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THE METABOLISM OF L-FUCOSE IN *ESCHERICHIA COLI* O111-B_4

by

Mohammad Ali Ghalambor

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in The
University of Michigan
1962

Doctoral Committee:

Assistant Professor Edward C. Heath, Chairman
Assistant Professor Harold J. Blumenthal
Professor G. Robert Greenberg
Professor Walter J. Nungester
Assistant Professor William G. Robinson

To Zahra

ACKNOWLEDGMENTS

I wish to express my sincere gratitude to:

Dr. Edward C. Heath for his eminent patience, guidance and most friendly and sincere cooperation, and especially for his encouragement throughout this work; Dr. Saul Roseman, Professor of Biological Chemistry, for his most helpful discussions and criticism; the members of my doctoral committee for their criticism and counsel.

The author wishes to acknowledge his great appreciation to Drs. Ivan F. Duff, Professor of Internal Medicine, and W. J. Nungester, Professor and Chairman of the Department of Bacteriology, for financial assistance.

Thanks and gratitude are due to The Ministry of Education of Iran, for offering me the opportunity to pursue my studies at The University of Michigan.

Acknowledgments are made to all the members of the Rackham Arthritis Research Unit for their helpful discussions and suggestions.

I am grateful to my wife, and my partents, for their constant patience, understanding, and encouragement.

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ABBREVIATIONS

ADP	adenosine-5'-diphosphate
A.S.	ammonium sulfate
ATP	adenosine-5'-triphosphate
Ca-P-Gel	calcium phosphate gel
CTP	cytidine-5'-triphosphate
DEAE-CELLULOSE	diethylaminoethyl-cellulose
DHA	dihydroxyacetone
DHAP	dihydroxyacetone phosphate
DPN	diphosphopyridine nucleotide
DPNH	reduced diphosphopyridine nucleotide
EDTA	ethylenediamine tetraacetate
FDP	D-fructose-1,6-diphosphate
L-Fu-1-P	L-fuculose-1-phosphate
D-GAP	D-glyceraldehyde-3-P
GDPF	guanosine diphosphate-L-fucose
GDPM	guanosine diphosphate-D-mannose
α-GPD	α-L-glycerophosphate dehydrogenase
GSH	glutathione
GTP	guanosine-5'-triphosphate
LDH	lactic dehydrogenase
Nudiki	nucleoside diphosphokinase
PEP	phosphoenolpyruvate

ABBREVIATIONS (Concluded)

PPi	inorganic pyrophosphate
D-Ru-5-P	D-ribulose-5-phosphate
TDPG	thymidine diphosphate-D-glucose
TCA	trichloroacetic acid
TDPR	thymidine diphosphate-L-rhamnose
TPN	triphosphopyridine nucleotide
TPNH	reduced triphosphopyridine nucleotide
Tris	tris (hydroxymethyl) aminomethane
TTP	thymidine-5'-triphosphate
UTP	uridine-5'-triphosphate
D-Xu-5-P	D-xylulose-5-phosphate

I. INTRODUCTION

Studies concerning the metabolism of L-fucose in microorganisms have been of interest since the mid-nineteen thirties (1), however, the individual biochemical steps were not investigated until the mid-nineteen fifties (2-4). Segal and Topper (5) have also demonstrated the utilization of L-fucose in human volunteers, but no information with regard to the pathway of catabolism of L-fucose in mammals has been reported.

Green and Cohen (3) demonstrated the enzymatic interconversion of L-fucose and L-fuculose in cell-free extracts of fucose-grown Escherichia coli Ba_{15} (first reaction in Figure 2). This work was followed by the finding of another enzyme, L-fuculose kinase, in cell-free preparations of Escherichia freundii (4), which catalyzed the phosphorylation of L-fuculose to form L-fuculose-1-P (second reaction in Figure 2).

Clarification of some aspects of the reaction catalyzed by L-fuculose kinase, and an investigation of the further metabolism of L-fuculose-1-phosphate were the primary objectives of this dissertation. Among the various bacteria which were examined for their ability to utilize L-fucose as their sole source of carbon and energy, including the genera Lactobacillus, Clostridium, Bacillus and Escherichia, Escherichia coli O111-B_4 was found to be an efficient system, and was selected as the organism for these investigations. Since no information was available concerning the metabolism of L-fucose by this particular organism, the

initial enzymatic steps involved in this process were determined before the further metablism of L-fuculose-1-phosphate was investigated. In a series of studies which were conducted in a manner similar to those of Green and Cohen, and Heath, the following enzymatic steps involved in the biosynthesis of L-fuculose-1-phosphate from L-fucose were established:

1. L-Fucose isomerase activity was demonstrated, by the ability of extracts to catalyze the conversion of L-fucose to L-fuculose. The latter compound was isolated and characterized.

2. Crude extracts of Escherichia coli O111-B_4 also possessed an L-fuculose kinase that catalyzed the phosphorylation of L-fuculose in the presence of ATP. L-Fuculose kinase was partially purified (35-80 fold) from the cell-free preparations of this organism and the product of the reaction it catalyzed was characterized as L-fuculose-1-phosphate.

With regard to the metabolism of L-fuculose-1-phosphate, Huang and Miller (6) employing cell-free extracts of L-fucose-grown Escherichia coli Ba_{15} as the enzyme source, suggested that L-fuculose-1-phosphate was first epimerized at position 3 (via D-xylulose-5-p;3-epimerase) to yield 6-deoxy-L-sorbose-1-phosphate which was subsequently cleaved to trioses by FDP aldolase (Figure 1). The foregoing contention, however, is not supported on the basis of the evidence obtained in the present studies; it is concluded that no epimerase is involved in the metabolism of L-fuculose-1-phosphate by Escherichia coli, and that the cleavage of L-fuculose-1-phosphate proceeds in a single step catalyzed by L-fuculose-

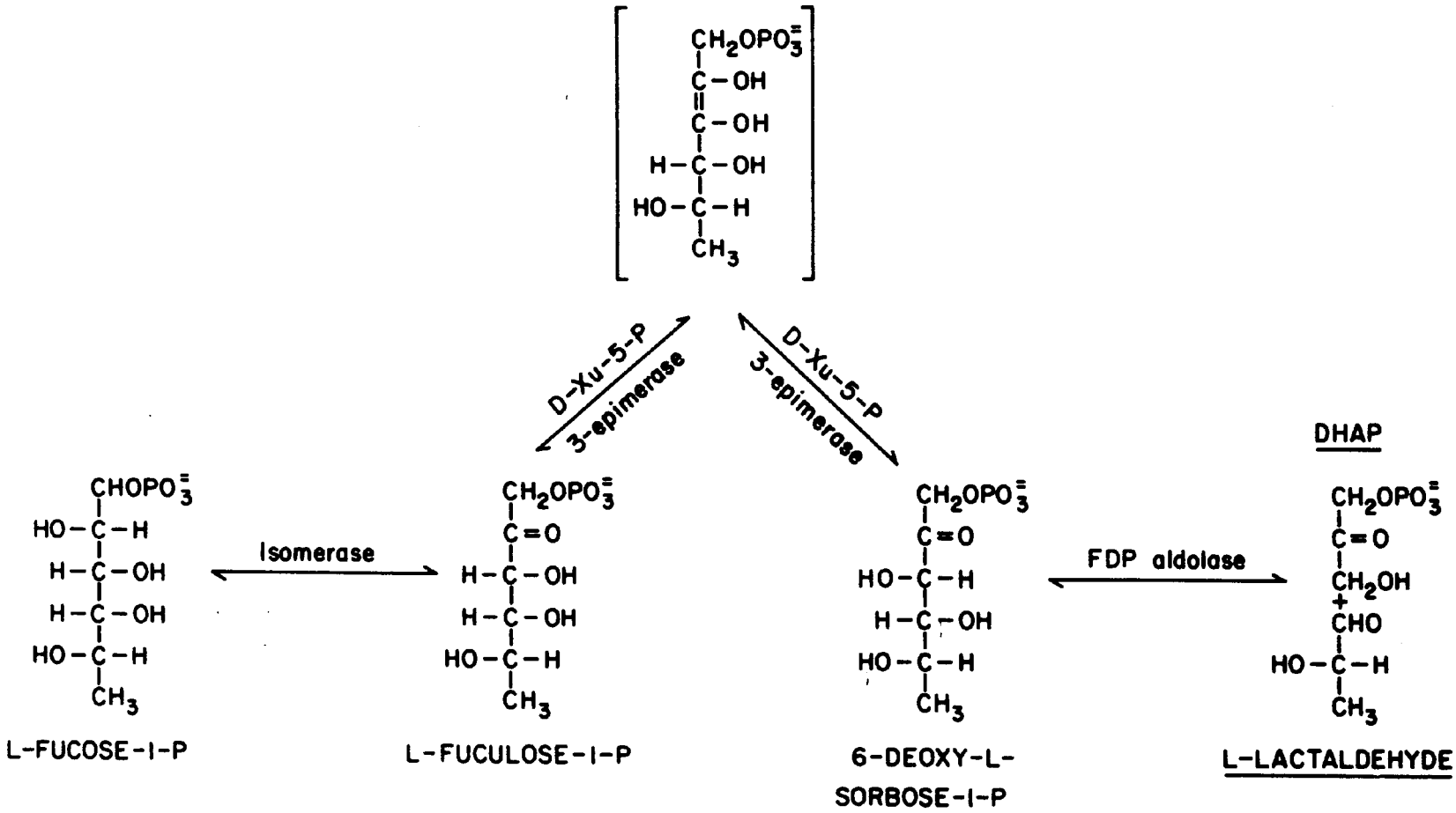
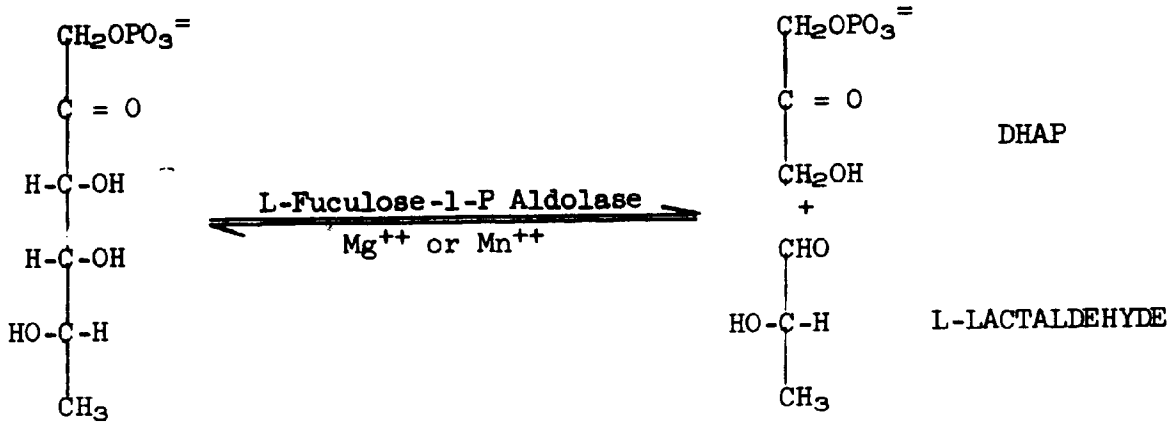


Figure 1. Pathway of the metabolism of L-fuculose-1-P as suggested by Huang and Miller (6).

l-phosphate aldolase as illustrated below:



L-FUCULOSE-1-P

Direct cleavage of L-fuculose-1-phosphate (7).

This enzyme has been partially purified (120-fold) from cell-free extracts of Escherichia coli O111-B_4 and has been demonstrated to be distinct from HDP aldolase and D-fructose-1-P aldolase on the basis of its substrate specificity.

On the basis of the data obtained in these studies, the over-all catabolism of L-fucose by Escherichia coli O111-B_4 may be presented by the sequence of reactions illustrated in Figure 2.

THE PATHWAY OF CATABOLISM OF L-FUCOSE BY *ESCHERICHIA COLI* O111-B4

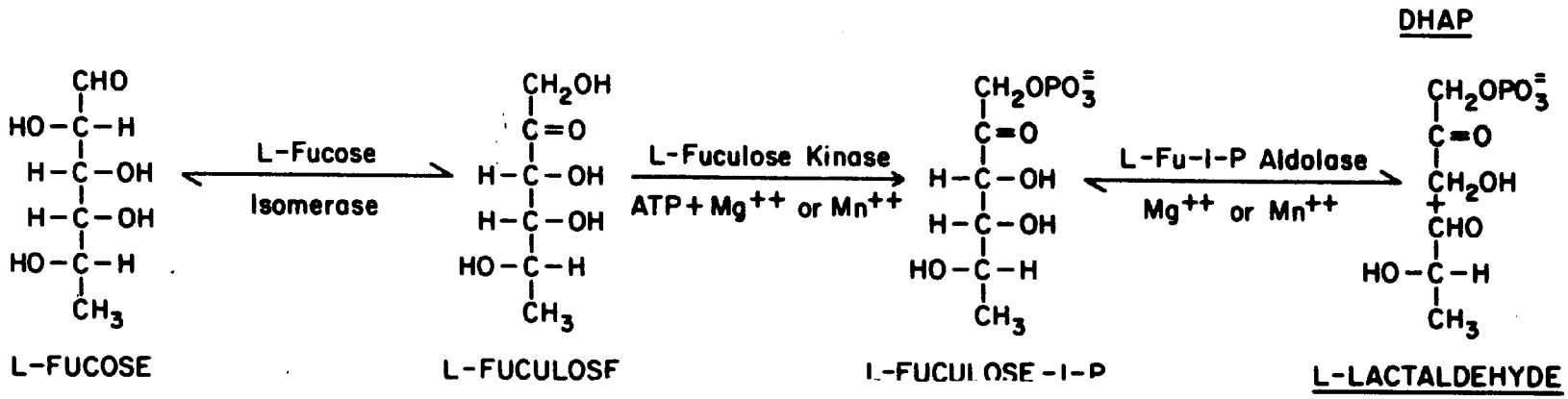


Figure 2. Pathway of the catabolism of L-fucose by Escherichia coli O111-B_4 as established by the present studies.

II. HISTORICAL

A. Occurrence

L-Fucose is also known by the following names: L-rhodeose and L-galactomethylose (8). Its chemical structure has been studied by Mayer and Tollens (9), Tollens and Rorive (10), and Hudson (11). The final characterization of the structure of L-fucose as 6-deoxy-L-galactose was made by Clark in 1922 (12).

L-Fucose is widely distributed in nature. In brown algae (Phaeophyceae), it exists in the form of a sulfated polysaccharide which is referred to as fucoidin (13-19). Fucoidin was first isolated from brown algae in 1913 by Kylin who also established the presence of L-fucose in this polymer (20,21). Fucoidin can be obtained from seaweed either by extraction of the dehydrated material with dilute mineral acids at room temperature (15,16), or by soaking in water (22), and isolated in the form of its calcium salt. The resulting crude fucoidin salt can be further purified by alcohol fractionation (15,16). The chemical nature of these fucose-containing polymers has been established by Percival and Ross (23,24), and Conchie and Percival (25), studying fucoidins isolated from various members of Phaeophyceae (Fucus vesiculosus, Laminaria cloustoni and Himanthalia lorea). On the basis of chemical degradation studies, the above investigators concluded that fucoidin is primarily a fucosan

monosulfate in which L-fucose-4-sulfate serves as the repeating unit. Furthermore, the L-fucopyranosyl-sulfate monomers are glycosidically bound together through α-1→2 linkages. The composition of L-fucose and sulfate in fucoidin has been shown to vary, depending on the purity of the preparation (24), the season of the year (26), and the species of Phaeophyceae (24); fucoidin preparations may contain 35-60% L-fucose and 30-40% sulfate. In addition to L-fucose, small quantities of other monosaccharides, such as D-xylose, D-galactose, and uronic acids have also been detected in several fucoidin preparations (14,23,24). Due to the abundance of fucoidin in the genera Laminaria (22,24,27), Gloiopeltis (28), Turbinaria (29), Kjellmaniella (30), and particularly, the members of the genera Fucus (8,15,18,24,25,31-34), Pelvetia (35-38), Ascophylum (8,39), Himanthalia (24), and Macrocystis (13,14), these marine algae are used as commercial sources of L-fucose.

For the manufacture of L-fucose, either fucoidin (13,14,35) or dehydrated seaweeds (8,12,36,38) are hydrolyzed with dilute mineral acids (at 100°C) and the liberated L-fucose is purified by either alcohol fractionation (35), or by converting it to its phenylhydrazone derivative (8,12,36,38). When L-fucose is isolated as its phenylhydrazone, the latter is first purified, subsequently decomposed by treatment with benzaldehyde, and the resulting free L-fucose is isolated and crystallized from ethanol. Chemically, L-fucose has been prepared by oxidation of L-fucitol (40).

In green algae (Chlorophyceae), L-fucose has been found as a constituent of the extracellular polysaccharides of Chlamydomonas ulvensis (41). In bacterial systems, L-fucose exists as a component of the extracellular polysaccharides of Aerobacter aerogenes (42,43), Aerobacter cloacae (44), Bacillus polymyxa (45), and Klebsiella aerogenes (46). It is also found as a constituent of capsular polysaccharides of Pseudomonas fluorescence (47), and in yeasts (48). In viruses, it has been detected in the polysaccharides obtained from influenza virus (PR_8 and LEE strains) (49).

In higher plants, L-fucose is present also as a component of polysaccharides. It has been found in the polysaccharides of sugar-beet pectin (50), hemicelluloses (B_1) of Vicia faba (51,52), in the gum Tragacanth (53,54), in the polysaccharides of Arabian cherry (55), and of plum leaf (56). It also occurs in the form of glycosides (saponins) in Gypsophila (graceful herbs) (57).

In the animal kingdom, L-fucose also has a broad distribution. It is a component of glycoproteins of blood serum (58,59), urinary secretions (59,60), and cerebrospinal fluid (61). It is present in the urinary gonadotropins (62), in the carbohydrate moiety of γ-globulins of some animals (63), as a constituent of reticular fibers of lymph nodes, lung and testis (64), renal reticulum (65), and as the free sugar in urinary secretions (66). The occurrence of L-fucose in oligosaccharides isolated from human milk was demonstrated by Kuhn (67,68), Montreuil (69), and Malpress (70). It is also present as a component of milk glycoproteins

(71). The presence of L-fucose as guanosine diphosphate-fucose in the milk (72), and mammary glands of several animals (72,73) has recently been established. Among neoplasmic tissues, L-fucose has been found in glycoproteins from human cancerous ascitic fluid (74) and in the so-called pathological fraction of sera from patients with multiple myeloma (75). It has also been detected in placental substances giving the skin reactions of cancer and pregnancy (76). In lower animals, L-fucose has been detected in the spawn jelly of amphibia (77,78), in the acrosome of guinea pig spermatozoon (79), and in the jelly-coat of the ovule of sea-urchin Arabacia lixula (80). In glycolipids isolated from oyster (81,82), L-fucose constitutes about 15% of the total carbohydrate, and in sea-cucumber Stichopus japanicus (83) it is present as the major component (60%) of a poly-L-fucose-sulfate-protein complex.

The ubiquity of L-fucose in the living organisms may represent some type of generalized function(s) performed by this sugar in the over-all maintenance of at least certain forms of life. These roles may be played at the structural, physiological or biochemical levels. Of utmost interest to immunochemists is the wide distribution of this compound in an important class of antigens, i.e., isoagglutinogens. Morgan and King (84), and Bray, Henry and Stacy (85) demonstrated the presence of L-fucose in purified polysaccharides with blood group type A activity obtained from hog gastric mucins. These findings were subsequently confirmed by Kabat et al. (86,87,90), and Partridge (89) who established the presence of L-fucose as a component of blood group A substance ob-

tained from human saliva and hog gastric lining. Kabat, et al. (86,90), Aminoff, et al. (91), Annison and Morgan (92), and Masamune, and Yohizawa (93) reported the occurrence of L-fucose in polysaccharides with blood group type O, H, Le^a, and B activity which had been isolated from various sources.

The role of L-fucose in the immunologic specificity of blood group substances has been studied by Morgan (94,95), Kabat (86,96,97), and Landsteiner (98) and their associates. It was known from the early studies of Landsteiner and Harte (99) on the chemical composition of blood group A, B, and O substances, that these antigens contained a common core in their polysaccharides consisting of [N-acetyl-D-glucosamine-N-acetyl-D-galactosamine-D-galactose] as the repeating unit. In addition, Bray et al. (85) by methylation studies demonstrated the presence of L-fucose as terminal residues in the polysaccharide chains of these blood group substances. These fucose residues could be removed by mild acid hydrolysis while causing no appreciable degradation of the basic polysaccharide chain (86). The relationship of L-fucose residues with immunological specificity of blood group substances was established by a series of studies conducted by Morgan, et al. (94,100,101). The basic discoveries that led to the conclusion that L-fucose was associated with the antigenic specificity of certain blood group substances are outlined as follows.

1. The release of L-fucose residues by mild acid hydrolysis of blood group A and O substances resulted in the loss of their respective blood group specificity (86).

2. Studying the inhibitory action of simple sugars on the specific immunologic reaction of H substance with its corresponding antisera from Anguilla anguilla (eel), Morgan, et al. (94) demonstrated that L-fucose and L-fucopyranosides served as potent inhibitors. This observation initiated the idea that L-fucose might be involved in the antigenic specificity of H substance. However, comparison of the extent of the inhibory effects of L-fucose and α-methyl-L-fucopyranoside on the immunologic reaction of H substance with anti-H sera, revealed that L-fucopyranoside gave a closer fit with anti-H antibody since it was found to be appreciably more effective than free L-fucose. This observation was subsequently confirmed by Morgan and Watkins (101) who demonstrated that the reaction of plant seed agglutinins (obtained from Lotus tetragonolobus) with human O cells also is inhibited by α-methyl-L-fucopyranoside. These findings led Morgan, et al., to suggest that L-fucose residues which are present as terminal groups in the polysaccharide chains of blood group H substance are glycosidically bound to the adjacent sugars, perhaps through α-linkages; furthermore, that an α-L-fucopyranosyl structure serves as the determinant of the blood group specificity.

3. Watkins (102), and Crumpton and Morgan (103) demonstrated that crude extracts of the protozoan Trichomonas foetus (102), and culture filtrates of Clostridium welchii (103) possessed several enzymes which destroyed the blood group activity of A, B, and H substances. Later, Watkins and Morgan (100) obtained a partially purified preparation from T. foetus which selectively inactivated H, but not A or B substances. This

enzyme preparation was shown to contain a specific fucosidase which catalyzed the hydrolytic cleavage of the glycosidic bond linking terminal L-fucose residues to the adjacent sugar moieties present in the polysaccharide chain of blood group H substance. Furthermore, the destructive effect of fucosidase on H substance could be inhibited by the addition of L-fucose (100). These observations further confirmed the conclusion drawn by Morgan et al. (94) concerning the role played by L-fucose in the immunological specificity of blood group H substance. The role of L-fucose as a determinant group in certain antigens has been established by actual chemical synthesis of artificial antigens containing terminally located L-fucose with proper configuration (104).

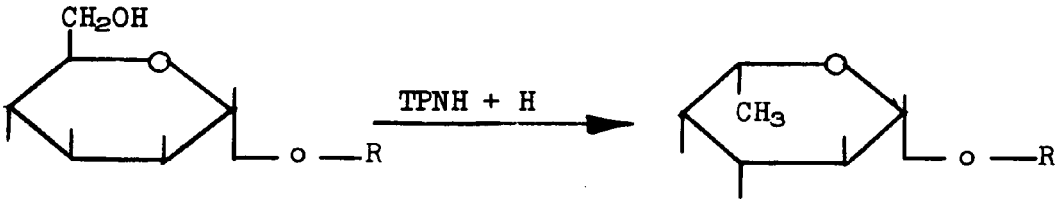
B. Metabolism

1. BIOSYNTHESIS

The biosynthesis of L-fucose was studied with isotopic methods independently and simultaneously by Wilkinson (46) in Klebsiella aerogenes, Segal and Topper (43) in Aerobacter aerogenes, and by Heath and Roseman (44) using Aerobacter cloacae as the organism. These investigators demonstrated that D-glucose-1-C^{14} was converted to L-fucose-1-C^{14}, and D-glucose-6-C^{14} was converted into L-fucose-6-C^{14}. These observations resulted in the conclusion that L-fucose is biosynthesized from D-glucose directly, without inversion or fragmentation of the molecule of D-glucose.

Although these findings indicated that D-glucose served as the precursor of L-fucose anabolism, they yielded no information concerning the

actual enzymatic mechanisms involved in this process. In 1958, Ginsburg and Kirkman (105) isolated guanosine diphosphate-L-fucose (GDPF) from aerobacter aerogenes. The same investigators (106-108) demonstrated that crude extracts of this organism catalyzed the conversion of guanosine diphosphate-D-mannose (GDPM) to GDPF as illustrated below:

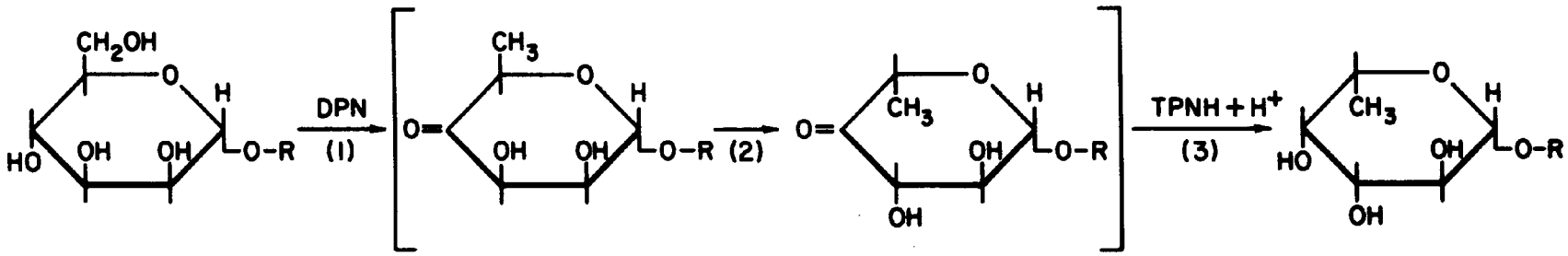


GDPM R = GDP GDPF

As can be seen in the above scheme, at least three steps are involved in this conversion: epimerizations at positions 3 and 5, and reduction of the molecule at carbon atom 6. The enzymatic mechanism involved in the conversion of GDPM to GDPF was suggested by Ginsburg (109), as shown in Figure 3.

The first indication of the biosynthesis of L-fucose in mammalian systems was the isolation of GDPF from ewe's milk (72) and mammary glands of various animals (73). Segal and Topper (5) were able to demonstrate the appearance of radioactive L-fucose in the milk of human volunteers to whom radioactive D-glucose was administered. These investigators concluded that D-glucose was converted into L-fucose without randomization or inversion of the carbon skeleton. Studies conducted by Foster and Ginsburg (110), however, demonstrated that in mammalian tissues GDPM is also converted into GDPF. Using rabbit lung tissues as the source of

THE PROPOSED MECHANISM OF ENZYMATIC CONVERSION OF GDPM TO GDPF



R = GDP

STEPS:

(1) Intramolecular oxidation-reduction (oxidation at C_{-4} and reduction at C_{-6}).

(2) Inversion of configuration at C_{-3} and C_{-5} via ene-diol formation.

(3) Stereospecific reduction at C_{-4}.

Figure 3. Mechanism of conversion of GDP-D-mannose to GDP-L-fucose in Aerobacter aerogenes as proposed by Ginsburg (109).

enzymes in their studies, these investigators postulated that the biosynthesis of L-fucose from D-glucose in mammalian systems follows a similar pattern as that established for bacteria (110); however, the mechanism of conversion of GDPM to GDPF remains to be established. Since the pathway for the biosynthesis of GDPM from D-glucose is known, the following sequence of enzymatic reactions were suggested to represent the enzymatic synthesis of L-fucose from D-glucose (110); this mechanism is compatible with the established pathways in bacteria and mammals (Figure 4).

2. CATABOLISM

The metabolic degradation of L-fucose by microorganisms was first studied in 1935 by **Tadokoro** (1) who demonstrated the accumulation of oxalic, lactic, formic, and glycolic acids, as the products of L-fucose fermentation by Aspergillus oryzae. In 1953, Kuhn et al. (111), in the course of their studies on metabolic pathways of Lactobacillus bifidus showed that lactic acid and acetic acid were the predominant products of L-fucose catabolism. Since these investigators were primarily concerned with the fermentation and the chemical nature of the products of L-fucose breakdown in living cells, no conclusion could be drawn regarding the nature of the enzymatic reactions involved.

In 1956, Green and Cohen (3) demonstrated the presence of the enzyme L-fuccse isomerase in cell-free preparations of L-fucose-grown Escherichia coli Ba_{15}, which catalyzed the interconversion of L-fucose and L-fuculose.

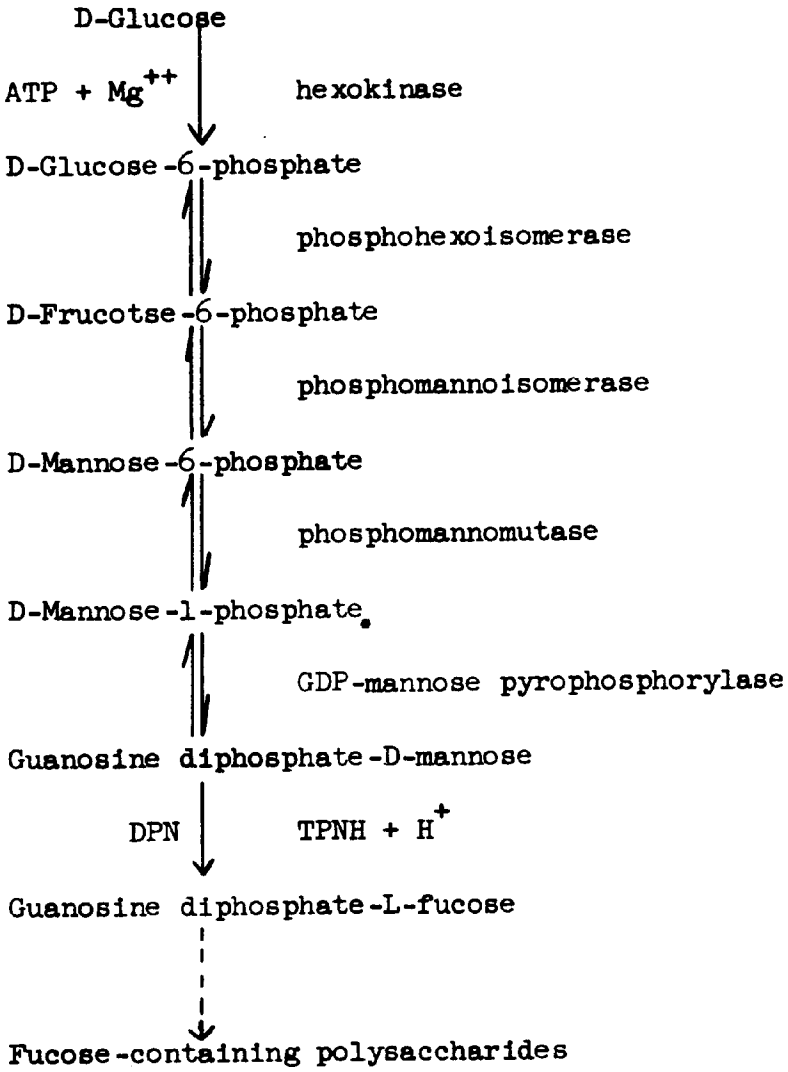


Figure 4. Pathway of biosynthesis of L-fucose from D-glucose as suggested by Foster and Ginsburg (110).

The product of the action of this enzyme on L-fucose was isolated and characterized as L-fuculose. Green and Cohen (3) suggested that their crude extracts also possessed an enzyme which could catalyze the phosphorylation of L-fuculose. Huang and Miller (6) employing crude extracts of the same organism, which had also been grown on L-fucose, concluded that both L-fucose and L-fuculose could be phosphorylated in the presence of ATP to yield L-fucose-1-phosphate, and L-fuculose-1-phosphate, respectively. These conclusions were entirely based upon the insufficient evidence of slight differences in the rates of phosphorylation of these two compounds. These authors noted the presence of L-fucose isomerase in the crude extracts of Escherichia coli Ba_{15}, therefore, the addition of either L-fucose or L-fuculose to such enzyme sources would rapidly result in an equilibrium mixture of both. Moreover, these investigators did not isolate any of the proposed products of phosphorylation, i.e., L-fucose-1-phosphate and L-fuculose-1-phosphate. In 1958, Heath (4) presented evidence for the presence of an enzyme in crude extracts of L-fucose-grown Escherichia freundii which catalyzed the phosphorylation of L-fuculose. This enzyme was partially purified, and the product of the reaction was characterized as L-fuculose-1-phosphate. An enzyme preparation free of L-fucose isomerase activity, phosphorylated L-fuculose, but not L-fucose.

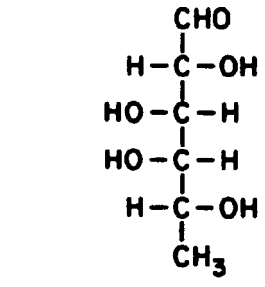
With regard to the further catabolism of L-fuculose-1-phosphate, Huang and Miller (6) reported the formation of DHAP from L-fucose, when the latter was incubated in the presence of ATP and crude extracts of L-

fucose-grown Escherichia coli Ba_{15}. Furthermore, they reported that the DHAP formation was stimulated by the addition to this system of a partially purified preparation of D-Xu-5-P,3-epimerase obtained from Lactobacillus plantarum. To interpret their results, these authors suggested the following sequence of reactions: L-fucose ⇢ L-fuculose ⟶ L-fuculose-1-P ⟶ 6-deoxy-L-sorbose-1-P ⟶ DHAP + L-lactaldehyde. They concluded that L-fuculose-1-P was epimerized at position 3 by D-Xu-5-P, 3-epimerase to the sorbose derivative which was subsequently cleaved by ordinary FDP-aldolase. These conclusions were mainly based on slight differences in kinetic data; none of the postulated intermediates in the pathway were isolated.

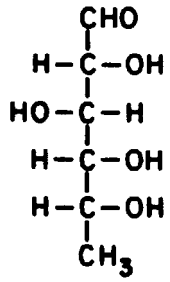
Segal and Topper (5), by parenteral administration of L-fucose-1-C^{14} to human volunteers, were able to demonstrate rapid release of the radioactivity in the respiratory CO_2, and in urine of these subjects. No attempts to establish the enzymatic mechanisms involved in the breakdown of L-fucose in man have been reported.

C. Natural Distribution and Metabolism of Other 6-Deoxyhexoses

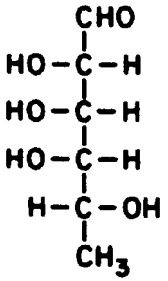
The structural formulae of some of the naturally occurring 6-deoxy sugars and their derivatives are illustrated in Figures 5 and 6; a brief description of these substances is presented below.



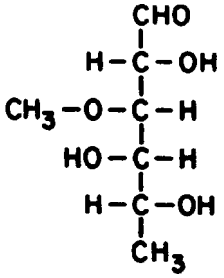
D-FUCOSE
(6-Deoxy-D-Galactose)



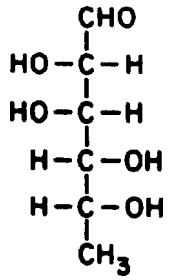
D-QUINOVOSE
(6-Deoxy-D-Glucose)



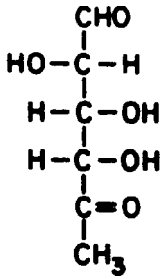
D-TALOMETHYLOSE
(6-Deoxy-D-Talose)



D-DIGITALOSE
(3-O-Methyl-D-Fucose)

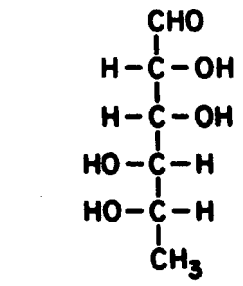


D-RHAMNOSE
(6-Deoxy-D-Mannose)

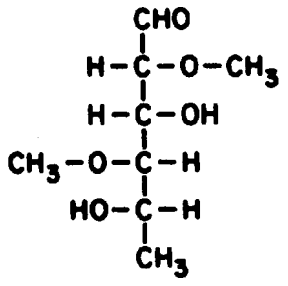


5-KETO-6-DEOXY-
D-ARABOHEXOSE

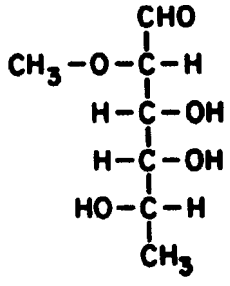
Figure 5. Structural formulae of the naturally occurring 6-deoxyhexoses.



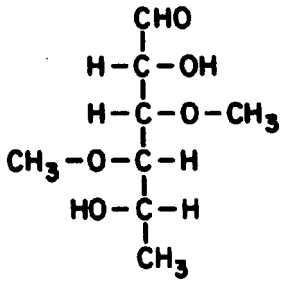
L-RHAMNOSE
(6-Deoxy-L-Mannose)



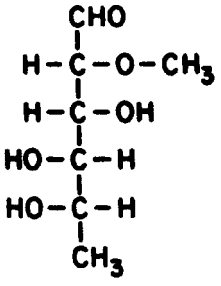
2,4-di-O-METHYL-
L-RHAMNOSE



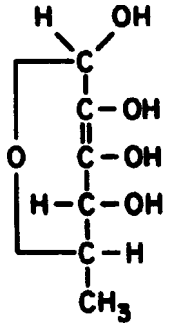
2-O-METHYL-
L-FUCOSE



*3,4-di-O-METHYL-
RHAMNOSE



*2-O-METHYL-
RHAMNOSE



ANGUSTOSE
(2-Keto-L-Fucopyranose)

*The D or L configuration has not been established.

Figure 6. Structural formulae of the naturally occurring 6-deoxyhexose derivatives.

1. D-FUCOSE AND ITS O-METHYL ETHER

a. D-Fucose

D-Fucose, also referred to as D-rhodeose (8,112) is present in the polysaccharides produced by the family Convolvulaceae, particularly Ipomea orizabensis (113) which produces Jalap resin (jalapin), and in Convolvulus scammonia (114) which produces a gum known as scammonium. In these polysaccharides which are used as purgatives, D-fucose is present in association with L-rhamnose, D-glucose, and long chain hydroxy acids (jalapinolic and convolvulinic acid) (115). It has also been found as a component of chartreusin, an antibiotic formed by Streptomyces chartreusis (116), and Streptomyces X-465 (117). In this antibiotic, D-fucose is present in the form of a glycoside in association with D-digitalose and an aglycon whose complete chemical structure has not been established (118). Field, et al. (119) in the course of their studies on the fermentation of the rare sugars by the members of the colon and aerogenes groups of bacteria, reported that the members of Escherichia and Aerobacter were able to utilize D-fucose as the sole source of carbon. Furthermore, they showed that acetic acid, ethanol, lactic acid,and succinic acid were the predominant products of fermentation.

b. D-Digitalose

D-Digitalose (3-O-methyl-D-fucose) has a broad distribution in glycosides which are present in the seeds of a family of plants known as

Strophanthus. Several of these glycosides have been isolated and characterized chemically; the representatives of these compounds are listed below: crystioside, obtained from Strophanthus intermedius (120); musaroside (from Strophanthus sarmentosus) (121); divaricoside and caudoside, obtained from Strophanthus caudatus (122); and arriagoside, walloside and grass-walloside, found in Strophanthus intermedius (123). D-Digitalose is present as a component of glycosides obtained from other sources, such as odoroside, which is found in Nerium odorum (124). All of these glycosides upon hydrolysis yield D-digitalose and an aglycon moiety. In Digitalis purpura (125), D-digitalose is present accompanied by glucose in the form of glycosides (digitalinum verum). In some of the antibiotics produced by Streptomyces (117,118), D-digitalose is found in association with D-fucose. The metabolism of D-digitalose is unknown.

