysis at various time intervals. For the analysis, the total volume of medium in the flask was measured, 2 ml. removed with a pipette and diluted to 50 ml. in a volumetric flask. From this, 5 ml. were used for the analysis, and pH values were determined by means of a glass electrode. The results are given table 8.

These figures showed that there was a definite relationship between the formation of reducing compound and the pH of the fermenting medium; the higher the buffered pH, the less reducing material formed. In the control series there were 251.5 mg. of reducing sugars formed, while there were only 148,8 mg. formed for the series buffered at pH 6.

Table 8

The Effect of pH upon l-Inositol Fermentation

(Blank = 10.1 ml. of 0.0948 N. $Na_2S_2O_3$)

Series	Time (days)	Vol. ml.	$Na_2S_2O_3$ ml. used	mg."ketose" per 0.2 ml.	mg."ketose" per flask	pH
0	1	10.0	0.39	0.57	2.85	--
	2.5	8.5	1.57	2.13	90.8	4.55
	4	8.6	3.98	4.45	191.7	5.6
	5	8.4	4.56	4.96	208.0	5.61
	6.25	8.1	5.90	6.15	249.0	5.57
	7.25	7.8	6.02	6.42	251.5	5.2
1	1	10.0	0.39	0.57	2.85	--
	2.5	9.5	0.72	1.00	47.5	5.62
	4	9.0	0.70	1.0	45.0	6.02
	5	9.0	1.42	1.80	81.0	5.88
	6.25	8.1	1.60	2.0	87.0	5.72
	7.25	8.6	1.64	2.05	88.0	5.30
2	1	10.0	0.39	0.57	2.85	--
	2.5	9.3	1.66	2.07	96.3	5.07
	4	9.2	2.14	2.60	119.8	5.57
	5	9.0	2.50	3.0	135.0	5.37
	6.25	8.5	2.93	3.43	145.8	5.2
	7.25	8.5	2.96	3.5	148.8	4.85
3	1	10.0	0.43	0.60	3.0	--
	2.5	9.5	2.46	3.0	142.4	4.82
	4	9.3	3.04	3.52	164.0	5.41
	5	9.2	3.08	3.57	164.5	5.12
	6.25	9.0	3.46	3.95	178.0	5.08
	7.25	8.4	3.78	4.25	178.5	4.80

Either approximately one-half of the i-inositol was fermented in the buffered medium or else the oxidation capacity of Acetobacter suboxydans was dependent on pH and only one hydroxyl group of i-inositol oxidized at pH 6; while two hydroxyl groups were oxidized in the normal fermentation. Further work is contemplated which should help to solve this problem.

Fermentability of Phospho-i-Inositol Compounds

Another possible method to locate the position of the carbonyl groups in "ketose" is to test the fermentability of various methyl ethers or phosphate esters of i-inositol. Anderson (1912) described the preparation of the barium salt of the tetraphosphoric acid ester of i-inositol by the reaction of phosphoric acid and i-inositol. Some of this salt was prepared according to his directions. A fermentation medium was made up containing 3 per cent of the barium salt, 0.5 per cent yeast extract, and inoculated with an active culture of Acetobacter suboxydans growing on i-inositol media. After an incubation period of ten days no additional reducing compound could be detected in the fermentation, conclusively showing the absence of carbonyl groups. This negative effect might be due to either the toxicity of the barium or to the partial insolubility of the salt.

Consequently the free phosphoric acid ester was prepared

by Anderson's method (1912). This free ester was obtained as an amorphous brown solid showing considerable acidity. After neutralization, about 50 ml. of a 3 per cent solution of this salt and 0.5 per cent yeast extract were prepared. The salt was partially but not completely soluble. After sterilization and cooling, some of the fermentation flasks were inoculated with an active culture of Acetobacter suboxydans. No fermentation occurred since, after ten days in the incubator, no additional reducible substances could be detected by analysis. The addition of 0.25 per cent sorbitol to the remaining fermentation flask with subsequent re-inoculation likewise resulted in no significant Acetobacter fermentation. Therefore the tetraphosphoric acid ester of i-inositol was not fermented by Acetobacter suboxydans. Although Butlin (1936) reported that a phosphatase enzyme capable of hydrolyzing hexose diphosphate was present in the organism.

It is noteworthy that experiments described later showed that the barium salt of the tetraphosphoric acid ester of i-inositol played the same role as Bios I in yeast growth stimulation.

The Effect of "Ketose" upon the Growth of Various Strains of Yeast

Since i-inositol is a compound of wide occurrence and great biological interest, and especially since it has

recently been proved by Eastcott (1928) to be an indispensable growth factor for various yeast species, the biological properties of i-inositol derivatives seems worth stressing. Dunning, Fulmer, Guymon and Underkofler (1938) state that, "If i-inositol could be biologically oxidized to keto-compounds, which might exist in reversible oxidation-reduction systems, some light may be thrown upon its role as Bios I". It would be of great significance should the keto-product, obtained from the fermentation of i-inositol by Acetobacter suboxydans, and which shall be referred to as "ketose" in this investigation, show Bios activity.

The Effect of "Ketose" on Yeast Growth

It was necessary to exclude the possibility that the possible stimulating effect of "ketose" on yeast growth might be due to the questionable presence of adsorbed yeast extract on the "ketose".

There was very little likelihood that such adsorbed material could survive the procedure involved in the preparation of a derivative, crystallization, and then the reconversion to the original "ketose" compound. Consequently, a phenylhydrazone derivative was prepared by treating "ketose" with phenylhydrazine in dilute acetic acid and then recrystallized. The "purified ketose" was recovered by hydrolysis of the hydrazone with benzaldehyde in the presence of a little benzoic acid as described by Posternak (1936).

The effect of the "purified" and "unpurified ketose"

on yeast growth is shown in table 9, made up of the following series:

0 - Control
1 - "Ketose"
2 - Purified "ketose"
3 - Bios II-A + "ketose"
4 - Bios II-A + purified "ketose"
5 - Bios II-B + "ketose"
6 - Bios II-B + purified "ketose"
7 - Bios II-A + Bios II-B + "ketose"
8 - Bios II-A + Bios II-B + purified "ketose"
9 - Bios II-A + Bios II-B + "ketose" + purified "ketose"
10 - Bios II-A + Bios II-B + "ketose" + i-inositol

Table 9

Comparison of the Effect of "Purified" and "Unpurified Ketose" on Yeast Growth (Yeast Strain No. 26. Red Filter)

Series	Photometer Reading Time in hours						
	0	12	24	48	72	96	120
0	93.6	93.3	90.2	75.3	62.6	50.7	42.5
1	93.7	93.5	87.1	69.0	56.3	48.4	40.2
2	93.6	93.4	87.0	68.3	56.5	48.7	40.4
3	93.8	92.0	78.2	55.1	45.2	32.4	24.0
4	93.4	91.5	79.0	55.5	45.2	32.1	24.2
5	93.4	91.7	76.5	52.4	40.5	30.6	22.0
6	93.4	91.1	78.5	53.1	41.7	29.9	21.2
7	93.5	89.5	72.5	43.8	30.7	20.2	14.0
8	93.3	89.4	72.2	43.9	30.7	20.6	13.3
9	92.8	89.2	68.7	43.3	31.3	19.1	13.7
10	93.3	89.2	70.3	43.4	30.5	20.2	15.0

It is evident from the results given above that "purified" and "unpurified ketose" react similarly and that the small differences existing are insignificant and due to experimental error. Therefore, we conclude that there was no adsorption of yeast extract on the "ketose". The

Subsequent experiments were performed with "unpurified ketose".

A keto-i-inositol compound had been prepared, therefore, although it possesses far different chemical properties, it still showed the Bios I activity of i-inositol. The oxidation of hydroxy groups to ketone groups does not alter the effect of i-inositol upon yeast growth. Further methodical chemical changes in the i-inositol molecule should throw light upon the configuration necessary for Bios I activity and perhaps finally, to synthesis of compounds with even greater activity than i-inositol.

The Effect of "Ketose" in Combination with Other Stimulants on the Growth of Yeast No. 5

Not only is it necessary to test the Bios activity of a new compound with several yeasts, but it is also expedient to test its action alone, and in combination with other yeast stimulants, if a proper conception of its capabilities is to be obtained. Since "ketose" is a derivative of i-inositol, one would expect that it might behave much like i-inositol as far as yeast growth was concerned. Likewise, the action of Bios II-A, β-alanine and pantothenic acid are similar and may be grouped together. Bios II-B and biotin behave alike and fall into another group. Various investigators, depending on the type of yeast studied and on the composition of the particular medium employed, have

reported on the necessity of Vitamins B_1, B_6 and l-leucine for maximum yeast growth. Consequently, these were grouped separately, but considered as necessary only in a group sense. With these relationships in mind, the following figure (12) was prepared systematically so that the effect of each chemical could be studied alone, and in combinations. "Ketose" occurs in the uneven series numbered above 11, in figure 12, while i-inositol is present in the even numbered series.

Figure 12 served as the key for the preparation of the subsequent fermentations and unless otherwise indicated "Series Number" refers to the same number on the figure with its accompanying composition.

Table 10 contains the results of photometer readings as a function of time for the complete series when tested with yeast strain No. 5.

Final photometer reading as a criterion for growth of yeast strain No. 5. The final photometer reading at 120 hours, varied from a reading of 15.8 for series No. 11 to 63.3 for that of No. 2. Intermediate between these two extreme values were the readings of the other 28 series. When these were arranged in order of final yields, it became evident that they readily fell into three main groups, the top one comprising 11 series ranging in photometer readings from 15.8 to 24.7. It was significant that each

Stimulant / Series	Bios II-A A_1	Pantothenic Acid A_2	β-Alanine A_3	Bios IIB B_1	Biotin B_2	i-Inositol C_1	Ketose C_2	Vitamin B_1 D	Vitamin B_6 E	l-Leucine F
Control	Basic Medium									
1	+									
2		+								
3			+							
4				+						
5					+					
6						+				
7							+			
8								+		
9									+	
10										+
11	+	+	+	+	+	+	+	+	+	+
12	+			+		+		+	+	
13	+			+			+	+	+	
14		+		+		+		+	+	
15		+		+			+	+	+	
16			+	+		+		+	+	+
17			+	+			+	+	+	+
18	+				+	+		+	+	
19	+				+		+	+	+	
20		+			+	+		+	+	
21		+			+		+	+	+	
22			+		+	+		+	+	+
23			+		+		+	+	+	+
24			+	+		+		+	+	
25			+		+		+	+	+	
26		+		+		+				
27		+		+			+			
28		+			+	+				
29		+			+		+			

Fig. 12. Key for the preparation of the subsequent fermentation series.

Table 10

Effect of Yeast Growth Stimulants Acting on Yeast Strain No. 5

Series	Photometer Reading Time in Hours						
	0	12	24	48	72	96	120
0	92.7	91.3	89.1	80.4	69.1	63.1	59.2
1	91.9	88.3	83.7	67.2	55.8	53.6	49.0
2	91.5	89.7	88.7	78.2	68.3	63.2	63.3
3	91.4	90.2	87.2	78.0	69.9	62.6	61.5
4	91.5	87.3	79.1	62.6	50.8	49.4	48.7
5	91.3	88.2	81.0	53.3	32.6	24.4	22.7
6	91.1	89.6	86.1	75.4	65.4	61.8	60.2
7	91.2	90.9	87.2	75.0	67.2	62.9	59.6
8	91.6	90.7	86.0	77.6	67.7	64.7	61.9
9	92.3	89.9	88.4	77.7	69.5	64.6	61.4
10	91.7	89.4	88.2	76.4	68.2	63.3	60.5
11	92.0	79.3	52.3	29.9	24.6	18.1	15.8
12	91.3	83.9	70.3	52.4	46.3	43.3	39.2
13	91.7	82.8	71.6	51.1	47.7	44.4	39.6
14	91.8	87.6	77.5	61.8	55.1	53.0	46.8
15	92.4	87.9	79.0	62.0	55.1	53.6	48.9
16	91.7	88.0	77.0	59.5	51.7	51.3	46.4
17	92.4	88.7	78.0	62.0	52.9	52.3	48.1
18	91.9	82.2	59.4	33.9	27.9	24.0	18.2
19	92.4	82.4	58.0	34.9	26.8	22.9	18.2
20	92.0	82.6	68.4	40.7	28.2	28.7	21.0
21	91.7	85.0	61.2	34.9	28.2	25.2	20.7
22	91.8	83.9	68.9	37.3	26.4	24.2	19.6
23	91.5	84.5	64.6	37.0	28.6	28.4	21.0
24	91.8	87.7	75.8	57.4	52.6	52.1	47.4
25	91.8	83.8	68.4	37.7	31.0	28.8	24.7
26	91.6	86.6	77.6	58.2	53.0	48.1	44.0
27	90.5	89.0	76.4	58.5	49.7	48.6	45.0
28	91.4	87.9	76.4	61.2	32.4	28.4	23.0
29	91.6	87.3	75.1	43.4	30.8	28.3	21.2

of these series contained biotin. The intermediate group was characterized by the presence of Bios II-A and Bios II-B, both singly and in combination. Its range included photometer readings 39.2 to 49.0 and also comprised 11 series. The low group was called the control group and was composed of the control series and those series having the other single constituents. The nine members gave photometer readings varying from 59.2 to 63.3.

Table 11 has been prepared to facilitate the comparison of the effects of the combinations of stimulants within the three general groups in regards to "Final yield" and "hours to average photometer reading".

As might be expected series 11 in the biotin group, containing all the stimulants, gave a considerable higher 120 hours yield. Substituting "ketose" for i-inositol in the presence of biotin, β-alanine, vitamins B_1, B_6 and l-leucine (series 22, 23) decreased the yield; the final readings were the same for t e paired series 18, 19, which contained biotin, Bios II-A, vitamins B_1 and B_6 and either i-inositol or "ketose". However, "ketose" induced a higher "final yield" in series 20, 21 containing in addition to biotin, pantothenic acid and vitamins B_1 and B_6, as also in series 28, 29 with the vitamins omitted. The stimulatory effect of the vitamins was shown by the increased yield in series 20, 21 in which they were present, over series 28, 29

Table 11

Comparison of "Final Yield" with the Criterion Average Photometer Reading as a Measure for Yeast Growth with Yeast Strain No. 5

Final Photometer Reading	Series	Average Photometer Reading	Hours to Average Photometer Reading
15.8	11	53.9	23
18.2	18	55.1	27
18.2	19	55.3	26
19.6	22	55.7	32
20.7	21	56.2	28
21.0	23	56.3	30
21.0	20	56.5	33
21.2	29	56.4	37
22.7	5	57.0	45
23.0	28	57.2	51
24.7	25	58.3	31
39.2	12	65.3	30
39.6	13	65.7	29
44.0	26	67.8	34
45.0	27	67.8	35
46.4	16	69.1	34
46.8	14	69.3	35
47.4	24	69.6	31
48.1	17	70.3	35
48.7	4	70.1	37
48.9	15	70.7	34
49.0	1	70.5	43
59.2	0	76.0	56
59.6	7	75.4	47
60.2	6	75.7	47
60.5	10	76.1	47
61.4	9	76.9	50
61.5	3	76.5	52
61.9	8	76.8	50
63.3	2	77.4	50

in which they were absent. A comparison of series 23 and 25 illustrated the positive action of l-leucine when added to biotin, β-alanine, "ketose" and vitamins B_1 and B_6.

In the similar series 18, 20, 25 containing i-inositol, it was evident that Bios II-A was more effective than pantothenic acid, which, in turn showed increased effect over β-alanine. Parallel series 19, 21, 23 and with "ketose" in place of i-inositol, similarly showed the same result.

The fact that biotin alone (series 5) was able to attain a final yield slightly greater than series 28, 25 was accredited to the fact that yeasts with sufficient time were able, in a limited way, to manufacture their own requisites. Furthermore, suspicion was cast on "final yield" as an adequate criterion for yeast growth.

In the middle, or the Bios II group, it was at once apparent that series 12, 13 gave relatively high final yields, thus showing that Bios II-A, Bios II-B, vitamins and either i-inositol or "ketose", were essential and sufficient for good yeast growth. A comparison of series 13, 14, 24 showed Bios II-A, pantothenic acid and β-alanine increased in effectiveness in the order given, when tested in conjunction with Bios II-B, i-inositol and vitamins B_1 and B_6. A partial companion series 13, 15 indicated also that Bios II-A was more stimulating than pantothenic acid when "ketose" was substituted for i-inositol. l-Leucine had a slight

stimulating effect in the presence of β-alanine, Bios II-B, i-inositol and vitamins as was indicated by series 16,24. Vitamins B_1 and B_6 when added to medium containing pantothenic acid, Bios II-B and either i-inositol or "ketose", produced a slight inhibitory reaction as evidenced by comparison of series 26, 14 and 27, 15.

Series 1 and 4, containing Bios II-A and Bios II-B respectively, comprised the lower members of the group, and both reached about the same final yield.