2. ANGUSTOSE

Structurally, it is 2-keto-L-fucopyranose (126,127). It is present as a component of angustomycin A (126-128), an antibiotic produced by Streptomyces hygrocopicus var. angustomyceticus (129). Angustomycin A which is active against Mycobacteria (129,130), is chemically 6-amino-9-(L-1-,2-fucopyranosyl)-purine (126,128); it is a structural analogue of adenosine. No information is available with regard to the metabolism of angustose.

3. O-METHYL-ETHERS OF L-FUCOSE AND RHAMNOSE

A Methyl derivative of L-fucose (2-O-methyl) is present as a con-

stituent of polysaccharides of plum leaf (56), and of sugar-beet pectin; (50) in both cases it is accompanied by the parent sugar, and L-rhamnose. This methyl-ether is also present as a component of mycosides, a class of glycolipids produced by Mycobacterium tuberculosis (131,132). MacLennan et al. (132), and Smith et al. (131) have isolated and identified three type-specific mycosides from various members of Mycobacterium, all of which contain O-methyl derivatives of 6-deoxy sugars; a brief description of these compounds is presented below.

a. Mycoside A

This compound which is characteristic of photochromogenic group of atypical members of Mycobacterium contains the sugars, 2-O-methyl-fucose, 2-O-methyl-rhamnose, and 2,4-di-O-methyl-rhamnose. These sugars are attached to the lipid moiety of mycoside A which has been identified as a mycocerosate of an aromatic alcohol. 2,4-di-O-methyl-rhamnose has been found as a component of Pneumococcus Type II polysaccharides (133,134).

b. Mycoside B

This glycolipid is produced by Mycobacterium tuberculosis var. bovis; its sugar component has been characterized as 2-O-methyl-rhamnose (132).

c. Mycoside C

Mycoside C is a peptide-glycolipid (135) produced by Mycobacterium tuberculosis var. avium; its structural units have been tentatively identified as: 6-deoxy-talose, 3,4-di-O-methyl-rhamnose, and a third com-

ponent which is possibly a mono-methyl ether of 6-deoxy-talose. The metabolism of the methyl ethers of 6-deoxy sugars is unknown.

4. 5-KETO-6-DEOXY-D-ARABOHEXOSE

This is the only known naturally occurring 5-keto derivative of a 6-deoxy-hexose. It is found in hygromycin A which is produced by Streptomyces hygrocopicus (136-140); in this antibiotic, it occurs glycosidically bound to one of the hydroxyl groups of a derivative of cinnamic acid (3,4-dihydroxy-methyl-cinnamic acid) (139). As for its metabolism, Elbein, et al. (140), employing isotopic techniques, have demonstrated that D-glucose is converted into 5-keto-6-deoxy-D-arabohexose by Streptomyces hygrocopicus without randomization of the isotope or inversion of the carbon chain. Thus, D-glucose-1-C^{14}, 2-C^{14} and 6-C^{14} were converted into 5-keto-6-deoxy-D-arabohexose labeled in C_1, C_2, and C_6, respectively.

5. QUINOVOSE

Quinovose (chinovose) is the 6-deoxy derivative of D-glucose which is also referred to as epirhamnose, isorhodeose and D-glucomethylse (8). It occurs naturally in Jalap resin (convolvulin) obtained from Tubera jalapa, and some other members of family Convolvulacea (141). On hydrolysis, this resin yields D-glucose, L-rhamnose, quinovose, and a long chain hydroxy acid (3,12-dihydroxyhexadecanoic acid). Quinovin (or chinovin), a glycoside obtained from the bark of various members of the family Cinchona, serves as a commerical source for production of quinvose (142). The metabolism of quinovose is not known.

6. L-RHAMNOSE

a. Occurrence

L-Rhamnose (6-deoxy-L-mannose), which is also known as L-mannomethylose and "isodulcit" (8), has a broad distribution in the plant kingdom. It is present as a component of polysaccharides of plum leaf (56), sugar-beet pectin (50), Acacia pycantha gum (143), and Acacia mollisina gum (144). The mucilages of Pangamia glabra (145), cress seeds, linseeds, Abelmoschus manihot (146), and Sisal pectic acids (147) also contain L-rhamnose. The mucilages obtained from the bark of Ulma fulva (slippery elm) (148,149) and flaxseeds (150,151) contain L-rhamnose as one of their major components; in these polymers, aldobionic acid (2-D-galacturonido-L-rhamnose) serves as the repeating unit (148,151). L-Rhamnose is present as a rhamnosan in the mucilages produced by Actinidia rufa (152), and in the free state in the poison sumac Rhus toxicodendron (153). It is broadly distributed in the natural flavonoids produced by many plants, especially members of two classes of plants, Rhamnaceae and Polyganaceae (154), and in tobacco (Nicotiana tobacum) (155), in which it occurs in the form of glycosides such as rutin (quercetin → glucose → rhamnose), quercitrin (quercetin → rhamnose), xanthorhamnin (rhamnetin → rhamninoside) all of which contain L-rhamnose and an aglycon. L-Rhamnose is also present as a constituent of flavonon glycosides of various citrus plants, especially Citrus sinensis (156) and Citrus paradisi (157), the most abundant of which are: naringin (naringenin → glucose → rhamnose)

and hesperidin (hesperetin → glucose → rhamnose).

L-Rhamnose can be prepared from either xanthorhamnin (158) or quercitrin (159,160). Quercitrin is obtained from the bark of Quercus coccinea var. tinctoria Gray in large amounts, and is commonly known as quercitron. Upon acid hydrolysis, quercitrin yields L-rhamnose, and quercetin (158,161). In bacteria, also, L-rhamnose is widely distributed. It is found as a component of the cell-wall polysaccharides of Mycobacterium tuberculosis var. hominis (162), where it occurs as terminal residues, and of Diplococcus pneumonia Types II, V and VI (163). Its isolation from the type-specific capsular polysaccharides of pneumococcus Type VII has been recently reported (164). It has been found in the endotoxins of Shigella sonnei, Shigella flexneri (165), and various species of Salmonella (166). Cell-walls (166-168) and type-specific polysaccharides of group D streptococci contain L-rhamnose as a major component (169). Recently, L-rhamnose has been found to occur in the form of thymidine diphosphate-L-rhamnose in Escherichia coli (170), Lactobacillus acidophilus (171), and Pseudomonas aeruginosa (172), Streptococcus faecalis (173,174), and in the form of uridine diphosphate-L-rhamnose in a non-capsulated Type II pneumococcus (175). In rhamnolipids produced by Pseudomonas aeruginosa (176,177), L-rhamnose is present in association with long chain hydroxy fatty acids (176,178,179).

b. Biosynthesis

The problem of biosynthesis of L-rhamnose has been studied intensively.

Hauser and Karnovsky (177) demonstrated that Pseudomonas aeruginosa cells synthesized a rhamnolipid when grown on glycerol, D-fructose or 1,2-propanediol as the sole carbon and energy sources. The study of distribution of radioactivity showed that when D-fructose-6-C^{14} was employed as the sole carbon source, L-rhamnose in the rhamnolipid was labeled exclusively in C_6, and when D-glucose-1-C^{14} was used, radioactivity was incorporated in both C_1 and C_6 of the molecule of L-rhamnose which was formed. On the basis of the failure of the resting cells of Pseudomanas aeruginosa to metabolize dihydroxyacetone and their ability to utilize lactaldehyde and pyruvaldehyde, these authors suggested that dihydroxyacetone in the phosphorylated form might be used by these cells, possibly through a condensation with lactaldehyde (in the presence of an aldolase with specificity for L-threo configuration), to form L-rhamnulose-1-phosphate which can be subsequently converted into L-rhamnose. They presented no evidence, however, to support this suggested pathway. Thus, the enzymatic steps involved in the biosynthesis of L-rhamnose remained unknown until recently.

The first evidence for the biosynthesis of L-rhamnose in intact cells was presented by Southard, et al. (168). Using isotopic techniques, these authors demonstrated that D-glucose was converted into L-rhamnose. Thus, D-glucose-1-C^{14} gave rise to L-rhamnose-1-C^{14} and D-glucose-6-C^{14} resulted in the incorporation of L-rhamnose-6-C^{14} in the cell walls of streptococci. These workers concluded that D-glucose is the major source for the carbon skeleton of the molecule of L-rhamnose.

The study of the pathway of biosynthesis of L-rhamnose was started by Okazaki *et al.* (170) who islated one of the intermediates of L-rhamnose biosynthesis, thymidine diphosphate-L-rhamnose (TDP-rhamnose), from *Escherichia coli*. This finding was confirmed by Okazaki (171), Kornfeld and Glaser (172), and Pazur and Shuey (173), working with *Lactobacillus acidophilus*, *Pseudomonas aeruginosa* and *Streptococcus faecalis*, respectively. Pazur and Shuey (174) succeeded in isolating two of the intermediates of L-rhamnose biosynthesis, TDP-rhamnose and TDP-glucose, from *Streptococcus faecalis*. Using crude extracts of this organism as the enzyme source, these authors suggested a possible pathway for L-rhamnose biosynthesis from D-glucose (Figure 7).

The final confirmation of the validity of this pathway was made simultaneously and independently by two groups of investigators, Kornfeld and Glaser (172, 180, 181), and Okazaki *et al.* (182) using *Pseudomans aeruginosa* and *Escherichia coli* B, respectively. Kornfeld and Glaser have isolated and partially purified two enzyme systems which catalyzed the following reactions:

1. α-D-glucose-1-P + TTP $\rightleftharpoons$ TDP-D-glucose + PPi

2. TDP-D-glucose + TPNH + H^+ $\xrightarrow{DPN}$ TDP-L-rhamnose + TPN + H_2O

The mechanism involved in the conversion of TDP-glucose into TDP-rhamnose was established by Glaser and Kornfeld (183). Using a partially purified enzyme system from the same organism, these authors suggested the mechanism illustrated in Figure 8.

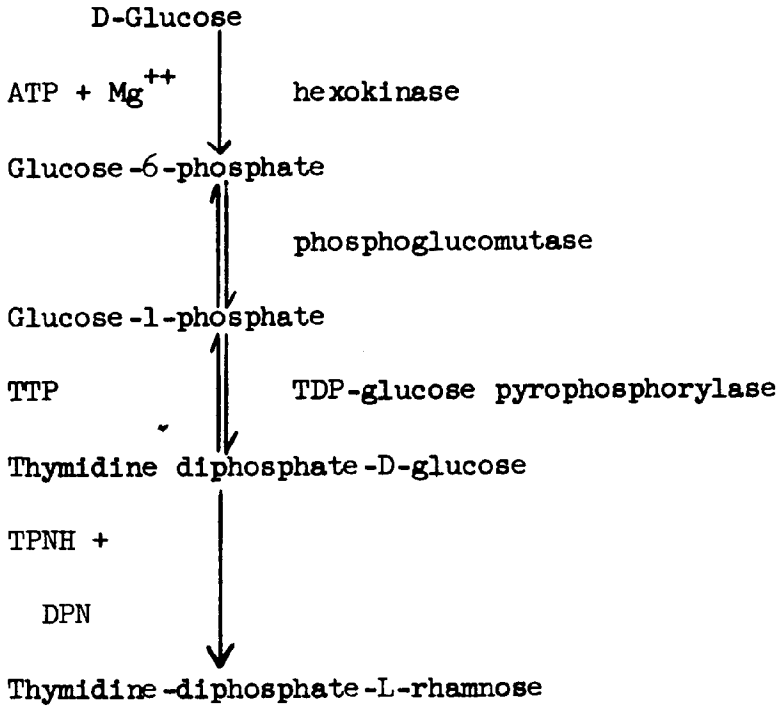
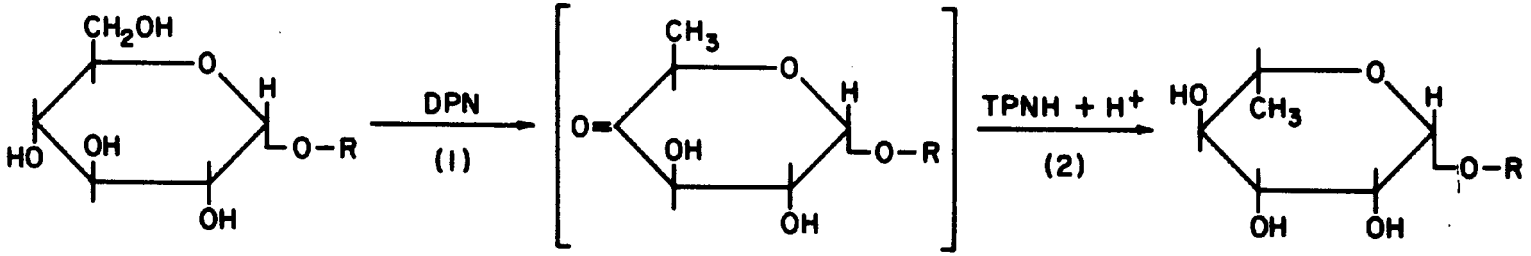


Figure 7. Pathway of biosynthesis of TDP-rhamnose as suggested by Pazur and Shuey (173,174).

THE PROPOSED MECHANISM OF ENZYMATIC CONVERSION OF TDPG TO TDPR



D-GLUCOSE

4-KETO-6-DEOXYHEXOSE

L-RHAMNOSE

R=TDP

STEPS:

(1) Oxidation at C_{-4} and reduction at C_{-6}

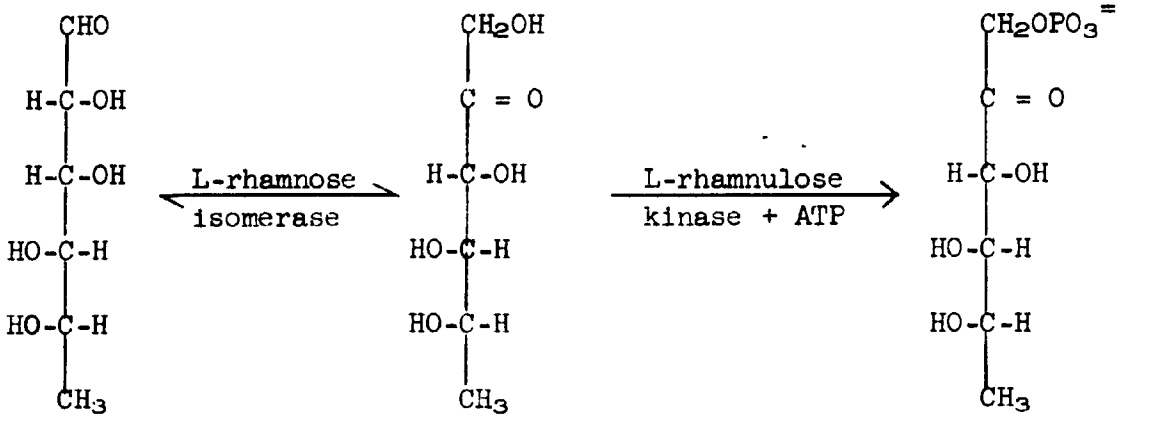
(2) Inversion of configuration at C_{-3} and C_{-5} followed by reduction at C_{-4}

Figure 8. Mechanism of conversion of TDP-D-glucose to TDP-L-rhamnose in Pseudomonas aeruginosa as proposed by Glaser and Kornfeld (183).

This pathway is proposed to involve an oxidation-reduction step, inversion of configuration at C_{-3}, and C_{-5}, followed by reduction at C_{-6}; this mechanism is analogous to that suggested for the conversion of GDPM to GDPF (109).

c. Catabolism

With respect to the enzymatic degradiation of L-rhamnose, Kluyver (184) postulated in 1937 that lactaldehyde was one of the intermediates in the catabolism of this compound by Bacterium rhamnosifermentans. In 1957, this postulate was confirmed by Englesberg (185), who isolated lactaldehyde as one of the products of fermentation of L-rhamnose by Pasteurella pestis. Wilson and Ajl (186), Tecce, et al. (187), and Englesberg (188), reported the presence of an enzyme (L-rhamnose isomerase) in the crude extracts of L-rhamnose-grown bacteria which could catalyze the interconversion of L-rhamnose and L-rhamnulose. These authors, also suggested that L-rhamnulose was phosphorylated by these extracts in the presence of ATP. Englesberg (189,190) and Wilson and Ajl (191,192), simultaneously and independently, suggested the following pathway for the catabolism of L-rhamnose by Pasteurella pestis and Escherichia coli, respectively.



L-RHAMNOSE L-RHAMNULOSE L-RHAMNULOSE-1-P

The presence of the enzymes involved in this pathway has been demonstrated in L-rhamnose-grown extracts of these two bacterial species although the second reaction has not been established. These atuthors suggested that the product of the kinase reaction was L-rhamnulose-1-P, although attempts to isolate and characterize the proposed product were not reported. L-Rhamnose utilization has recently been demonstrated in a variety of bacterial species (193), but the enzymatic steps involved have not been established.

7. D-RHAMNOSE

D-Rhamnose (6-deoxy-D-mannose), has been recently detected as a component of polysaccharides of certain Gram negative bacteria (194). Markovitz (195) reported that the same organisms could convert GDPM to GDP-rhamnose. However, the identity of the sugar moiety of the proposed product as rhamnose is questionable.

Palleroni and Doudoroff demonstrated that D-rhamnose could be isomerized by mannose isomerase to yield D-rhamnulose (196,197). Huang and

Miller (198) suggested that D-rhamnulose could be phosphorylated by a kinase present in rat liver homogenates, presumably to form D-rhamnulose-(1-phosphate). This conclusion was based solely on the observation that when the product of the kinase was incubated with FDP aldolase, it gave rise to alkali-labile phosphate (supposedly DHAP). However, the validity of these conclusions remains to be established.

8. D-TALOMETHYLOSE

The occurrence of D-talomethylose (6-deoxy-D-talose) in mycoside C (131) and bacterial polysaccharides (194) has been recently reported. Little information is available concerning its metabolism. Markovitz (195) suggested that GDPM was converted to GDP-talomethylose by organisms which contained D-talomethylose as a component of their cell walls, and further speculated that GDP-talomethylose might be an intermediate in the biosynthesis of talomethylose. However, these suggestions have not been established.

D. Conclusion

The similarity of the metabolic pathways of L-fucose and L-rhamnose in microorganisms suggests that this may represent the general pattern of catabolism of 6-deoxyhexoses in these systems. Not only do the mechanisms of the enzymatic reactions involved in the biosynthesis and catabolism of these two substances follow rather similar patterns, but also

the development of ideas and approaches to these problems present some general resemblances.

1. Both L-fucose and L-rhamnose are biosynthesized at the nucleotide level, from GDPM and TDPG, respectively. These nucleotides undergo similar changes (intramolecular oxidation-reduction, inversion of configuration at C_{-3}, and C_{-5} followed by reduction at C_{-6} in the presence of TPNH) in order to be converted to GDPF and TDPR, respectively.

2. In their catabolism, the first step involves isomerization to the corresponding ketoses followed by phosphorylation. It will remain for future investigations to determine if other 6-deoxyhexoses undergo metabolic changes similar to those described for L-fucose and L-rhamnose. The suggestion has already been made that this might be the case for the biosynthesis of the 6-deoxyhexoses, D-rhamnose and 6-deoxy-D-talose (195).

III. MATERIALS AND METHODS

A. Organism

1. STOCK CULTURES

A culture of Escherichia coli O111-B_4 was kindly supplied by Dr. P. L. Kendrick of this university. Stock cultures of this organism were prepared by growth on a synthetic medium containing 2% agar and 0.5% L-fucose. The cultures were incubated at 37° for 24 hours and stored at 4°. For the maintenance of the stock culture, transfers were made every two months.

2. CULTIVATION CONDITIONS

Two types of liquid media were employed in these studies:

a. A complex medium (C-medium) which contained the following (in gm/liter): yeast extract (Difco), 6.0; K_2HPO_4, 11.0; KH_2PO_4, 8.5 and a carbon source (D-glucose and/or L-fucose), 5.0.

b. A synthetic medium (S-medium) with the following composition (in gm/liter): NH_4Cl, 5.0; Na_2HPO_4, 10; KH_2PO_4, 3.0; K_2SO_4, 1.0; NaCl, 1.0; $MgSO_4 \cdot 7H_2O$, 0.20; $CaCl_2 \cdot 6H_2O$, 0.02; $FeSO_4 \cdot 7H_2O$, 0.001 and L-fucose, 5.0.

Sugars were autoclaved separately as 25% solutions and added aseptically to the sterile media. When solid media were desired, 2 gm of agar were added to 100 ml of the liquid medium.

Unless otherwise stated, all incubations were carried out at 37° on a New Brunswick, Model V rotary action shaker (one inch stroke, oscillation of 200 times per min). For the preparation of actively growing cells, 125 ml Erlenmeyer flasks containing 25 ml of liquid medium were inoculated with a loopfull of stock culture. Two successive 24 hour transfers were made in fresh liquid media (using 1% inocula). The final culture obtained was used to inoculate agar slants. Primary inocula were obtained by transfer of a loopfull from freshly prepared agar slants to 50 ml of liquid medium contained in 250 ml Erlenmeyer flasks. Final inocula (5%) were made to 2-liter flasks containing 1 liter of liquid medium. Growth of the cultures were followed by the measurement of turbidity, using zero time turbidity of the cultures as the control on samples removed at various intervals. Whenever necessary, L-fucose utilization during growth was estimated (199) by the determination of its concentration in these samples using L-fucose-less media as the blank. Cells were harvested after 12-16 hours by centrifugation at 4°, washed twice with 0.15 M KCl solution yielding approximately 5 gm of wet cell paste per liter of medium. Since enzymes of interest (L-fucose isomerase, L-fuculose kinase, and L-fu-1-P aldolase) were stable in intact cells for extensive periods of time, the cells were stored in lusteroid tubes at -20° and used as desired.

3. SELECTION OF MEDIUM FOR ENZYMATIC STUDIES

Comparative studies were made on the total L-fuculose kinase activ-

ity of Escherichia coli cells grown in the following media.

a. C-Medium containing 0.2% D-glucose and 0.5% L-fucose.

b. C-Medium supplemented with 0.5% L-fucose.

c. S-Medium containing 0.5% L-fucose as the sole source of carbon.

As illustrated (Figures 9, 10, 11), complete dissimilation of L-fucose occurred in all of these media. Cells of various growth phases were harvested and used for the preparation of crude extracts; the latter were subsequently assayed for their L-fuculose kinase activity. The comparison of the results obtained (Table 1) indicated that extracts prepared from early stationary-phase cells exhibited the greatest amount of kinase activity in all cases. Although, there was no appreciable difference in the specific activities of the kinase preparations obtained from cells grown in various media, the S-medium was selected for cultivation of this organism.

4. PREPARATION OF CRUDE EXTRACTS

A comparative study was made on the L-fuculose kinase activity of cell-free extracts prepared by the following methods.

a. Grinding With Alumina

Wet cell paste (1 gm) was suspended in 1.0 ml of 0.1M potassium phosphate buffer, pH 7.0, and ground in a mortar (previously cooled to 4°) for 5 min with 3 gm of alumina (Alcoa A-301). Then 4 ml of cold water were added to the disrupted cell suspension, and alumina and cell debris were removed by centrifugation at 25,000xg for 10 min at 4°.

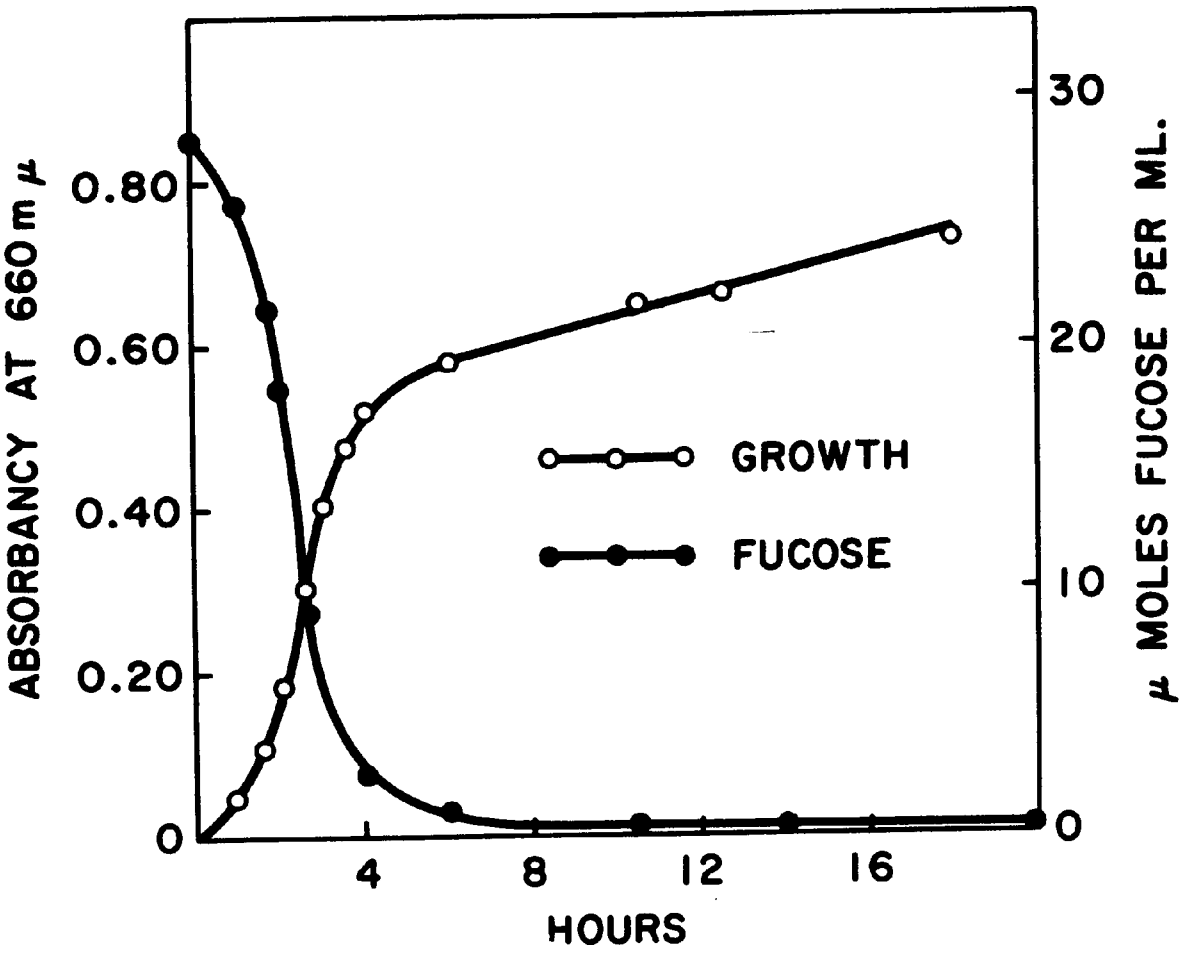


Figure 9. Disappearance of L-fucose during growth of a culture of Escherichia coli O111-B_4 in the complex medium supplemented with 0.5% L-fucose. Control: turbidity of inoculated culture at zero time.

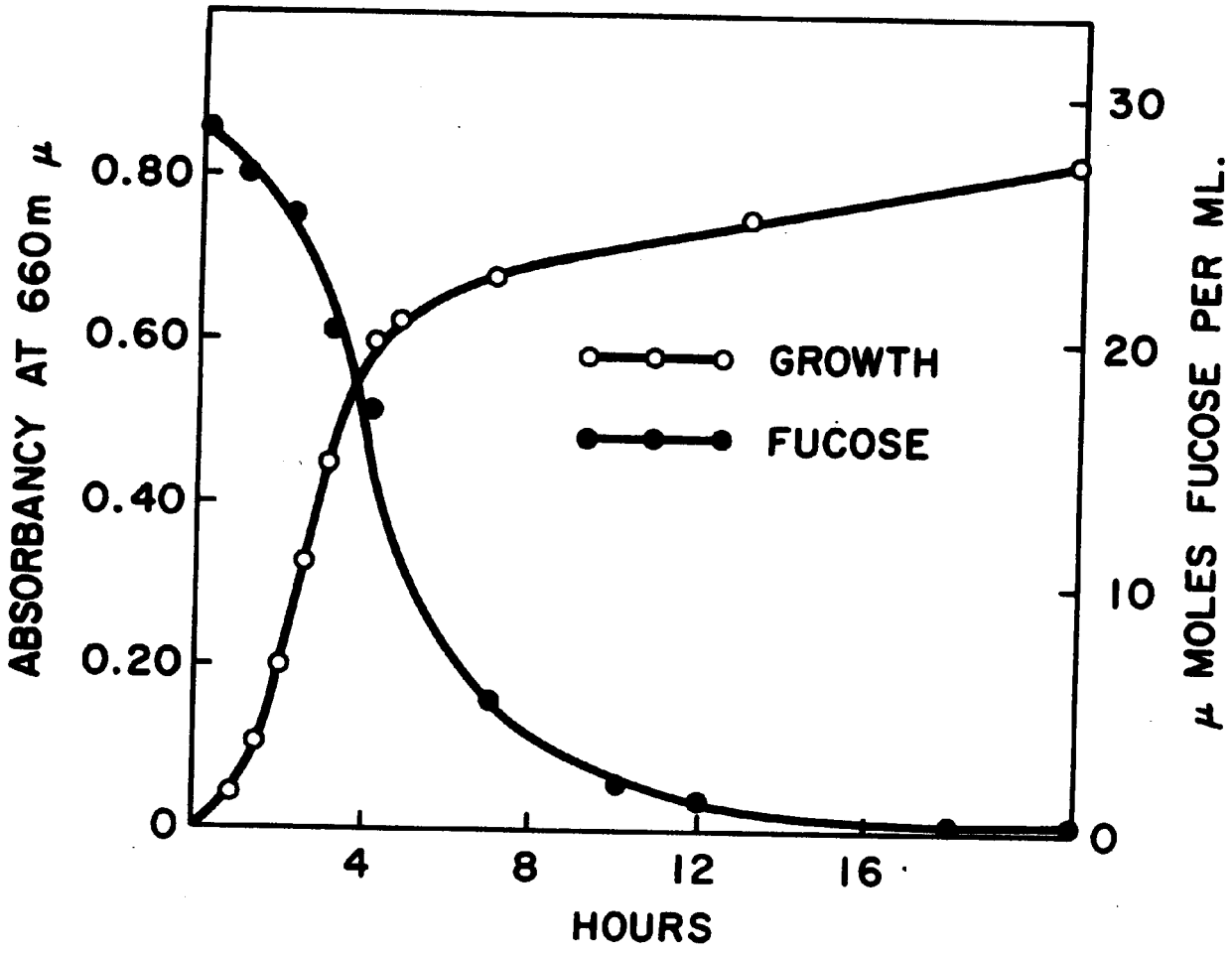


Figure 10. Disappearance of L-fucose during growth of a culture of Escherichia coli O111-B_4 in the complex medium supplemented with 0.5% L-fucose + 0.2% D-glucose. Control: turbidity of inoculated culture at zero time.

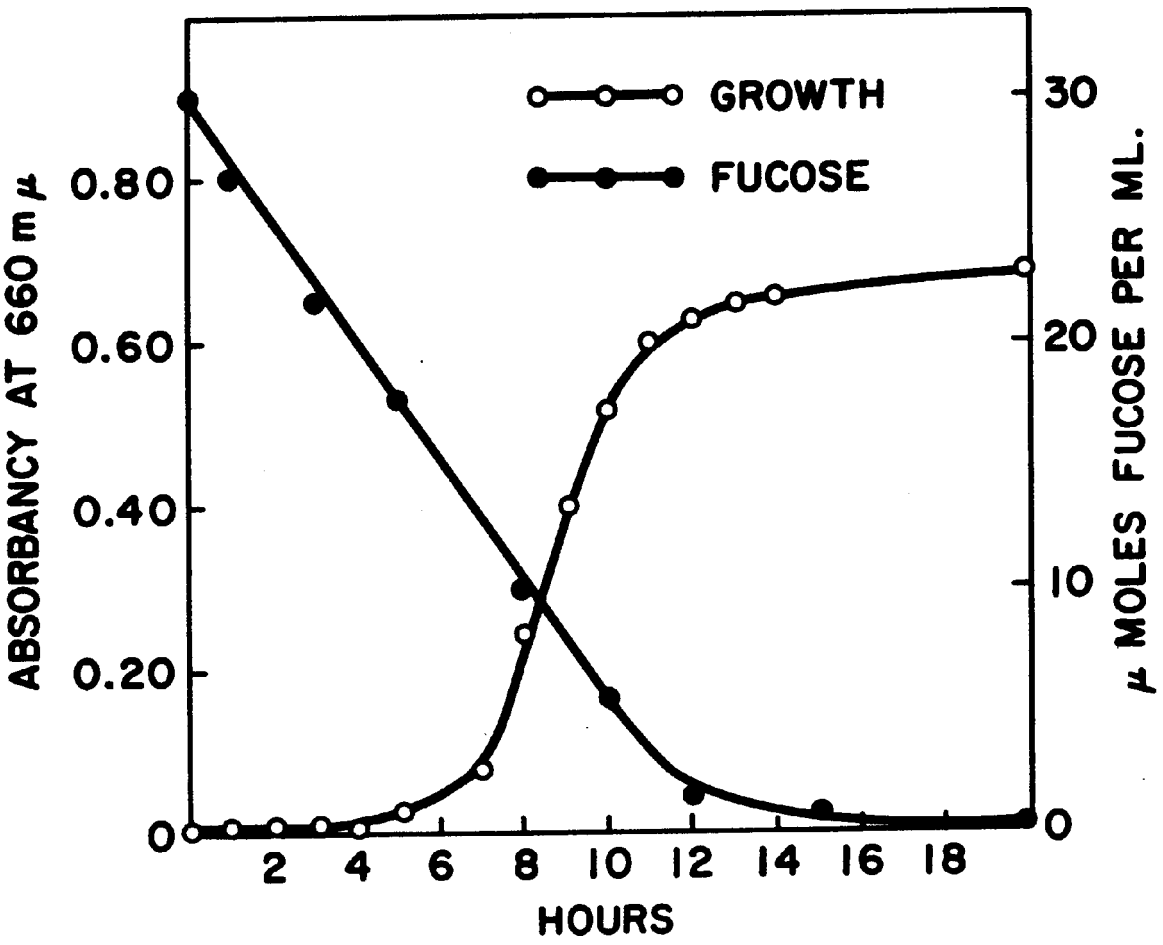


Figure 11. Disappearance of L-fucose during growth of a culture of Escherichia coli O111-B_4 in the synthetic medium containing 0.5% L-fucose as the sole carbon source. Control: turbidity of the inoculated culture at zero time.

TABLE 1

COMPARISON OF L-FUCULOSE KINASE ACTIVITY OF CRUDE EXTRACTS OBTAINED FROM CELLS GROWN UNDER VARIOUS CONDITIONS

Medium	Age of Culture (hr)	Total Protein (mg)	Total Units	Specific Activity x 10^3
C-Medium + 0.5% Fucose	3.0	160	4.4	27.5
	4.0	300	6.8	22.6
	6.0	544	27.0	49.6
	26.0	613	21.5	35.1
C-Medium + 0.5% Fucose + 0.2% Glucose	3.0	375	6.8	18.1
	4.0	440	10.0	22.7
	7.0[a]	---	---	---
	26.0	600	27.6	46.0
S-Medium + 0.5%	10.0	225	3.75	16.6
	12.0	292	8.80	30.1
	15.0	348	16.80	48.3
	24.0	392	8.10	20.7

[a]Lost during experiment.

For each type of medium tested in this experiment, a set of 4-two-liter flasks containing 1 liter of medium each were inoculated (1% inoculum) with an actively growing culture which had been grown on the same type of medium. Growth and L-fucose disappearance were measured in samples removed at various time intervals. L-Fuculose kinase activity was estimated by the ($Ba(OH)_2$-$ZnSO_4$, method), (200) in crude extracts prepared by sonic oscillation from cells at approximately the following phases of growth: acceleration phase, log phase, early stationary phase, and after 20-26 hours of growth. At any particular phase, one flask from each set was employed.

b. Nossal Disintegration

Two gm of wet cell paste were suspended in 5 ml of 0.1M potassium phosphate buffer, pH 7.0 and placed in an 8 ml Nossal cell along with 8 gm of acid-washed glass beads, and disrupted in Nossal mechanical disintegrator for two 30-sec periods with cooling in ice between the periods. The disrupted cell suspension obtained was centrifuged at 25,000xg for 10 min at 4°, the residue was washed with 5 ml of the same buffer, centrifuged,and the supernatant fluids were combined.

c. Sonic Oscillation

Five gm of wet cell paste were suspended in 20 ml of 0.1M potassium phosphate buffer, pH 7.0, and disrupted in a Raytheon 10KC sonic oscillator (cooled with running tap water) for 15 min. The disrupted cell suspension obtained was centrifuged at 25,000xg for 10 min at 4°; the pellet was washed with 5 ml of 0.1M phosphate buffer, pH 7.0, and the supernatant solutions were combined. The comparison of the results obtained (Table 2) demonstrated that sonic oscillation was superior to the other methods employed with respect to total activity of L-fuculose kinase (per gm of cells) and specific activity. Consequently, sonic oscillation was selcted as the method of preparation of crude extracts throughout this study, except that 0.02M potassium phosphate buffer, pH 7.0 was substituted for 0.1M potassium phosphate buffer, pH 7.0, since the former was found to be more suitable for subsequent enzyme purifications.

TABLE 2

COMPARISON OF L-FUCULOSE KINASE ACTIVITY IN EXTRACTS PREPARED BY VARIOUS METHODS

Methods of Extraction	Gm Wet Paste	Volume (ml)	Protein mg/ml	Units/gm Wet Paste	Specific Activity x 100
Alumina Grinding	1	6	4.7	1.89	6.7
Mechanical Shaking	2	12	6.8	2.50	6.1
Sonic Oscillation	5	30	23.8	25.20	17.6

L-Fuculose kinase activity was assayed by the $ZnSO_4$-$Ba(OH)_2$ procedure (200) on the cell-free preparations. See the text for details of this assay method.