A yeast stimulant might be defined as any material which causes an increase in yeast growth over that which occurs in the control; similarly, a toxic material causes a decrease in yeast growth compared to the control. In the control group, series 7, 6, 10, 9, 3, 8 and 2, were below series 0 (basal medium) and therefore "ketose", i-inositol, l-leucine, vitamin B_6, β-alanine, vitamin B_1 and pantothenic acid exerted a toxic effect under the above test conditions.

"Hours to average photometer reading" as a criterion for yeast growth. One other point to be considered, besides the final yield, was something concerning the rate of growth or the rate at which stimulation occurred. As a criterion of this rate of growth, the "hours to average photometer reading" was chosen. If one were given the "final yield" and the "hours to average photometer reading" for any particular series, a fair approximation to the correct

growth curve could be drawn.

Furthermore, it is important as well as obvious that even though some stimulants are of much greater value than others as far as final yield is concerned; there is also something to be said for those stimulants which initiate a high rate of growth. In the industrial production of micro-organisms or their products where time, with accompanying overhead, is the important element it is the logarithmic phase that is the point of interest and the total crop may not be important. In other instances time is not an important element and the increase in total crop is the goal. A comparison of the affects of materials upon the growth of micro-organisms in different growth phases has been discussed in detail by Fulmer (1926).

The term "average photometer reading" may be defined as one-half the sum of the initial and the final photometer readings; and was determined by means of the following formula,

$$P_{av} = \frac{P_o + P_{120}}{2}$$

where P_{av} is the average photometer reading, P_o the initial photometer reading and P_{120} the final photometer reading.

To calculate the "hours average photometer reading" a curve was drawn for each series and the time required to reach the average photometer reading was read directly from the graph.

When this new criterion of yeast growth was employed, as shown in table 11, it was at once apparent that series listed in order of "final yield" need not coincide with a list determined by "hours to average photometer reading". Since no definite groupings could be detected, the complete series was compared as a whole.

Contrary to the "final yield" criterion, the control series (basal medium) gave the lowest growth rate and therefore all other series must be considered as containing stimulants. Bios II-B alone (series 4) gave higher final yields and acted a little more rapidly than did Bios II-A (series 1). Biotin (series 5), although potent in producing high yields was very slow to assert itself in the absence of other stimulants. Series 6 and 7 contained i-inositol and "ketose" respectively and both required the same time to attain their average photometer readings.

Bios II-A came first, panothenic acid second and β-alanine last, (series 19, 21 and 25), in a comparison of their influence upon growth rates in series where biotin, "ketose" and vitamins were present in each. Since 22 had l-leucine added, it was not possible to directly compare it with 18 and 20, which showed that Bios II-A exerted a greater initial stimulus than pantothenic acid when "ketose" was present in place of i-inositol, the other components being the same. Pantothenic acid conditioned a lower rate of growth than β-alanine and both were lower than Bios II-A

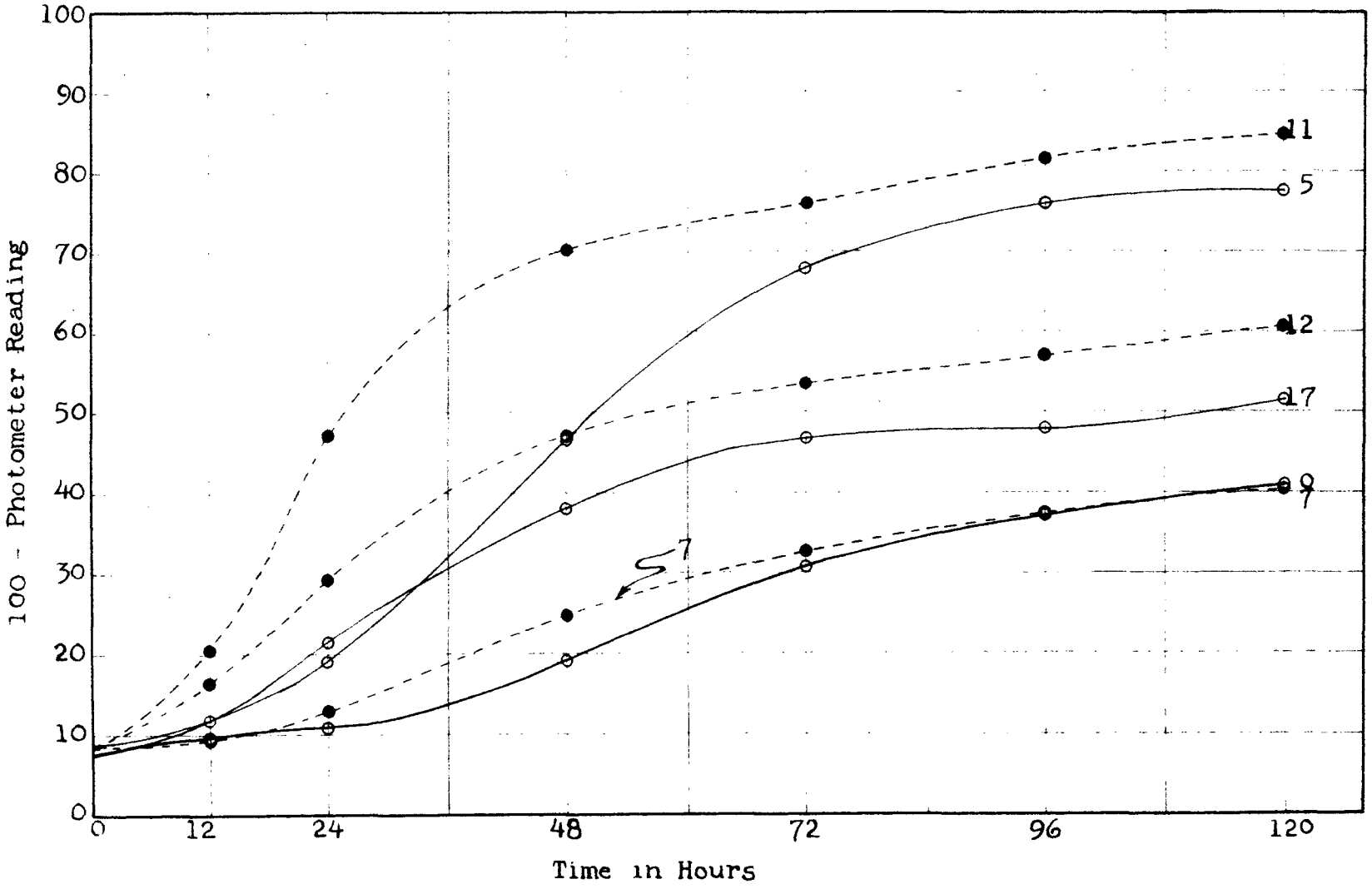


Fig. 13. Typical curves representing the growth of yeast No. 5 in the presence of stimulants.

when tested with Bios II-B, i-inositol and vitamins, (series 12, 24, 14). The replacement of i-inositol by "ketose" also showed Bios II-A as superior to pantothenic acid (series 13, 15).

Series pairs 17, 23; 16, 22; 14, 20; 12, 18 and 15, 21 indicated that Bios II-B, in the concentrations employed, could not adequately replace biotin. However, when pairs 27, 29 and 26, 18 were examined, it was found that Bios II-B plus pantothenic acid and either i-inositol or "ketose", gave higher growth rates than those series which substituted biotin for Bios II-B. In other words Bios II-B gave a higher growth rate than biotin when vitamins were not added to the series.

Greater growth rates were exhibited when vitamins were added, as shown by the pairs, 15, 27; 20, 28 and 21, 29. In the presence of pantothenic acid, Bios II-B and i-inositol however, no increase was noted (series 26, 14). A comparison of series 26 and 24 showed that β-alanine plus vitamins was more potent than pantothenic acid alone, both in the presence of Bios II-B and i-inositol. The addition of l-leucine, to medium containing "ketose", gave a slight increase in growth rate; but a toxic action was noted when i-inositol was substituted for "ketose" (series 23, 25; 24, 16).

A study of the paired "ketose" and i-inositol series showed that in the following pairs the "ketose" series gave the higher yield (22, 23; 21, 22; 12, 13 and 28, 29) while the only exception was pair 14, 15, which was reversed by

one hour. Thus it appeared that "ketose" played the same role in yeast stimulation as i-inositol, but acted a little more rapidly when tested with yeast strain 5.

The Effect of Yeast Growth Stimulants upon Yeast Strain No. 10

Fermentation series were prepared as indicated on figure 12 and inoculated with a 48 hour active culture of yeast strain No. 10. The data for yeast growth with time as determined by regular photometer readings are presented in table 12.