B. Materials Obtained From Other Sources

The following materials were obtained from commerical sources: crystalline ADP, ATP, CTP, DHAP, DPN, DPNH, GSH, TPN, TPNH, UTP, D-arabinose, ascorbic acid, DHA, D-fructose, D-frucose-1-P, D-fructose-1-6-di-P, L-fucose, D-galactose, D-glucose, D-glucose-6-P, D- and DL-glyceraldehyde, DL-glyceraldehyde-3-P, glycolaldehyde, pyruvaldehyde (Union Carbide Chemical Company), glycolic acid, D-lyxose, D-mannose, PEP, D-ribose, L-sorbose, D-, L- and DL-threonine, D-xylose, nucleoside diphophokinase, potato acid phosphatase, α-glycerophosphate dehydrogenase and rabbit muscle lactic dehydrogenase.[1]

1. The product from Worthington Laboratories contains pyruvate kinase.

Other materials used in the present study were as follows: calcium phosphate gel was an aged preparation (about 9 months) which had been prepared as described (327); DEAE cellulose, type 40 (Brown Co.); powdered cellulose, (Whatman standard grde); alumina (Alcoa, A-301); glass beads, type 100-5005 (Minnesota Mining and Manufacturing Co.); Whatman, Schleicher and Schuell filter paper with various grades of thickness. The following ion-exchange resins were the products of The Dow Chemical Co.: Dowex-1 Cl^- (200-400 mesh, 8% cross-linked) and Dowex-50 H^+ (200-400 mesh, 8% cross-linked). Dowex-1 Cl^- was converted into $CO_3^=$ or formate forms, and Dowex-50 H^+ was converted into the K^+ form by conventional techniques. Mixed-bed ion-exchange resin consisted of a mixture of equal volumes of Dowex-1 $CO_3^=$ and Dowex-50 H^+.

I wish to thank the following individuals for their generous gifts of the indicated compounds: Drs. R. H. Abeles, DL-glyceraldehyde; B. W. Agranoff, ITP; H. J. Blumenthal, L-rhamnose and a pure sample of FDP, A. D. Elbein, D- and L-isomers of 3,6-dideoxygalactose; G. R. Greenberg, L-arabinose and GTP; E. C. Heath, crystalline FDP aldolase and glyceraldehyde-3-P-dehydrogenase; B. L. Horecker, D-ribulose-1,5-di-P, J.K.N. Jones, L-sorbose-1-P.and 6-deoxy-L-sorbose; G. W. Jourdian, lithium lactate; T. Reichstein, L-fuculose-o-nitrophenylhydrazone; H. Renis, D-psicose; W. G. Robinson, pyruvaldehyde and crystalline DL-lactaldehyde; S. Roseman, crystalline D-tagatose; G. M. Savage, psicofuranine; and W. A. Wood, D-xylulose and L-ribulose-o-nitrophenylhydrazone.

C. Materials Prepared

1. PREPARATION OF KETOSES

a. L-Fuculose

L-Fuculose was prepared from L-fucose by chemical and enzymatic methods as follows.

(1) Chemical Method.—Isomerization of L-fucose to L-fuculose was carried out according to the method of Reichstein (201). Four gm of L-fucose were dissolved in 40 ml of anhydrous pyridine, and refluxed for 8 hours (at about 115°) in a round bottom flask with a drying tube attached to it. Pyridine was removed by distillation in vacuo, followed by azeotropic distillation with water several times. After the dry syrup was dissolved in 15 ml of absolute ethanol and allowed to stand at 4° for 4 days, the fucose crystallized. The crystals were harvested, washed with absolute ethanol,and the washings were added to the filtrate. The resulting solution was concentrated in vacuo to 8 ml (at 35° to 40°) and stored at 4° for 18-20 hours; this procedure was repeated twice and the fucose crystals recovered in all of the steps were combined (total weight = 3.23 gm).

The amount of L-fuculose formed in this procedure was determined on an aliquot of the mother liquor by the cysteine-carbazole method (202) (the yield was 8.6% of theoretical) and the solution was concentrated in vacuo to dryness. The residue was dissolved in a small amount of water, deionized with mixed-bed ion-exchange resin, and further purified by

chromatography on a powdered cellulose column (see "Chromatographic procedures") using methylethylketone-propionic acid-water (15:5:6 v/v/v) as the developing solvent. L-Fuculose emerged from the column between 300-450 ml of effluent. The proper fractions were combined, the solvent was evaporated in vacuo, and the residue was dissolved in water and extracted three times with 100 ml portions of ether and the organic layer discarded. The aqueous phase was deionized with mixed-bed ion-exchange resin and filtered. An aliquot of the filtrate was chromatographed on Whatman No. 1 filter paper (using the same solvent as mentioned above), which showed the presence of fuculose as well as fucose. Analyses of an aliquot of the filtrate for methylpentose (199) and ketose (202) indicated a ratio of fuculose to fucose of 3 to 1. For final purification of fuculose from the mixture of fucose and fuculose, the following experiments were conducted.

(a) A portion of the mixture was concentrated to a syrup and treated with o-nitrophenylhydrazine according to the procedure of Reichstein (201). The o-nitrophenylhydrazones of fucose and fuculose were separated on the basis of the differences in their solubility in absolute ethanol (the L-fucose derivative is very slightly soluble in cold absolute ethanol, whereas, the L-fuculose derivative is soluble) and were identified according to their melting points (Table 3).

(b) Another portion of the mixture was purified by chromatography on Whatman 3MM using ethylacetate-acetic acid-water (3:1:3 v/v/v) as the developing solvent. The faster moving band, which corresponded to fuculose

TABLE 3

MELTING POINTS OF O-NITROPHENYLHYDRAZONES OF L-FUCOSE AND L-FUCULOSE

Compound	M.P.	Reported M.P.
L-Fucose	181-182° (With Decomposition)	182-183° (With Decomposition) (201)
L-Fuculose[a]	162-163°	161-162° (201)
Product of Isomerase	161-162°	162-163° (2)

[a]Prepared by isomerization of L-fucose in pyridine.

(TCA-orcinol positive, 203), was eluted with water, deionized,and rechromatographed on paper to assure its purity. By colorimetric (methylpentose to ketose ratio was 1:1) and chromatographic criteria, the eluted material behaved in the same manner as a pure sample of fuculose, with no detectable contamination with fucose. The final yield of pure L-fuculose was approximately 4% of the theoretical amount.

(2) Enzymatic Method.—The chemical isomerization described above yields an equilibrium mixture containing approximately 90% fucose and 10% fuculose; since the separation and purification of L-fuculose from this mixture involves some technical difficulties, the enzymatic procedure of Green and Cohen (3) was adopted. This method is based on the isomerization of L-fucose by L-fucose isomerase. The above authors demonstrated that the equilibrium of this reaction ($K = \frac{[\text{L-Fuculose}]}{[\text{L-Fucose}]} = 0.125$, pH 8.0) was shifted in favor of L-fuculose, when the incubation was carried out in the presence of borate buffer (which forms a complex with

fuculose more readily than fucose). Furthermore, they indicated that by using the proper ratio of fucose to borate concentration (approximately 1:40), the reaction reached an equilibrium at which 85% of the total methylpentose was present as fuculose. In the present study, crude extracts of fucose-grown Escherichia coli O111-B_4 prepared by sonic oscillation were used as the source of L-fucose isomerase. The extracts were assayed for the presence of isomerase as described (see "Enzyme assays"). The formation of L-fuculose from L-fucose gave linear responses with time of incubation and the concentration of isomerase. In a typical experiment for the large-scale preparation of L-fuculose, an incubation mixture was prepared containing the following (in millimoles): L-fucose, 6.0; borate buffer (boric acid-sodium borate) pH 8.0, 250; and 30 ml of crude extract (27 mg protein/ml) in a final volume of 2.5 liters. The following controls were included: (a) An enzyme-less control, containing 250 μmoles of buffer (the same as above) and 6.0 μmoles of L-fucose in a total volume of 2.5 ml. (b) A substrate-less control, which contained 250 μmoles of buffer (the same as above) and 30 μl of the above crude extract in a final volume of 2.5 ml. The mixtures were incubated at 37°, and the reaction was followed by determination of the ketose formed (202) in 0.10 ml aliquots removed from each of the controls and the flask with the complete reaction mixture (incubated flask) at various time intervals. The values obtained for the optical densities of the incubated flask (after subtraction of the sum of the values of the two controls from the value of the corresponding incubated sample) were plotted against time of incu-

bation (Figure 12). After about 3 hours the reaction reached equilibrium with approximately 70% conversion of L-fucose to L-fuculose. The L-fuculose formed was isolated as follows: the incubation mixture was treated with 200 ml of 0.15M $ZnSO_4$ followed by adjustment of the pH to 6.8 with 0.3N $Ba(OH)_2$. After removal of the inactive protein by centrifugation, the clear supernatant fluid was concentrated to dryness in vacuo. The residue was dissolved in 800 ml of absolute methanol and the borate was removed by distillation of the methylborate in vacuo; this procedure was repeated 5 times, until a syrupy material was obtained. The syrup was dissolved in water and deionized with mixed-bed resin. Analysis of methylpentose (199) and ketose (202) content of the resulting solution gave the following results (in millimoles): total methylpentose, 4.60 and ketose, 4.10. The components of this mixture were separated by column and paper chromatography, and by preparation of o-nitrophenylhydrazone derivatives as previously described.

The purified product obtained by the enzymatic isomerization described was characterized as L-fuculose by its o-nitrophenylhydrazone derivative the melting point of which was identical to that of an authentic sample of this compound (Table 3). The product was found to be homogeneous by paper chromatography in three solvent systems (A, B, and I), and by analyses which indicated a ratio of methylpentose to ketose of 1:1.

b. D-Psicose

D-Psicose was prepared from psicofuranine by modification of the pro-

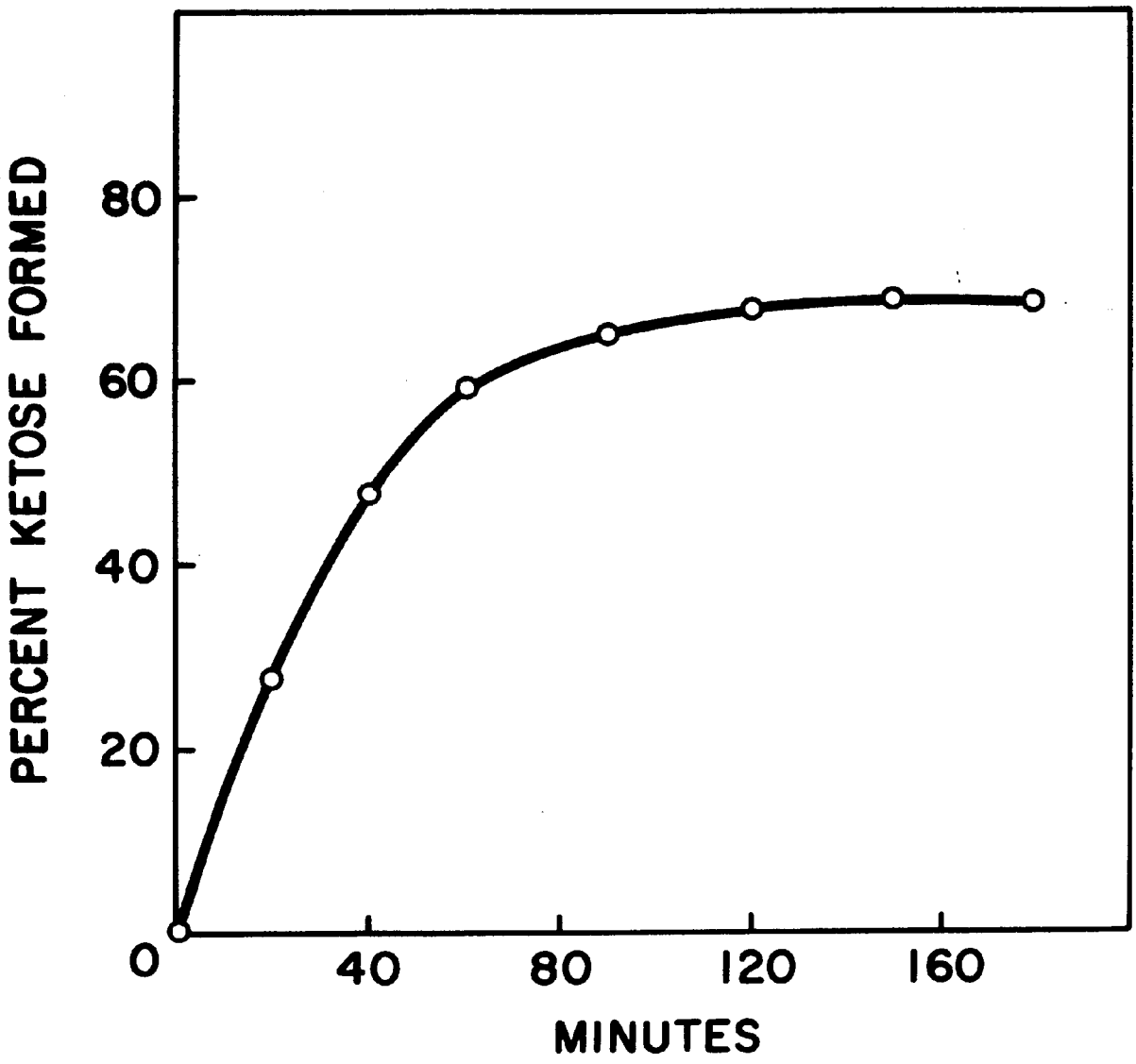


Figure 12. Formation of L-fuculose from L-fucose by L-fucose isomerase in the presence of borate buffer. Two-0.1 ml aliquots were removed at each interval, and their fuculose content was determined by the cysteine-carbazole method (202). See text for details of the experiment.

cedure described by Schroeder et al. (204). Psicofuranine (1 gm) was hydrolyzed in 1.14N H_2SO_4 by incubation for 6 hours at 25°, followed by 3 hours at 37°. The hydrolysate was deionized with mixed-bed resin and filtered. The psicose recovered in the neutral filtrate appeared to be homogeneous by paper chromatography in four solvent systems (A, B, D, and G). A sample of it was converted to its phenylosazone derivative the physical properties of which were identical to those reported for the authentic phenyl-D-psicosazone (205, Table 4).

c. Other Ketoses

All other ketoses were prepared from their corresponding aldoses according to the method of Reichstein (201) with the following modifications.

1. After isomerization in pyridine, the remaining aldoses were selectively oxidized to their corresponding acids by treatment with bromine water (206) at room temperature for 20 min in the presence of 1M potassium bicarbonate buffer, pH 7.2. After filtration, the excess bromine was evaporated in vacuo by taking the solution to dryness. The residue was dissolved in water, the resulting solution containing the sugar acid and the ketose was deionized, and the sugar acids removed by treatment with mixed-bed resin.

2. The ketose present in the resin filtrate was further purified by paper chromatography on Schliecher and Schuell 589 Green Ribbon paper, using ethylacetate-acetic acid-water (3:1:3) as the developing solvent.

In all cases, the ketoses were differentiated from their corresponding aldoses on the basis of the following criteria: (a) greater rate of migration on the paper crhomatograms; (b) characteristic color with TCA-orcinol reagent (203); and (c) preparation of appropriate derivatives, after elution from the paper chromatograms.

The following ketoses were prepared by the described procedure: D- and L-ribulose were prepared from D- and L-arabinose respectively, and a portion of each was converted to its o-nitrophenylhydrazone derivative according to the procedure of Glatthaar and Reichstein (207) with the following modifications: after the heating step, the reaction mixture was stored at 4° for 24 hours, followed by storage at -20° for a period of 8-10 hours. The crude product was purified by washing with water, twice with absolute ethanol, once with anhydrous diethyl ether, and dried in vacuo (yield was 35-50% of the theoretical quantity). This procedure would usually yield a product with a high degree of purity, however, a recrystallization from 50% ethanol in water was included to assure the constancy of the melting point. Final yield was 20-35% of the theoretical quantity. D-Xylulose and L-rhamnulose were prepared from D-xylose and L-rhamnose, respectively, and were characterized by the preparation of their phenylosazone derivatives. The general procedure described by Shriner and Fuson (208) was employed for the preparation of the osazones of the sugars, but in order to improve the yield the following modifications were made.

(i) The reaction was carried out in the presence of potassium acetate buffer, pH 4.5 (209) in a final concentration of 4M (210).

(ii) The optimum heating period (in boiling water bath) was determined for each sugar. This was found to be: 7 to 10 min in the case of D-psicose and D-tagatose; 5-7 min in the case of D-xylulose and D-fructose, and 15-20 min for D-galactose and L-rhamnulose.

(iii) After heating, the tubes were stored overnight at 4°. The crystals were harvested by filtration, and purified by washing successively with water, 25% ethanol, followed by absolute ethanol and anhydrous ether. Recrystallization from ethanol-water resulted in a material whose melting point remained constant upon further recrystallization. A summary of the properties of the ketose derivatives prepared is shown in Table 4.

2. SYNTHESIS OF o-NITROPHENYLHYDRAZINE

o-Nitrophenylhydrazine was prepared from nitraniline (211) according to the following procedure.

Step 1. o-Nitraniline (100 gm) was dissolved by warming in 1000 ml of concentrated HCl, and the solution allowed to cool, upon which the hydrochloride salt partially separated.

Step 2. The solution of o-nitraniline hydrochloride was diazotized by the addition of sodium nitrite solution (50 gm $NaNO_2$ in 300 ml water) at 0°, and allowed to stand at room temperature for 1 hour.

TABLE 4

PHYSICAL CHARACTERISTICS OF THE KETOSES PREPARED[a]

Compound	Derivative	Free Sugar			Properties of the Derivatives					
		Optical Rotation[b]			Optical Rotation[c]			M.P.[d]		
		Found	Reported		Found	Reported		Found	Reported[d]	
D-Fructose	Phenylosazone	-92°	-92°	(212)[e]	-89°	-89°	(213)	204-205°	208°	(213)
D-Psicose	Phenylosazone	+4.6°	+4.7°	(205)	-78.1°	-78.1°	(205)	162-163°	162-163°	(205)
L-Rhamnulose	Phenylosazone	-	-14.3°	(197)	-	+107°	(213)	182-183°	184°	(213)
D-Tagatose	Phenylosazone	-5°	-5°	(214)	+110°	+110°	(213)	192-193°	190°	(213)
D-Xylulose	Phenylosazone	-	-36.8°	(215)	-	-70°	(213)	167-168°	165-167°	(216)
D-Ribulose	o-Nitrophenyl-hydrazone	-15.8°	-15.8°	(217)	-43.9°	-47.5°	(217)	164-165°	164-165°	(218)
L-Ribulose	o-Nitrophenyl-hydrazone	+16°	+16°	(219)	+43°	+48.3°	(207)	163-164°	164-164°	(218)

[a]Except D-fructose and D-tagatose all of the ketoses were prepared as described.

[b]$[\alpha]_D^{25}$ in water.

[c]$[\alpha]_D^{25}$ in pyridine in the case of phenylosazones and in methanol in the case of o-nitrophenylhydrazones.

[d]Corrected M.P., whereas the melting points reported in the present studies are uncorrected.

[e]The figures in parentheses are references.

Step 3. The diazocompound was reduced by dropwise addition of a solution of stannous chloride (320 gm in 320 ml concentrated HCl) at 0°. The yellow precipitate which formed was separated from the mother liquor by filtration and washed with water. The resulting double stannous salt of o-nitrophenylhydrazine was dissolved in hot water and the solution was treated with H_2S. The dark colored precipitate of stannous sulfide was removed by filtration and the clear yellow filtrate was allowed to stand overnight at 4°. The yellow platelets of o-nitrophenylhydrazine hydrochloride were harvested by filtration and dried by dessication over $CaCl_2$.

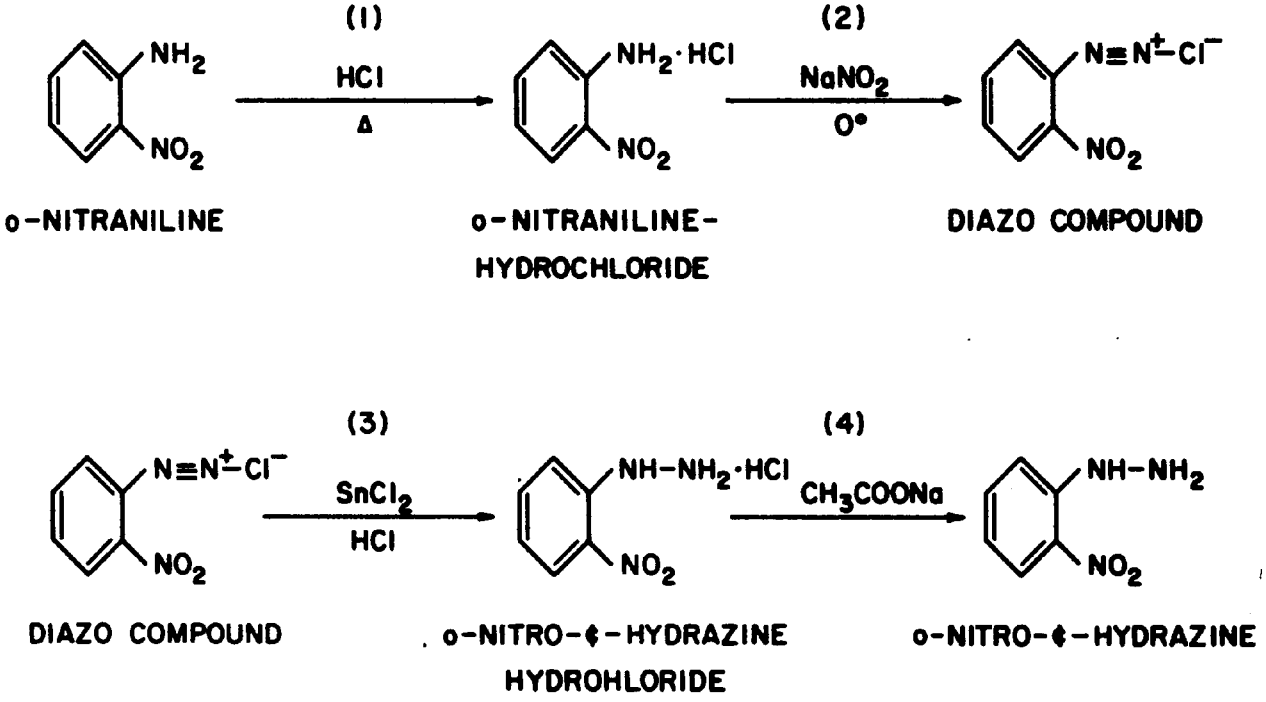
Step 4. Conversion of the o-nitrophenylhydrazine hydrochloride salt to its free base was accomplished by treatment of a hot solution of the salt with an excess of an aqueous solution of sodium acetate, followed by cooling. The free base precipitated voluminously as fine red needles which were harvested by filtration, washed with water, and dried in vacuo. Recrystallization from benzene, followed by a second recrystallization from 50% ethanol, yielded pure o-nitrophenylhydrazine as silky, bright red needles melting point of which remained constant upon further recrystallization (m.p., 90-91°; m.p. reported, 90°). The yield was approximately 60% of the theoretical. The sequence of the chemical reactions involved in this procedure are formulated in Figure 13.

3. PREPARATION OF ALDEHYDES

a. Lactaldehyde

Lactaldehyde was prepared by two methods.

PREPARATION OF o-NITROPHENYLHYDRAZINE FROM o-NITRANILINE



STEPS:

(1) Preparation of o-nitraniline hydrochloride.

(2) Diazotization.

(3) Reduction of diazo compound.

(4) Conversion of o-nitro-¢-hydrazine hydrochloride to its free base.

Figure 13. Preparation of o-nitrophenylhydrazine from o-nitraniline.

(1) Threonine Degradation.—D-, L- and DL-lactaldehyde were prepared by ninhydrin degradation of L-, D- and DL-threonine, respectively, according to the method of Huff and Rudney (220) with the following modifications: after the heating step, the preparation was deionized with mixed-bed resin until all of the colored material was removed.

(2) Pyruvaldehyde Reduction.—DL-Lactaldehyde was prepared from pyruvaldehyde according to the method of Hough and Jones (221) by the following modifications: after the acid hydrolysis of DL-lactaldehyde dibutyl acetal, the resulting solution was taken to dryness in vacuo. The viscous solution of DL-lactaldehyde obtained was dehydrated by dissolving it in absolute ethanol, followed by evaporation in vacuo. This procedure was repeated twice and the syrup was stored at 4° for 48 hours in the absence of air and dried under reduced pressure, after which time DL-lactaldehyde partially crystallized. The crystals were harvested, washed with ether, and dried in vacuo. The crystals obtained exhibited a melting point of 107-108° (reported 107°). The various lactaldehyde preparations appeared to be homogeneous by paper chromatography in several solvent systems (Table 5). The locations of the spots on the chromatograms were revealed by an aniline-TCA spray (222) which yielded a deep orange color with lactaldehyde. Lactaldehyde solutions were dedimerized wtih acid (223), adjusted to pH 6.8 by the addition of alkali, and stored at -20°.

The lactaldehyde samples obtained by various methods were characterized by the preparation of the following derivatives.

TABLE 5

PAPER CHROMATOGRAPHY[a] OF LACTALDEHYDE PREPARED BY VARIOUS METHODS

Lactaldehyde Preparation	Solvent A	Solvent J	Solvent E
Known DL-Lactaldehyde	0.78	0.86	0.83
Ninhydrine Degradation of D-Threonine	0.77	0.85	0.83
Prepared From Pyruvaldehyde	0.78	0.86	0.82

[a]Refer to "Chromatographic procedures" for details.

(a) 2,4-Dinitro-phenylhydrazone derivatives of lactaldehyde preparations were prepared by the method of Neuberg (224). The crude products were washed with water, followed by absolute ethanol, and recrystallized from an ethylacetate-petroleum ether mixture.

(b) 2,4-Dinitro-phenylosazone derivatives of known DL-, D- and DL-lactaldehyde (prepared from pyruvaldehyde) and pyruvaldehyde were prepared by treatment of the aldehydes with 2,4-dinitro-phenylhydrazine (in 2N HCl) at room temperature for 2 hours, followed by gentle heating on a steam bath for a few minutes. The mixture was maintained at room temperature for a few hours and stored overnight at 4°. The resulting crystals were harvested, washed with water, followed by absolute ethanol, and recrystallized from ethyl acetate to constant melting point.

(c) Dimedon derivatives of various lactaldehyde preparations were prepared by treating these preparations with dimedon in the presence of potassium acetate buffer, pH 4.5. Gentle heating on a steam bath for

5-10 min, and subsequent incubation at room temperature for a few hours was followed by storage at 4° for 2-3 days. This procedure was found to be necessary in order to obtain high yields. Washing of the resulting crystals with cold water followed by ethanol yielded samples of a high degree of purity. As a routine procedure, however, these derivatives were recrystallized from methanol to constant melting point. The metling points of the derivatives of the various lactaldehyde samples prepared were identical to those obtained for an authentic sample of crystalline DL-lactaldehyde (Table 6).

b. D- and L-Glyceraldehyde

D- and L-glyceraldehyde were prepared by lead tetraacetate (Matheson, Coleman and Bell) oxidation of D-fructose and L-sorbose, respectively by the method of Perlin and Brice (225). The sequence of reactions involved are shown in Figure 14. The various glyceraldehyde preparations were characterized as their dimedon derivatives (225); samples of commerical D- and DL-glyceraldehyde were also treated in the same manner to serve as means of comparison. The dimedon derivatives obtained were washed with water followed by cold absolute ethanol, and recrystallized from absolute ethanol to constant melting point. The physical properties of the various glyceraldehyde samples prepared were identical to those reported by Perlin, et al. (225, Table 7). To confirm the identity of these preparations as glyceraldehyde, an aliquot of each was chromatographed along with authentic samples in several solvent systems (Table 8); by these criteria,

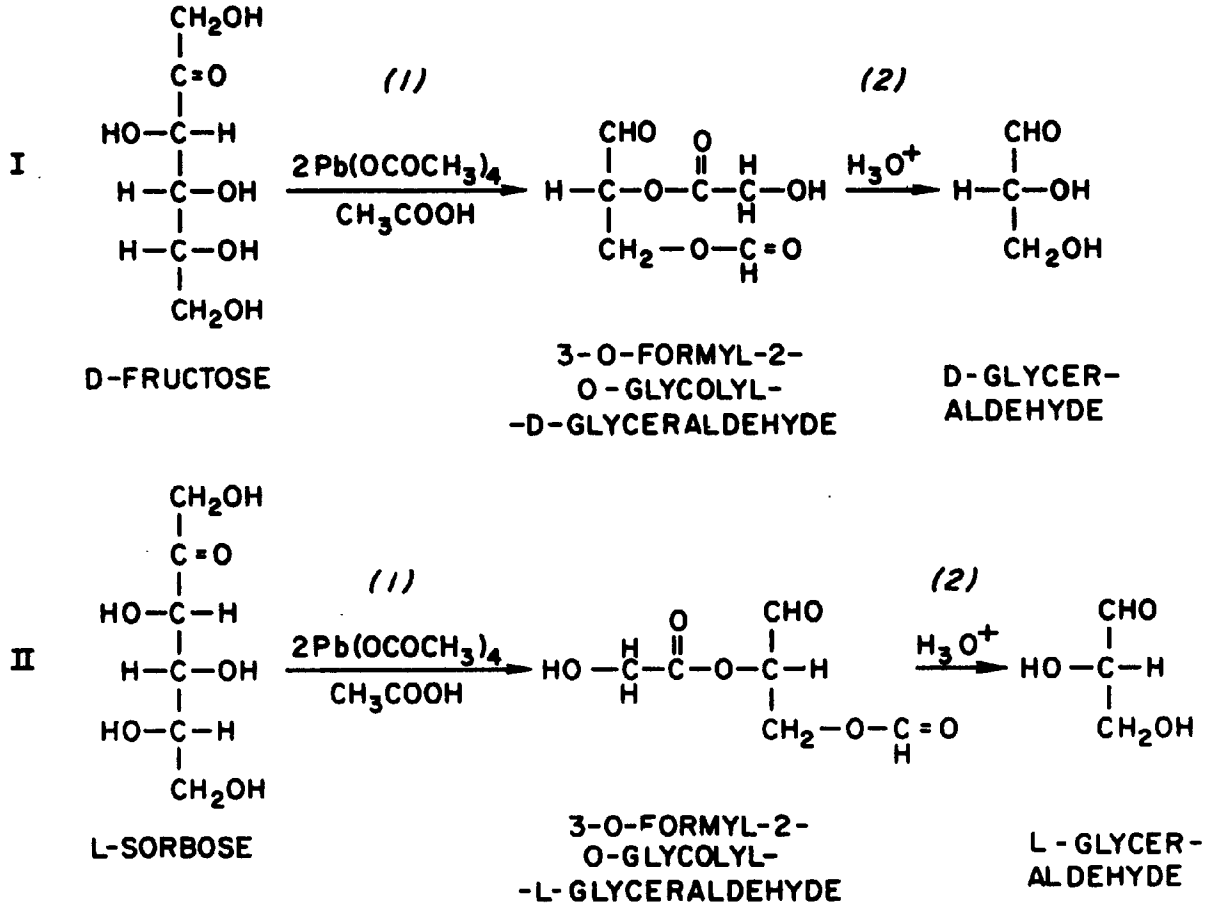
TABLE 6

MELTING POINTS[a] OF VARIOUS LACTALDEHYDE DERIVATIVES

Derivatives	Crystalline DL	Prepared From D-Threonine	Prepared From Pyruvaldehyde	Reported M.P.
2,4-Dinitro-Phenylhydrazone	155-156°	154-156°	155-156°	157° (224)
2,4-Dinitro-Phenylosazone	299-300°	297-299°	298-299°	298° (226)
Dimedon	181-182°	180-182°	181-182°	---

[a]All the melting points except those reported in the literature are uncorrected.

PREPARATION OF D & L-GLYCERALDEHYDE



REACTIONS

(1) Oxidative degradation of ketose with lead tetraacetate.
(2) Acid hydrolysis of the diester of glyceraldehyde.

Figure 14. Preparation of D- and L-glyceraldehyde from D-fructose and L-sorbose respectively.

TABLE 7

PHYSICAL PROPERTIES OF GLYCERALDEHYDE-DIMEDON DERIVATIVES

Compound	Melting Points		Optical Rotations	
	Found[a]	Reported	Found $[\alpha]_D^{24}$[b]	Reported $[\alpha]_D^{27}$
Commerical DL-Glyceraldehyde	203-205°	---	---	0
Synthetic D-Glyceraldehyde	202-204°	194-200° (225)	+204	+208 (225)
Synthetic L-Glyceraldehyde	204-205°	197-198° (225)	-200	-210 (225)

[a]Uncorrected melting points.

[b]Optical rotations were performed on 0.5% solutions of the derivatives in absolute ethanol.

TABLE 8

PAPER CHROMATOGRAPHY[a] OF VARIOUS GLYCERALDEHYDE PREPARATIONS

Preparations	Solvent A $R_{fructose}$	Solvent B $R_{fructose}$
D-Fructose	1.00	1.00
Commerical DL-Glyceraldehyde	1.51	2.10
Commerical D-Glyceraldehyde	1.50	2.10
Synthetic L-Glyceraldehyde	1.51	2.00
Synthetic D-Glyceraldehyde	1.51	2.10

[a]Refer to "Chromatographic procedures" for details.

they appeared to be homogeneous with no detectable amounts of ketohexose from which they had been derived. Glyceraldehyde solutions were depolymerized by allowing their aqueous solutions to stand at room temperature for 2-4 days (227), and the resulting solutions were stored at -20°.

4. PREPARATION OF POTASSIUM SALTS OF PHOSPHATE ESTERS

a. DL-Glyceraldehyde-3-P

DL-Glyceraldehyde-3-P-diethylacetal (Barium) salt was converted to DL-glyceraldehyde-3-P by treatment with Dowex-50 H^+, followed by heating the suspension in a boiling water bath for three min. After centrifugation, the supernatant solution containing the free DL-glyceraldehyde-3-P was adjusted to pH 6.8 by the addition of 1N KOH, and stored at -20°. The procedure for pH adjustment and the conditions of the storage were

applied to all of the phosphate esters which will be discussed.

b. DHAP

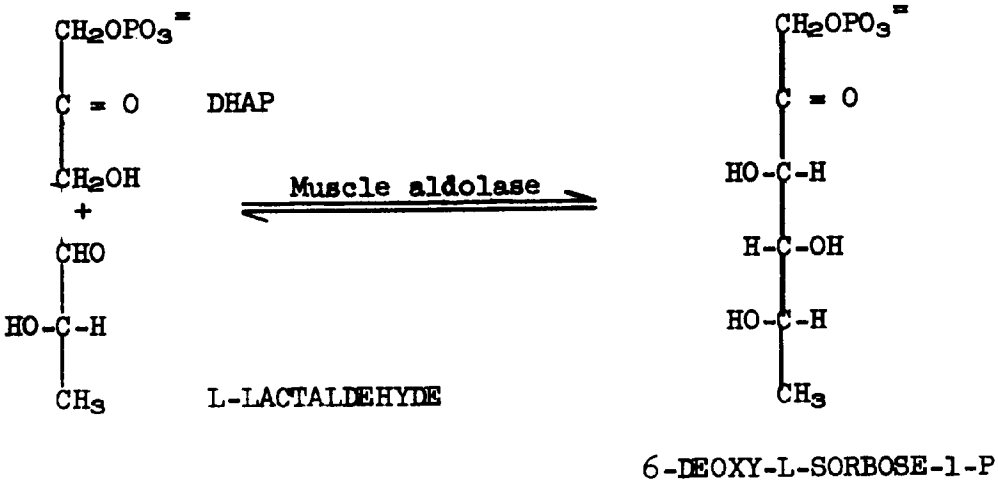
Dihydroxyacetone-phosphate, cyclohexylamine salt, dimethyl ketal·H_2O was converted to DHAP by treatment with Dowex-50 H^+, followed by incubation at 38-40° for 4 hours.

c. Other Sugar-Phosphates

D-Fructose-1-P was prepared by treatment of D-fructose-1-P, dicyclohexylamine salt with Dowex-50 H^+ followed by centrifugation. The resulting solution was adjusted to pH 6.8 by the addition of 1·0N KOH. For the preparation of solutions of FDP, D-glucose-6-P, PEP, L-sorbose-1-P and D-Ru-diP, the corresponding barium salts of the phosphate esters were dissolved in dilute acetic acid. Sufficient amounts of 0.1M K_2SO_4 were added until no further barium sulfate precipitate was formed. The resulting suspension was centrifuged, the precipitate washed several times with water, and washings and the supernatant solution were combined. The resulting solution which contained the phosphate ester in the form of its potassium salt was treated as described previously.

5. ENZYMATIC SYNTHESIS OF 6-DEOXY-L-SORBOSE-1-P

6-Deoxy-L-sorbose-1-P was prepared by condensation of DHAP with L-lactaldehyde in the presence of muscle aldolase according to the method of Hough and Jones (221). This reaction is illustrated by the following formula:



The incubation mixture contained the following (in μmoles): L-lactaldehyde, 100; DHAP, 100; Tris buffer, pH 7.5, 500; and 3.0 mg of crystalline muscle aldolase in a final volume of 10 ml. An aliquot was removed from the incubation mixture at zero time and heated at 100° for one min to serve as the control. Another control which contained no L-lactaldehyde was included. The mixture was incubated at room temperature and the reaction was followed by determination of the ketohexose formed (228) in aliquots removed from the incubation mixture at various intervals. Approximately 60 μmoles of product were formed after 4 hours. The ketose-P was isolated in the form of its barium salt in a manner similar to that employed for the isolation of L-fuculose-1-P (see "Preparation of L-fuculose-1-P"). The final yield of purified product, based on the organic phosphorus (229) and the ketohexose analysis (228) was 50 μmoles. To confirm the identity of the ketose moiety of this product as 6-deoxy-L-sorbose, a portion of the product was treated with potato acid phosphatase and chromatographed in Solvent A. A single component

was observed on the chromatogram which corresponded to an authentic sample of 6-deoxy-L-sorbose (R_f, 0.41), and gave an orange color with the TCA-orcinol reagent (203) which is characteristic of 6-deoxy-L-sorbose. In the same solvent, L-fuculose has an R_f value of 0.37, and with TCA-orcinol reagent yields a bright yellow spot which fluoresces under ultraviolet light.

D. Analytical Procedures

1. COLORIMETRIC METHODS

Unless otherwise indicated, all the colorimetric determinations outlined below were conducted as originally described, except that in cases where higher sensitivity was desired, the volume of the reaction mixture was reduced while the same proportion of various ingradients were used. The following compounds were determined by colorimetric procedures.

Methylpentoses were measured by the cysteine-sufuric acid method of Dische and Shettles(199), using L-fucose as the standard. This method will not differentiate between aldoses and ketoses.

Ketoses were determined by the cysteine-carbazole procedure of Dische and Borenfreund (202). The absorbancies of chromogens produced by pentuloses, methylpentuloses and ketohexoses were measured at 540, 545, and 560 mμ, respectively (2,189,230). Where free sugars are not available in crystalline for m, the following crystalline derivatives were used as standards: L-ribulose-o-nitrophenylhydrazone for pentuloses, and L-

fuculose-o-nitrophenylhydrazone for methylpentuloses.

The following incubation periods (at 37°) were found to be appropriate for the development of maximum color of the indicated compounds: ribulose, 15 min; psicose and tagatose, 30 min; and xylulose, 60 min. In the case of fructose and sorbose, maximum color production required a prolonged incubation period. However, since a standard identical to each one was employed for comparison, a 2 hour incubation period was used.

Ketohexoses were measured by the cysteine-sulfuric acid method of Dische and Devi (228). Since this method is specific for ketohexoses, it was possible to determine these compounds selectively when present in solutions containing a mixture of various ketoses.

Aldohexoses were determined by the anthrone procedure (231). Pentoses were measured by the orcinol method of Mejbaum (232). Tetroses were estimated by the D-fructose-cysteine-sufuric acid method of Dische and Dische (233).