Since "final yield" and "hours to average photometer reading", are the criteria to be used in this study for determining the effect of the stimulants on yeast growth, these data have been arranged for convenience in table 13.

Final photometer reading as a criterion for growth of yeast strain 10. The series again fall into the three distinct groups of biotin, Bios II and control when the final photometer readings are considered. The division in the biotin and Bios II groups occur between series 22 and 13 where there is a jump in photometer reading from 19.2 to 25.0. Beginning the control group was series 2 with a reading of 41.5 as compared with series 14--the last member of the Bios II group--which had a reading of 34.2.

Figure 14 further shows this distinct separation into definite groups by a comparison of a few examples from each division.

When "final yields" were compared in the biotin group

Table 12

Effect of Yeast Growth Stimulants Acting on Yeast Strain No. 10

Series	Photometer Reading: Time in Hours						
	0	12	24	48	72	96	120
0	91.5	90.6	88.5	75.3	62.8	47.0	43.2
1	91.3	90.0	83.9	58.0	45.0	35.6	31.5
2	91.2	89.7	89.2	74.1	56.0	44.1	41.5
3	91.0	90.4	87.1	66.7	56.7	45.3	42.7
4	91.5	91.3	88.6	62.6	44.4	32.1	29.4
5	91.3	89.9	84.5	47.8	25.6	19.8	16.0
6	91.3	90.7	88.2	73.2	59.6	46.7	41.9
7	91.6	90.6	89.2	71.2	57.7	44.2	41.9
8	91.6	90.9	90.1	78.1	64.0	49.6	45.6
9	91.4	90.6	88.3	78.0	64.4	47.3	43.0
10	91.1	90.1	88.7	77.7	63.9	46.4	42.0
11	91.0	88.6	61.4	28.5	19.4	13.1	11.0
12	91.0	88.0	79.5	50.2	38.5	31.1	27.0
13	90.7	89.2	77.6	49.6	38.4	28.4	25.0
14	91.8	89.8	79.1	55.9	45.7	38.1	34.2
15	91.6	89.7	79.4	54.0	42.3	36.9	32.9
16	91.9	89.6	78.8	53.6	42.8	37.7	34.1
17	91.3	89.5	78.5	52.2	43.5	38.0	33.2
18	91.9	89.8	81.8	35.4	26.2	21.4	16.7
19	91.6	89.9	78.8	32.5	24.6	19.4	14.4
20	92.0	89.9	83.9	40.9	26.4	20.7	16.8
21	91.8	89.8	74.9	33.0	23.7	20.8	16.5
22	92.0	90.4	83.9	49.2	27.6	22.4	19.2
23	92.1	90.0	83.1	36.9	26.5	20.7	18.1
24	91.5	89.9	79.2	50.2	42.5	36.6	33.1
25	91.7	90.3	79.9	31.8	24.2	20.9	17.8
26	91.8	89.7	78.4	47.8	38.0	32.7	30.4
27	91.8	90.0	78.6	48.2	37.8	30.9	30.0
28	91.4	90.3	85.3	48.4	20.2	16.0	13.9
29	91.6	89.9	80.1	34.4	20.5	17.4	14.6

Table 13

Comparison of "Final Yield" with the Criterion Average Photometer Reading as a Measure for Yeast Growth with Yeast Strain No. 10

Final Photometer Reading	Series	Average Photometer Reading	Hours to Average Photometer Reading
11.0	11	51.0	27
13.9	28	52.6	46
14.4	19	53.0	33
14.6	29	53.1	38
16.0	5	53.7	44
16.5	21	54.2	32
16.7	18	54.3	35
16.8	20	54.4	39
17.8	25	54.7	31
18.1	23	55.1	35
19.2	22	55.6	45
25.0	13	57.9	38
27.0	12	59.0	40
29.4	4	60.5	50
30.0	27	60.9	35
30.4	26	61.1	35
31.5	1	61.4	44
32.9	15	62.2	40
33.1	24	62.3	35
33.2	17	62.3	36
34.1	16	63.0	37
34.2	14	63.0	39
41.5	2	66.4	58
41.9	6	66.6	60
41.9	7	66.8	59
42.0	10	66.5	67
42.7	3	66.8	48
43.0	9	67.2	67
43.2	0	67.3	61
45.6	8	68.6	65

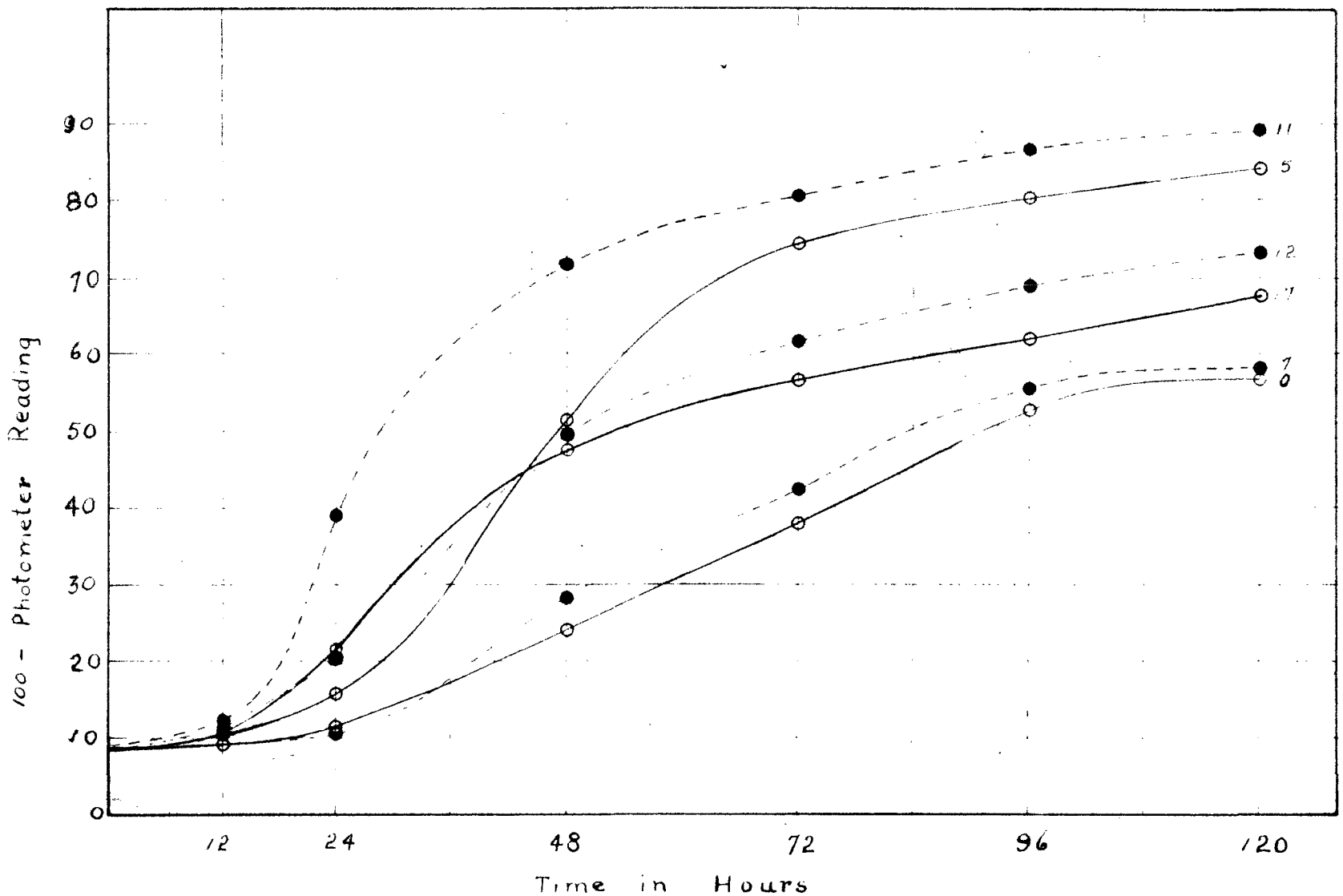


Fig. 14. Typical curves representing the growth of yeast No. 10 in the presence of stimulants.

it was apparent as well as expected that series 11, containing all the stimulants, gave the highest "final yield". The fact that series 11 was higher than any of the others may be due to the presence of vitamins B_1 and B_6. These vitamins did not appear to stimulate any other series, but it was interesting that in combination with all the other stimulants the effect was quite noticeable.

With one exception (series 28, 29) the paired series containing i-inositol and "ketose" seemed to indicate that "ketose" induced a slightly higher "final yield". However, in some cases this difference did not appear to be of great significance. The series 28, 29 contained pantothenic acid which might indicate that in combination with this particular stimulant i-inositol gave higher yields than "ketose".

The effect of l-leucine, as indicated by series 23, 25, was apparently nil when added to medium containing biotin, β-alanine, i-inositol and vitamins.

Series 19, 21 and 25 showed that Bios II-A induced a higher yield than pantothenic acid and that they were both higher than β-alanine. Biotin, "ketose", vitamins B_1 and B_6 were also present in the medium.

The high yield of biotin alone (series 5) again showed that yeasts, given a constituent of Bios, could either manufacture the other required Bios components, or else they could bring different enzymatic reactions into play so as to render unnecessary additional stimulants.

The next group comprised eleven series and included photometer readings 25 to 34, and was called the Bios II group. Particularly noticeable was the fact that series 12, 13 which contained Bios II-A, Bios II-B and either i-inositol or "ketose", did not compare with those series containing biotin although all the constituents supposedly necessary for good yeast growth were present.

In every case, a comparison of the paired i-inositol and "ketose" series revealed the fact that "ketose" produced a higher "final yield" than i-inositol (series 12, 13; 26, 27; 14, 15; and 16, 17).

Series containing vitamins gave less growth than comparable series without the vitamins as noticed in series pairs 27, 15 and 26, 14. The addition of l-leucine to medium containing β-alanine, Bios II-B, i-inositol and vitamins likewise produced a lower final yield (series 24, 16).

Both Bios II-A and Bios II-B (series 1 and 4), used singly, seemed to have rather a stimulating effect as far as final growth was concerned.

Comparing series 14, 24 and 12 it was noticeable that pantothenic acid was not as good a stimulant as its precursor β-alanine, while Bios II-A was far better than either.

In the control group all the stimulants were employed singly with the exception of Bios II-A, Bios II-B and biotin.

There was a range from slight stimulation for pantothenic acid to a slight depressing effect due to vitamin B_1. It was again interesting that "ketose" and i-inositol (series 7 and 6) were exactly the same.

"Hours to average photometer reading" as a criterion for growth of yeast strain 10. When "hours to average photometer reading" was employed as the criterion for yeast growth, new significant facts emerged. Considering the control series first, the striking feature was the high initial stimulus of β-alanine (series 3), although it was not appreciably different from the control at the 120 hour reading. Series 8, 9, and 10 contained vitamins B_1, B_6 and l-leucine and since their rates of growth were below series 0 (basal medium) they must be classed as depressants under these conditions. Bios II-A (series 1) gave a very high initial stimulus compared to its "final yield" while biotin (series 5) acted very slowly when compared to its high "final yield". "Ketose" (series 7) was ahead of i-inositol (series 6) by one hour.

Although series 11 contained all the stimulants and attained 50 per cent photometer reading first, series 25, 21 and 19 were next in order, and showed that with "ketose", vitamins and biotin, the variable component, β-alanine, was one hour faster than pantothenic acid, which was ahead of Bios II-A. Series 23 was next in order, but due to

l-leucine the initial stimulus was less than series 25. Similarily, a comparison of series 24, 16 showed the depressing effect of l-leucine in the presence of biotin, β-alanine, vitamins and i-inositol.

The order β-alanine, pantothenic acid, Bios II-A was substantiated by series 24, 14 and 12 where they were tested as addends with Bios II-B, i-inositol and vitamins B_1 and B_6.

Biotin reacts more quickly than Bios II-B in the concentrations employed in this study as was showed by the paired series 17, 23; 16, 22; 14, 20; 12, 18; and 15, 21. However a comparison of series 27, 29 and 26, 28 indicated that Bios II-B gave a greater initial stimulus than biotin when tested with pantothenic acid and either "ketose" or i-inositol in the absence of vitamins.

Series 14, 15 (which was reversed by one hour) was the only exception to the general statement that "ketose" induced higher growth rates than i-inositol when tested with yeast strain No. 10 as shown by series pairs 28, 29; 20, 21; 12, 13 and 22, 23.

Vitamins alone did not appear to increase the growth rate, but when added with other stimulants, a positive effect was noticed upon comparing series 28, 20; 29, 21; and 20, 28; a negative effect appeared when vitamins were added to medium containing pantothenic acid and Bios II-B (series 14, 26).

With yeast strain 10 "ketose" showed greater Bios I activity than i-inositol itself in the same concentrations.

It definitely appeared to give greater initial stimuli. The results are similar to those obtained with yeast strain 5, but are much more definite and therefore more significant.

The Effect of Yeast Stimulants upon the Growth of Yeast Strain No. 26.

Yeast strain No. 26 was next chosen as the test organism for the various stimulants and the data are reported in table 14.

Since the "final photometer reading" and "hours to average photometer reading" were the criteria employed in this investigation to measure yeast growth, table 15 was prepared and the series listed in order of decreasing final photometer reading. The time required for a given series to reach its average photometer reading was included in the same table so that direct comparison of the two criteria could be facilitated.

Final photometer reading as a criterion for stimulation of yeast strain 26. In the biotin group the photometer readings range from 7.9 from series 19, and to 12.9 for series 5. Between the biotin and Bios II groups there was a jump in readings from 12.9 to 17.4. The eleven members of the Bios II group extended from series 12 to series 1, or from photometer readings 17.4 to 28.9. Series 10 was first in the control group with a final yield of 43.1, a jump of 12.2 per cent from series 1 of the Bios II group. The lowest "final

Table 14

Effect of Yeast Growth Stimulants Acting on Yeast Strain No. 26

Series	Photometer Reading: Time in Hours						
	0	12	24	48	72	96	120
0	91.0	90.4	87.2	76.3	64.5	51.8	45.8
1	91.7	88.6	80.4	58.0	44.5	37.3	28.9
2	90.6	90.2	87.8	70.9	57.9	52.0	43.5
3	90.7	90.4	86.6	70.9	61.7	55.3	46.6
4	91.3	88.9	81.5	60.1	38.9	28.2	27.1
5	91.7	88.7	81.1	47.4	27.0	17.5	12.9
6	90.8	90.2	86.1	73.2	59.5	53.7	43.9
7	91.3	90.0	84.5	68.8	57.2	50.3	44.2
8	90.8	90.4	86.6	78.2	66.7	56.0	49.0
9	90.8	90.3	86.8	74.5	64.0	53.9	45.4
10	90.8	89.9	87.3	72.3	60.1	49.9	43.1
11	91.0	78.1	43.2	24.1	15.1	10.9	8.5
12	91.0	85.1	66.0	47.2	34.1	23.8	17.4
13	90.8	85.9	68.5	46.7	34.1	25.9	18.5
14	91.6	87.7	75.9	57.9	44.1	32.0	27.0
15	91.9	87.3	77.4	56.3	42.2	32.1	23.7
16	91.8	86.7	80.3	56.4	42.9	34.6	28.5
17	90.8	86.9	75.1	55.3	43.9	34.1	26.8
18	91.0	86.5	56.5	29.3	17.9	11.0	8.4
19	89.9	85.5	56.5	28.9	16.7	10.5	7.9
20	91.4	86.0	53.9	33.4	19.6	13.6	11.0
21	91.3	86.0	58.0	32.3	18.4	12.6	10.3
22	91.3	86.3	53.0	32.0	20.0	14.0	11.0
23	91.1	85.7	54.5	30.4	19.2	13.1	10.6
24	91.8	87.4	75.6	55.6	44.6	33.6	27.2
25	90.8	86.1	54.0	32.5	19.2	13.1	10.7
26	91.0	87.0	71.8	53.6	38.8	31.6	24.6
27	89.8	88.4	76.0	50.4	41.1	32.2	26.7
28	91.4	87.8	56.3	27.3	14.9	9.8	8.1
29	91.0	87.8	66.9	34.6	19.2	13.1	10.3

Table 15

Comparison of "Final Yield" with the Criterion Average Photometer Reading as a Measure for Yeast Growth with Yeast Strain No. 26

Final Photometer Reading	Series	Average Photometer Reading	Hours to Average Photometer Reading
7.9	19	48.9	29
8.1	28	49.8	28
8.4	18	49.7	29
8.5	11	49.0	19
10.3	29	50.6	37
10.3	21	50.8	30
10.6	23	50.9	27
10.7	25	50.8	25
11.0	20	51.2	26
11.0	22	51.1	25
12.9	5	52.3	44
17.4	12	54.2	38
18.5	13	54.6	38
23.7	15	57.8	46
24.6	26	57.8	42
26.7	27	58.2	37
26.8	17	58.8	42
27.0	14	59.3	45
27.1	4	59.2	49
27.2	24	59.5	43
28.5	16	60.2	44
28.9	1	60.3	45
43.1	10	67.0	58
43.5	2	67.0	54
43.9	6	67.4	57
44.2	7	67.7	50
45.4	9	68.1	63
45.8	0	68.4	64
46.6	3	68.7	52
49.0	8	69.9	66

yield" was 49.0 given by series 8 containing vitamin B_1.