Lactaldehyde was determined by the Barker-Summerson proceudre (234). The copper-lime ($Ca(OH)_2$-$CuSO_4$) step was included in order to obtain maximum color, as recommended by Huff (223). The extinction coefficient of lactaldehyde preparations were approximately 80% of that obtained for lithium lactate under similar conditions.

Glyceraldehyde was determined by the method of Park and Johnson (235). Glycolic acid was measured by the chromotropic acid procedure (236). 1, 2-Propanediol was estimated by the ninhydrin method of Jones and Riddick (237). Phosphorus was determined by the method of Fiske and SubbaRow

(238) or by the method of Chen, et al. (229).

2. SPECTROPHOTOMETRIC METHODS

Bacterial growth (in liquid cultures) was estimated by the determination of absorbancies (660 mμ) of samples removed from the culture at various intervals. The absorbancy of the inoculated culture at zero time was used as the control, and the absorbancy of the uninoculated culture as the blank.

Protein was determined by the spectrophotometric method of Waddel (239). In a preliminary study, a comparison was made between this method and the procedure of Lowry, et al. (240) using standard solutions of crystalline bovine albumin and crude extracts of Escherichia coli O111-B_4. The results obtained by these two methods agreed within experimental error.

The reduced forms of pyridine nucleotides (DPNH and TPNH) were determined spectrophotometrically at 340 mμ. The optical density changes were used to estimate the reduced nucleotides, using a molar absorbancy index of 6.22×10^3 (241).

ADP was measured according to the method of Kornberg and Pricer (242). DHAP was determined enzymatically in the presence of α-GPD and DPNH (243,244). D-Glyceraldehyde-3-P was determined spectrophotometrically in the presence of D-glyceraldehyde-3-P dehydrogenase and DPN (245).

3. INSTRUMENTS

Colorimetric and spectrophotometric measurements were made in a Beckman Model DU spectrophotometer. The continuous rate determination of enzyme reactions were conducted in a Beckman Model DU spectrophotometer equipped with a Gilford automatic recorder and cuvette positioner. The frequency of changing of the samples was 5 sec (at 5 sec-intervals, cuvettes were displaced automatically) so that when using a set of four cuvettes, each cuvette would be read at 20 sec intervals. The sensitivity of the instrument could be adjusted so that 0.005 units of optical density could be measured with precision. When higher sensitivities were desired, a special set of quartz cuvettes with a total capcity of 0.50 ml were substituted for 1.0 ml quartz cuvettes. Since it was found that with this instrument 0.2-0.5 ml total volume could be used without varying the precision of the measurements, as a routine procedure 0.25 ml total volume was employed which increased the sensitivity four-fold as compared with 1.0 ml cuvettes. Under these conditions, 0.001 μmole of DPNH gave an optical density of 0.025 at 340 mμ, which could be measured with high precision. Spectra were determined with a Cary Model 14 spectrophotometer equipped with an automatic recorder. Optical rotations were measured in a Model 180 Rudolph visual polarimeter. The author is indebted to Miss Janet C. Leutz and Mr. Jack J. Distler of the Rackham Arthritis Research Unit for their technical assistance in this regard. Crude extracts of bacteria were prepared in a Raytheon 10 KC sonic oscillator.

Melting point determinations were made in a Fischer-Jones hot stage melting point apparatus. Unless otherwise stated, all the melting points reported in the present studies are uncorrected. I wish to acknowledge Dr. Eugene E. Dekker of this University for the use of this instrument.

E. Enzymatic Procedures

1. L-FUCOSE ISOMERASE

a. Assay

L-Fucose isomerase activity was measured by the determination of L-fuculose formed in the presence of L-fucose. The incubation mixture contained 2.0 μmoles of L-fucose, 50 μmoles of Tris hydrochloride buffer, pH 7.8 and appropriate amounts of L-fucose isomerase in a total volume of 0.5 ml. The following controls were included: (a) an enzyme-less control, in order to correct for the non-enzymatic isomerization of L-fucose under the conditions of the assay and (b) a substrate-less control. The tubes were incubated at 37°. At various time intervals (5, 10 and 20 min), aliquots (0.1 to 0.2-ml) were removed from the tubes and their ketose content was determined by the cysteine-carbazole method (202). For the estimation of L-fuculose, the absorbancy values of the two controls were subtracted from that of the complete system, and the corrected value obtained was converted into μmoles by comparison with a standard curve which was run simultaneously.

2. L-FUCULOSE KINASE

a. Method of Assay

L-Fuculose kinase activity was measured by the following methods.

(1) Ba(OH)-$ZnSO_4$ Method.—For crude preparations, where interfering materials were present, the $Ba(OH)_2$-$ZnSO_4$ procedure was employed (200). The phosphorylation of L-fuculose was estimated by the determination of the residual methylpentose after removal of the phosphate esters by $Ba(OH)_2$-$ZnSO_4$ precipitation. The incubation mixture contained the following: 2.5 μmoles each of L-fuculose, ATP, KF and $MgCl_2$; 25 μmoles of Tris buffer, pH 7.8; and appropriate amounts of L-fuculose kinase in a total volume of 0.5 ml. A control with no ATP was included, and the tubes were incubated at 37°. At various time intervals an aliquot (0.05 to 0.1 ml) was removed from each tube and placed in a tube containing 0.5 ml of a 0.15M solution of $ZnSO_4$; after mixing, 0.5 ml of a 0.3N solution of $Ba(OH)_2$ was added. The tubes were shaken vigorously, allowed to stand for a few minutes, and the precipitate was removed by centrifugation. Aliquots of the clear supernatant fluids were used for the determination of the remaining methylpentose. The difference between the amount of methylpentose in the control and the amount remaining in the complete system was used to estimate the amount of L-fuculose which was phosphorylated.

(2) Spectrophotometric Method.--For more purified preparations, where a spectrophotometric method could be applied, L-fuculose kinase activity was measured by determining the rate of ADP formation from ATP in the pre-

sence of L-fuculose. The reaction mixture (0.5 ml) contained the following (in μmoles): L-fuculose, 2.0; ATP, 5.0; $MgCl_2$, 5.0; GSH, 5.0; potassium fluoride, 5.0; Tris buffer, pH 7.8, 50; and appropriate amounts of L-fuculose kinase. A control containing all of the above ingredients except L-fuculose was included to determine ATPase activity. Following incubation at 37° for 10 min, the mixture was heated in a boiling water bath for 1 min, cooled to room temperature and centrifuged.

ADP in the supernatant fluid was determined spectrophotometrically at 340 mμ by adding an aliquot (0.05-0.10 ml) to a cuvette containing the following (in μmoles): DPNH, 0.2; PEP, 0.5, phosphate buffer, pH 7.0, 10; and $MgCl_2$, 5.0. The initial absorbancies at 340 mμ were determined; 5 μl of a solution of LDH (containing 50 μg of enzyme) were added to each cuvette and the optical density changes were followed. The difference between the initial and final absorbancy values were used to estimate the amount of ADP present. The theoretical basis of this assay is illustrated in Figure 15. A unit of activity is defined as the amount of enzyme required to catalyze the formation of 1 μmole of ADP per minute under the conditions described. Specific activity is expressed in terms of the number of units per mg of protein.

b. Purification Procedure

Cell-free extracts of Escherichia coli O111-B_4 were prepared by sonic oscillation as described previously (see "Preparation of crude extracts"). Unless otherwise stated, all operations were conducted at temperatures

ASSAY OF L-FUCULOSE KINASE

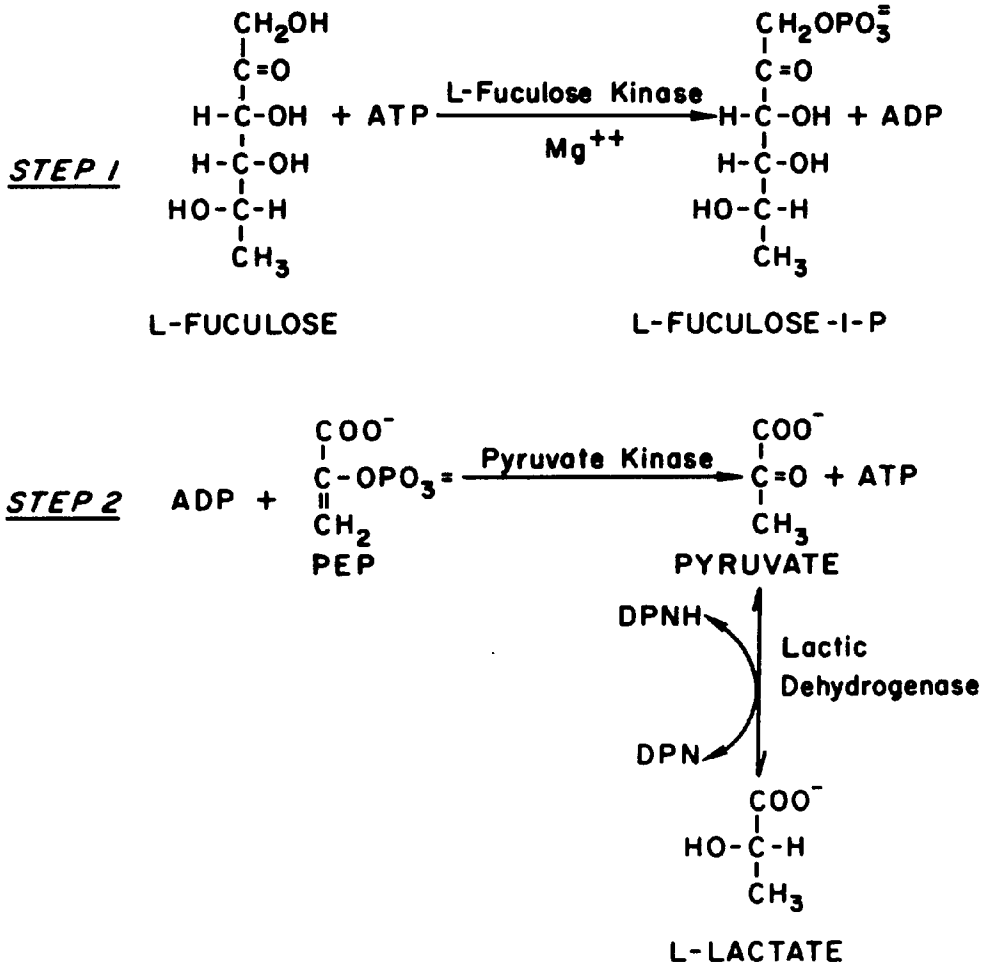


Figure 15. Basis of spectrophotometric assay of L-fuculose kinase.

ranging from 0° to 4°.

Protamine sulfate fractionation. As excess protamine sulfate precipitated the enzyme, pilot tests with small aliquots of the crude extract were used to determine the optimum amount. In a typical experiment, 50 ml of crude extract were diluted with 0.02M phosphate buffer, pH 7.0, to give a protein concentration of about 26 mg/ml. To the diluted extract were added, with gentle stirring, 30 ml of a 2 percent solution of protamine sulfate, and the mixture was allowed to stand for 5 minutes. The suspension was centrifuged at 30,000xg for 10 min and the supernatant solution (93 ml) was diluted with phosphate buffer to give a protein concentration of 2.2 mg/ml.

Ammonium sulfate fractionation. The diluted protamine sulfate supernatant solution (180 ml) was treated with 35 gm of solid ammonium sulfate, the suspension stirred for 5 minutes, centrifuged at 30,000xg for 5 min, and the small precipitate discarded. The supernatant solution was treated with an additional 10 gm of ammonium sulfate, and after stirring for 5 min, the suspension was centrifuged at 30,000xg for 10 min. The precipitate, containing the majority of L-fuculose kinase activity, was dissolved in 0.02M potassium phosphate buffer, pH 7.0, yielding a final volume of 9.25 ml. This preparation was at least 33-fold purified (see footnote Table 9); the purification procedure is summarized in Table 9. There was no detectable L-fucose isomerase in the preparation and L-fucose would not serve as substrate for the phosphorylation reaction. Traces of ATPase activity were detectable in the preparation.

TABLE 9

PURIFICATION OF L-FUCULOSE KINASE

Fraction	Mg Protein	Total Units	Specific Activity x 10^2	Percent Recovery
Crude Extract	1850	242	2.9	---[a]
Protamine Sulfate	400	539	13.4	100
Ammonium Sulfate	41.6	202	96.6	74
Ammonium Sulfate[b]	16.2	12.9	80	100
Dialyzed Fraction	15.7	9.3	59	72
Calcium-Phosphate	6.7	8.6	128	67

[a]Because of interfering enzymes in the crude extracts, it was impossible to accurately determine the total amount of L-fuculose kinase present. The specific activity of the enzyme in the crude extract therefore, is estimated from the total activity recovered in the protamine sulfate supernatant fluid.

[b]A separate experiment, see text for details.

In certain instances, that is in substrate specificity studies, where further purification of L-fuculose kinase was necessary, another purification step was included. Since this step was not performed on the above preparation, it is described separately below.

1.6 ml (16.2 mg protein) of an ammonium sulfate fraction (specific activity 0.8) was dialyzed against 50 volumes of 0.01M potassium phosphate buffer, pH 7.0, containing 0.01M GSH, for 4 hours at 4°. The dialyzed sample was centrifuged,and the supernatant fluid made up to 3.0 ml with water. To the resulting solution, equal volumes of 0.02M phosphate buf-

fer, pH 7.0, and of a suspension of calcium phosphate gel (containing 7.5 gm of dry weight/ml) were added. Following 5 min at 0°, the mixture was centrifuged, and the supernatant fluid which contained the majority of L-fuculose kinase activity was saved. Results of this purification step are summarized in the lower portion of Table 9. Although some losses of activity occurred upon dialysis, the treatment with calcium phosphate gel resulted in at least 2-fold purification.

The stability of this fraction (33-fold purified) was tested as follows: an aliquot was dialyzed against 50 volumes of 0.01M potassium phosphate buffer, pH 7.0, containing 0.01M cysteine HCl for 15 hours at 4°; this treatment resulted in 30-40% loss of activity. To 1.0 ml of the undialyzed preparation was added 0.5 ml of a 0.05M solution of GSH, pH 6.9. The sample was divided into six aliquots, five of which were stored at -20°, and one at 4°. The kinase activity was measured in samples stored at these two temperatures after 1, 3, 9, and 11 days. Storage at 4° resulted in 20% loss in activity after 24 hours, and complete loss of activity after 11 days, while the enzyme was completely stable at -20° after 11 days of storage (20% of activity was lost after 4 months at -20°). Repeated freezing and thawing of the preparations did, however, result in considerable losses in activity. Four cycles of freezing and thawing resulted in 40% loss of activity.

3. L-FUCULOSE-1-P ALDOLASE

a. Method of Assay

The cleavage of L-fuculose-1-P could be followed by determining either dihydroxyacetone-P or lactaldehyde. For routine assay of the enzymes, dihydroxyacetone-P was measured spectrophotometrically using α-glycerol-P dehydrogenase and DPNH (Figure 16). In crude preparations, when DPNH oxidase was present, the assay was carried out in two steps. The enzyme was incubated with L-fuculose-1-P, the reaction stopped by heating, and an aliquot removed for determination of the dihydroxyacetone-P. In more highly purified preparations, L-fuculose-1-P, L-fuculose-1-P aldolase, α-glycerol-P dehydrogenase,and DPNH were combined in a single cuvette and the formation of DHAP was followed continuously.

In the two-step assay, the complete system contained the following (in μmoles): L-fuculose-1-P, 0.2 to 0.5; Tris buffer, pH 7.2, 5-10; potassium fluoride, 5; and appropriate amounts of L-fuculose-1-P aldolase in a total volume of 0.05-0.10 ml. Controls containing all the above ingredients except L-fuculose-1-P were included. Following 5-20 min of incubation at 37°, the tubes were placed in a boiling water bath for 1 min, cooled,and the following additions were made: 0.10 ml of a solution of L-fuculose-1-P (containing 0.2 to 0.5 μmoles, depending on the amount used in the complete system) was added to the control and 0.10 ml of water to the incubated tube and mixed. After centrifugation, the amount of DHAP in the supernatant fluids was determined as follows: An aliquot (0.05-0.10

TWO STEP ASSAY OF L-FU-1-P ALDOLASE

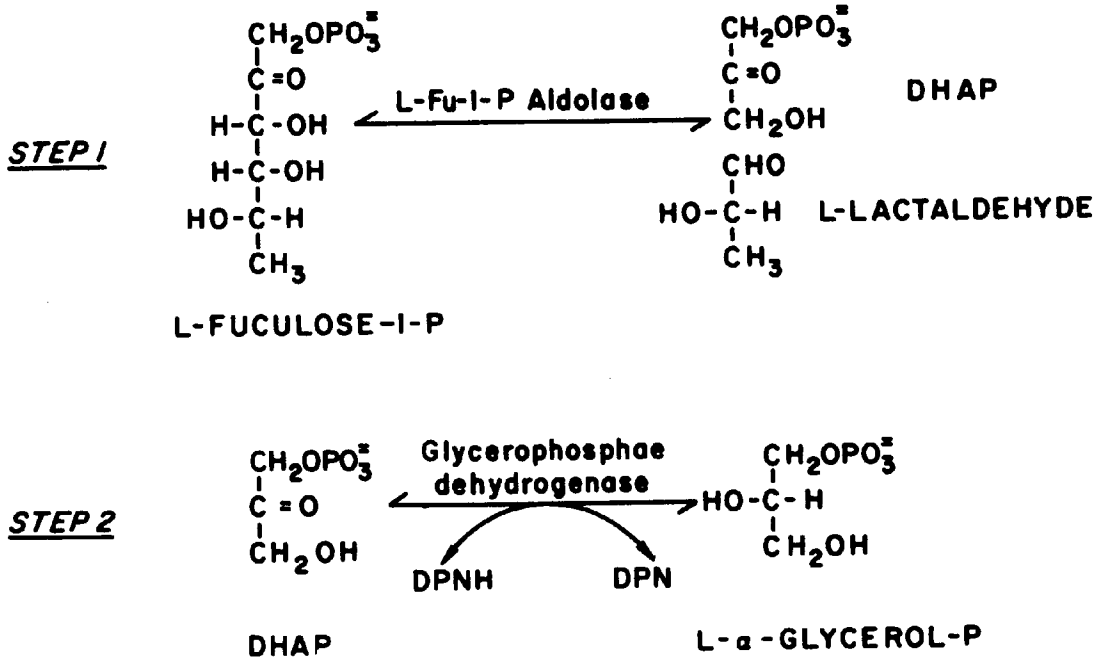


Figure 16. Basis of spectrophotometric assay of L-fuculose-1-P aldolase.

ml) of each supernatant solution was placed in a 0.5 ml quartz cuvette containing 0.05-0.10 μmoles of DPNH and 5 μmoles of Tris buffer, pH 7.2, and the final volume was made up to 0.25 ml with water. Following measurement of the initial absorbancies at 340 mμ, 5 μl (1-5 μgm) of a solution of α-GPD were added to each cuvette, and the absorbancies were followed until there was no further change. The difference between the initial and final absorbancy values were used to calculate the quantity of DHAP in the sample.

For the single-step assay, incubation mixtures were prepared as follows (in μmoles): L-fuculose-1-P, 0.25 to 0.5; Tris buffer, pH 7.2, 5; potassium fluoride, 5; DPNH, 0.05-0.1; and 1-5 μgm of α-glycerol-P dehydrogenase in a final volume of 0.25 ml. A control cuvette was included that contained all of the above ingredients except L-fuculose-1-P; this control was used to correct for residual amounts of DPNH oxidase present in the enzyme preparations. After obtaining initial absorbancies at 340 mμ, L-fuculose-1-P aldolase (5-10 μl) was added to all of the cuvettes, and the optical densities were determined continuously for 10-20 min with the recording spectrophotometer described (see "Analytical procedures").

A unit of enzyme is defined as the quantity that catalyzes the formation of 1 μmole of dihydroxyacetone-P per min under the conditions of the two-step assay. The single-step assay is useful in that it gives continuous rate measurements. However, since accurate temperature control was not possible (the temperature varying between 23° and 30°),

this method was used only when relative values were desired as indicated in the text. Specific activity is defined as units of enzyme per mg of protein.

b. Purification Procedure

Cell-free extracts of L-fucose-grown Escherichia coli O111-B_4 were prepared by sonic oscillation (see "Preparation of crude extracts"). The enzyme preparations were maintained between 0° and 4° during purification. Purification procedure consisted of the following steps.

Protamine sulfate treatment. A 10 ml aliquot of the crude extract (36 mg protein per ml) was diluted with 0.02M potassium phosphate buffer, pH 7.0, to a final protein concentration of 20 mg/ml. This solution was then treated with 2 ml of a 2% solution of protamine sulfate, allowed to stand for 5 min, and the precipitate removed by centrifugation at 25,000x g for 10 min.

Calcium phosphate gel I. The supernatant fluid (19 ml) was diluted to 38 ml with 0.02M phosphate buffer, pH 7.0, and slowly added, with stirring, to 36 ml of calcium phosphate gel suspension (containing 10 gm/ml). The mixture was occasionally stirred for 5 min, centrifuged at 2,000xg for 5 min, and the residue discarded.

Ammonium sulfate fraction. To the supernatant fluid (68 ml) were added 40 ml of 1M potassium acetate buffer, pH 5.5, followed by 12 ml of water and 32 gm of solid ammonium sulfate. The mixture was stirred until the ammonium sulfate dissolved and was then allowed to stand for 5 min.

After centrifugation at 25,000xg for 5 min, the inactive precipitate was discarded. Solid ammonium sulfate (23 gm) were slowly added with continuous stirring to the supernatant fluid, the mixture stirred for 5 min more, and centrifuged at 25,000xg for 5 min. The resulting precipitate was dissolved in 1.5 ml of 0.02M phosphate buffer, pH 7.0, yielding a solution containing 6 mg protein per ml.

Isoelectric precipitation. The ammonium sulfate fraction was dialyzed against 300 volumes of 0.01M acetate buffer, pH 5.5, for 3 hours. The resulting precipitate was removed by centrifugation at 25,000xg for 5 min and discarded. The supernatant fluid contained 1.3 mg of protein per ml.

Calcium phosphate gel II. The dialyzed fraction from the preceding step was diluted with 3 ml of 0.02M phosphate buffer, pH 7.0, to give a final concentration of 0.42 mg of protein per ml, and then mixed with 1.5 ml of calcium phosphate gel suspension (10 mg/ml). The resulting suspension was occasionally stirred for 5 min, centrifuged at 3,000xg for 5 min, and the supernatant fluid retained.

Using these fractionation procedures, L-fuculose-1-P aldolase was purified approximately 120-fold with about 70% recovery. These data are summarized in Table 10. The purified preparations were completely free of DPNH oxidase activity. At no stage of purification was there any indication that more than a single protein was required for the reaction. The enzyme was relatively stable when stored at -16°; only 10-20% of the activity was lost over a period of several months of storage with repeated

TABLE 10

PURIFICATION OF L-FUCULOSE-1-P ALDOLASE

Fraction	Mg Protein	Total Units	Specific Acticity x 10^2	Percent Recovery
Crude Extract	363.0	35.5	---[a]	---[a]
Protamine Sulfate	131.1	40.8	3.1	100
Calcium Phosphate I	61.2	35.4	5.7	90
Ammonium Sulfate	9.1	30.7	33.8	75
Isoelectric Precipitation	1.9	32.4	169	80
Calcium Phosphate II	0.7	28.6	400	70

See text for conditions of assay.

[a]Interfering materials in the crude extract prevented accurate estimation of the activity, thus the specific activities and recoveries are compared with those of the protamine sulfate fraction.

freezing and thawing of the preparation. The purified L-fuculose-1-P aldolase preparation was slightly contaminated with FDP-aldolase; the ratio of activity of L-fuculose-1-P aldolase to that of FDP-aldolase in the crude preparations was approximately 1, while the ratios in the purified preparations were generally 10-20. It was impossible to exclude FDP-aldolase activity entirely, even in the most purified preparations. It was found that an appreciable amount of FDP-aldolase activity was removed in the second calcium phosphate gel treatment; the ratio of L-fuculose-1-P aldolase activity to that of FDP-aldolase in such preparations could be raised to 50 by an additional treatment with calcium

phosphate gel and/or DEAE cellulose chromatography. Two experiments in which FDP-aldolase activity and L-fuculose-1-P aldolase activity were followed during purification are described below.

Calcium phosphate gel: A partially purified preparation (isoelectric precipitation step, 22.5 ml) of L-fuculose-1-P aldolase was adjusted to pH 6.8 by the addition of 0.1M phosphate buffer, pH 7.0, and treated with 20 ml of calcium phosphate suspension (10 mg/ml) in a final volume of 45 ml. The mixture was placed in an ice bath for 5 min, stirred occasionally, and centrifuged. The supernatant fluid contained the bulk of the L-fuculose-1-P aldolase activity (Table 11). By this procedure the $\frac{\text{L-fuculose-1-P aldolase activity}}{\text{FDP-aldolase activity}}$ ratio was increased approximately 7-fold.

DEAE cellulose chromatography: A preparation (1 ml, isoelectric precipitation step) of L-fuculose-1-P aldolase was applied to a column containing 2 ml of DEAE cellulose, which had been equilibrated with 0.01M phosphate buffer, pH 7.0, and washed with 4 ml of water. The washings were combined and assayed for FDP-aldolase and L-fuculose-1-P aldolase activities. The majority of L-fuculose-1-P aldolase activity was recovered in the eluate, while an appreciable portion of FDP-aldolase activity adsorbed to the column under the conditions described (Table 12). In the final preparation the ratio of $\frac{\text{L-fuculose-1-P aldolase activity}}{\text{FDP-aldolase activity}}$ was increased as high as 11-fold.

TABLE 11

REMOVAL OF FDP ALDOLASE ACTIVITY WITH CALCIUM PHOSPHATE GEL

Preparation	Activity[a] With		Activity With L-Fu-1-P / Activity With FDP
	L-Fu-1-P	FDP	
Experiment I			
Before Treatment	0.33	0.25	1.32
After Treatment	0.32	0.35	9.10
Experiment II			
Before Treatment	7.42	1.91	3.9
After Treatment	7.01	0.26	27

[a]Activity = Total units of enzyme activity with either L-fuculose-1-P or FDP.

TABLE 12

REMOVAL OF FDP ALDOLASE ACTIVITY WITH DEAE CELLULOSE

Preparation	Activity[a] With		Activity With L-Fu-1-P / Activity With FDP
	L-Fu-1-P	FDP	
Experiment I			
Before Fractionation	12.3	4.90	2.5
After Fractionation	10.5	0.32	33
Experiment II			
Before Fractionation	12.3	4.90	2.5
After Fractionation	9.0	0.5	18

[a]Activity = Total units of enzyme activity with either L-fuculose-1-P or FDP.

4. LACTALDEHYDE REDUCTASE

The presence of lactaldehyde reductase was detected in crude preparations (Ammonium sulfate step) of L-fuculose-1-P aldolase; such preparations were employed as a source of lactaldehyde reductase with no further purification.

a. Method of Assay

Lactaldehyde reductase activity was measured by the spectrophotometric determination of DPNH oxidation in the presence of lactaldehyde. The assay was conducted in a single-step in 0.5 ml quartz cuvettes. The incubation mixture (0.25 ml) contained the following (in μmoles): lactaldehyde, 1.0; DPNH, 0.05; and Tris buffer, pH 7.2, 10. A control containing all the above ingredients except lactaldehyde was included to determine DPNH oxidase activity of the preparation. The initial absorbancies at 340 mμ were determined, and the reaction was initiated by the addition of appropriate amounts of lactaldehyde reductase to all cuvettes.

5. MISCELLANEOUS ENZYMATIC PROCEDURES

Acid phosphatase was employed for dephosphorylation of sugar-phosphates. The incubation mixture contained the following (in μmoles): sugar-phosphate, 2-5; $MgCl_2$, 10; potassium acetate buffer, pH 5.0, 200; and sufficient amounts of commerical potato acid phosphatase in a total volume of 1.0 ml. A control tube containing all the above ingredients with D-glucose-6-P as the substrate (to determine the activity of acid phosphatase), and a substrate-less control were included. The mixtures

were incubated at 37°, and the reaction was followed by determination of inorganic phosphorus (229) in aliquots (0.05-0.10 ml) removed from each tube at various intervals. In order to obtain the free sugars, the incubation mixtures were deproteinized by the $Ba(OH)_2$-$ZnSO_4$ procedure (200). Following centrifugation, the clear supernatant fluids were deionized and concentrated *in vacuo*.

F. Chromatographic Procedures

1. PAPER CHROMATOGRAPHY

Compounds to be identified were chromatographed on Whatman No. 1 or Schleicher and Schuell 589 Blue Ribbon filter paper by the descending-one-dimensional method. For preparative purposes, especially in case of separation of ketoses from their corresponding aldoses, Whatman 3 MM, or Schleicher and Schuell 589 Green Ribbon filter paper were employed. The materials to be separated were streaked on sheets of paper (18 x 22 in.) with the aid of a capillary pipette and air dried. The paper was equilibrated with the solvent system prior to developing. After development in the solvent (to be specified), the sheets were dried, a guide strip was cut from the lateral sides of each paper, and the locations of the sugras were revealed by treating the strips with a suitable reagent. After determination of the area of each band on the chromatogram, the bands were cut off, and the sugars were eluted with water. The eluates were collected, deionized with mixed-bed resin, concentrated *in vacuo*

and analyzed. To assure homogeneity of the separated compounds, aliquots of these compounds were rechromatographed along with suitable standards.

The solvent systems employed in these studies are listed in Table 13. The following reagents were employed for the detection of the indicated compounds on chromatograms: silver reagent (246), reducing compounds; aniline-TCA reagent (222), aldehydes; TCA-orcinol reagent (203), ketoses; and ammonium molybdate reagent (247) for phosphorus.

The results are expressed either as R_f or as relative R_f values; these values are defined as follows:

$$R_f = \frac{\text{(distance from starting line to the center of the zone)}}{\text{(distance from starting line to the solvent front)}}$$

$$\text{Relative } R_f = \frac{\text{(distance travelled by the substance in question)}}{\text{(distance travelled by a reference substance)}}$$

However, since the relative R_f values were more reliable (248) they were used more often for the expression of the results. $R_{Glucose}$ values of compounds chromatographed in various solvent systems are shown in Table 14.

2. COLUMN CHROMATOGRAPHY

a. Cellulose Column

Free sugars (in the preparation of ketoses from their corresponding aldoses) were separated by chromatography on a powdered cellulose column (249); such columns were prepared as follows: cellulose powder (standard grade) was suspended in acetone, homogenized in a Waring blendor for about

TABLE 13

SOLVENT SYSTEMS USED IN CHROMATOGRAPHIC PROCEDURES

Solvent	Composition
A	Ethylacetate-acetic acid-water (3:1:3)[a]
B	Butanol-pyridine-water (3:1:1)
C	Butanol-ethanol-water (3:1:1)
D	Methylethylketone-propionic acid-water (15:5:6)
E	Butanol-pyridine-water (4:6:1)
F	Pyridine-ethylacetate-water (4:3:1)
G	88% phenol in water (commercial analytical grade)
H	Isoproponal-water (4:1)
I	Butanol-ethonal-water (3:3:1)
J	Methanol-NH_4OH-water (6:1:3)

[a]The proportions are expressed in volume (under standard conditions).

TABLE 14

$R_{GLUCOSE}$ VALUES OF COMPOUNDS CHROMATOGRAPHED IN VARIOUS SOLVENT SYSTEMS

Compound	Solvent							
	A	B	C	D	E	F	G	H
Glucose[a]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Arabinose	1.70	1.25	1.50	1.47	1.00	1.00	1.25	1.40
Fructose	1.45	1.25	1.35	1.25	1.1	1.00	1.20	--
Fucose	2.10	1.70	2.10	1.87	1.15	1.10	1.45	1.78
Fuculose	3.80	2.60	3.2	3.00	1.45	1.34	2.05	2.24
Glyceraldehyde	3.10	2.80	2.65	2.4	1.35	--	1.95	--
Lactaldehyde	8.10	4.00	5.6	5.2	1.67	1.50	2.85	2.76
Psicose	2.20	1.70	1.85	1.67	1.25	1.20	1.43	1.84
Rhamnulose	3.5	2.85	3.10	2.65	1.40	1.34	2.1	2.24
Ribulose	2.90	2.05	2.40	2.35	1.30	1.17	1.75	1.8
6-Deoxy-Sorbose	4.10	2.70	3.30	2.85	1.53	1.43	2.20	--
Sorbose	1.30	1.15	1.20	1.13	1.05	1.1	1.07	1.13
Tagatose	1.60	1.45	1.50	1.33	1.16	1.2	1.30	1.28
Xylose	1.90	1.50	1.85	1.60	1.30	1.05	1.40	1.23
Xylulose	2.70	2.15	2.57	2.10	1.1	1.20	1.85	1.70

[a]The R_f values of glucose (when Whatman No. 1 filter paper was used) in the solvents indicated were as follows: 0.1, A; 0.2, B; 0.14, C; 0.15, D; 0.51, E; 0.56, F; 0.32, G; and 0.29, H.

5 min,placed in a column (40 cm x 9.6 cm^2) in several portions,and allowed to settle by gravity. The column was equilibrated with the solvent system, either solvent system A or D as specified,by allowing the solvent to run through the column for several hours. The mixtures to be separated were concentrated to a syrup, dissolved in sufficient amount of the same solvent, and applied to the column. The column was then developed with the solvent at room temperature at a rate of approximately 1 ml/min. Ten ml fractions were collected with an automatic fraction collector. Two aliquots were removed from each fraction, spotted on strips of Whatman No. 1 filter paper,and air dried. One of the strips was developed with silver reagent (246) for reducing sugar test; the other was used for the detection of ketose by the TCA-orcinol method (203). The sugars were obtained as syrups, by combining appropriate fractions, followed by extraction with ethyl ether and concentration of the aqueous phase in vacuo. The syrup was dissolved in small amounts of water, deionized with mixed-bed resin, and chromatographed on paper to assure its homogeneity.

b. Ion-Exchange Column

Ketose phosphates were isolated on Dowex-1-formate resin columns as follows: columns (25 cm x 3.5 cm^2) were prepared by allowing the resin to settle by gravity and were washed with water for several hours, until the washings had a pH of 4.0 to 4.5. The solution containing the mixture of phosphate esters (pH 3.0-3.5) was applied to the column, the latter was washed with two volumes of water and fractionated (at 4°) by gradient

elution with 200 ml of water in a constant volume mixing chamber and 1 liter of 0.4N formic acid containing 0.1M sodium formate in the reservoir. Fractions (10 ml) were collected on an automatic fraction collector and an aliquot of each was used for the detection of ketoses (203). When quantitative study of the elution pattern was desired, ketose determination (202) was made on aliquots removed from the fractions. The fractions containing ketose phosphates were pooled and adjusted to pH 6.5 by the addition of 10N KOH. The ketose phosphates were converted to their barium salts by the addition of 3 equivalents of barium acetate, and 4 volumes of absolute ethanol were added. After storage at 4° for several hours, the white preceipitate was harvested by centrifugation, washed with 80% ethanol, absolute ethanol and ether, and dried in vacuo. The crude preparation obtained was purified by dissolving it in dilute acetic acid, the resulting solution was treated with Norit A to remove the nucleotides (this was done in the case of L-fuculose-1-P preparation by the kinase reaction), and the phosphates were reprectipitated in the form of barium salt as described.

Phosphoglycolic acid (obtained as a product of periodate oxidation of ketose-phosphates studied) was isolated on a column (15 cm x 0.8 cm^2) containing Dowex-1 Cl^- resin by gradient elution with 500 ml of 0.05N HCl in a constant volume mixing vessel and 1 liter of 0.4N HCl in the reservoir. Fractions (5 ml) were collected, and the position of phosphoglycolic acid was determined by the chromatropic acid test (236) on

aliquots removed from the fractions. Phosphoglycolate was recovered by combination of the appropriate fractions.

IV. RESULTS

A. L-Fucose Isomerase

The presence of L-fucose isomerase in crude extracts of L-fucose-grown Escherichia coli O111-B_4 was demonstrated by the characterization of the product formed, when such extracts were incubated with L-fucose. The product of isomerization of L-fucose was identical to L-fuculose on the basis of the following criteria.

1. Equimolar ratios were obtained for methylpentose, ketose, and ketohexose as determined by:

   a. Cysteine-sulfuric acid method of Dische and Shettles (199).

   b. Cysteine-carbazole reaction of Dische and Borenfreund (202).

   c. Cysteine-sulfuric acid test of Dische and Devi (228).

These results indicated that the product of isomerase was a 6-deoxy-ketohexose.

2. In the cysteine-carbazole reaction, the product of isomerase yielded a chromogen with a spectrum indistinguishable from that obtained for authentic L-fuculose under similar conditions (Figure 17).

3. Paper chromatography of the product in various solvent systems revealed a single component, which exhibited relative mobilities identical to those found for authentic L-fuculose (Table 15).

4. Final characterization of the product of isomerase was made by the preparation of its o-nitrophenylhydrazone derivative the properties

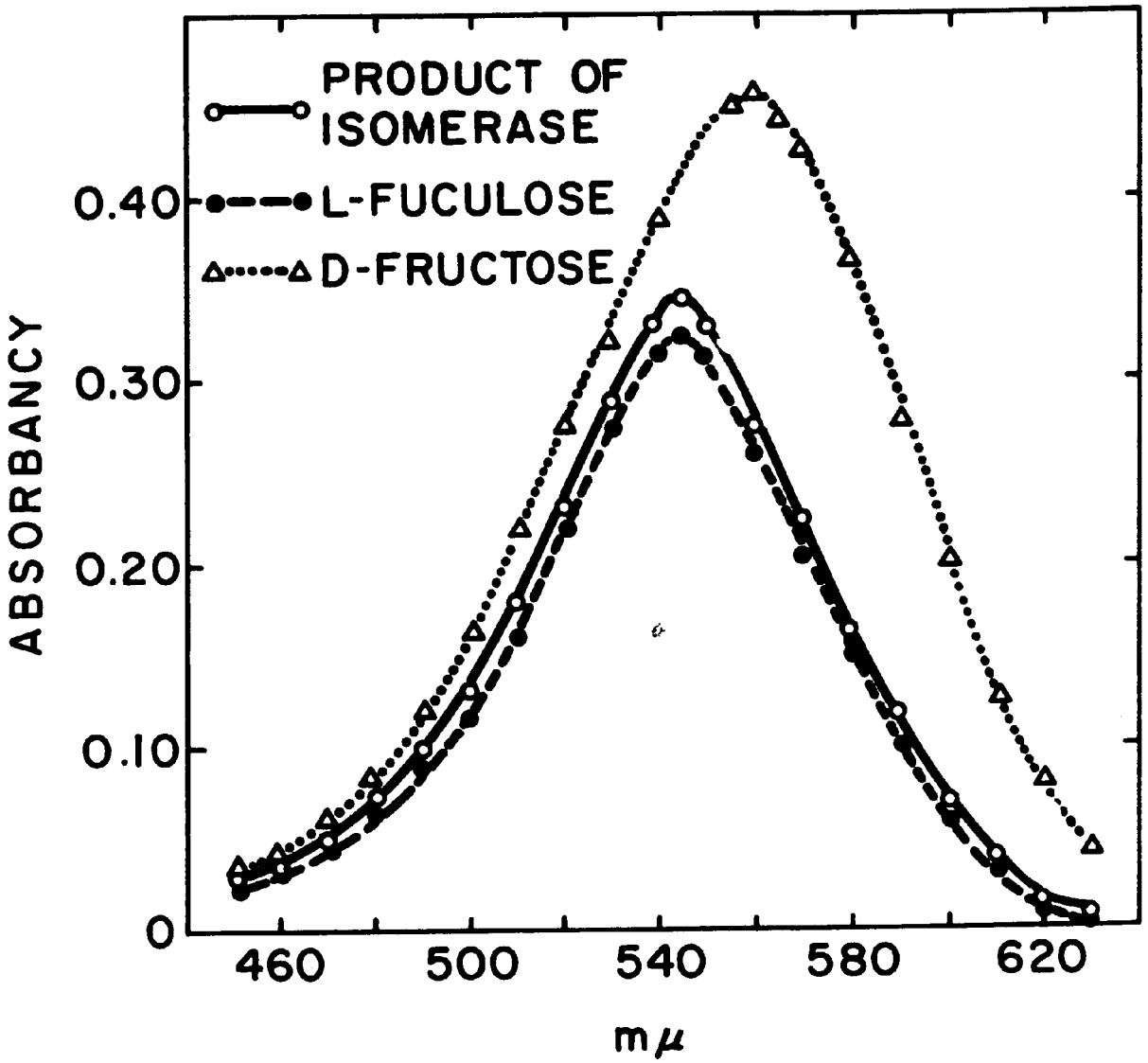


Figure 17. Absorption spectra of chromogens produced by various ketoses in the cysteine-carbazole reaction (202).