Typical examples were selected from each group and plotted in figure 15. In graph form, the large differential between the three groups showed up well. It should be noted that biotin had a very slow initial stimulus, but finally attained a relatively high final yield.

Series 19, 28, 18, and 11 in the order named, gave the best "final yields", although the difference was small and not significant. The combination of biotin and Bios II-A was very effective upon yeast strain 26, since it was present in three (19, 18 and 11) of the top four series and not present in any other series in the biotin group. That Bios II-A was a better stimulant than pantothenic acid under the same test conditions with yeast strain 26 was shown in series 19, 21 and 18, 20. β-alanine, a moiety of pantothenic acid, did not adequately replace either pantothenic acid or Bios II-A (series 19, 21 and 25). Vitamins added to "ketose" gave no noticeable effect, (series 29, 21), however an increase in photometer readings from 8.1 to 11.0 occurred when vitamins B_1 and B_6 were added to medium containing biotin, pantothenic acid and i-inositol (series 28, 20), thus showing that vitamins were secondary in importance to more potent yeast stimulants.

Practically the same "final yield" was noted for series 23, 25, and hence l-leucine, the variable, produced neither stimulating nor depressing effects.

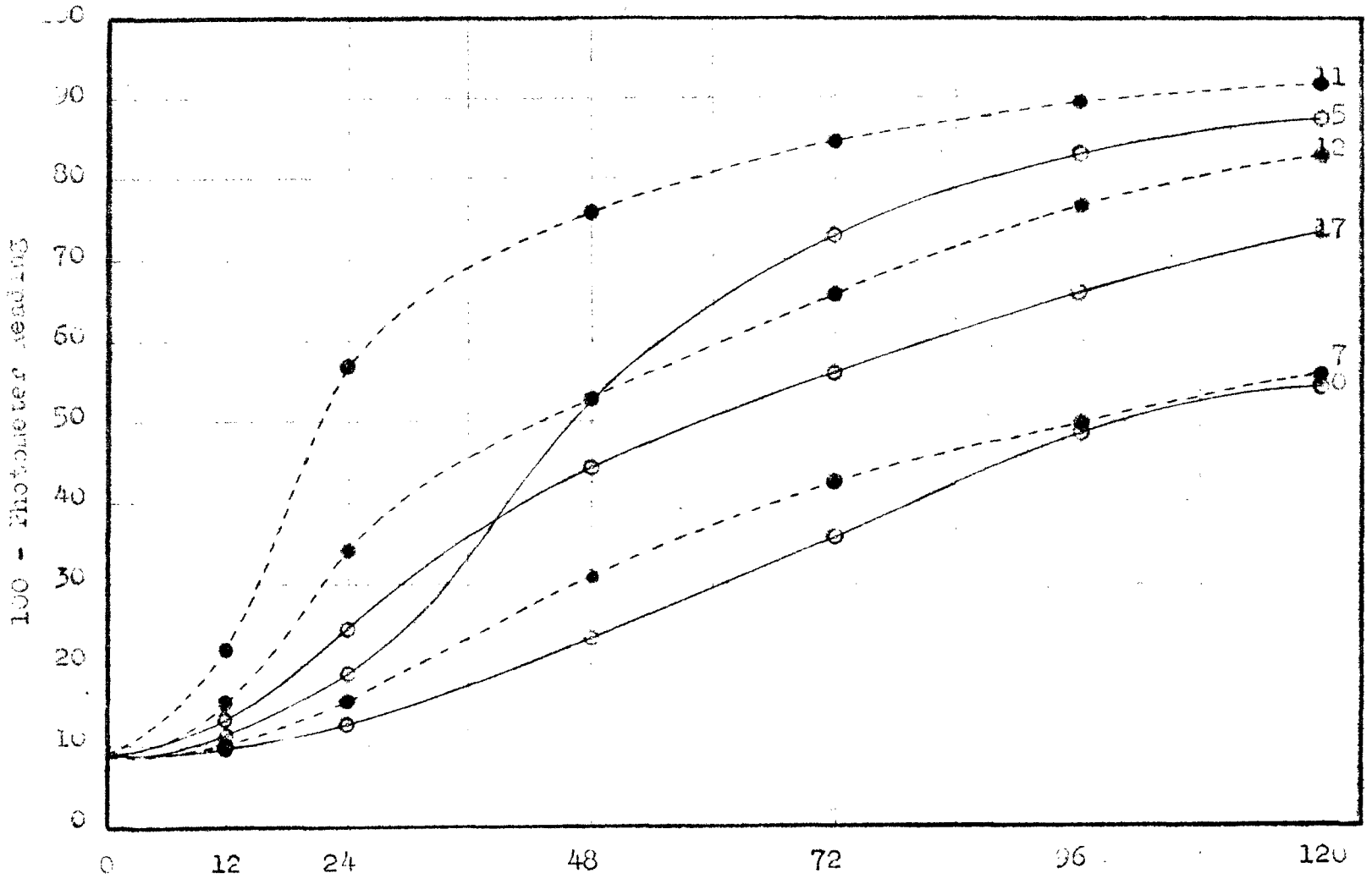


Fig. 15. Typical curves representing the growth of yeast No. 26 in the presence of stimulants.

i-Inositol, in combination with biotin and pantothenic acid, produced greater yeast growth than "ketose" in the same combinations (series 28, 29). Although series 28, 29 showed i-inositol more active than "ketose", the remaining paired series in the biotin group (18, 19; 20, 21; and 22, 23) showed that the "ketose" series gave the highest yields.

Series 5 contained biotin alone and was the last member of the group. Thus it was apparent that biotin in the concentrations employed for this study was the stimulant responsible for the high "final yield" in the biotin group.

Series 12, 13 of the middle group produced relatively high final photometer readings, thus showing again that Bios II-A was a powerful stimulant when present with Bios II-B or its equivalent. Pantothenic acid in the same combinations was more effective than its precursor β-alanine; but neither could match the great effect produced by Bios II-A (series 12, 14 and 24). The presence of "ketose" in the medium did not change this order of effectiveness (series 13, 15). Bios II-A (series 1) and Bios II-B (series 2) separately gave low yields, but when added together with either Bios I or "ketose", greatly increased the final yields.

The addition of vitamins caused an increase in growth when "ketose" was present (series 15, 27), but a decrease was observed in the presence of i-inositol (series 14, 26). Likewise no increased growth could be determined when l-leucine was added to β-alanine, Bios II-B, vitamins B_1

and B_6, and i-inositol (series 24, 16).

In the paired series, "ketose" caused a higher yield in series 14, 15 and 16, 17; while lower yields were observed in series 12, 13 and 26, 27.

Series 10, 2, 6, 7 and 9 in the control group and containing respectively, l-leucine, pantothenic acid, i-inositol, "ketose" and vitamin B_6, each exerted a stimulating effect and produced higher "final yields" than the control (series 0). Conversely however, β-alanine and vitamin B_1 gave a slight inhibitory effect. "Ketose" (series 7) and i-inositol (series 6) produced about equal stimulation.

"Hours to average photometer reading" as a measure of stimulation upon yeast strain 26. It was significant that the first ten series in order of "hours to average photometer reading" all contained biotin (series 11, 25, 22, 20, 23, 28, 19, 18, 21, and 29). However, series 5 containing biotin alone was eighteenth in order, which proved that biotin must have other stimulants present for maximum effect. By itself, the initial stimulus was slow.

Yeast strain No. 26 also reacted differently in the order of effectiveness of Bios II-A, pantothenic acid and β-alanine when added to medium containing biotin, "ketose", and vitamins. β-alanine caused the highest initial stimulus to be followed by Bios II-A and then pantothenic acid (series 25, 21 and 19). The order of effectiveness was again changed in medium containing Bios II-B, i-inositol and vitamins.

The addition of Bios II-A was most effective, pantothenic acid least effective, and β-alanine intermediate between the two (series 12, 24, and 14).