TABLE 15

PAPER CHROMATOGRAPHY[a] OF PRODUCTS OF L-FUCOSE ISOMERASE AND L-FUCULOSE KINASE[b]

Compounds	Solvent A	Solvent B	Solvent D	Solvent E	Solvent I
Fucose	1.00	1.00	1.00	1.00	1.00
Fuculose	1.81	1.53	1.54	1.37	1.61
Product of Isomerase	1.80	1.53	--	--	1.60
Product of Kinase	--	1.54	1.55	1.37	1.61
6-Deoxy-L-Sorbose	1.94	1.60	1.52	1.33	--
Rhamnulose	1.67	--	1.41	1.21	--

[a]Results expressed as R_{fucose} values.

[b]Product of kinase was treated with acid phosphatase prior to chromatography.

of which were identical to those of an authentic sample of L-fuculose-o-nitrophenylhydrazone (Table 3).

B. L-Fuculose Kinase

1. PROPERTIES OF THE ENZYME

Effect of time of incubation: As illustrated in Figure 18, the rate of ADP formation was proportional to the time of incubation for at least 15 min.

Effect of enzyme concentration: The rate of ADP formation was linear over 8-fold range of protein concentration (Figure 19).

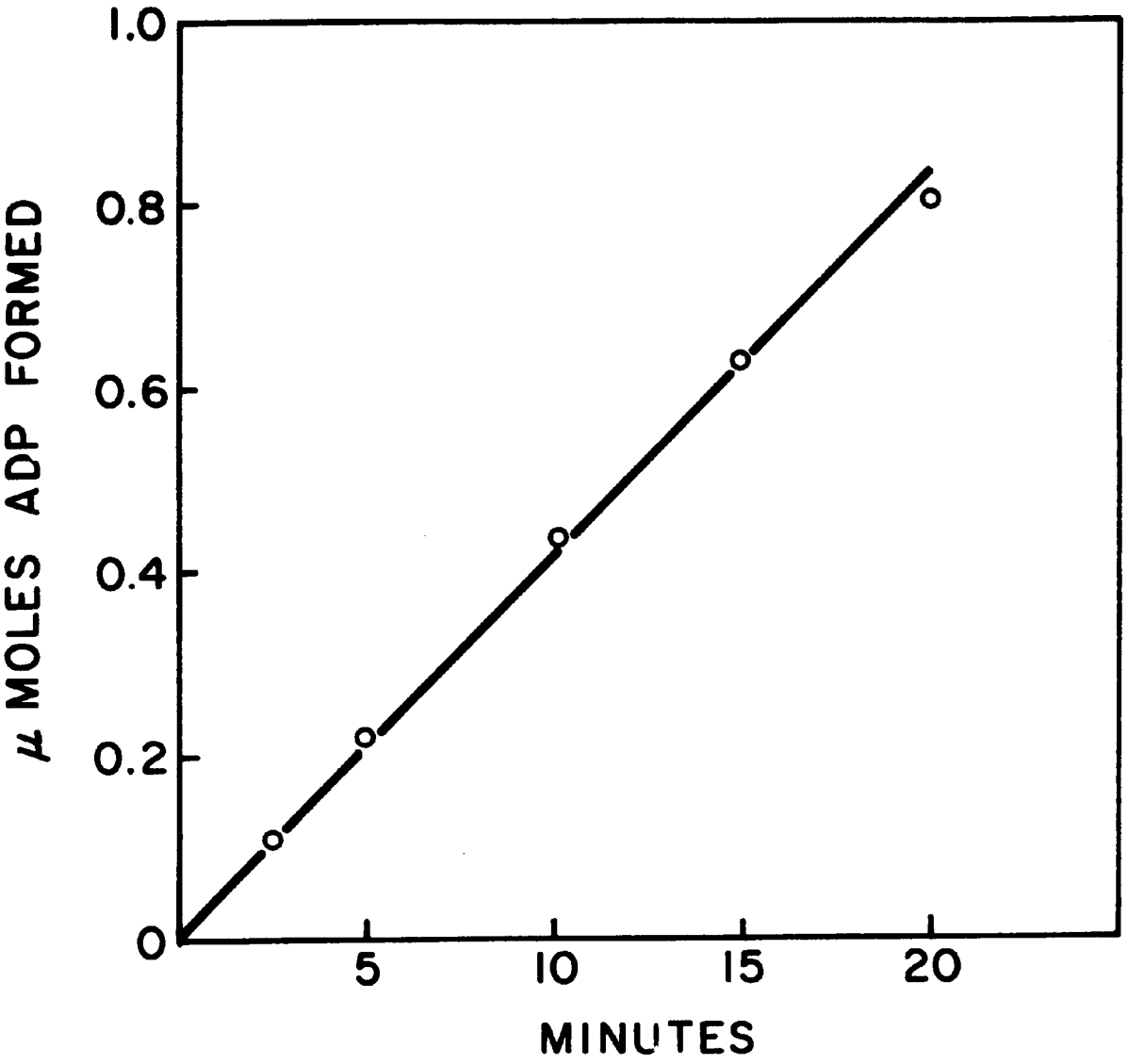


Figure 18. Effect of time of incubation on L-fuculose kinase reaction. The incubation mixture contained the following (in μmoles): ATP, 5.0; $MgCl_2$, 5.0; GSH, 5.0; KF, 5.0; L-fuculose, 4.0; Tris buffer pH 7.8, 50; and 0.5 units of L-fuculose kinase (A.S. step) in a final volume of 0.75 ml. Aliquots were removed at various intervals and assayed by the spectrophotometric method.

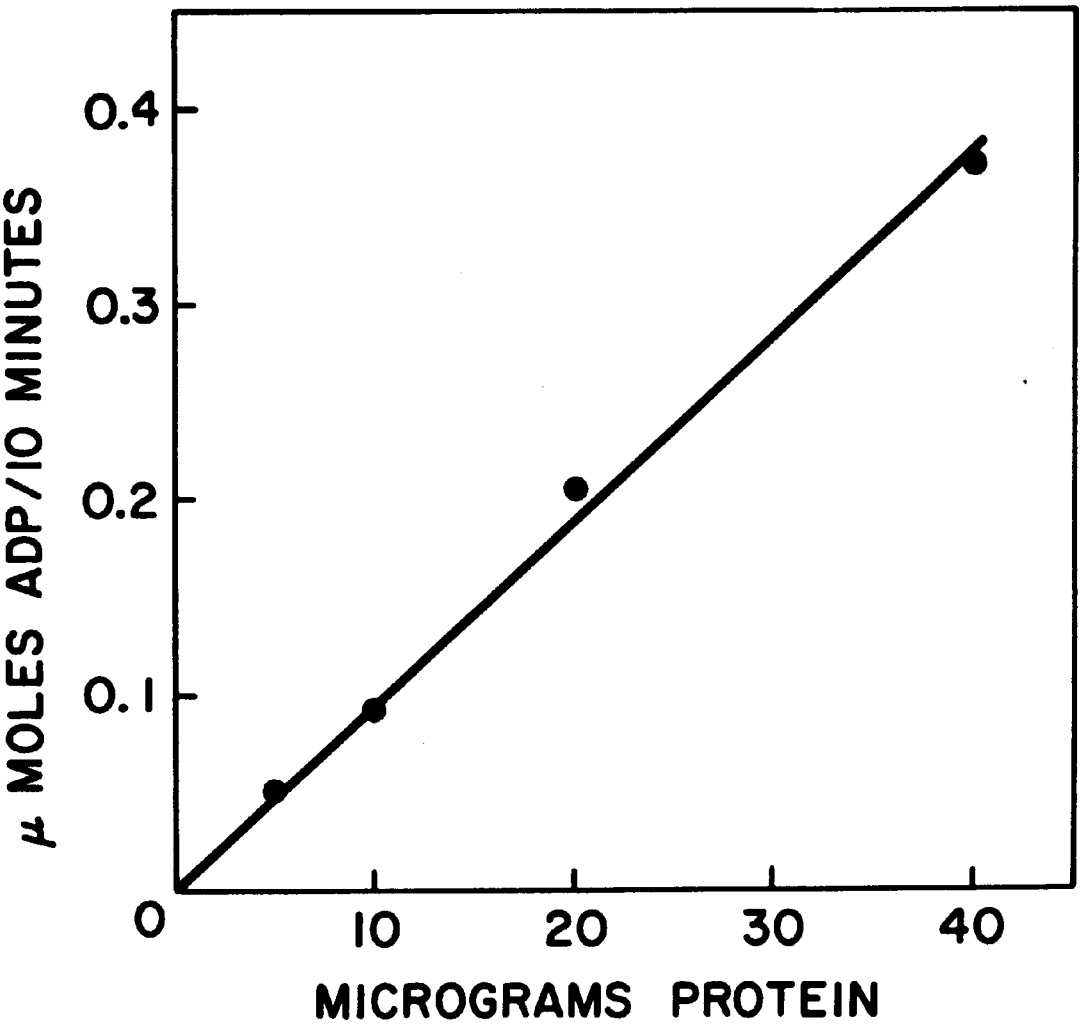


Figure 19. The reaction rate as a function of L-fuculose kinase concentration. Conditions were the same as those described for the spectrophotometric method, except that the kinase concentration was varied.

Effect of pH: The dependence of the rate of ADP formation on the pH of the incubation mixture is shown in Figure 20. The enzyme exhibited optimum activity at pH 7.8.

Effect of substrate concentration: The influence of L-fuculose on the reaction rate is illustrated in Figure 21. When plotted according to the method of Lineweaver and Burk (250), the calculated Km value was found to be approximately 1.4×10^{-3}M.

Effect of cations: L-Fuculose kinase exhibited an absolute requirement for a divalent cation. Purified preparations of the enzyme were stimulated at least 20-fold by the addition of Mg^{++} or Mn^{++}, while monovalent cations had little or no effect. As illustrated in Table 16, Mg^{++} and Mn^{++} exhibited the same magnitude of stimulation and the Km value obtained was approximately 1×10^{-3}M (Figure 22).

Nucleotide specificity: As illustrated in Table 17, ATP was the only nucleotide which could serve as the phosphoryl donor in this reaction; ITP would partially substitute for ATP, while other nucleotides tested were found to be relatively inactive.

Effect of ATP concentration: The influence of the concentration of ATP on L-fuculose kinase activity is illustrated in Figure 23. The calculated Km value was found to be approximately 1.2×10^{-3}M.

Stoichiometry: Stoichiometry of the reaction of L-fuculose kinase shown in Table 18, indicates that for each mole of L-fuculose which disappeared, one mole of L-fuculose-1-P and one mole of ADP were formed.

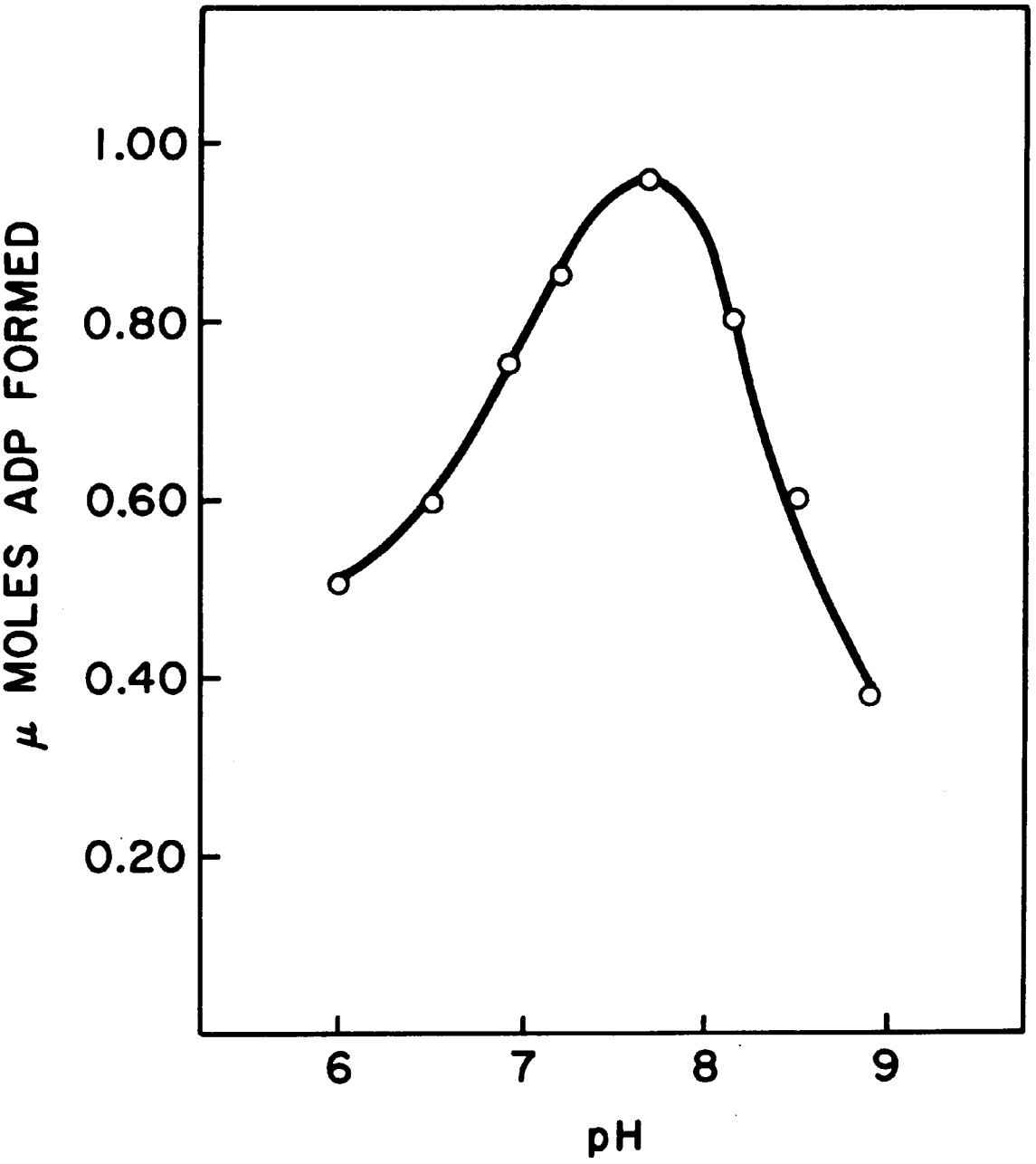


Figure 20. Effect of pH on the rate of L-fuculose kinase reaction. Conditions were the same as those described for the spectrophotometric method, except that the pH of the incubation mixture was varied; Tris buffer was used above pH 7 and potassium phosphate buffer was used below pH 7. The pH values indicated on the curve represent average values of the pH of the incubation mixture before and after incubation period.

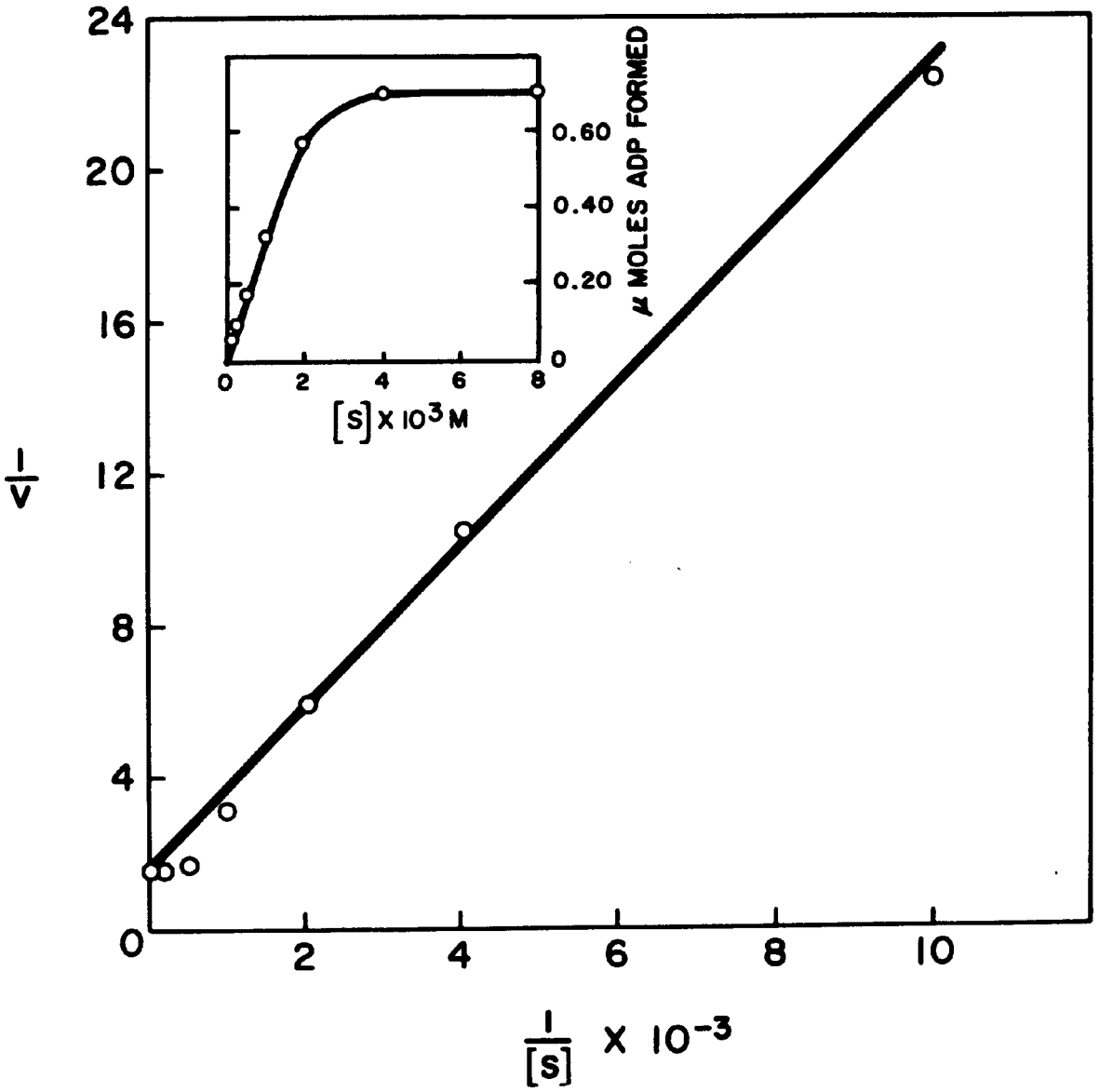


Figure 21. Effect of L-fuculose concentration on the rate of L-fuculose kinase reaction. Conditions were the same as those described for the spectrophotometric method, except that L-fuculose concentration was varied.

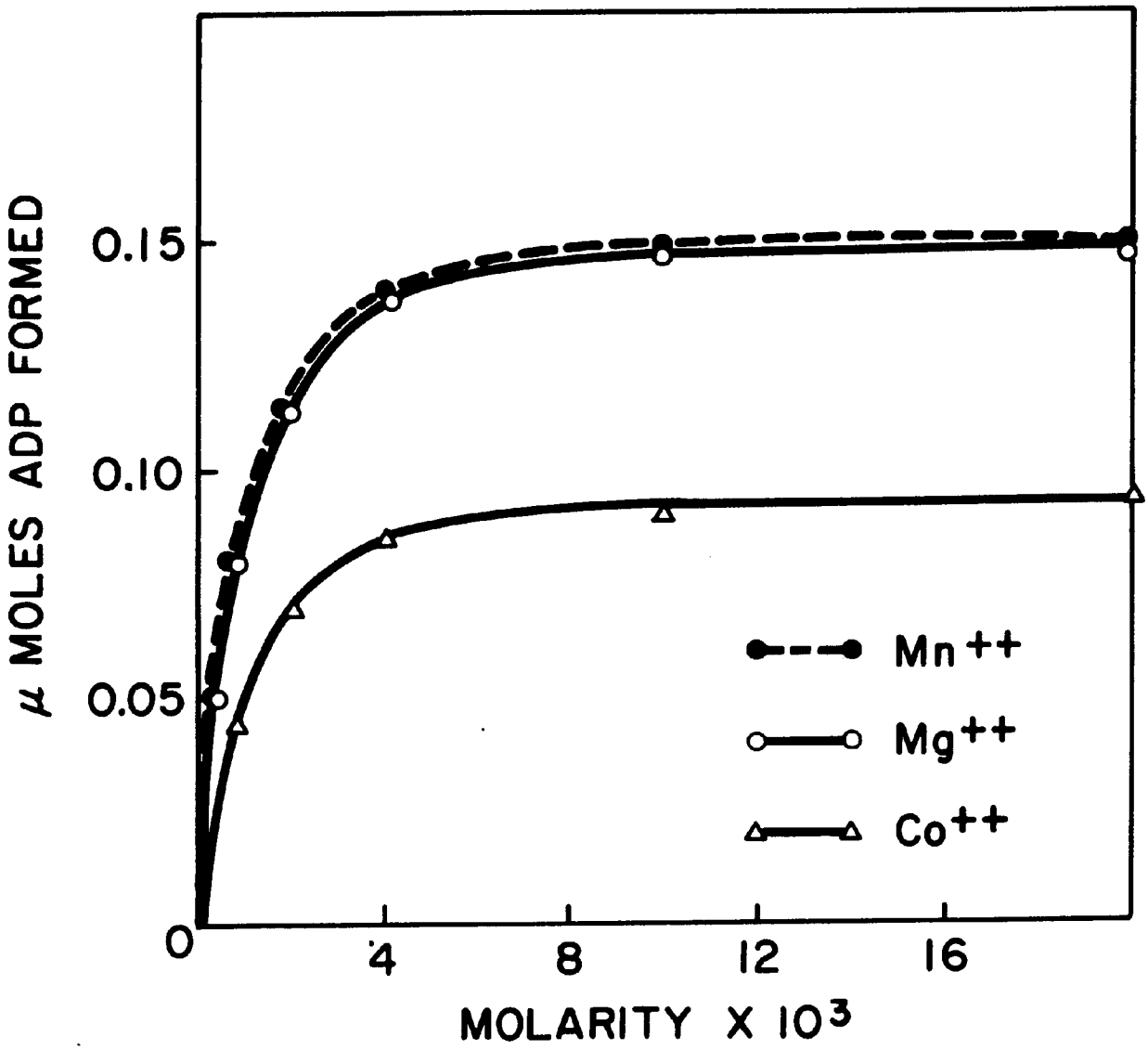


Figure 22. Effect of cation concentration on the rate of L-fuculose kinase reaction. The incubation mixture contained the following (in μmoles): L-fuculose, 1.0; ATP, 2.5; KF, 1.25; GSH, 2.5; Tris buffer, pH 7.8, 10; L-fuculose kinase 40 μg (Ca-P-Gel step); and varying amounts of cations in a final volume of 0.25 ml.

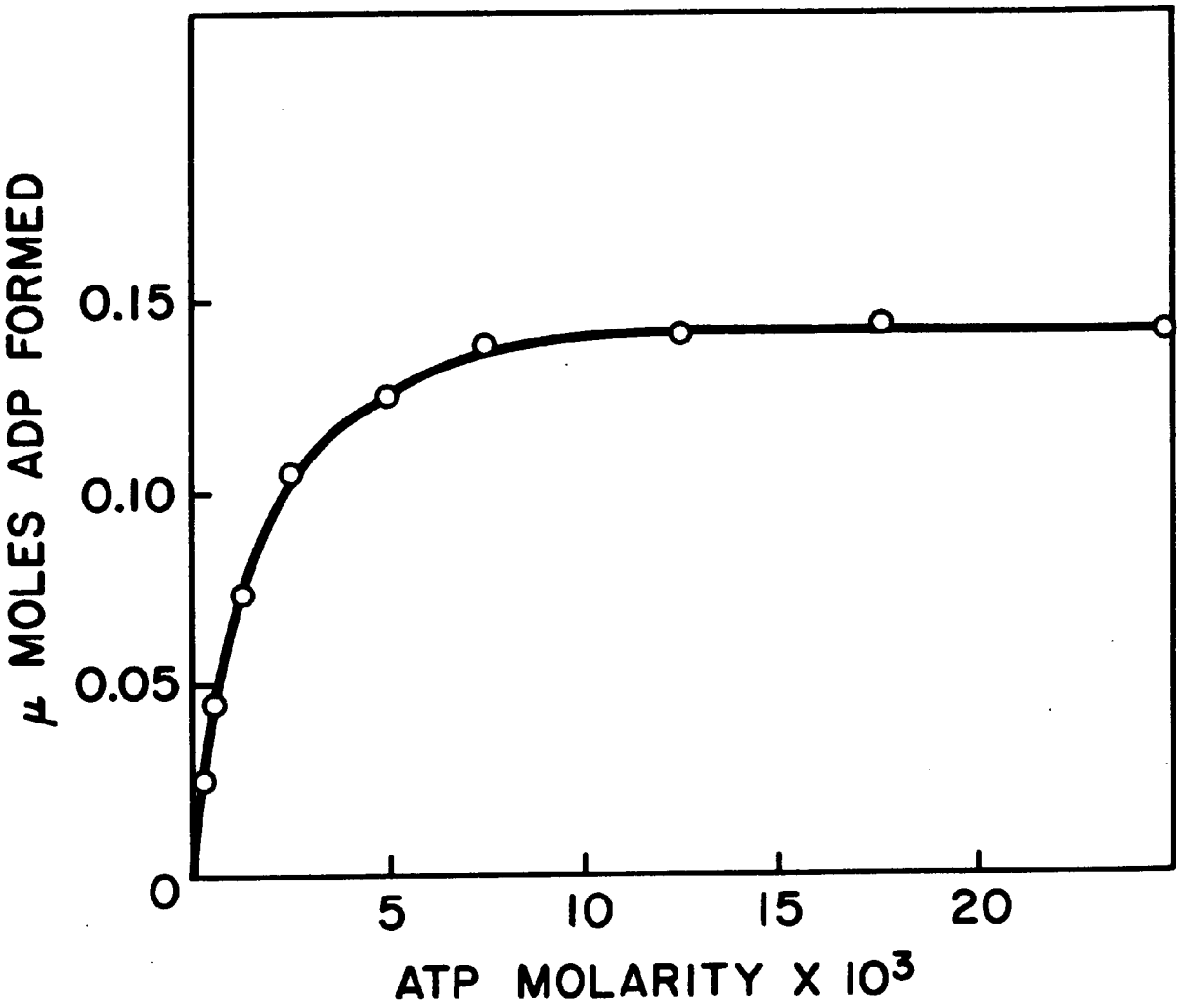


Figure 23. Effect of ATP concentration on the rate of L-fuculose kinase reaction. The incubation mixture contained the following (in μmoles): L-fuculose, 1.0; GSH, 2.5; $MgCl_2$, 2.5, KF, 1.25; Tris buffer, pH 7.8 10; 40 μg of L-fuculose kinase (Ca-P-Gel step); and varying amounts of ATP in a final volume of 0.20 ml.

TABLE 16

CATION REQUIREMENT OF L-FUCULOSE KINASE

Cation	μMoles ADP Formed	Relative Activity
Mg^{++}	0.120	100
Mn^{++}	0.120	100
Co^{++}	0.080	67
Ca^{++}	0.036	30
K^{+}	0.012	10
Na^{+}	0.012	10
No Metal	0.005	4

The incubation mixture contained the following (in μmoles): L-fuculose, 1.0 ATP, 2.5; GSH, 2.5; KF, 1.25; Tris pH 7.8, 10; 20 μg of L-fuculose kinase, and 2.5 μmoles of each of the cations in the form of their chloride salts in a final volume of 0.25 ml. Incubation was carried out at 37° for 10 min, and the ADP formed was determined as previously described.

TABLE 17

NUCLEOTIDE SPECIFICITY OF L-FUCULOSE KINASE

Nucleotide	Percent Activity
ATP	100
ITP	10
CTP	0
GTP	0
UTP	0

The incubation mixture contained the following (in μmoles): L-fuculose, 1.0; GSH, 5.0, KF 2.5; MG^{++}, 2.5; Tris pH 7.8, 20; L-fuculose kinase 100 μg,and 2.5 μmoles of nucleotide triphosphate in a final volume of 0.25 ml. Following 10 min incubation at 37°, the reaction was stopped by heating the tubes in boiling water-bath for 1 min followed by centrifugation. The supernatant fluid was assayed for nucleotide di-phosphate formed as described, except that 0.2 μmoles of ATP and 50 μg of nudiki were added to each of the cuvettes.

TABLE 18

STOICHIOMETRY OF L-FUCULOSE KINASE REACTION

Compound	μMoles	Ratios
L-Fuculose	-1.16	1.00
L-Fuculose-1-P	+1.11	0.96
ADP	+1.09	0.94

The incubation mixture contained the following (in μmoles): L-fuculose, 4.0; GSH, 10; KF, 5.0; ATP, 5.0; $MgCl_2$, 5.0; Tris buffer, pH 7.8, 50; and 0.2 mg of a partially purified preparation of L-fuculose kinase in a final volume of 1.0 ml. Two controls were included.

1. An L-fuculose-less control (L-fuculose was added after the incubation period) to correct for ATPase activity.

2. A zero time control which contained heat-inactivated enzyme to correct for the possible non-enzymatic changes of L-fuculose under the conditions of the assay. Following 15 min incubation at 37°, the tubes were heated in a boiling water bath for 1 min, cooled, and centrifuged. The components of the incubation mixture were determined as follows: ADP was determined in each of two aliquots (0.05 and 0.10 ml) from each of the tubes by the method of Kornberg and Pricer (242). To determine the amount of the remaining L-fuculose and L-fuculose-1-P formed, 0.4 ml aliquots of each of the tubes were applied to columns containing 2 ml of Dowex-1-formate resin, washed with 10 ml of water and then eluted with 10 ml of 0.4 N formic acid containing 0.1 N ammonium formate. L-Fuculose and L-fuculose-1-P were determined by two methods (202, 228) in the water wash and the eluate respectively. To confirm the results obtained by column chromatography, 0.10 ml aliquots of each of the tubes were treated with the $Ba(OH)_2$-$ZnSO_4$ method (200), and the amount of L-fuculose present in the supernatant solutions was determined (202, 228). The amount of L-fuculose found by this method corresponded to the amount that was not retained by the resin (above).

Substrate specificity: L-Fuculose exhibited maximum activity with the enzyme, although sugars such as D-glucose, D-fructose, D-mannose, D-ribulose,and D-xylulose also demonstrated appreciable activity. The fact that other sugars could serve as substrate for this enzyme can be explained either on the basis of a rather broad substrate specificity, or the presence of other enzymes in this preparation which can catalyze the phosphorylation of these sugars; i.e., hexokinase which could account for the phosphorylation of D-glucose, D-fructose,and D-mannose. The interesting aspect of substrate specificity of L-fuculose kinase is the observation that it does not catalyze the phosphorylation of the diastereoisomers of L-fuculose, such as L-rhamnulose and 6-deoxy-L-sorbose. Similar results have been obtained by Eagon (193). In his studies on L-fucose and L-rhamnose metabolism by microorganisms, this author reported that the crude extracts of L-fucose-grown bacteria contained a kinase which could specifically phosphorylate L-fuculose, but not L-rhamnulose, the situation was reversed when they were grown on L-rhamnose. Moreover, the L-fuculose kinase preparation exhibited no detectable activity with other isomers of L-fucuolse, such as L-rhamnose and L-fucose; this point will be discussed in more detail (see "Discussion"). The results of this experiment are shown in Table 19.

2. PREPARATION OF L-FUCULOSE-1-P

In order to characterize the product of the kinase reaction, large scale incubation mixtures (150 ml each) were prepared containing the

TABLE 19

SUGAR SPECIFICITY OF L-FUCULOSE KINASE[a]

Substrate	μMoles ADP Formed	Relative Activity
L-Fuculose	0.210	100
D-Glucose	0.135	64
D-Mannose	0.135	64
D-Fructose	0.097	46
D-Ribulose	0.078	37
D-Xylulose	0.074	35
D-Galactose	0.025	12
D-Arabinose	0.00	0
D-Ribose	0.00	0
D-Xylose	0.00	0
D-Psicose	0.00	0
D-Tagatose	0.00	0
3,6-Dideoxy-D-Galactose	0.00	0
3,6-Dideoxy-L-Galactose	0.00	0
L-Arabinose	0.00	0
L-Fucose	0.00	0
L-Rhamnose	0.00	0
L-Rhamnulose	0.00	0
L-Sorbose	0.00	0
6-Deoxy-L-Sorbose	0.00	0

[a]Incubation mixture contained the following (in μmoles): ATP, 5.0; substrate, 2.0; GSH, 5.0; Mg^{++}, 5.0; KF, 2.5; Tris buffer, pH 7.8, 25; and 20 μg of L-fuculose kinase (calcium-phosphate step) in a final volume of 0.25 ml. Controls without substrate were included. After 15 min incubation at 37°, the tubes were heated in a boiling water bath for 1 min, cooled, 2.0 μmoles of substrate were added to the control, and the volume of the mixtures were adjusted to 0.40 ml with water. Following centrifugation, aliquots (100 μl) from supernatant fluids were used for ADP determination as described.

following (in mmoles): L-fuculose, 1.5; ATP, 2.0; Tris buffer, pH 7.8, 4.0; KF, 1.0; GSH, 1.0; and 50 mg L-fuculose kinase (ammonium sulfate fraction). A control was included which contained the following (in μmoles): ATP, 20; Tris buffer, pH 7.8, 40; KF, 10; GSH, 10; and 0.5 mg of L-fuculose kinase (the same as above) in a final volume of 1.5 ml. The mixtures were incubated at 37°, and the reaction was followed by the determination of ADP in aliquots (0.025-0.05 ml) removed from each mixture at various intervals. As illustrated in Figure 24, the reaction reached a maximum after about 5 hours of incubation, at which time approximately 750 μmoles of L-fuculose-1-P had been formed (based on the ADP assay). An aliquot of the incubation mixture was assayed by the $Ba(OH)_2$-$ZnSO_4$ procedure (200); the amount of phosphorylated product by this method was estimated to be 780 μmoles.

The ketose-phosphates were isolated as follows: the reaction mixture was adjusted to pH 3.5 by the addition of a solution of 50% TCA, and the precipitate was removed by centrifugation. The supernatant fluid was extracted with three 100 ml portions of ethyl ether and the aqueous phase was fractionated on a column (30 cm x 3.5 cm^2) of Dowex-1-formate resin and eluted as described (see "Chromatographic procedures"). Two aliquots were removed from each fraction, the ketose content of one was determined by the cysteine-carbazole (202), and the other aliquot was used for the determination of ketohexose by the cysteine-sulfuric acid (228) method. L-Fuculose-1-P emerged from the column in a symmetrical peak between 350-450 ml of effluent (Figure 25). The appropriate frac-

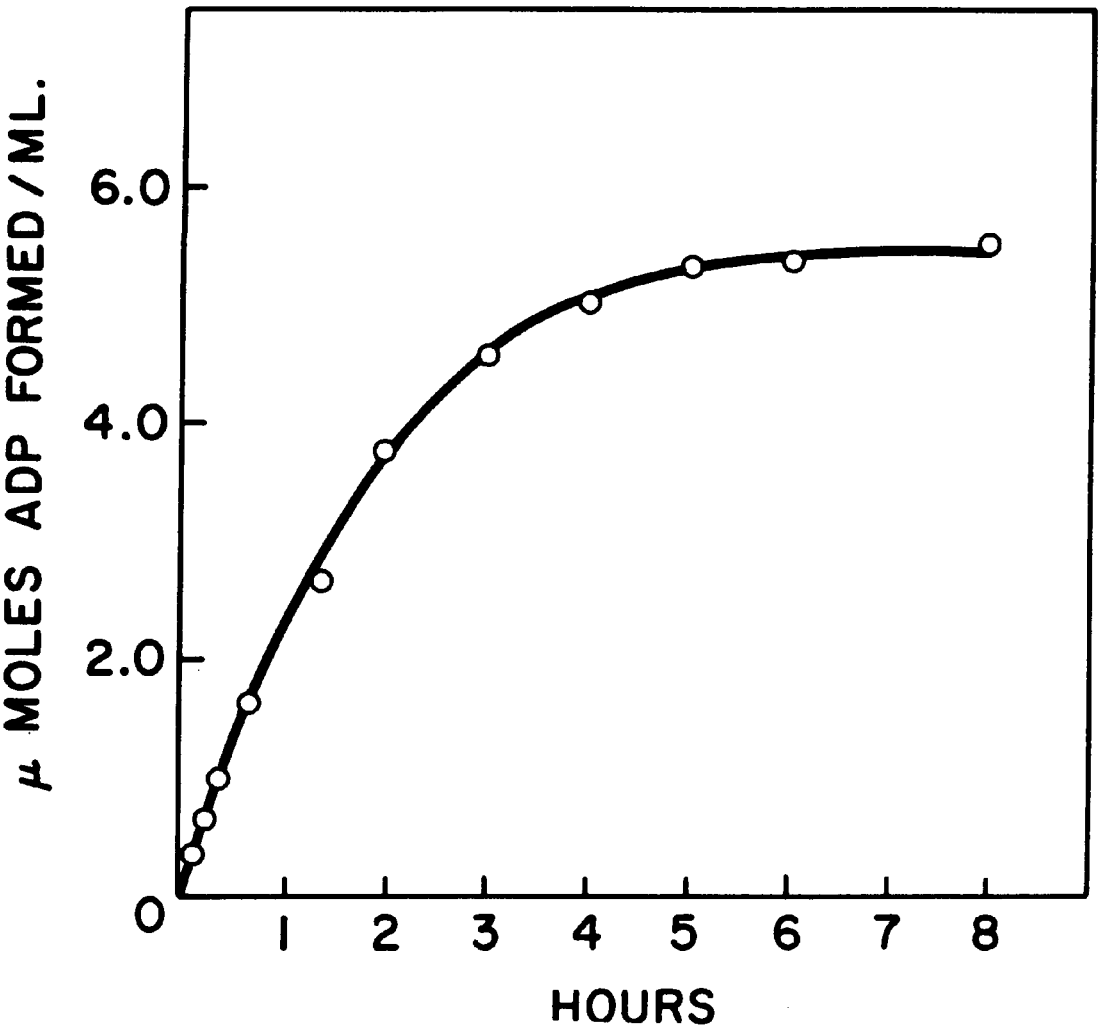


Figure 24. The large-scale preparation of L-fuculose-1-P.

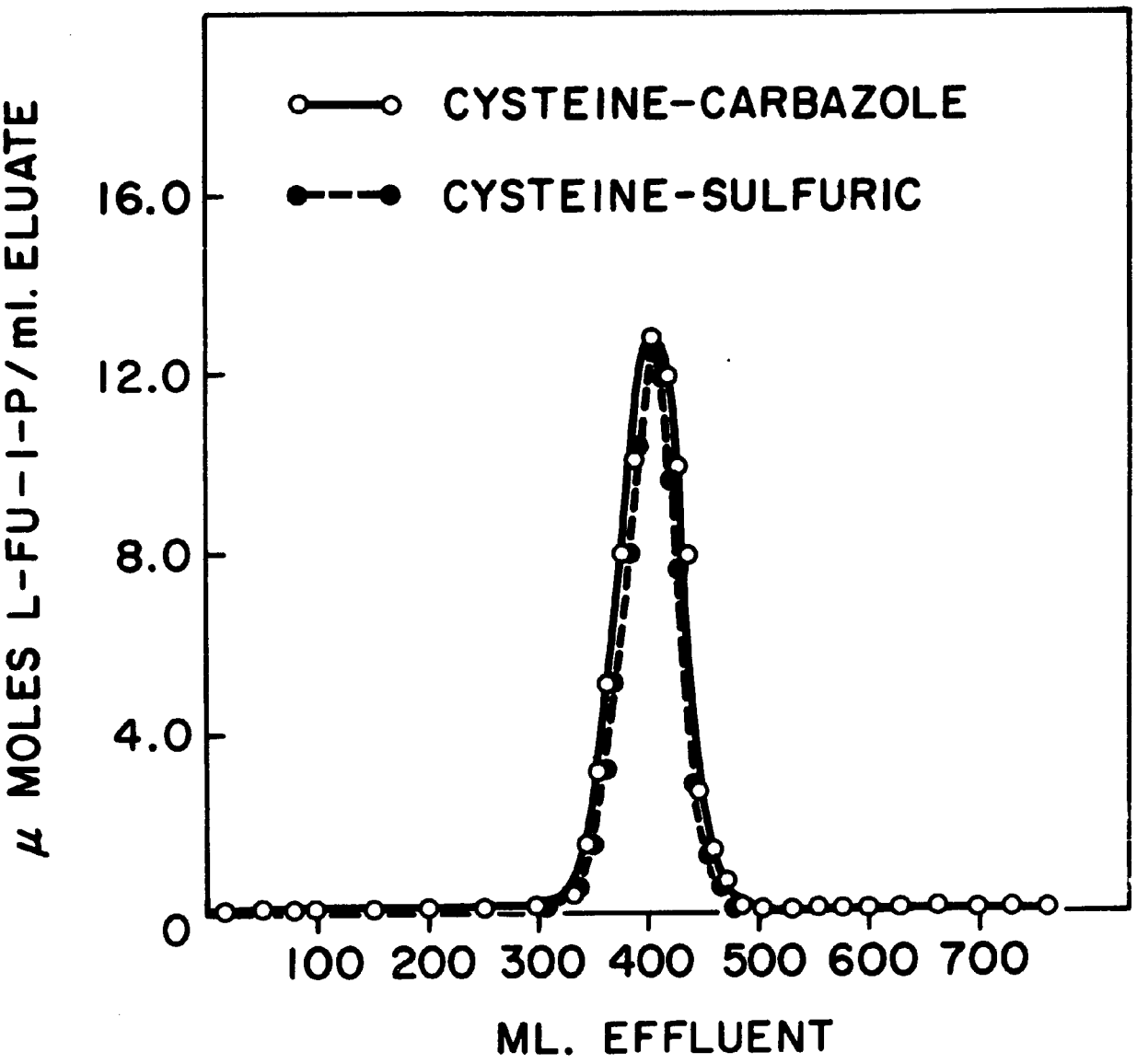


Figure 25. Elution pattern of L-fuculose-1-P. See text for details.

tions were combined, the ketose phosphate was obtained in the form of its barium salt (as described), and purified as follows: the barium salt was dissolved in dilute acetic acid, treated with charcoal (acid-washed Norit A) to remove small quantities of contaminating nucleotides, and the ketose-phosphate was reprecipitated as barium salt. The yield was approximately 650 μmoles.

To ascertain the homogeneity of the product, one μmole of it was streaked on Whatman 3 MM filter paper and subjected to paper electrophoresis (1000 volts/25 cm effective length) in 1% borate buffer, pH 7.0 for 20 min. After air drying, two guide strips were cut from lateral sides of the paper, one was sprayed with the TCA-orcinol reagent (203), and the other was treated with the phosphorus reagent of Axelrod and Bandursky (247). A single superimposable band was observed in each case indicating that the product was a ketose-phosphate.

3. CHARACTERIZATION OF THE REACTION PRODUCT

a. Analysis

The product of phosphorylation of L-fuculose was analyzed for inorganic phosphorus, total phosphorus, methylpentose, ketose and ketohexose. The results obtained (Table 20) indicated that the product was a 6-deoxy-ketohexose monophosphate.

To identify the sugar moiety of the product, an aliquot (5 μmoles) was treated with acid phosphatase and the free sugar was chromatographed in several solvent systems. In all instances the product behaved as an

TABLE 20

ANALYSIS OF L-FUCULOSE-1-P FORMED BY L-FUCULOSE KINASE

Analyses	μMoles/ml	Molar Ratios
Inorganic P	0.00	0.00
Total P	4.80	1.00
Methyl Pentose	4.50	0.94
Ketose	4.70	0.98
Ketohexose	4.65	0.97

Determinations were conducted as follows: phosphorus, by the method of Fiske-SubbaRow (238); methylpentose, by the cysteine-sulfuric acid test of Dische and Shettle (199); ketose, by the cysteine-carbazole procedure (202); and ketohexose by the cysteine-sulfuric acid method of Dische and Devi (228).

authentic sample of L-fuculose (Table 15), and when observed under the ultra-violet lamp after spraying with the TCA-orcinol reagent (203), showed the bright yellow fluorescence typical of L-fuculose. Furthermore, in the cysteine-sulfuric acid method for ketohexoses (228), the dephosphorylated product yielded a chromagen the spectrum of which was indistinguishable from that obtained for an authentic sample of L-fuculose (Figure 26). These data indicated that the product of phosphorylation of L-fuculose was L-fuculose-phosphate.

b. Acid Hydrolysis

The lability of the phosphate ester to acid hydrolysis is illustrated in Figure 27. Approximately 50% of the organic phosphorus of

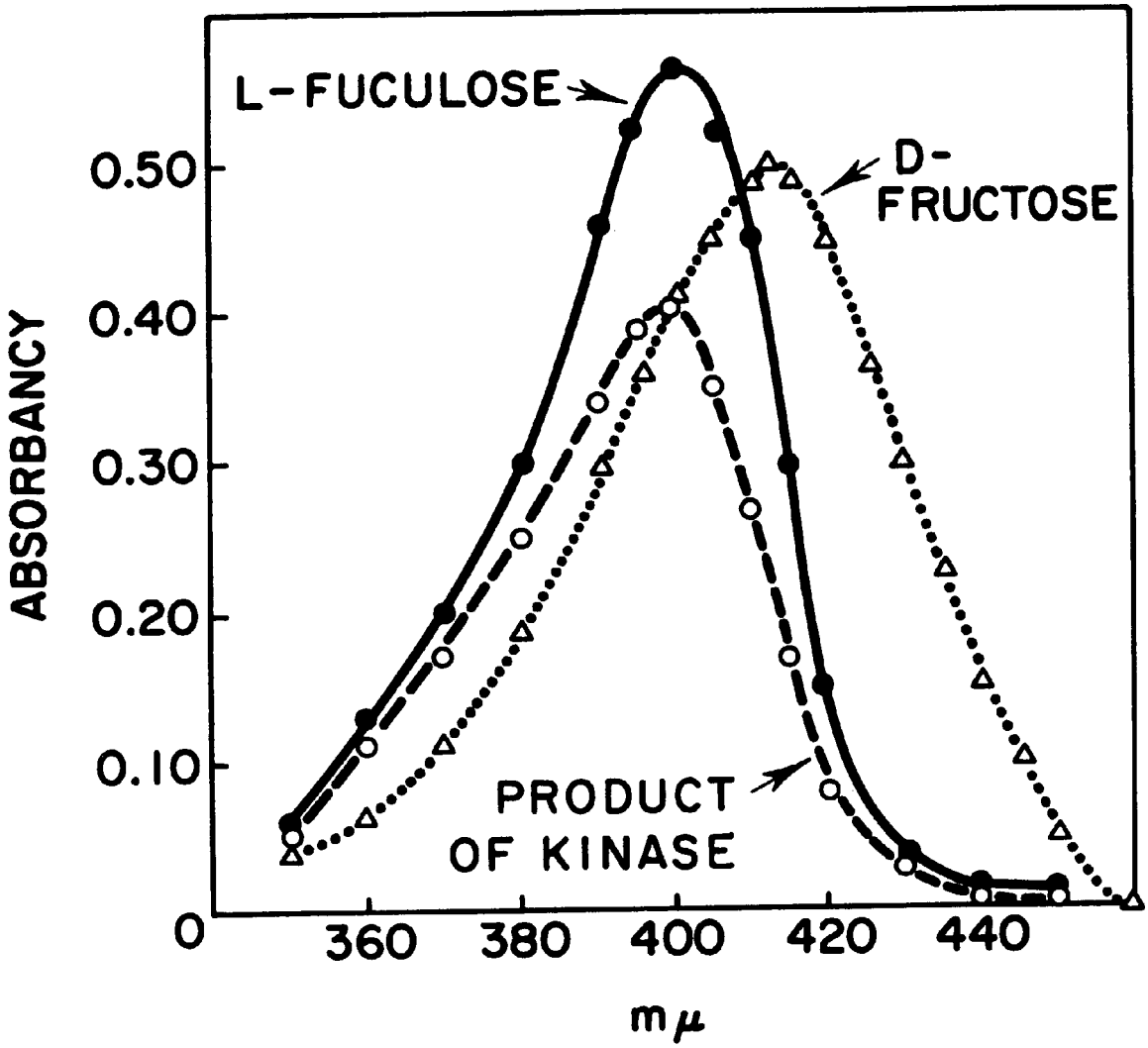


Figure 26. Absorption spectra of chromogens produced by various ketoses in the cysteine-sulfuric method of Dische and Devi (228).

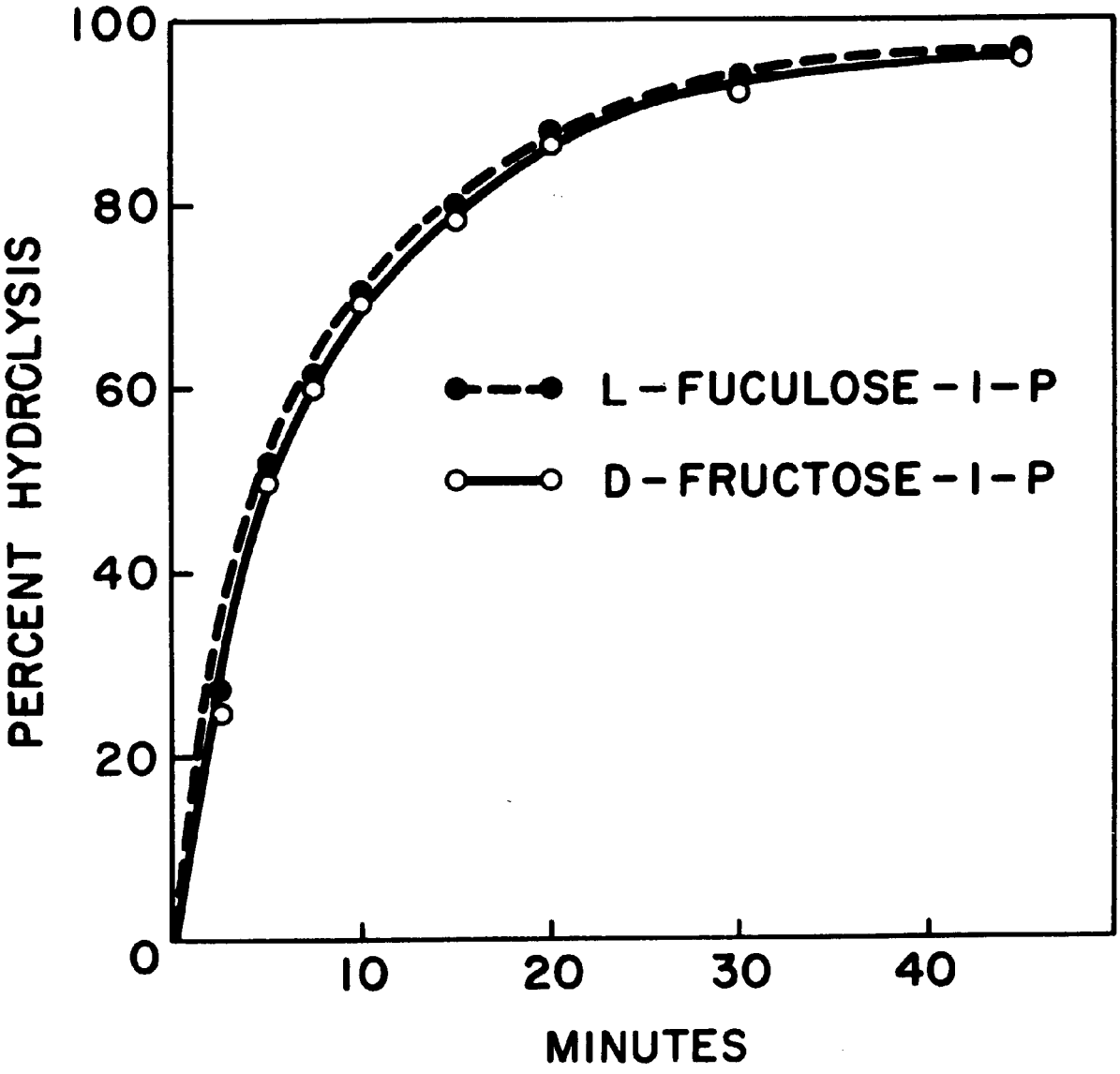


Figure 27. Acid hydrolysis of L-fuculose-1-P and D-fructose-1-P. The reaction mixture contained 2.5 μmoles of either L-fuculose-1-P or D-fructose-1-P in a total volume of 2.0 ml of 1N HCl. At zero time two 0.2 ml aliquots were removed from each reaction mixture, one of which was used for the determination of total phosphorus, and the other for inorganic phosphorus determination (229). The amount of inorganic phosphorus found in each case was negligible. Tubes were placed in a boiling water bath, and the course of hydrolysis was followed by determination of the inorganic phosphorus liberated. At various intervals an aliquot (0.2 ml) was removed from each of the reaction mixtures, immediately transferred to a tube containing 5.0 ml of ice-cold water, and the resulting solution was used for the determination of inorganic phosphorus. The amount of total phosphorus at zero time was taken as 100%, and the percent hydrolysis calculated as (inorganic phosphorus at each interval/total phosphorus at zero time) x 100 is plotted vs incubation time.

L-fuculose-phosphate was liberated in 5 min at 100° in 1N HCl. For comparison, D-fructose-1-P was subjected to the same treatment simultaneously; it exhibited a half-life of about 5 min. These results are in agreement with Tanko and Robinson's (251) finding that 55% of the organic phosphorus of D-fructose-1-P was liberated after 5 min under similar conditions. It must be mentioned that the half-life of hydrolysis of phosphate group of D-fructose-6-P requires a much longer period under the same conditions.

c. Periodate Oxidation

In order to confirm the position of the phosphate group, the product was subjected to periodate oxidation as follows: the reaction mixture contained 7.5 μmoles of L-fuculose-1-P and 200 μmoles of sodium periodate in a final volume of 4.0 ml. Following 4 hours of incubation in the dark, excess periodate was destroyed by the addition 100 μmoles of glucose. The oxidation products were applied to a column (15 cm x 0.8 cm^2) of Dowex-1 Cl^- resin, washed with 3 volumes of water, and eluted as previously described (see "Chromatographic procedures"). Aliquots of the fractions (5 ml) collected were tested for phosphoglycolic acid by the chromotropic acid procedure (236). Phosphoglycolic acid emerged from the column in fractions 9-17 which were pooled and concentrated in vacuo. A total of 5.8 μmoles of phosphoglycolic acid were recovered from the periodate oxidation products which corresponded to approximately 77% recovery. The molar ratios of organic phosphorus, and glycolic acid in the product

were found to be 1.0:0.97, indicating that the phosphate containing product obtained from periodate oxidation of L-fuculose-1-P was indeed phosphoglycolic acid. These results established the location of phosphate group in the 1 position of the molecule of ketose. Had the position of phosphate group been on any carbon atom other than 1, phosphoglycolic acid would not have been formed upon periodate oxidation. As illustrated in Figure 28, the complete periodate oxidation (4) of L-fuculose-1-P results in the formation of the following compounds (in moles): phosphoglycolic acid, 1; formic acid, 2; and acetaldehyde, 1. However, L-fuculose-5-P under the same conditions would yield the following (in moles): formaldehyde, 1; carbon dioxide, 1; formic acid, 1; and phospholactaldehyde, 1. Since phosphoglycolic acid will be formed upon periodate oxidation of ketose-phosphates only when the phosphate group is in the 1 position, therefore, it is concluded that the product of phosphorylation of L-fuculose is indeed L-fuculose-1-phosphate.

C. L-Fuculose-1-P Aldolase

1. PROPERTIES OF THE ENZYME

Effect of time of incubation: The dependence of cleavage of L-fuculose-1-P on the incubation time is illustrated in Figure 29. Single-step assay procedure gave a linear response for at least 20 min, whereas the two-step assay method gave linear results for about 15 min.

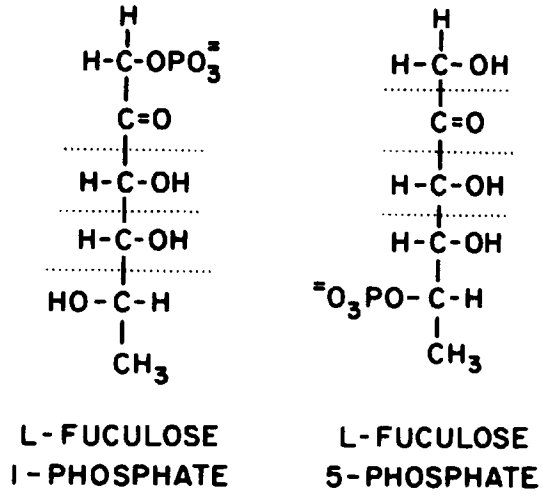


Figure 28. Periodate cleavage of L-fuculose-phosphates. See text for details.

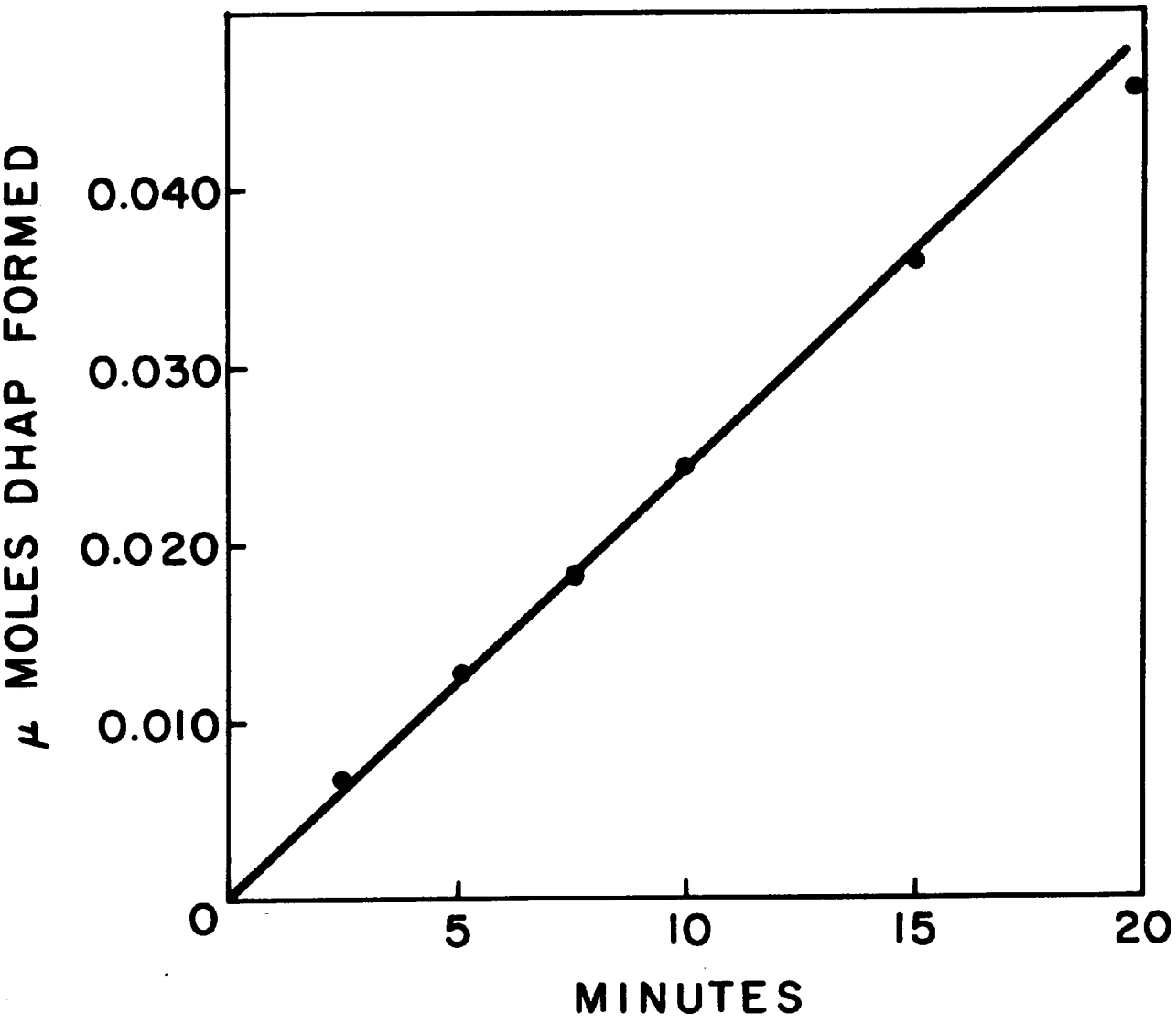


Figure 29. Effect of time of incubation on L-fuculose-1-P aldolase reaction. The incubation mixture contained the following (in μmoles): L-fu-1-P, 0.5; Tris buffer, pH 7.2, 10; DPNH, 0.10; crystalline α-GPD, 0.10 mg; and sufficient amounts of L-fuculose-1-P aldolase in a total volume of 0.30 ml. The rate determination was conducted by the single-step assay procedure.

Effect of enzyme concentration: As illustrated in Figure 30, the rate of DHAP formation was linear over 10-fold range of protein concentration.

Effect of pH of incubation: Figure 31, illustrates the influence of pH on the rate of the reaction of L-fuculose-1-P aldolase. As indicated, the pH optimum of the enzyme was found to be approximately 7.2.

Effect of substrate concentration: The dependence of the rate of DHAP formation on L-fuculose-1-P concentration is shown in Figure 32. When plotted according to the method of Lineweaver and Burk (250), the Km value was estimated to be 7×10^{-4}M.

Stoichiometry: The results of stoichiometry studies are shown in Table 21. As indicated, for each mole of L-fuculose-1-P which was cleaved, 1 mole of DHAP and 1 mole of lactaldehyde were formed.

Effect of cations: The cation requirement of L-fuculose-1-P aldolase is shown in Table 22. As indicated, approximately 50% of the activity was lost when L-fuculose-1-P aldolase preparations were dialyzed against distilled water, while the loss of activity exceeded 90% when the dialysis was carried out in the presence of EDTA. These dialyzed preparations could be reactivated (as high as 75%) by the inclusion of divalent cations (8×10^{-3}M) in the incubation mixtures, whereas, monovalent cations had little or no effect.

Effect of cysteine: The effect of cysteine HCl on crude preparations of L-fuculose-1-P aldolase is shown in Figure 33. These preparations contained both FDP and L-fuculose-1-P aldolase activity. The ratio

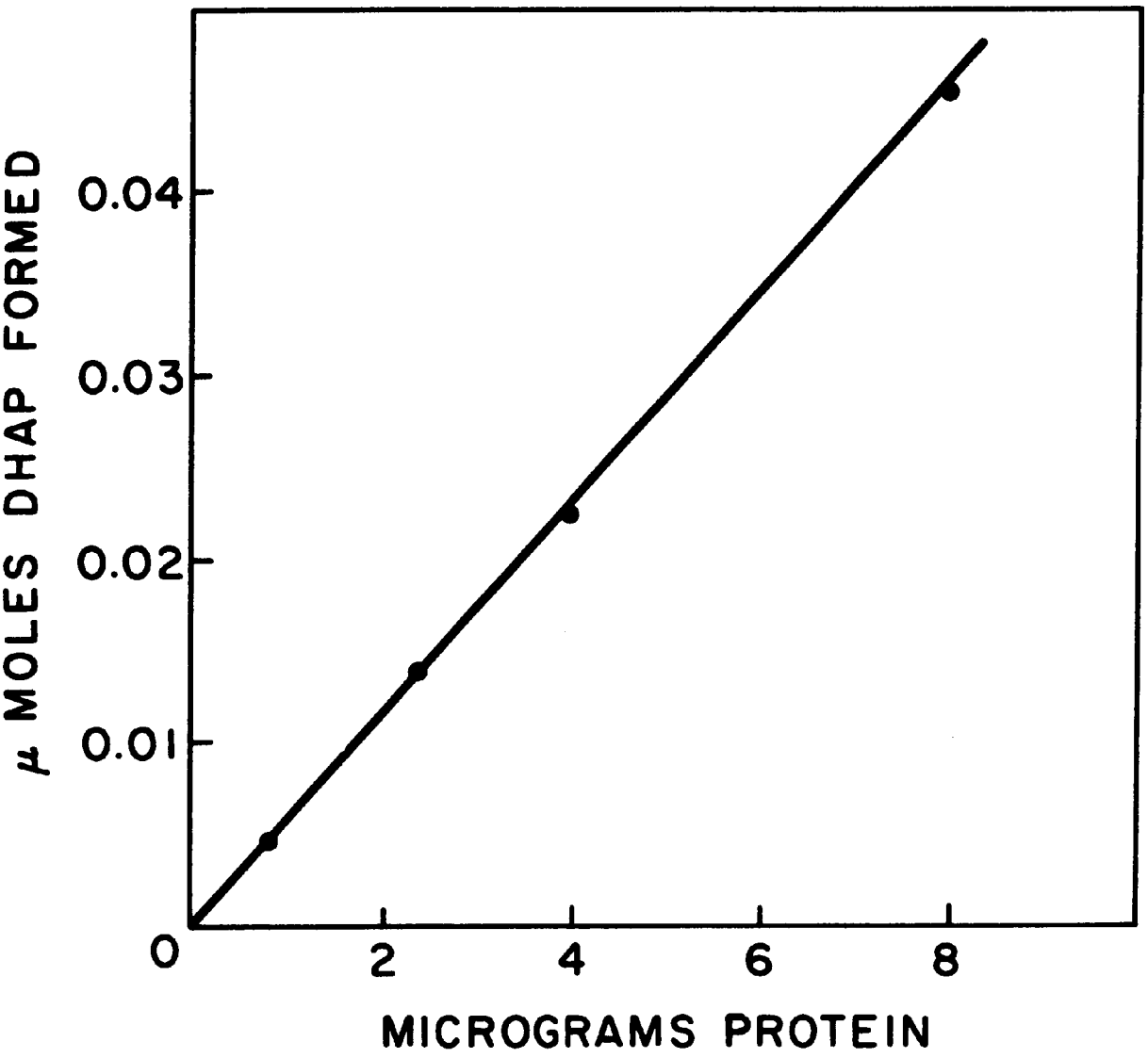


Figure 30. The reaction rate as a function of L-fuculose-1-P aldolase concentration. Conditions were as described in the text for the single-step assay, except that the enzyme concentration was varied.

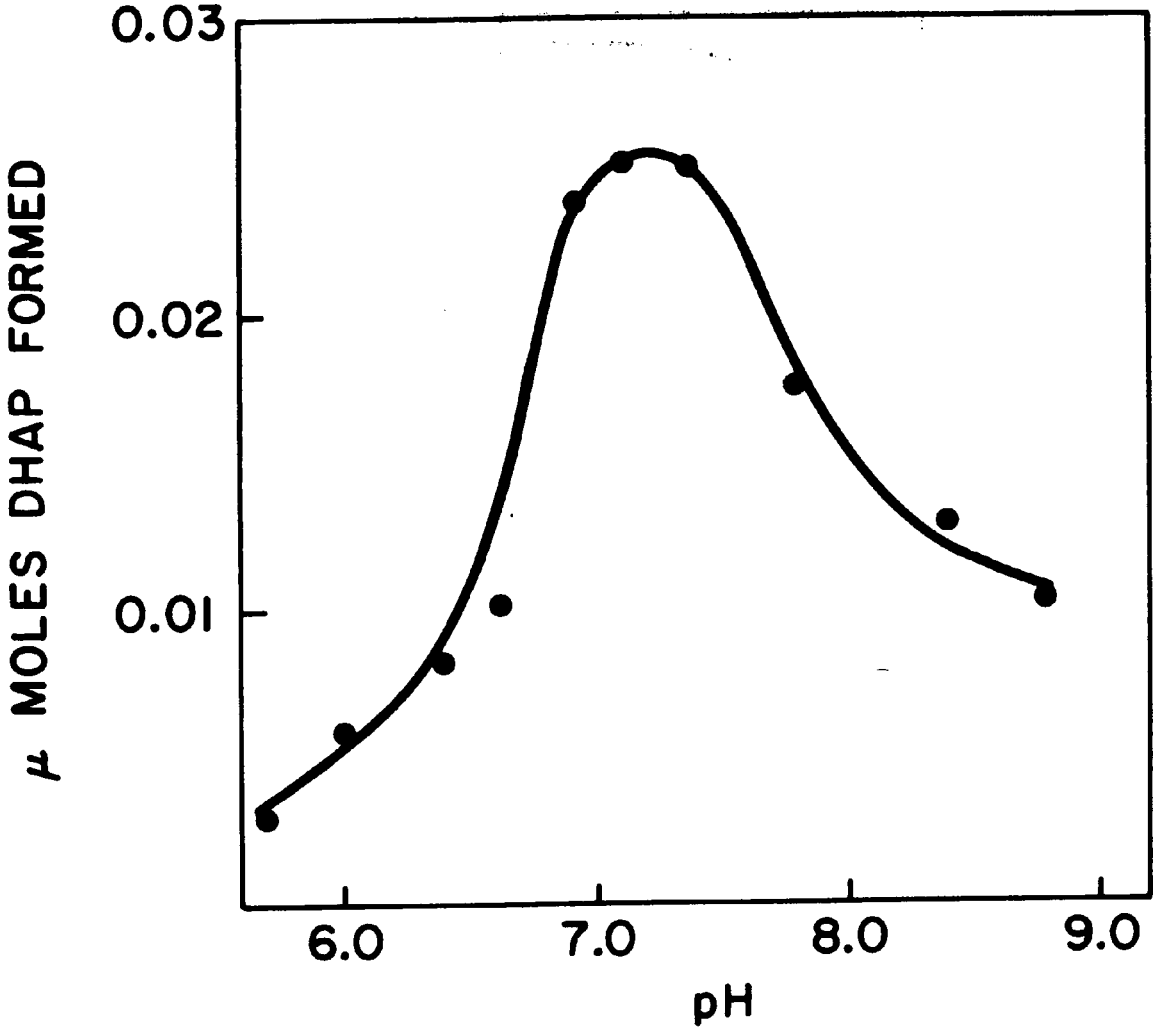


Figure 31. Effect of pH on the rate of L-fuculose-1-P aldolase reaction. Two-step assay procedure was applied.

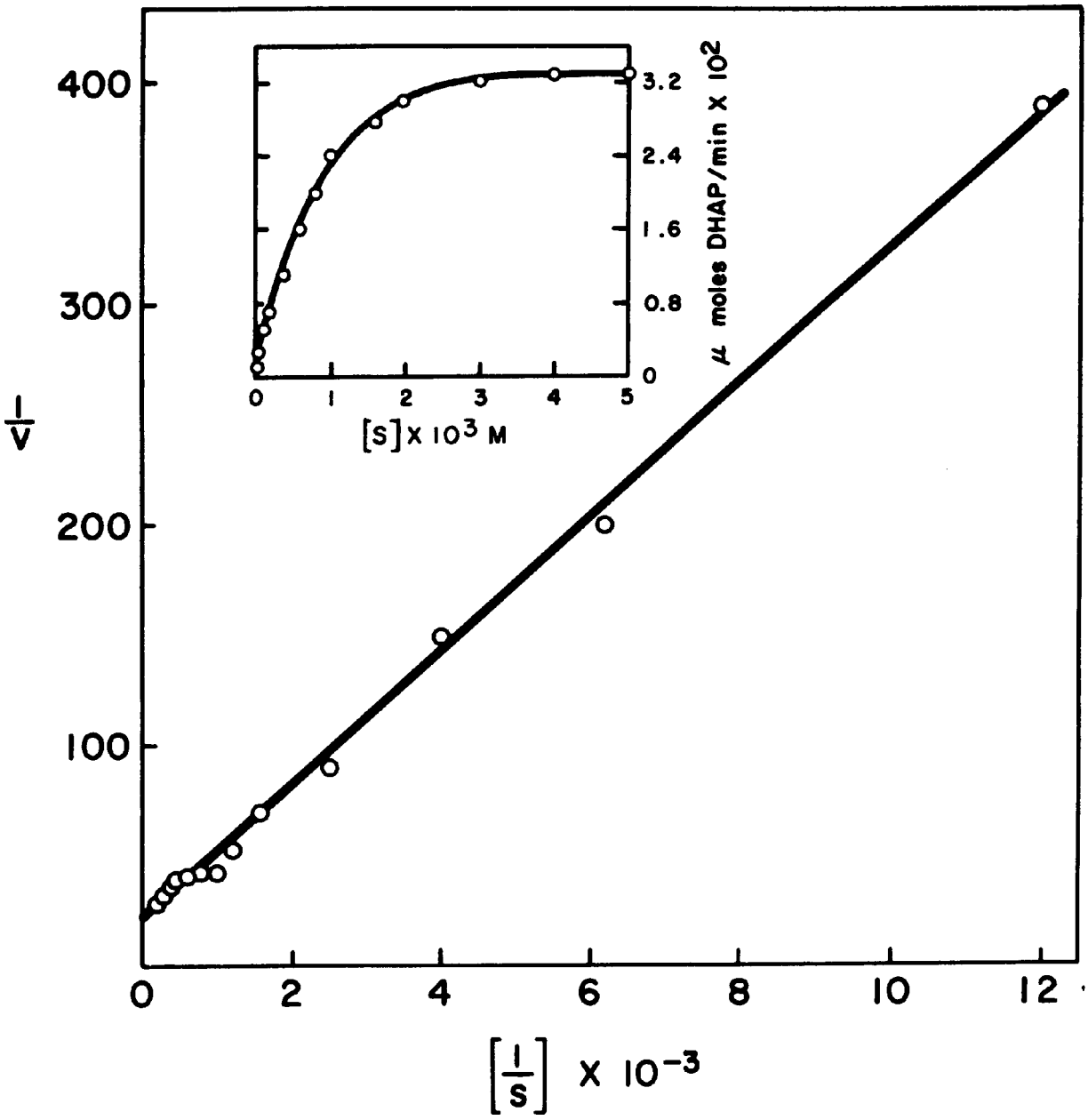


Figure 32. Effect of L-fu-1-P on the reaction rate of L-fuculose-1-P aldolase. Conditions were as described in the text for the single-step assay, except that the substrate concentration was varied.

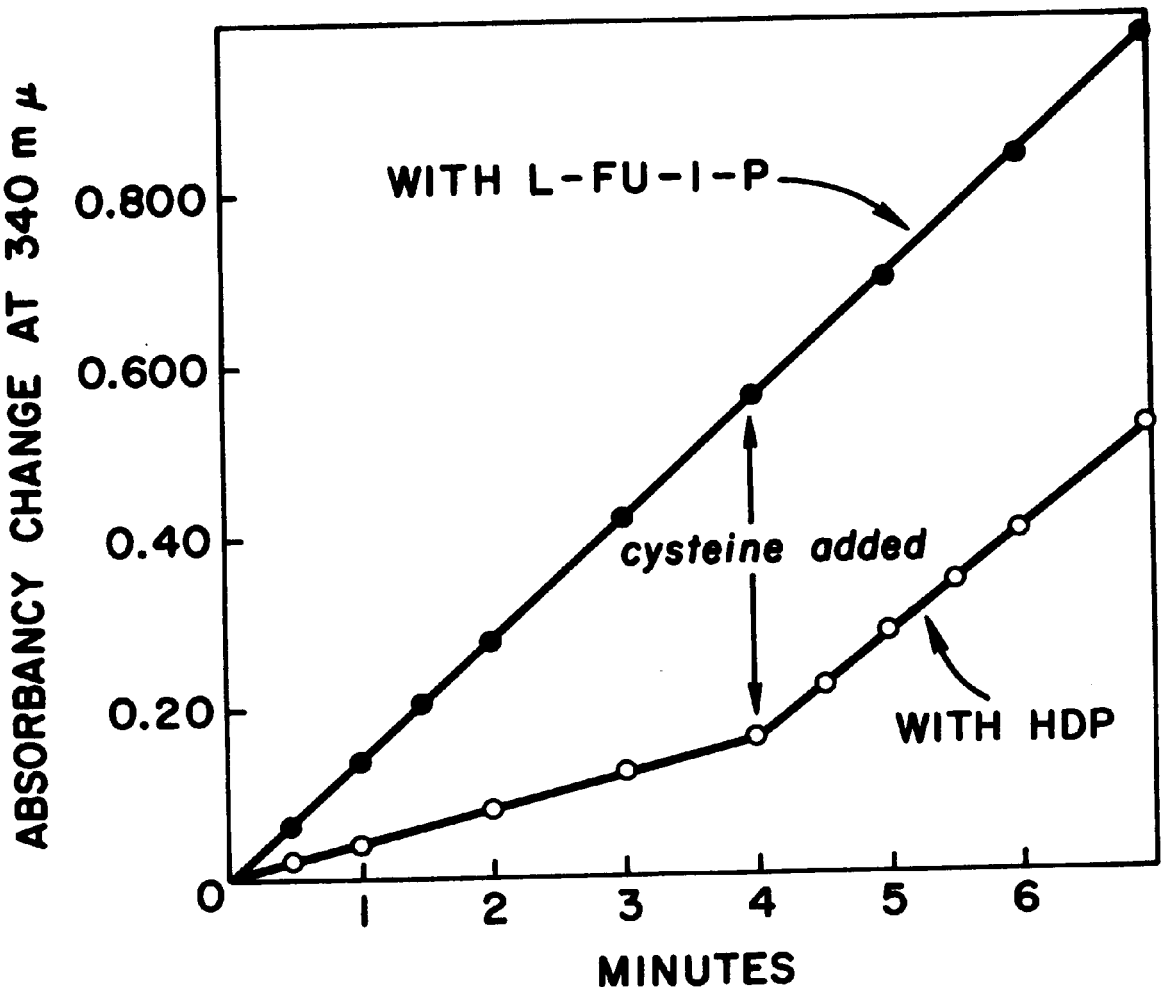


Figure 33. Effect of cysteine on L-fuculose-1-P aldolase and FDP-aldolase of Escherichia coli O111-B_4. The incubation mixture contained the following (in μmoles): L-fu-1-P, or FDP, 0.50; DPNH, 0.10; Tris buffer, pH 7.2, 10; 20 μg of L-fuculose-1-P aldolase preparations (isoelectric precipitation step contained FDP-aldolase also); and 0.10 mg of α-GPD in a total volume of 0.40 ml. The rate determinations were conducted continuously (as described for the single-step assay) for 4 min at which time 5.0 μmoles of cysteine were added to each cuvette.