The addition of vitamins caused a stimulating effect with either "ketose" (series 29, 21) or i-inositol (series 28, 20) present in biotin-pantothenic-"ketose" medium. If Bios II-B was substituted for biotin, the vitamins caused no increase in rate of growth (series 26, 14). Perhaps this distinction was due to the crude Bios II-B solution containing some vitamins, while biotin was prepared from a purified concentrate. However, l-leucine showed a depressing reaction and series containing it gave a lower growth rate than its paired series (series 25, 23 and 24, 16). l-Leucine alone (series 10) gave a slight stimulating effect as compared with the vitamin B_1 and B_6 series (series 8 and 9), which were practically the same as the control (series 0).

Although "ketose" alone (series 7) gave a considerable higher rate of growth than i-inositol alone (series 6), in the paired series containing i-inositol and "ketose", series pairs 22, 23; 18, 19; 28, 29; 20, 21; 14, 15 and 12, 13 showed faster growth when i-inositol was present, while only one pair (series 16, 17) showed "ketose" as the most effective.

Series 26, 24 suggested that β-alanine plus vitamins B_1 and B_6 could equal the effect of pantothenic acid when each was added to medium containing Bios II-B and i-inositol.

Bios II-A (series 1) produced a greater growth rate than Bios II-B (series 4) although Bios II-B gave a better "final yield". β -alanine and pantothenic acid (series 3 and 2) also caused good initial stimulus, but not equal to Bios II-A.

An examination of these results obtained from three different yeasts revealed the fact that "ketose" definitely does show Bios I activity. In the majority of combinations its effect is greater than that for i-inositol; in some combinations its effect is less. Although the degree to which this activity was manifest depended upon the yeast strain employed as the test organism and upon the particular combination of stimulants under observation, "ketose" played a role in yeast growth stimulants similar to that of i-inositol. A change in the chemical formula of a known Bios component has resulted in a product of slightly greater stimulating capacity.

The Effect of Some Phospho-i-Inositol Compounds Upon Yeast Growth

Tetra-Phosphoric Ester of i-Inositol as a Yeast Growth Stimulant

Acetobacter suboxydans, as shown previously was unable to oxidize either the free ester or the barium salt of the tetraphosphoric acid ester of i-inoisitol. This was very interesting since it showed that this organism could neither

attack the configuration of the phosphoric-i-inositol nor could it hydrolyze the phospho groups by means of a phosphatase enzyme. In other words, Acetobacter suboxydans was specific in its requirements and attacked only the free i-inositol. Although it has been known for some time that i-inositol or Bios I (Eastcott) (1928) exerted a stimulating effect on yeast, no mention was found as to the nature of i-inositol derivatives upon yeast growth. i-Inositol occurs in nature mainly as its phophorous esters, so it would be interesting to test whether or not yeast cells possess a phosphatase enzyme capable of attacking this phosphate ester or whether it is necessary to supply Bios I as pure i-inositol.

Consequently a small amount of the barium salt of the tetraphosphoric acid ester of i-inositol, as prepared earlier in this study, was weighed out and dissolved in water to form the stock solution. Sufficient hydrochloric acid was added to give a clear homogenous mixture. The stock solution contained 0.200 grams of salt per 100 ml. and 3.5 ml. of this solution were added to every series of 175 ml. each as indicated in the following fermentation. Series were prepared as follows and yeast growth data, a function of time, are shown in table 16.

0 - Control
1 - i-Inositol
2 - Salt
3 - Bios II-A + i-inositol
4 - Bios II-A + Salt
5 - Bios II-B + i-inositol

6 - Bios II-B + Salt
7 - Bios II-A + Bios II-B + i-inositol
8 - Bios II-A + Bios II-B + Salt
9 - Bios II-A + Bios II-B + i-inositol + Salt

Table 16

Effect of the Barium Salt of the Tetraphosphoric Ester of i-Inositol Upon the Growth of Yeast Strain No. 26

Series	Photometer reading Time in hours						
	0	12	24	48	72	96	120
0	91.5	90.0	85.2	67.3	52.4	41.7	37.0
1	91.2	89.5	83.8	64.6	51.1	45.6	38.1
2	85.6	84.5	81.1	64.0	49.6	41.2	30.9
3	90.7	85.9	75.0	51.3	39.0	29.1	23.0
4	85.4	81.9	70.4	47.4	37.0	27.8	21.1
5	89.9	85.2	69.5	48.8	38.2	29.2	20.4
6	85.5	83.2	71.7	48.0	35.8	26.3	20.4
7	89.8	84.7	65.4	42.4	30.0	20.3	14.2
8	84.3	79.7	63.0	38.1	27.0	18.2	13.2
9	85.2	80.4	61.2	38.6	27.5	19.1	14.2
10	90.9	89.6	85.7	66.0	52.7	41.0	33.2

These results showed that i-inositol alone gave no stimulation but that the salt gave a sudden increase toward the end of the fermentation and produced a higher "final yield" than the control series. Throughout, it seemed that the series containing the salt gave a slightly higher yield than the companion series with i-inositol. This slight effect may be due either to the extra amount of phosphorus added with the salt or to the decrease in pH associated with the hydrochloric acid necessary for the solution of the salt. The significant thing however, was that the salt was able to play the same role as i-inositol as a yeast growth

stimulant. Comparative readings were given when either or both Bios II-A and Bios II-B were present in the medium.

The Effect of Calcium Phytate Upon Yeast Growth

Phytin is a natural constituent of most of the cereal grains as well as other seeds. It can be prepared from the steep water obtained in the manufacture of starch from corn by precipitation with milk of lime until the desired pH value is reached. By this method Bartow and Walker (1938) succeeded in obtaining white crystalline calcium phytate. The following experiment was performed with some of this material. Hydrochloric acid was necessary to dissolve the calcium phytate in the stock solution which contained 0.8 grams of salt per 100 ml.. In the test fermentations 1.75 ml. of this solution were added for each series of 175 ml. Series were prepared as follows and the results recorded in table 17.

0 - Control
1 - i-inositol
2 - phytin
3 - Bios II-A
4 - Bios II-A + i-inositol
5 - Bios II-A + phytin
6 - Bios II-B
7 - Bios II-B + i-inositol
8 - Bios II-B + phytin
9 - Bios II-A + Bios II-B + i-inositol
10 - Bios II-A + Bios II-B + phytin
11 - Bios II-A + Bios II-B + i-inositol + phytin

Table 17

Effect of Phytin on the Growth of Yeast Strain No. 26

Series	Photometer reading Time in hours						
	0	12	24	48	72	96	120
0	91.0	90.2	85.7	76.6	61.5	48.4	44.1
1	91.0	89.9	82.7	67.2	54.4	48.0	38.4
2	87.6	88.7	79.4	60.5	49.2	36.1	28.3
3	91.2	89.4	74.4	52.5	39.4	31.8	23.3
4	91.2	87.9	68.7	49.2	39.4	31.4	24.2
5	90.7	87.8	70.2	45.4	35.1	26.1	18.6
6	91.1	88.4	77.8	51.7	40.0	31.6	24.1
7	90.7	87.6	69.9	49.5	39.2	31.8	23.9
8	91.0	88.0	72.5	47.3	34.5	26.0	19.8
9	90.8	86.7	60.4	38.7	28.0	20.7	14.6
10	90.7	88.5	64.8	34.6	22.7	15.5	12.8
11	90.0	86.6	62.5	35.5	24.8	17.7	13.1

It was evident from the above table that the calcium phytate had some Bios present as an impurity. However, it is also shown that calcium phytate may be substituted for i-inositol as Bios I, since greater readings were given when either or both Bios II-A and Bios II-B were added.

These studies should be considered as preliminary and not necessarily representing final data. A more detailed and comprehensive investigation will be continued in these laboratories.

DISCUSSION

Chemical Properties of "Ketose"

The complexity of the problem of positive identification of the "ketoses" is seen from the fact that there exists the possibility of four monoketo-i-inositols and nine diketo-i-inositols. Neither Posternak (1936) nor Kluyver and Boezaardt (1939), who claimed to have prepared monoketo-i-inositol, attempted its identification among the four possible monoketo-i-inositols. "Ketose" is not inosose, but it may be a mixture of mono- and diketo-i-inositols, of unknown configuration, whose physical properties are so similar that they cannot be separated by crystallization. However, suitable derivatives might be prepared wherein the properties of the two compounds show enough variation to permit separation. The original keto-compounds could be recovered by hydrolysis. This type of work is in progress in which the acetyl derivative has been chosen as most likely to be successful.

The experimental work presented in this thesis strongly suggests that "ketose" is predominantly a diketo-i-inositol. Quantitative hydrogenation with its counterpart quantitative oxidation, exclude the monoketo-inosose conclusion of Kluyver. Likewise, the relatively small amount

of reducing compound formed in phosphate buffered medium indicates that the difference in results in this work and that of Kluyver and Boezaardt (1939) might be in the effect of pH upon the i-inositol fermentation, or the absence of small amounts of sorbitol from their medium.