TABLE 21

STOICHIOMETRY OF L-FUCULOSE-1-P ALDOLASE REACTION

Compounds	μMoles	Ratios
Experiment I		
L-Fuculose-1-P	-0.96	1.00
DHAP	+1.06	1.10
L-Lactaldehyde	+0.96	1.00
Experiment II		
L-Fuculose-1-P	-0.80	1.00
DHAP	+0.78	0.97
L-Lactaldehyde	+0.78	0.97

The incubation mixture contained the following (μmoles in a final volume of 3.0 ml): L-fuculose-1-P, 3.0; DPHN, 1.5; potassium fluoride, 20; Tris buffer, pH 7.2, 100; L-fuculose-1-P aldolase, 25 μg; and 0.1 mg of α-glycerol-P dehydrogenase. After incubation for 30 min at room temperature, the components of the incubation mixture were determined as follows: L-fuculose-1-P was determined by the specific keto-hexose method of Dische (228); the cysteine-sulfuric acid reagent does not react with ketotrioses. Dihydroxyacetone-P was determined enzymatically in the reaction mixture as it was formed. For the determination of L-lactaldehyde, an aliquot of the incubation mixture was deionized with mixed-bed ion exchange resin, and the lactaldehyde content of the neutral supernatant fluid was determined by the Barker-Summerson method (234).

TABLE 22

THE EFFECT OF DIALYSIS AND CATIONS ON L-FUCULOSE-1-P ALDOLASE

Fractions	Cations Added	Percent Activity	Specific Activity x 10^3
Undialyzed	---	100	314
Undialyzed	Mg^{++}	95	310
Undialyzed	Mn^{++}	100	314
(a)[a] Distilled Water	---	42	260
(b) Phosphate Buffer	---	58	360
(c) Phosphate Buffer + EDTA	---	16	98
(d) EDTA	---	6.7	38
EDTA	Mn^{++}	75	430
EDTA	Ca^{++}	54	300
EDTA	Mg^{++}	52	310
EDTA	K^{+}	16	100
EDTA	Na^{+}	16	100

[a]Purified preparations of L-fuculose-1-P aldolase were dialyzed at 4° for 12 hours against 200 volumes of the following solutions: (a) distilled water; (b) potassium phosphate buffer (0.01M), pH 7.0; (c) solution (b) containing 0.005M sodium EDTA; and (d) sodium EDTA (0.005M), pH 7.0. The dialyzed enzyme solutions were centrifuged and the L-fuculose-1-P aldolase activity in each case was assayed in each of two aliquots (5 µl and 10 µl) of the supernatant fluids. As a control for the effect of cations, aliquots from the undialyzed preparation were treated simultaneously in the same manner as the dialyzed preparations. The cations were added as their chloride salts. The conditions of the assay were the same as the single-step method, except that KF was not included in the incubation mixture.

of $\frac{\text{FDP-aldolase activity}}{\text{L-Fu-1-P aldolase activity}}$ was 1:3; the addition of cysteine to the incubation mixture stimulated the FDP-aldolase activity, while it had no influence on the rate of the reaction of L-fuculose-1-P aldolase. A quantitative study of the effect of cysteine on this crude preparation indicated that the stimulation of the FDP-aldolase activity was dependent on the concentration of cysteine in the incubation mixtures, whereas, the activity of L-fuculose-1-P aldolase remained constant over 20-fold range of cysteine concentration (Table 23). The necessity of sulfhydryl compounds for the maximum activity of FDP-aldolases of Escherichia coli (252), Clostridium perfringens (253), and Brucella suis (254) have also been reported. Stimulation by sulfhydryl containing compounds has also been observed with other aldolases (255).

2. CHARACTERIZATION OF THE REACTION PRODUCTS

a. DHAP

DHAP was characterized primarily on the basis of its reactivity with crystalline α-GPD in the presence of DPNH. In addition, chromatography of the cleavage products of L-fuculose-1-P (in Solvent J = methanol, 6; ammonium hydroxide, 1; water, 3) yielded a component which gave a positive test for organic phosphorus (247) and its relative mobility corresponded to that of authentic DHAP ($R_{\text{fuculose-1-P}} = 0.8$).

b. L-Lactaldehyde

The characterization of L-lactaldehyde, as the other product of the

TABLE 23

THE EFFECT OF CYSTEINE ON THE RATE OF REACTION OF L-FU-1-P ALDOLASE

Cysteine (μMoles/ml)	Percent Activity With	
	FDP	L-Fu-1-P
No Cysteine	20	100
1.25	30	100
2.50	40	100
6.25	48	---
12.50	73	100
25.00	80	100

The incubation mixture contained the following (in μmoles): substrate (FDP or L-Fu-1-P), 1.0; Tris buffer, pH 7.2, 10; crude L-fuculose-1-P aldolase preparation, 10 μl; and varying amounts of cysteine HCl (neutralized to pH 6.8) in a total volume of 0.40 ml. After measurement of initial absorbancies at 340 mμ, 5 μl of a solution of α-GPD was added to each cuvette, and the reaction was followed for 10 min.

reaction, was complicated by its instability in neutral and alkaline solutions (223). After deionization, the neutral product gave a positive Barker-Summerson color reaction (234). Paper chromatography indicated a single component which corresponded in all cases to authentic lactaldehyde (Solvent A; R_f, 0.78; Solvent J; Rf, 0.87). It was possible to prepare a derivative of the lactaldehyde formed in the incubation mixture by trapping it with hydrazine (256), and subsequently converting this material to the bis-2,4-dinitrophenylhydrazone derivative. For the preparation of this derivative the following incubation mixture was

prepared (μmoles in a final volume of 50 ml); L-fuculose-1-P, 210; Tris buffer, pH 7.0, 1500; hydrazine sulfate, 1,000; and L-fuculose-1-P aldolase, 2.0 mg. After incubation for one hour at 37°, the incubation mixture was deproteinized by the addition of one-fifth volume of 50% trichloracetic acid, and centrifuged. To this solution (60 ml) were added 50 ml of 2N HCl containing 200 mg of 2,4-dinitrophenylhydrazine, and the mixture was maintained at room temperature for 5 hours, heated on a steam bath for 30 min,and stored overnight at 4°. The resulting solid was harvested, washed with water, and extracted with ethyl acetate. The ethyl acetate soluble material was concentrated in vacuo ,and the bis-2,4-dinitrophenylhydrazone derivative was precipitated with petroleum ether. The crystals thus obtained were dried and exhibited a decomposition point (297-299°: uncorrected) which corresponded to that reported for the authentic bis-2,4-dinitrophenylhydrazone derivative of lactaldehyde (226). In addition, on admixture of the unknown with the authentic lactaldehyde derivative, the decomposition point remained the same. Since this derivative was optically inactive, it was not possible to ascertain the configuration of the lactaldehyde obtained from the enzymatic reaction. However, further studies described below (see "Reversibility of the reaction") indicate that the product formed by cleavage of L-fuculose-1-P is indeed L-lactaldehyde.

3. REVERSIBILITY AND EQUILIBRIUM

Preliminary experiments indicated that a ketohexose-P was formed

when L-lactaldehyde and dihydroxyacetone-P were incubated with the purified L-fuculose-1-P aldolase. An aldol-type condensation of these two substances could result in the formation of one or more 6-deoxy ketose 1-phosphates, depending upon the specificity of the enzyme. To resolve this question, the products of the reaction were treated with potato acid phosphatase and subjected to paper chromatography in 2 solvent systems. When the developing solvent was Solvent B, a single component was obtained which corresponded in mobility to L-fuculose ($R_{glucose}$, 2.3). In addition, the compound was clearly distinguishable from 6-deoxy-L-sorbose ($R_{glucose}$, 2.46) and D-fructose ($R_{glucose}$, 1.32), and when observed under the ultra-violet lamp after spraying with the trichloracetic acid-orcinol reagent (203), showed the bright yellow fluorescence typical of L-fuculose. In Solvent A (using Schleicher and Schuel 589 Blue Ribbon filter paper), L-fuculose and 6-deoxy-L-sorbose exhibited R_f values of 0.34 and 0.39 respectively. There was no indication on these paper chromatograms of the presence of any ketohexose other than L-fuculose in the incubation mixture.

A quantitative study of the equilibrium is shown in Figure 34, starting with either L-fuculose-1-P,or with L-lactaldehyde and dihydroxyacetone-P. Under these conditions (0.002M substrate), the equilibrium mixtures obtained in both cases consisted of approximately 60% L-fuculose-1-P and 40% of the cleavage products. The average value for the equilibrium constant in the direction of cleavage:

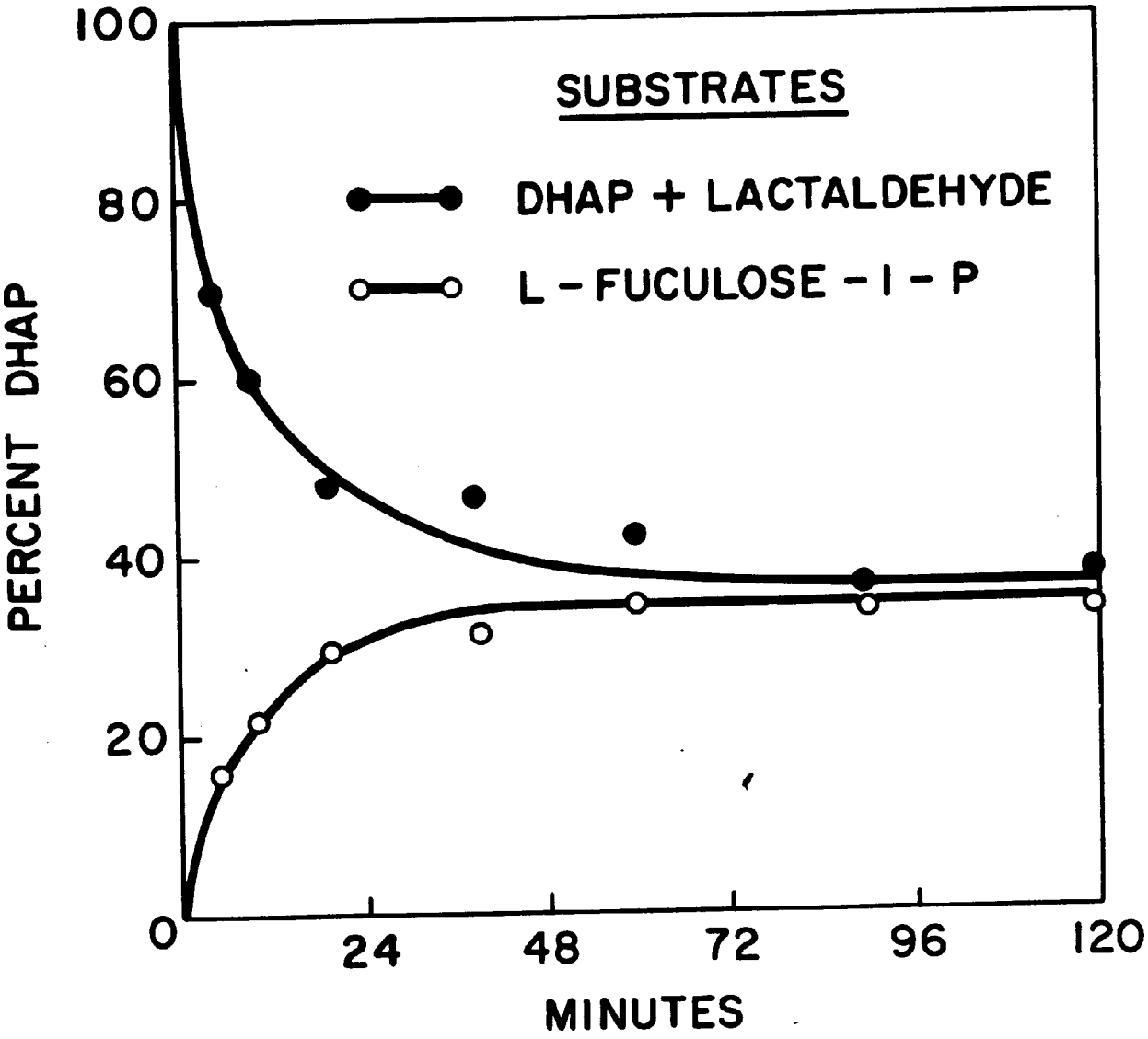


Figure 34. Reversibility and equilibrium studies. Incubation mixtures contained the following (in μmoles); either L-fuculose-1-P, 1; or dihydroxyacetone-P, 1, and L-lactaldehyde, 1; Tris buffer, pH 7.2, 25; and 50 μg of L-fuculose-1-P aldolase (calcium phosphate gel II), in a total volume of 0.5 ml. The mixtures were incubated at 37°, two separate aliquots were removed from each incubation mixture at the times indicated, and heated in a boiling water bath for 1 min. One of the aliquots was used for the determination of L-fuculose-1-P (228) and the other aliquot was used for the determination of dihydroxyacetone-P (see text).

$$\left(K = \frac{[\text{dihydroxyacetone-P}][\text{L-lactaldehyde}]}{[\text{L-fuculose-1-P}]}\right)$$

was 4.6×10^{-4}M at 37°.

4. SPECIFICITY STUDIES

L-Fuculose-1-P aldolase was tested for its ability to catalyze the cleavage of a variety of ketose phosphates. The data illustrated in Table 24 indicate that, of the compounds tested, only L-fuculose-1-P was cleaved significantly, demonstrating a requirement at least for the cis configuration of hydroxyl groups at C-3,4, and distinguishing this enzyme from FDP-aldolase. The only other available ketose-1-P with a cis C-3,4 configuration was D-ribulose-1,5-diP which was essentially inactive in the system. However, the inability of the enzyme to catalyze the cleavage of this compound may be due to the fact that the latter possesses a second phosphate group in the C-5 position (see below).

Further evidence to support the contention that the enzymatic cleavage of L-fuculose-1-P does not involve FDP-aldolase is presented in Figure 35. Under these conditions, incubation of L-fuculose-1-P aldolase with D-fructose-1,6-diP resulted in no significant cleavage, while L-fuculose-1-P rapidly yielded dihydroxyacetone-P. When crystalline muscle aldolase was added to each of these incubation mixtures, there was a rapid cleavage of fructose-diP while there was no influence on the rate of cleavage of L-fuculose-1-P.

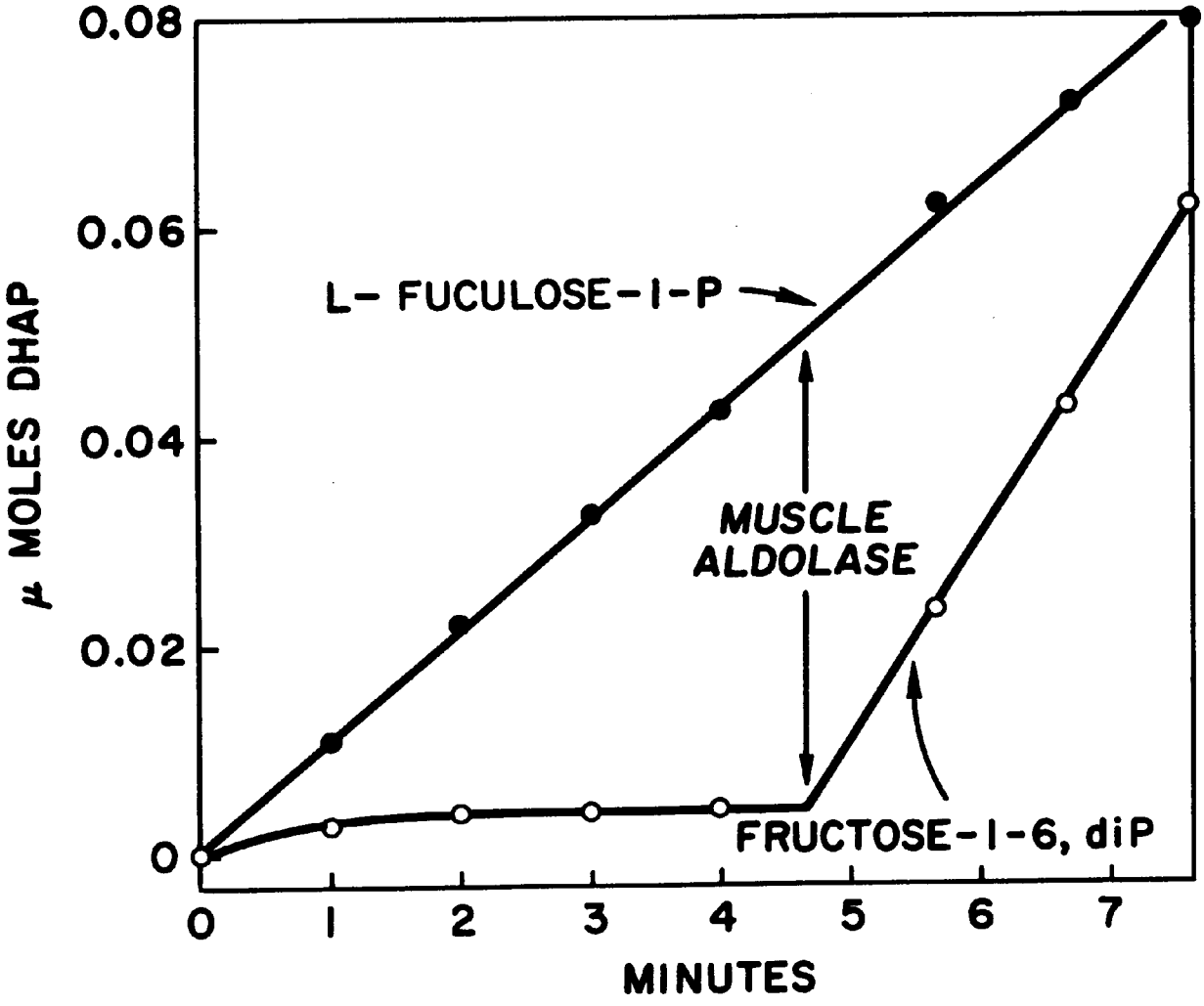


Figure 35. Specificity distinction between L-fuculose-1-P and FDP-aldolase. Incubation mixtures contained the following (in μmoles): L-fuculose-1-P, 0.2 (solid circles); D-fructose-1, 6-diP, 0.2 (open circles); DPNH, 0.1; Tris buffer, pH 7.2, 10; 100 μg of α-glycerol-P dehydrogenase; the reaction was initiated by the addition of 8 μg of L-fuculose-1-P aldolase (Calcium Phosphate Gel II). The final volume was 0.4 ml. At the time indicated by the arrow, 1.0 μg of HDP-aldolase was added to each mixture.

TABLE 24

SUGAR PHOSPHATE SPECIFICITY

Compound	C-3,4 Configuration	Relative Activity
L-Fuculose-1-P	cis	100
D-Fructose 1,6-diP	trans	4
6-Deoxy-L-Sorbose-1-P	trans	5
L-Sorbose-1-P	trans	0
D-Fructose-1-P	trans	4
D-Ribulose 1,5-diP	cis	6

Incubation mixtures contained the following in μmoles: ketose phosphate, 0.25; Tris buffer, pH 7.2, 10; DPNH, 0.06; 25 μg of α-glycerol-P dehydrogenase; and 2 μg of L-fuculose-1-P aldolase (calcium phosphate II), in a total volume of 0.25 ml. Initial absorbancies at 340 mμ were measured, the reaction initiated by adding the aldolase, and the absorbancies followed continuously for 10 minutes.

Specificity studies on L-fuculose-1-P aldolase in the direction of condensation were undertaken to determine whether or not aldehydes other than L-lactaldehyde would serve as substrate. The results of these studies are shown in Table 25. In addition to L-lactaldehyde, D-lactaldehyde, glycolaldehyde, and D- or L-glyceraldehyde also served as substrates for the enzyme while formaldehyde, acetaldehyde, and D-flyceraldehyde-3-P were inactive. The inability of D-glyceraldehyde-3-P, formaldehyde, and acetaldehyde to act as substrate with this enzyme contrasts with the aldehyde specificity of FDP-aldolase (257-260).

TABLE 25

ALDEHYDE SPECIFICITY

Compound	Relative Activity
L-Lactaldehyde	100
D-Lactaldehyde	73
Formaldehyde	<0.5
Acetaldehyde	<0.5
Glycolaldehyde	10
D-Glyceraldehyde	33
L-Glyceraldehyde	33
D-Glyceraldehyde-3-Phosphate	<0.2

Incubation mixtures contained the following (in μmoles): aldehyde, 2.5 (however, compounds which exhibited no detectable activity under the standard conditions, were tested at substrate levels up to 25 μmoles per incubation); dihydroxyacetone-P, 2; Tris buffer, pH 7.2, 25; and 5 μg of L-fuculose-1-P aldolase (calcium phosphate gel II), in a total volume of 0.25 ml. At appropriate intervals, aliquots were removed, heated in a boiling water bath for 1 min, and assayed as follows: (1) All samples were assayed for the remaining dihydroxyacetone-P by the method described in the text. (2) Mixtures containing 3-carbon aldehydes were analyzed for ketohexose content by the method of Dische and Devi (228). (3) Mixtures containing acetaldehyde and glycolaldehyde were assayed for ketopentose content by the method of Mejbaum (232). (4) Mixtures containing formaldehyde were assayed for keto-tetrose content by the method of Dische and Dische (233).

In instances where dihydroxyacetone-P disappearance and the formation of a condensation product were detected, the values obtained agreed within experimental error, and the averages of these values are presented in the table.

As the specificity of L-fuculose-1-P aldolase in the direction of cleavage sppeared to require the cis configuration of hydroxyls at C-3,4 of the ketose phosphate, it was of interest to determine whether or not this specificity was retained when aldehydes other than lactaldehyde were substrates. Preliminary experiments were conducted to determine whether or not the ketose phosphates formed as a result of the condensation of dihydroxyacetone-P with D-lactaldehyde, D-glyceraldehyde, L-glyceraldehyde, and glycolaldehyde possessed the cis configuration of hydroxyl

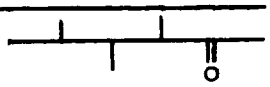
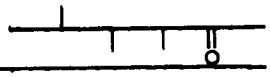
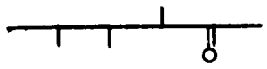
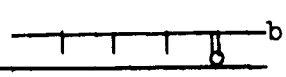
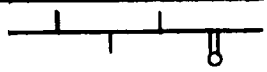
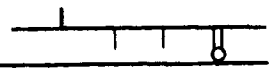
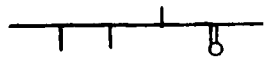
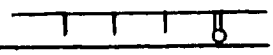
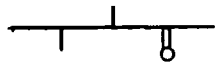
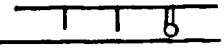
groups at C-3,4. Thus, the ketose phosphates resulting from the condensation of dihydroxyacetone-P and these aldehydes in the presence of L-fuculose-1-P aldolase were isolated, the phosphate groups removed with potato acid phosphatase, and the resulting sugars characterized by colorimetric methods as well as by paper chromatography. For comparison, similar experiments were conducted in which FDP-aldolase was substituted for L-fuculose-1-P aldolase. These data are summarized in Table 26. It can be seen that each aldehyde gave a single ketose phosphate, corresponding to the product expected if L-fuculose-1-P aldolase was indeed specific for the cis configuration of hydroxyl groups at C-3,4 and formed only a single diastereoisomer. Further characterization of these ketose phosphates was achieved by bisynthesis of sufficient quantities of each and preparation of appropriate derivatives (see below).

5. ENZYMATIC SYNTHESIS OF NEW KETOSE-1-PHOSPHATES

The study of the reversibility of the L-fuculose-1-P aldolase reaction indicated that this enzyme was specific for the cis hydroxyl groups at C-3,4 of the molecule of ketose-phosphate, and that a single diastereoisomer was formed (see Table 26). These data indicated (by chromatographic criteria), that the products of condensation of DHAP with D-glyceraldehyde, L-glyceraldehyde, and glycolaldehyde were D-psicose-1-P, L-tagatose-1-P,and D-ribulose-1-P, respectively. In order to characterize these products of condensation more definitively, large-scale preparations of these ketose-phosphates were undertaken. For the synthesis of the compounds indicated, the following incubation mixtures were prepared (in μmoles).

TABLE 26

CONDENSATION PRODUCTS WITH VARIOUS ALDEHYDES[a]

Aldehyde	Enzyme	Product R_{Gluc}	Standards		
			R_{Gluc}	Name	Configuration
L-Lactald.	H	2.48	2.46	6-Deoxy-L-Sorbose	
	F	2.30	2.30	L-Fuculose	
D-Lactald.	H	2.32	2.30	D-Rhamnulose	
	F	2.48	--	----	 [b]
L-Glycerald.	H	1.23	1.2	L-Sorbose	
	F	1.42	1.42	L-Tagatose	
D-Glycerald.	H	1.37	1.32	D-Fructose	
	F	1.74	1.72	D-Psicose	
Glycolald.	H	2.65	2.65	D-Xylulose	
	F	2.20	2.24	D-Ribulose	

[a]The following abbreviations were used in this table: L-Lactald., L-Lactaldehyde; D-Lactald., D-lactaldehyde; L-Glycerald., L-glyceraldehyde; D-Glycerald., D-glyceraldehyde; Glycolald., glycolaldehyde.

Incubation mixtures contained the following (in μmoles) in a final volumes of 1.0 ml: dihydroxyacetone-P, 5; aldehyde, 5; Tris buffer, pH 7.2, 50; and either L-fuculose-1-P aldolase (F), 12 μg, or HDP-aldolase (H), 5 μg. Incubation was conducted at 37° for 1 hour and each mixture was applied to a column containing 2 ml of Dowex-1-formate resin (200 to 400 mesh), washed with 5 column volumes of water, and the sugar phosphates were eluted with 0.4 N formic acid containing 0.1M ammonium formate. Fractions (3 ml) were collected and analyzed for ketose phosphate as indicated in the text; the fractions containing the product (usually numbers 3 to 7) were pooled, adjusted to pH 6.8, and concentrated in vacuo to 1 ml. The sugar phosphates were then treated with

TABLE 26 (Concluded)

acid phosphatase, deionized with mixed-bed resin, and chromatographed (Schleicher and Schuell, 589 (Blue Ribbon paper) with n-butanol; pyridine; water (3:1:3) as the developing solvent. (Multiple development technique was applied.) The ketoses were detected on the chromatograms with the trichloroacetic acid-orcinol reagent (203); with each aldehyde used, only a single spot was detectable on the chromatogram. Similar results were obtained when ethyl acetate; acetic acid; water (3:1:3) was the developing solvent.

[b]Authentic 6-deoxy-psicose was not available for comparison with the product formed in this case. The configuration presented in the table therefore is inferred by analogy with the results obtained with the other aldehydes.

D-Ribulose-1-P: Glycolaldehyde, 200; DHAP, 200; Tris buffer, pH 7.2, 2000; KF, 200; and 12 mg of L-fuculose-1-P aldolase (calcium phosphate II) in a total volume of 48 ml.

D-Psicose-1-P: D-Glyceraldehyde, 200; DHAP, 200; Tris buffer, pH 7.2, 2000; KF, 200; and 12 mg of L-fuculose-1-P aldolase (calcium phosphate II) in a total volume of 43 ml.

L-Tagatose-1-P: L-Glyceraldehyde, 200; DHAP, 180; Tris buffer, 2000; KF, 200; and 9 mg of L-fuculose-1-P aldolase (clacium phosphate gel II) in a final volume of 40 ml.

All of the mixtures were incubated at 37°, and the reaction was followed by removal of aliquots from each incubation mixture at various time intervals (0, 30, 90, 120, and 150 min) and determination of the ketose-phosphates synthesized as follows: the ketohexoses were determined by the method of Dische and Devi (228), and ketopentoses by the cysteine-carbazole method (202) (values were determined at 540 mμ after 1 hour to eliminate the interference of the color formed by DHAP with that of ribulose). After 2 hours of incubation, the maximum ketose formation was attained in all cases. On the basis of the above colorimetric methods, the following amounts of ketose-phosphates were synthesized (in μmoles): D-ribulose-1-P, 150; D-psicose-1-P, 140; and L-tagatose-1-P, 135.

The ketose-phosphates were isolated as follows: the incubation mixtures were adjusted to pH 3.0 by the addition of TCA, and the precipitates were removed by centrifugation. Each supernatant fluid was extracted

with three 100 ml portions of ether to remove TCA and then fractionated on a column (25 cm x 3.5 cm^2) containing Dowex-1-formate and eluted as described (see "Chromatographic procedure), except in this case the eluent consisted of 0.4N formic acid containing 0.1M ammonium formate. The fractions containing the ketose-phosphates (175 ml to 250 ml in each instance) were pooled and adjusted to pH 6.5 with potassium hydroxide. The ketose-phosphates were precipitated as barium salts and purified as described. Each of the preparations was dissolved in water, and barium was removed by treatment with potassium sulfate. Analysis of the solutions with cysteine-carbazole method for ketoses (202) indicated the following yields of the products (in μmoles): D-psicose-1-P, 120; L-tagatose-1-P, 130; and D-ribulose-1-P, 140.

Final characterization of the condensation products as the 1-phosphate esters of D-ribulose, D-psicose and L-tagatose was conducted as follows.

a. Analysis

Each of the products of condensation indicated a ketose (cysteine-carbazole method, 202) to organic phosphorus (229) ratio of 1.0 to 1.0 (within experimental error), with no detectable amounts of inorganic phosphorus (Table 27). In addition, the products of condensation of D- and L-glyceraldehyde with DHAP were analyzed by the cysteine-sulfuric acid method (228), whose values corresponded to those obtained by cysteine-carbazole test, indicating that the products were ketohexose-phosphates.

TABLE 27

ANALYSIS[a] OF ENZYMATICALLY SYNTHESIZED SUGAR-PHOSPHATES

Compounds	Phosphorus		Ketose	Ketohexose	Pentose
	Inorganic	Total			
D-Psicose-1-P	0.00	1.10	1.20	1.24	---
L-Tagatose-1-P	0.00	1.18	1.24	1.30	---
D-Ribulose-1-P	0.00	1.10	1.16	---	1.06

[a]The analyses are presented in terms of μmoles. See the text for details.

The product of condensation of glycolaldehyde was tested by the orcinol reaction for pentoses (232). The amount of pentose determined in this reaction corresponded to that found in cysteine-carbazole method, indicating that this product was a pentulose-phosphate. Furthermore, in this colorimetric reaction, the ratio of absorbancy at 540 to the absorbancy at 670 was 0.85 (found for known D-ribulose, 0.83), while xylulose yielded a ratio of 0.45. Anderson and Wood (261), and Stumpf, et al. (262) reported the following $\frac{540 \text{ absorbancy}}{670 \text{ absorbancy}}$ ratios for the indicated compounds: ribulose, 0.8-0.95 and xylulose, 0.42-0.48.

b. Periodate Oxidation

In order to establish the position of phosphate group, the products of condensation (10 μmoles of each) were subjected to periodate oxidation; the phosphate-containing product in each instance was isolated by chromatography on Dowex-1 Cl^- resin, and characterized as phosphoglycolic

acid as previously described (see "Chromatographic procedures"). A summary of the data obtained is shown in Table 28. As indicated, a minimum of 70% recovery of phosphoglycolic acid in each case demonstrated that the condensation products were indeed ketose-1-phosphates.

c. Preparation of Derivatives

The unequivocal identification of the condensation products was achieved by the preparation of an appropriate derivative of each one, followed by its characterization. Thus, 220 μmoles of the condensation product of D-glyceraldehyde with DHAP, 165 μmoles of product of condensation of glycolaldehyde with DHAP, and 260 μmoles of condensation product of L-glycerlaldehyde with DHAP were treated with an excess of potato acid phosphatase. At various intervals an aliquot from each of the incubation mixtures was treated with $Ba(OH)_2$-$ZnSO_4$ method (200), and the amount of ketose remaining in the supernatant fluid determined as a measure of the extent of dephosphorylation. After 5 hours of incubation, more than 90% of the ketohexose phosphates, and approximately 80% of the pentulose-phosphate had been dephosphorylated. The reactions were stopped by the addition of an equal volume of a solution of 0.15M $ZnSO_4$, followed by an equal volume of a 0.3N solution of $Ba(OH)_2$. The precipitate was removed by centrifugation, and the clear supernatant fluids were deionized and concentrated in vacuo. The recoveries (in μmoles) of D-psicose, L-tagatose, and D-ribulose were: 196, 250, and 140, respectively. The free sugars were chromatographed in bands on Schleicher and Schuell 589 Green

TABLE 28

ANALYSIS[a] OF PHOSPHOGLYCOLATE OBTAINED FROM PERIODATE OXIDATION OF ENZYMATICALLY SYNTHESIZED SUGAR-PHOSPHATES

Compound	Phosphorous		Glycolic Acid	Molar Ratios of $\frac{\text{Glycolate}}{\text{P}}$	Percent Recovery of Phosphoglycolate
	Inorganic	Total			
D-Fructose-1-P[b]	0.00	0.76	0.81	1.07	75
D-Psicose-1-P	0.00	0.83	0.86	1.03	71
L-Tagatose-1-P	0.00	0.83	0.94	1.13	72
D-Ribulose-1-P	0.00	0.86	0.95	1.10	77

[a]The analyses are presented in terms of μmoles. See the text for details.

[b]D-Fructose-1-P was used for comparison.

Phosphorus was determined by the method of Chen, et al. (229), and glycolic acid by the chromtropic acid procedure (236). Recoveries are based on organic phosphorus values.

Ribbon filter paper using solvent system B. After two successive developments, the sugars were located on the chromatograms and eluted with water in the manner described (see "Chromatographic procedures"). A single band was detected on the chromatogram in each instance which accounted for approximately 90% of the material originally applied to the paper. On the basis of cysteine-carbazole test (202), approximately 170 μmoles of D-psicose, 230 μmoles of L-tagatose, and 120 μmoles of D-ribulose were recovered. The solutions were concentrated in vacuo to small volumes and their optical rotations determined. The ketohexoses were converted to their phenylosazone derivatives, and D-ribulose was converted to its o-nitrophenylhydrazone derivative as described.

d. Characterization of the Derivatives

(1) Product of Condensation of DHAP With Glycolaldehyde.—All of the possible products of condensations of DHAP with glycolaldehyde are illustrated in Figure 36. These are: D-ribulose-1-P, L-ribulose-1-P, D-xylulose-1-P, and L-xylulose-1-P. The product expected when this reaction is catalyzed by FDP-aldolase would be D-xylulose-1-P (216,263,264), while the condensation of DHAP with glycolaldehyde in the presence of L-fuculose-1-P aldolase yielded a single diastereoisomer which was characterized as D-ribulose-1-P (Table 29). As indicated, the free sugar of this product (referred to as unknown in the table) exhibited the fast color development in the cysteine-carbazole reaction which is typical of ribulose (2,230,265), in contrast to xylulose which requires a long period

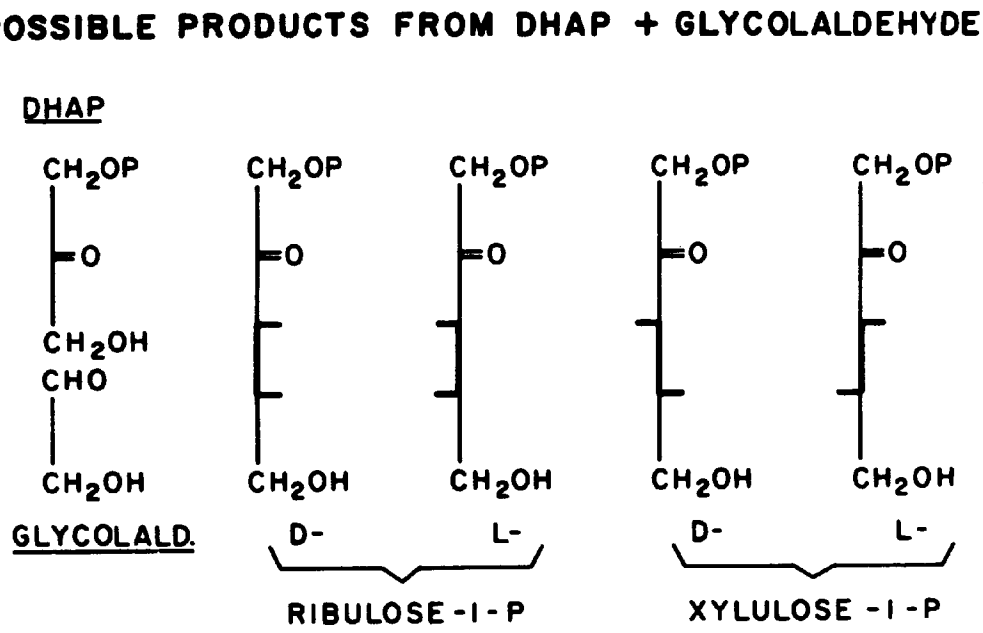


Figure 36. All of the possible diastereoisomers formed by aldol condensation of DHAP with glycolaldehyde.

TABLE 29

CHARACTERIZATION OF ENZYMATICALLY SYNTHESIZED RIBULOSE (-1-P)

Compound	Sugar		o-Nitro-Phenylhydrazone	
	Color Development in Cysteine-Carbazole	$[\alpha]_D^{26}$ [a]	M.P.	$[\alpha]_D^{25}$ [b]
Unknown	< 20 min	-14.5°	164-165°	-42.9°
D-Ribulose	< 20 min	-15.8°	164-165°	-43.7°
L-Ribulose	< 20 min	+16°	163-164°	+43°
D-Xylulose	> 80 min	-36.8°	---	---
L-Xylulose	---	+36.8°	---	---

[a] in water, c = 1.7.

[b] in methanol, c = .3.

The data for D- and L-xylulose have been obtained from the literature (262).

of time (about 100 min) under the same conditions (261,266). A rate study of the development of maximum color in this reaction is illustrated in Figure 37. As indicated, the development of maximum color with the unknown compound and with authentic ribulose required less than 20 min. The optical rotation of the free sugar, the melting point and the optical rotation of its o-nitrophenylhydrazone derivative corresponded to those found and reported for D-ribulose. Furthermore, these data clearly differentiated the unknown product from all of the other possible diastereoisomers (see Table 29).