An indirect method of attack was resorted to, in testing the fermentability of certain phospho-i-inositol compounds, whose configuration may or may not be known at present. The tetraphosphoric acid ester of i-inositol did not ferment, thus indicating that Acetobacter suboxydans did not have a phosphatase enzyme capable of hydrolyzing the phosphate groups. Previous experiments have shown that phytin, a hexaphosphoric acid ester was not attacked. Future experiments of this type should be performed on various methyl ethers of the i-inositols, both natural and synthetic.

Methylation of "kebose" with dimethyl-sulphate in a hot alkaline medium might have significant possibilities in determining the actual chemical nature of the compound. Previous attempts to identify oxidative products have been unsuccessful due to the lability of the hydroxyl and carbonyl groups. The protection of the hydroxyl groups by the formation of methoxy derivatives should permit a break in the chain adjacent to a carbonyl group upon subjection to suitable oxidizing agents. From a consideration of the configuration and nature of the oxidized

fragments the chemical structure of "ketose" could be postulated.

Biological Properties of "Ketose"

The degree of yeast stimulation in the presence of various combinations of known stimulants was dependent on the particular strain of Saccharomyces cervisiae employed. In accordance with Lesh, Underkofler and Fulmer (1938) yeast strain 26, belonging to the third group, appeared to be least susceptible to stimulation by substances containing Bios I activity. Yeast strain 10 showed the greatest sensitivity to Bios I compounds, while strain 5 was next in order. Since it was unlikely that the yeasts studied during this investigation are completely representative of all the strains of Saccharomyces cervisiae, the writer does not contend that the results outlined in this section will apply to other members of the three groups discussed by Lesh, Underkofler and Fulmer (1938), but they should assist somewhat in this connection. The fact that certain yeasts behave similarly with one criterion does not necessarily mean that they react similarly to all criteria.

Table 18 contains the fermentation series for the different yeasts arranged in order of "final yield" and "hours to average photometer reading". It was at once evident that the order in which the series occurred varied with each yeast and with the criterion employed. Series II

Table 18

A Comparison of the Series Order Based on "Final Yield" and "Hours to Average Photometer Reading" for Yeasts No. 5, 10 and 26

Final Yield			: Hours to Average Photometer Reading		
Yeast 5	Yeast 10	Yeast 26	: Yeast 5	Yeast 10	Yeast 26
11	11	19	11	11	11
18	28	28	19	25	25
19	19	18	18	21	22
22	29	11	21	19	20
21	5	29	13	23	23
23	21	21	12	18	28
20	18	23	23	27	19
29	20	25	24	26	18
5	25	20	25	24	21
28	23	22	22	17	29
25	22	5	20	16	27
12	13	12	16	29	12
13	12	13	15	13	13
26	4	15	26	20	17
27	27	26	27	14	26
16	26	27	14	12	24
14	1	17	17	15	16
24	15	14	29	5	5
17	24	4	4	1	1
4	17	24	1	22	14
15	16	16	5	28	15
1	14	1	7	3	4
0	2	10	6	4	7
7	7	2	10	2	3
6	6	6	2	7	2
10	10	7	8	6	6
9	3	9	9	0	10
3	9	0	28	8	9
8	00	3	3	9	0
2	8	8	0	10	8

was most consistent by showing the highest "final yield" and required the shortest time to reach its average photometer reading for each of the three yeast strains.

An examination of parallel series 19, 21 and 25 containing Bios II-A, pantothenic acid and β-alanine respectively in addition to biotin, "ketose", vitamins B_1 and B_6; revealed the fact that Bios II-A was most potent in producing high final growths, to be followed by pantothenic acid and then β-alanine. This order was the same for each of the three yeasts. A similar parallel series, composed of 12, 14, and 24 and containing Bios II-B, i-inositol, and vitamins B_1 and B_6 also exhibited the same order of effectiveness with Bios II-A, pantothenic acid and β-alanine. The three yeasts investigated gave the same results as far as final photometric readings were concerned. In general then, Bios II-A was more effective than either pantothenic acid or β-alanine in causing a high final yeast crop. Pantothenic acid was better than β-alanine.

Where the hours necessary to reach average photometer reading was the criterion employed upon the above parallel series, it was at once evident that the order of effectiveness had changed. Yeast No. 10 showed greatest initial stimulation upon the addition of β-alanine. Pantothenic acid and Bios II-B were effective in that order when either i-inositol or "ketose" was present. Bios II-A initiated a greater growth rate for both series when tested upon yeast

No. 5. Pantothenic acid was next in the "ketose" series, but β-alanine achieved second place in the i-inositol series. When yeast strain 26 was used as the test organism, β-alanine initiated the highest growth rate when "ketose" was present, while Bios II-A was the most effective in the presence of i-inositol.

It was significant that the top five series under yeast strain No. 10 all contained "ketose", thus showing the greater stimulating effect of "ketose" over i-inositol. In only one case (14, 15) did a series pair show greater stimulation for the i-inositol series than for "ketose", and this was reversed by only one hour.

Rainbow and Bishop (1939), tested specimens of a number of rare chemicals for Bios activity. Quebrachitol, the methyl ether of i-inositol, was among those tested and found to contain no Bios activity. This was very interesting in the light of the positive results exhibited by "ketose" in this investigation. The methyl ether of i-inositol was inactive but the carbonyl derivative did not destroy its Bios I activity. Inasmuch as "ketose" was an oxidized product of i-inositol it seemed that i-inositol acted as Bios I through some oxidation reduction equilibrium reaction, involving the oxidation of one or more hydroxyl groups.

Eastcott (1928) in reporting the identification of Bios I as i-inositol, stated that the i-inositol added in her studies

could be quantitatively recovered by hydrolyzing the yeast crop. Some i-inositol was also recovered from yeast grown in solution containing only sugar and salts, but the amount was less. Thus, although the above author quantitatively recovered the i-inositol added, it would still be possible for some "ketose" to be formed and to function in reversible oxidation-reduction systems in the role of Bios I. Also, the method of recovery may include "ketose" too. No mention was made as to the reducing power of the recovered i-inositol. This equilibrium will be further investigated in these laboratories.

Not only may yeast species vary in their requirements of i-inositol, but different races might differ in their needs of Bios II-A. For some, Bios II-A was the most effective stimulant, for others either pantothenic acid or β-alanine showed greater stimulation. This variability in the yeast strains suggests that Bios may be of a coenzyme nature. Thus a change in the source of Bios I or Bios II-A available to the yeast would probably necessitate bringing into action a new enzyme or set of enzymes in order for assimilation to take place in the cell. A change in the enzyme would involve an appropriate change in the coenzyme and the yeast would show a difference in Bios requirements. The same reasoning would explain the results obtained by Williams and Rohrman (1936) relating to the difference in the pantothenic acid effect when aspartic acid and asparagine were employed as the source of nitrogen.

SUMMARY AND CONCLUSIONS

1. Several experiments were described which deal with the chemical configuration of the fermentation product ("ketose") obtained from the fermentation of i-inositol by Acetobacter suboxydans. Quantitative hydrogenation and oxidation data combine to show that the compound is predominately a diketo-i-inositol, although a small percentage of a monoketo-i-inositol compound might be present as an impurity.

2. A procedure has been described using hot alkaline dimethyl sulphate to methlyate "ketose". This protection of the hydroxyl groups should provide a new and a very useful tool for determining the structure and configuration of "ketose".

3. Phospho-i-inositol compounds such as the tetra- and hexa-phosphoric acid esters of i-inositol were not oxidized by Acetobacter suboxydans. However, in yeast studies they were able to play the same role as i-inositol in the Bios complex. These data indicate that the Acetobacter organism is unable to utilize certain phosphate esters of i-inositol although the yeast cell possesses the ability to either utilize them directly or hydrolyze them to units which are assimilable.

4. "Ketose" was capable of stimulating yeast growth

alone and in a variety of combinations with nine known stimulants when tested upon three types of Saccharomyces cerevisiae. The compound played approximately the same role as i-inositol or Bios I, but in the majority of cases, a greater initial stimulation was observed for "ketose" series. A reversible oxidation-reduction system between "ketose" and i-inositol could explain the "ketose" activity.

5. A photometer method for measuring yeast growth was described and calibrated by direct microscopic count. Curves were drawn permitting data conversion from one to the other. It was also found that yeast cells in suspension conform to Beer's law until counts for over four hundred were reached.

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