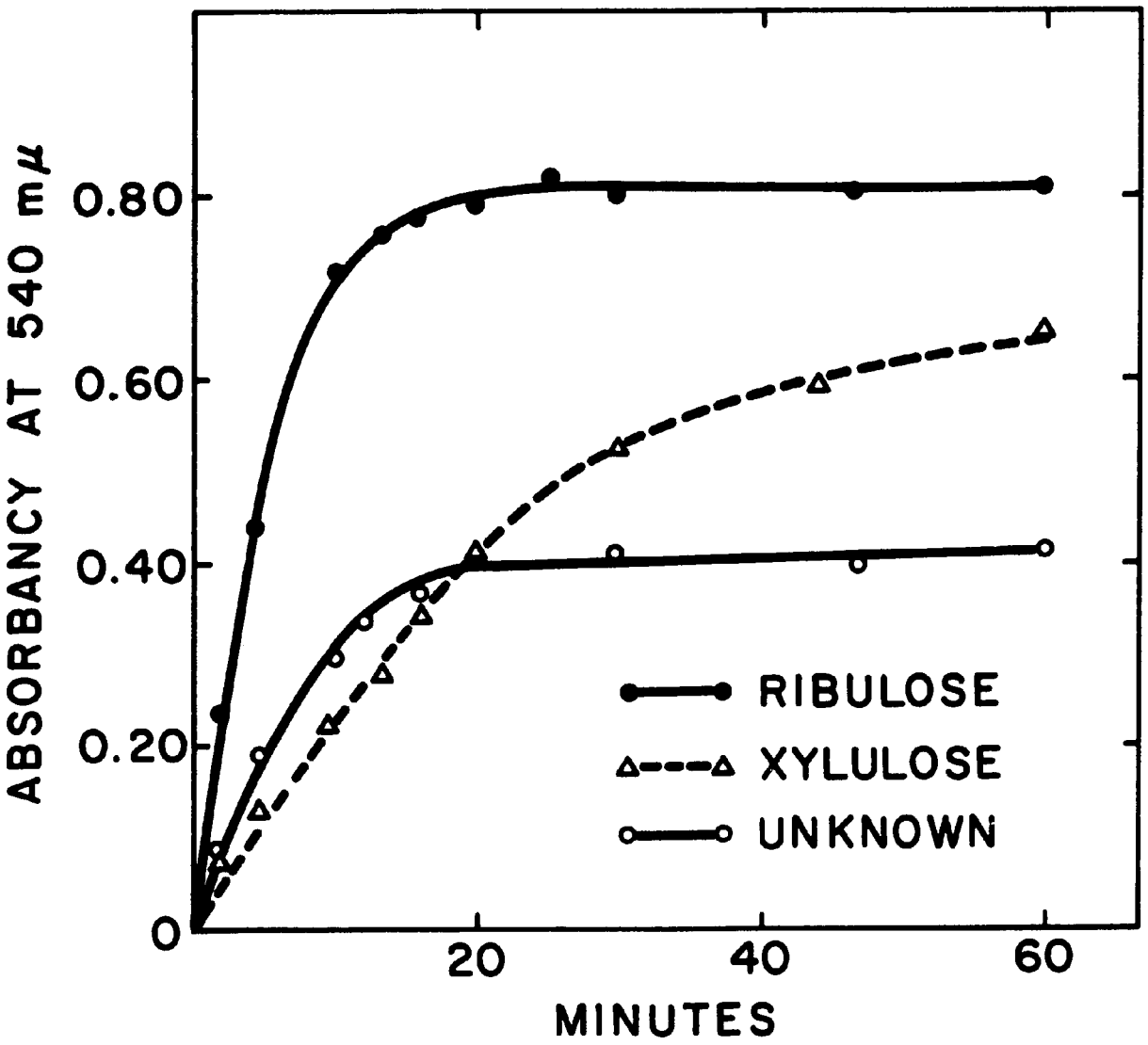


Figure 37. Distinction between ribulose and xylulose on the basis of the rate of color development in the cysteine-carbazole reaction (202).

(2) Product of Condensation of DHAP With D-Glyceraldehyde.—As illustrated in Figure 38, the 4 possible products of condensation of DHAP with D-glyceraldehyde are: 1-phosphate esters of D-psicose, D-tagatose, D-fructose,and D-sorbose. The expected product of the reaction of FDP-aldolase in this case is D-fructose-1-P (267,268), whereas, when the condensation of DHAP with D-glyceraldehyde was catalyzed by L-fuculose-1-P aldolase, a single component was formed which was characterized as D-psicose-1-P (Table 30). The optical rotation of the free sugar, referred to as unknown in the table, and the time required for maximum color development in the cysteine-carbazole test (202) corresponded to those of an authentic sample of D-psicose. The melting point and the specific rotation of the phenylosazone of the unknown compound were also found to be identical to those found for a known sample of phenyl-D-psicosazone. These criteria clearly distinguish the unknown from the other possible diastereoisomers (see Table 30).

(3) Product of Condensation of DHAP With L-Glyceraldehyde.—All of the possible diastereoisomers formed by condensation of DHAP with L-glyceraldehyde are shown in Figure 39. As indicated, these are 1-phosphate esters of L-tagatose, L-psicose, L-sorbose,and L-fructose. The product expected when the condensation is catalyzed by FDP-aldolase would be L-sorbose-1-P (267,268). However, the catalysis of this reaction by L-fuculose-1-P aldolase resulted in the formation of a single diastereoisomer which was characterized as L-tagatose-1-P (Table 31).

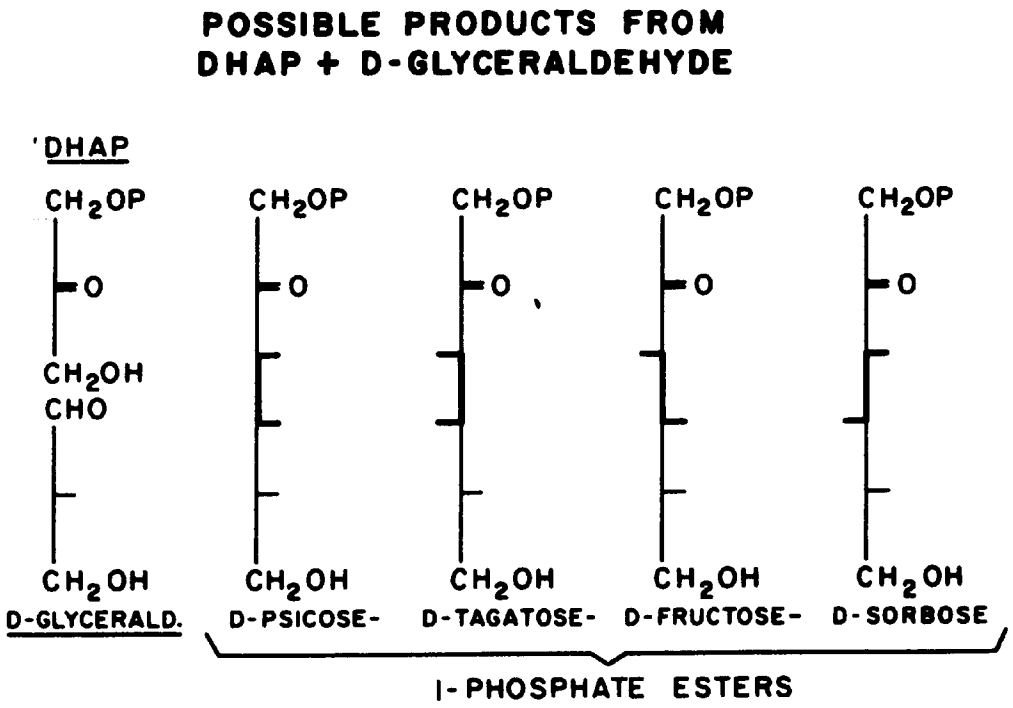


Figure 38. All of the possible diastereoisomers formed by aldol condensation of DHAP with D-glyceraldehyde.

TABLE 30

CHARACTERIZATION OF ENZYMATICALLY SYNTHESIZED PSICOSE (-1-P)

Compound	Sugar		Phenylosazone	
	Color Development in Cysteine-Carbazole	$[\alpha]_D^{25}$ [a]	M.P.	$[\alpha]_D^{25}$ [b]
Unknown	< 40 min	+4.6°	162-164°	-78.4°
D-Psicose	< 40 min	+4.7°	162-163°	-78.1°
D-Tagatose	< 60 min	-5°	192-193°	+110°
D-Fructose	> 90 min	-92°	204-205°	-89°
D-Sorbose	> 90 min	+43°	157°	+12.9°

[a] in water.

[b] in pyridine, except for D-sorbose which has been done in ethanol-pyridine mixture.

The data for D-sorbose were obtained from the literature (269).

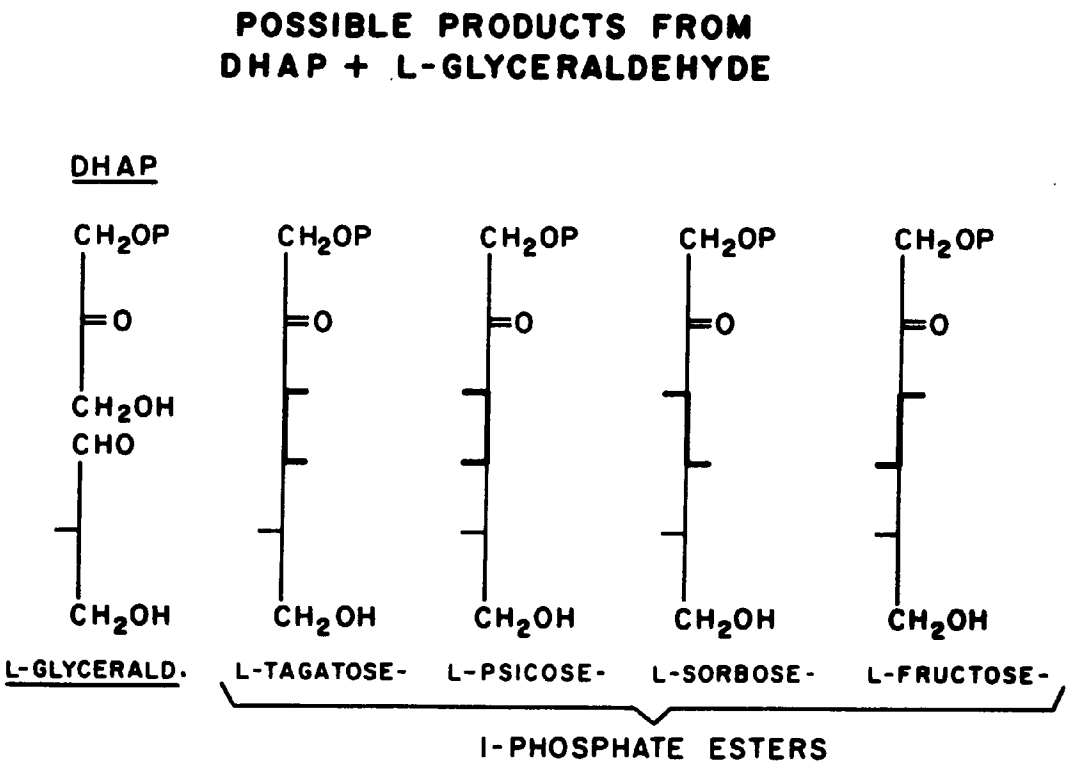


Figure 39. All of the possible diastereoisomers formed by aldol condensation of DHAP with L-glyceraldehyde.

TABLE 31

CHARACTERIZATION OF ENZYMATICALLY SYNTHESIZED TAGATOSE (-1-P)

Compound	Sugar		Phenylosazone	
	Color Development in Cysteine-Carbazole	$[\alpha]_D^{25}$ [a]	M.P.	$[\alpha]_D^{25}$ [b]
Unknown	< 60 min	+5°	189-190°	-108°
D-Tagatose	< 60 min	-5°	192-193°	+110°
L-Psicose	---	-4.7°	173-174°	+78°
L-Sorbose	> 90 min	-43°	157°	-12.9°
L-Fructose[c]	---	+92°	205°	+89°

[a] in water, c = 3.

[b] in pyridine, c = 0.37, except for L-sorbose which has been done in pyridine-ethanol mixture.

[c] Values derived from D-fructose.

The data for L-psicose (270) and L-sorbose (271,272) were obtained from the literature.

The sugar moiety of this compound referred to as unknown in Table 31 gave an optical rotation identical in magnitude and opposite in sign to that found for an authentic sample of D-tagatose. In the cysteine-carbazole test (202), the time required for its maximum color corresponded to tagatose and was distinct from that found for L-sorbose. The phenylosazone of this unknown and phenyl-D-tagatosazone exhibited the same melting points, and their specific rotations were identical in magnitude and opposite in sign. On the basis of these data, it is concluded that the product of condensation of DHAP with L-glyceraldehyde is L-tagatose -1-P.

D. Lactaldehyde Reductase

1. PROPERTIES OF ENZYME

Effect of time of incubation: As illustrated in Figure 40, the oxidation of DPNH in the presence of lactaldehyde reductase gave linear response with the time of incubation for at least 15 min.

Effect of enzyme concentration: The rate of DPNH oxidation was linear over 10-fold range of protein concentration (Figure 41).

Pyridine nucleotide specificity: The effects of TPNH and DPNH on the reaction velocity of lactaldehyde reductase are illustrated in Figure 42. As indicated, the rate of oxidation of DPNH is approximately two times faster than that of TPNH. This observation may be explained by the following 3 possibilities.

a. The enzyme may possess a broad specificity for pyridine nucleotides, thus it is active with both DPNH and TPNH.

b. The observation that DPNH is oxidized at a higher rate may mean that this enzyme is specific for DPNH only, and the ability of TPNH to serve as a cofactor in this system might be due to the presence of a transhydrogenase which transforms TPNH to DPNH according to the following reaction:

$$TPNH + H^+ \xleftarrow{DPN} DPNH + H^+ + TPN$$

Thus, the presence of DPNH in catalytic amounts in a system containing TPNH and enzyme may result in quantitative conversion of TPNH to DPNH, since the latter is utilized as it is formed.

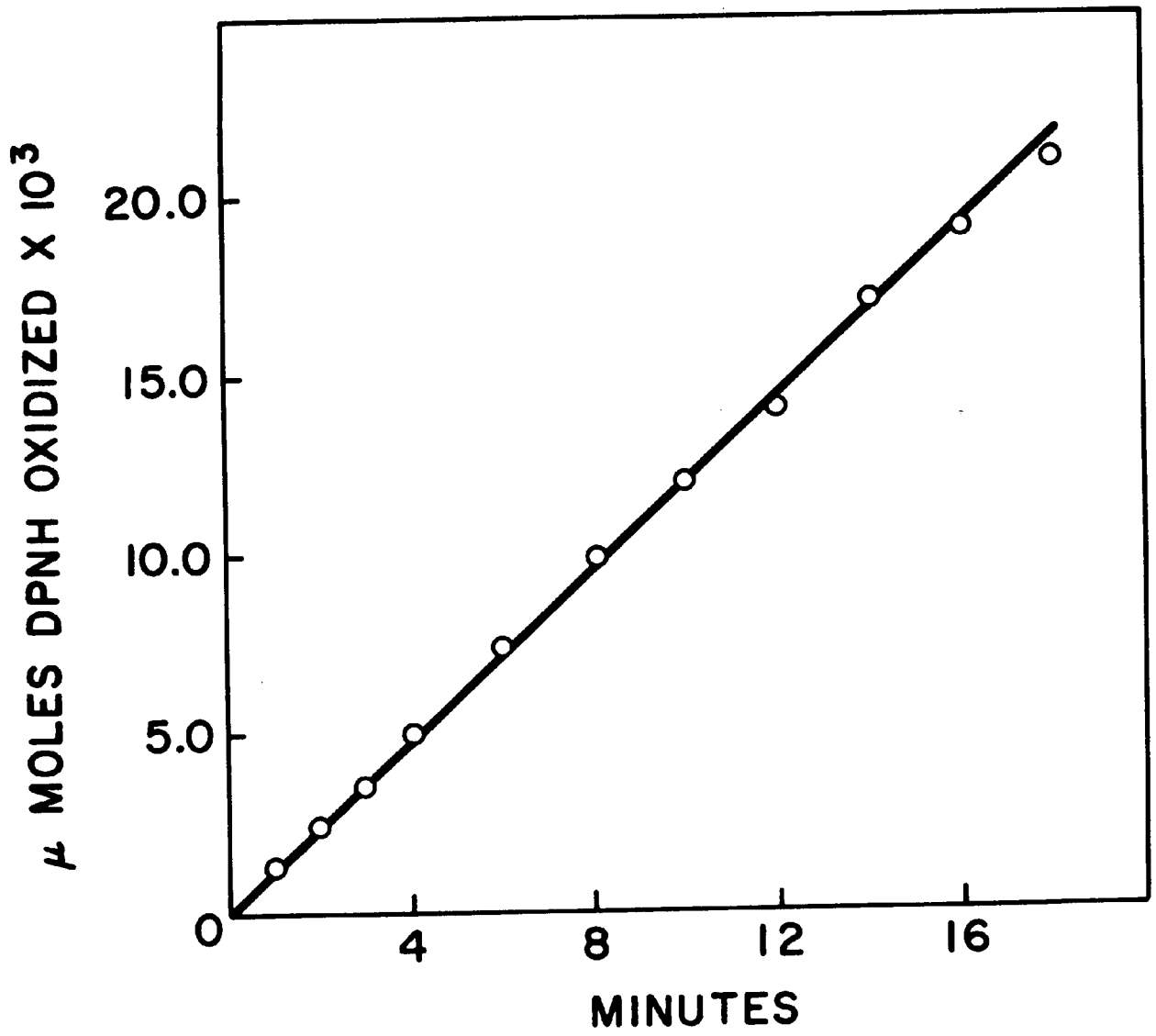


Figure 40. The effect of incubation time on the rate of lactaldehye reductase. Incubation mixture contained the following (in μmoles): L-lactaldehyde, 2.0; DPNH, 0.05, Tris pH 7.0, 10;and 25 μg protein in a total volume of 0.25 ml.

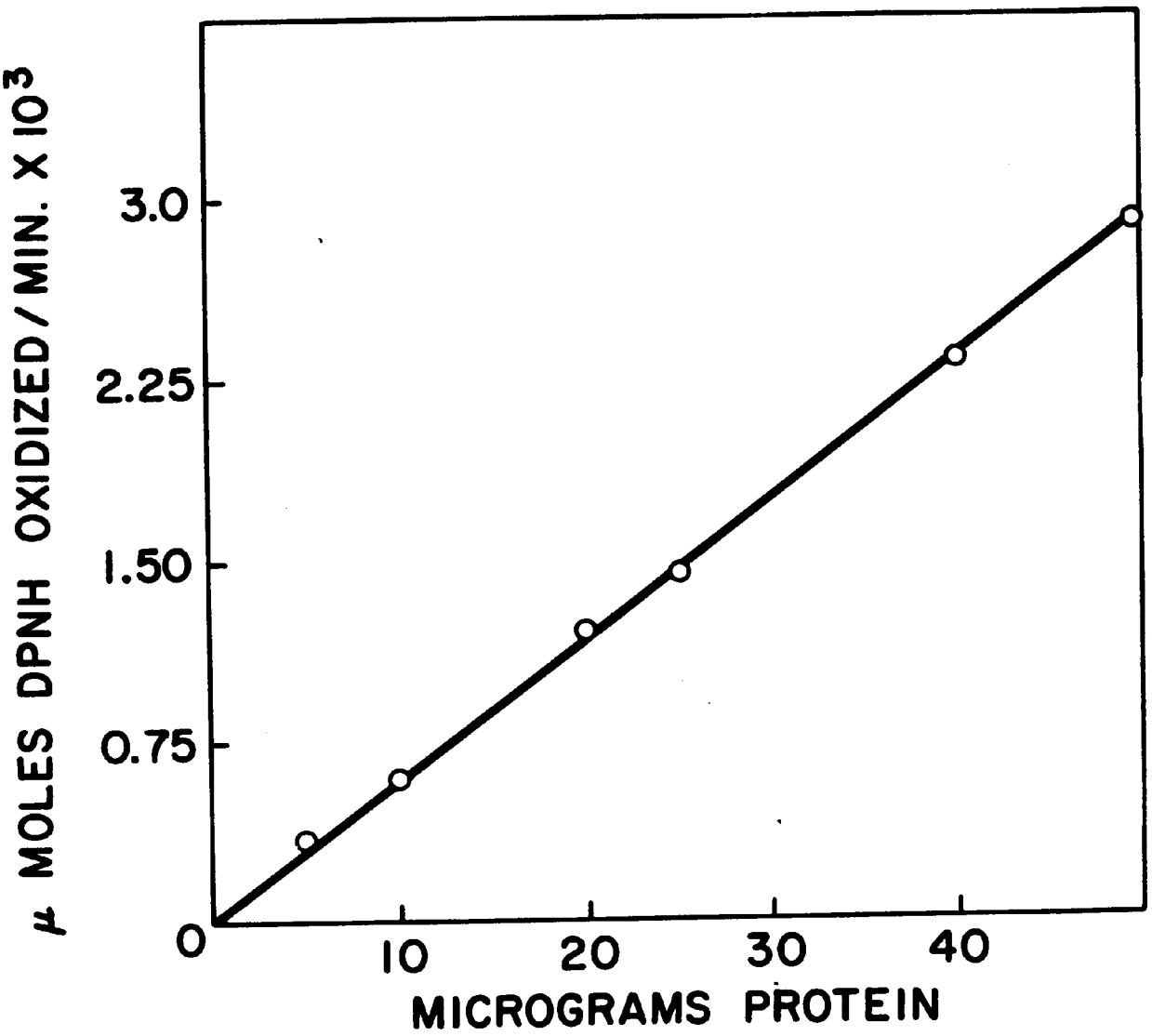


Figure 41. The reaction rate as a function of lactaldehyde reductase concentration. The incubation mixture contained the following (in μmoles): L-lactaldehyde, 2.0; Tris pH 7.0, 10; DPNH, 0.05 and varying amounts of protein in a total volume of 0.25 ml.

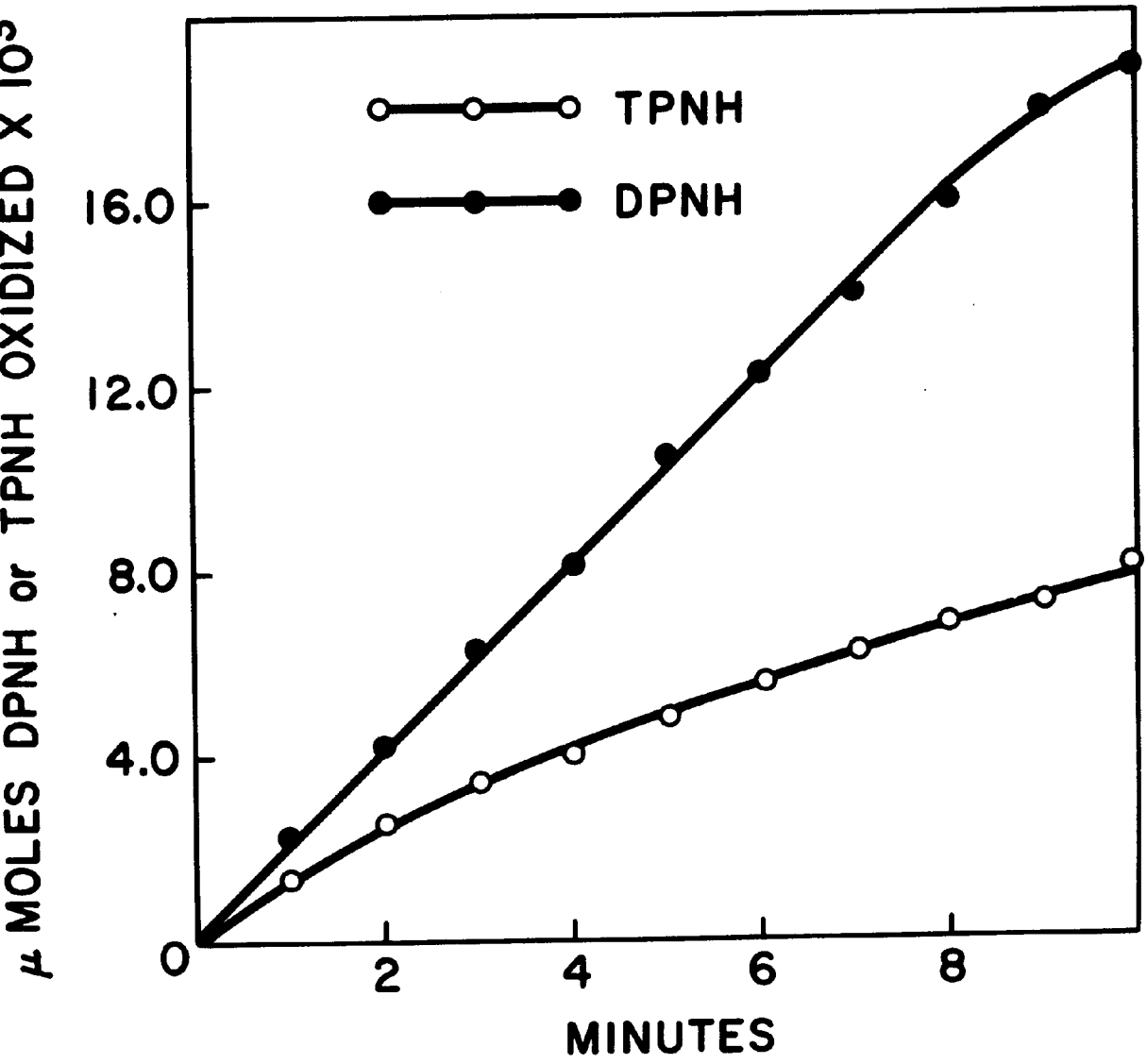


Figure 42. Pyridine nucleotide specificity of lactaldehyde reductase. Incubation mixture contained the following (in μmoles): L-lactaldehyde, 2.0; TPNH or DPNH, 0.05; Tris buffer pH 7.0, 10; and 40 μg of lactaldehyde reductase in a final volume of 0.25 ml.

c. There may be two different lactaldehyde reductases, one of which requires DPNH and the other TPNH. In fact, a TPNH-dependent lactaldehyde reductase has already been described for mammalian systems (328). However, the establishment of the pyridine nucleotide specificity requires more elaborate experiments, using more highly purified enzyme preparations than those employed in these studies.

2. IDENTIFICATION OF THE REACTION PRODUCT

When D- or L-lactaldehyde were incubated with lactaldehyde reductase in the presence of DPNH and the incubation mixture was treated with the Jones and Riddick colorimetric reaction (237), a positive test was observed. Although this reaction is not strictly specific for 1,2-propanediol (propionaldehyde also reacts in this test yielding a color identical to that formed by 1,2-propanediol), the chromogen formed by the product of lactaldehyde reductase gave a spectrum similar to that of 1,2-propanediol (maximum absorption at 595 mμ).

Furthermore, it was found that 1,2-propanediol could also serve as substrate for this enzyme. Thus, when 1,2-propanediol was incubated with lactaldehyde reductase in the presence of DPN, the formation of DPNH was linear with time of incubation for at least 12 min (Figure 43). These observations may suggest that this enzyme preparation contains an enzyme that catalyzes the reversible reduction of lactaldehyde to 1,2-propanediol as shown in the following equation.

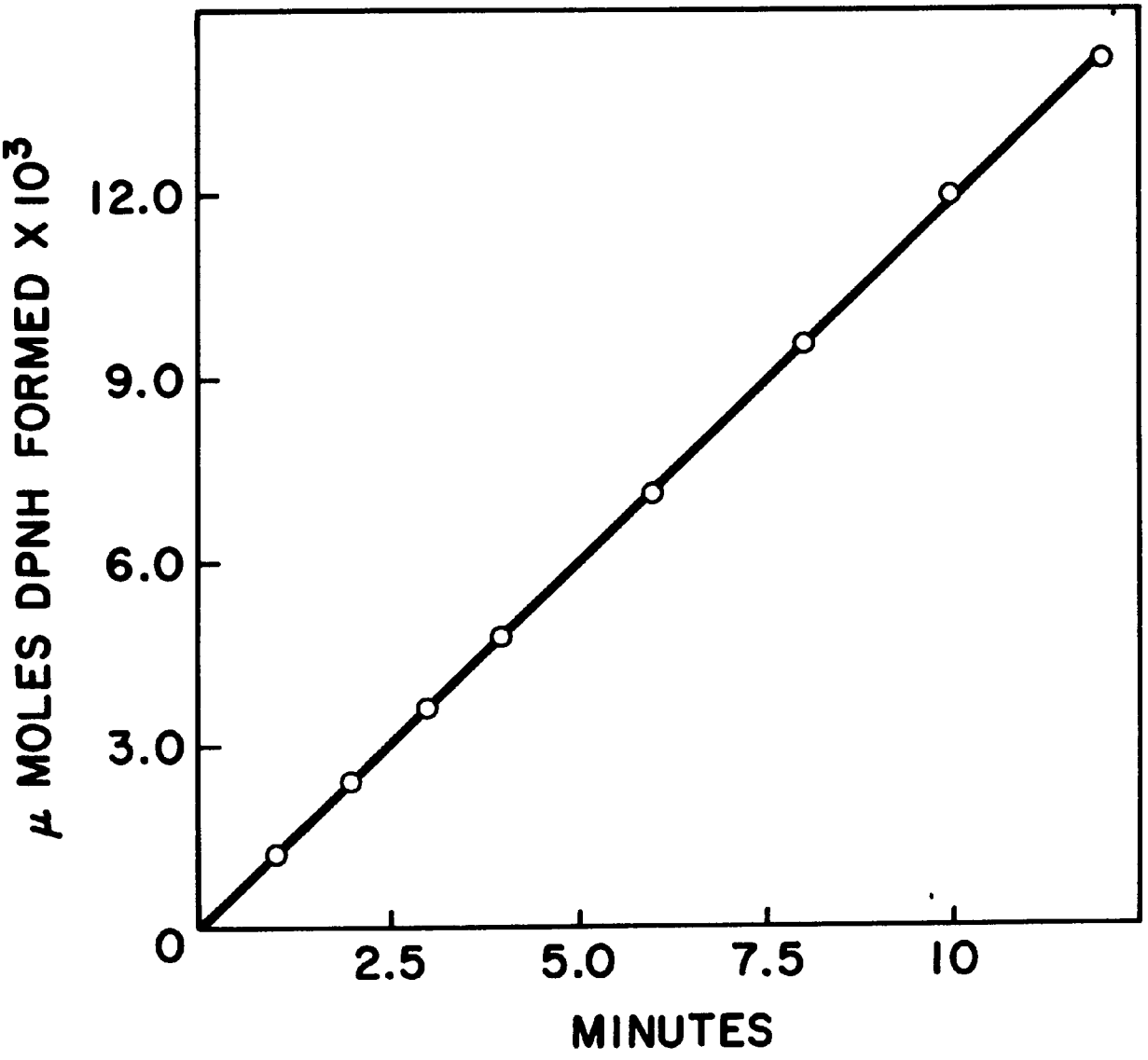
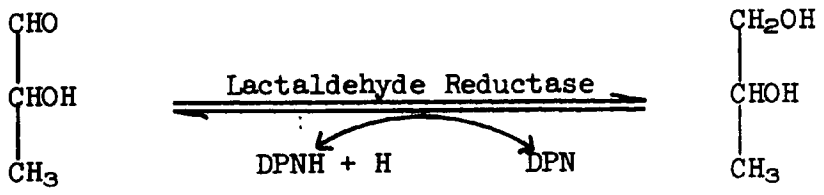


Figure 43. Oxidation of 1,2-propanediol by lactaldehyde reductase in the presence of DPN. Incubation mixture contained the following (in μmoles): 1,2-propanediol, 1.0; DPN, 1.0; Tris buffer, pH 7.0, 10 and 100 μg of lactaldehyde reductase in a total volume of 0.25 ml.



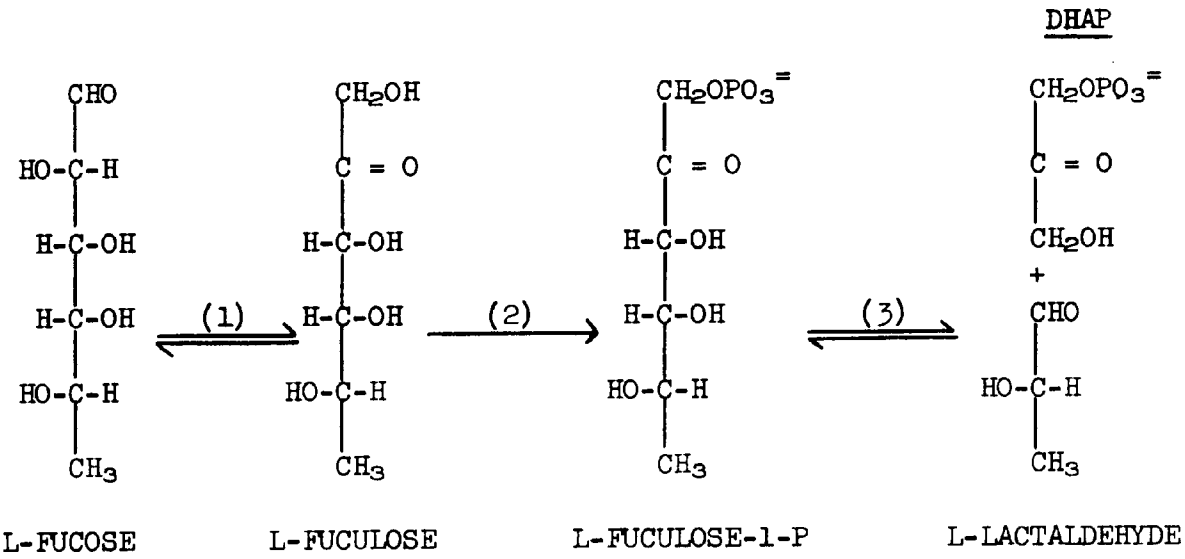
LACTALDEHYDE 1,2-PROPANEDIOL

However, there is no evidence to rule out the possibility of the presence of two separate enzymes, one of which catalyzes the oxidation of 1,2-propanediol, and another that catalyzes the reduction of lactaldehyde. Therefore, no speculation can be made with regard to the existance of any relationship between lactaldehyde reduction and 1,2-propanediol oxidation by this sytem.

It appears that the partially purified lactaldehyde reductase described in the present studies is distinct from ordinary alcohol dehydrogenase, on the basis of the fact that acetaldehyde was reduced at only 5% the rate of D-, or L-lactaldehyde. Other aldehydes tested with the present enzyme including pyruvaldehyde and DL-glyceraldehyde were also found to be relatively inactive. In addition, the bacterial lactaldehyde reductase reported here is also distinct from the mammalian lactaldehyde reductase described by Gupta and Robinson (328), since the latter specifically reduces D-lactaldehyde, but not the L-isomer.

V. DISCUSSION

The study of L-fucose catabolism in Escherichia coli O111-B_4 (grown on L-fucose) has resulted in the elucidation of a pathway by which this substance is degraded to three-carbon fragments. This pathway involves the following sequence of reactions:



(1) L-Fucose Isomerase
(2) L-Fuculose Kinase + ATP + Mg^{++} or Mn^{++}
(3) L-Fuculose-1-P Aldolase + Mg^{++} or Mn^{++}

The present section will be devoted to a discussion of the findings reported in this thesis and a general discussion concerning aldolases with particular regard to the following aspects: (a) inducibility of the enzymes involved; (b) fucose-fuculose isomerization; (c) specificity of L-fuculose kinase; (d) the aldol cleavage of L-fuculose-1-P; (e) mechanism of action of aldolases; (f) specificity of enzyme-catalyzed aldol condensations; (g) comparison of the substrate specificity of aldolases; and (h) biosynthesis of new ketose phosphates.

A. Inducibility of the Enzymes

Green and Cohen (3) have demonstrated that the presence of L-fucose in the culture medium was a prerequisite for the dissimilation of this compound by Escherichia coli Ba_{15}. These authors concluded that the enzyme responsible for the initial step in the utilization of L-fucose (i.e., L-fucose isomerase) was an inducible enzyme; furthermore, they suggested that the catabolism of L-fucose may involve more than one inducible enzyme.

In the present study, it has been observed that the enzymes involved in the catabolism of L-fucose (i.e., L-fucose isomerase, L-fuculose kinase and L-fuculose-1-P aldolase) are detectable only in the organisms which had been grown on L-fucose. In a particular experiment concerned with L-fuculose kinase, it was discovered that the specific activity of the enzyme preparation remained constant as long as L-fucose was present in the medium. Furthermore, these enzymes have not been detected in glucose-grown organisms. Although these observations may indicate that the enzymes of L-fucose catabolism are inducible, it remains to be established whether the induction is a single-step phenomenon (i.e., the gene loci which control the synthesis of these enzymes are linked) or it takes place via the process of sequential induction.

Similar studies have been conducted by Englesberg (189,198) and Wilson and Ajl (191) concerning L-rhamnose catabolism. These authors demonstrated that the presence of L-rhamnose in culture medium was nec-

essary for the formation of L-rhamnose isomerase and L-rhamnulose kinase by Escherichia coli and P. pestis, respectively. Moreover, it was shown that the induction of the enzymes of L-rhamnose catabolism was a single-step phenomenon, since L-rhamnulose could also induce the formation of both L-rhamnose isomerase and L-rhamnulose kinase. Several inducible isomerases have been established for pentoses, such as: L-arabinose (273-275), D-xylose (276-278), L-lyxose (261),and D-arabinose (2).

A variety of microorganisms may not contain all the enzymes (in sufficient quantities) required for their growth under specific conditions. These organisms, however, frequently possess the genetic capacity to synthesize enzymes when they are grown in the presence of a substance referred to as the "inducer." The inducer does not necessarily have to be the natural substrate of the enzyme, however, it must possess certain structural characteristics to satisfy the substrate specificity requirements of the enzyme (279-281). Recent studies on the genetic aspects of the enzyme β-D-galactosidase of Escherichia coli K_{12} have elucidated some interesting aspects of induced enzymes and the process of enzyme induction. The capacity of synthesis of an inducible enzyme is genetically controlled as established by the conversion of lactose negative (lack the capacity to synthesize enzymes required for lactose metabolism, i.e., galactoside-permease and β-D-galactosidase) strains of Escherichia coli to lactose positive (those which are able to synthesize the enzymes involved in lactose metabolism). This conversion has been achieved by two genetic methods: transduction and recombination which are briefly

described below.

1. Transduction of lac^+ genetic determinants into lac^- strains of Escherichia coli K_{12} by phage P_1 (previously grown in lac^+ cells) was accompanied by the acquisition in the transduced cells of the ability to synthesize β-galactosidase, Luria, et al. (282,283); moreover, this acquired synthetic capacity in the transduced cells was retained as a stable genetic character. With regard to the inducible enzymes involved in L-rhamnose metabolism, Englesberg, et al. (190) demonstrated that protorophs of Salmonella typhosa which are incapable of utilizing L-rhamnose (Rh^-) could be transduced to Rh^+ (capable of forming enzymes required for L-rhamnose catabolism) by phage PLT_{22} which had been previously grown on Salmonella typhimurium.

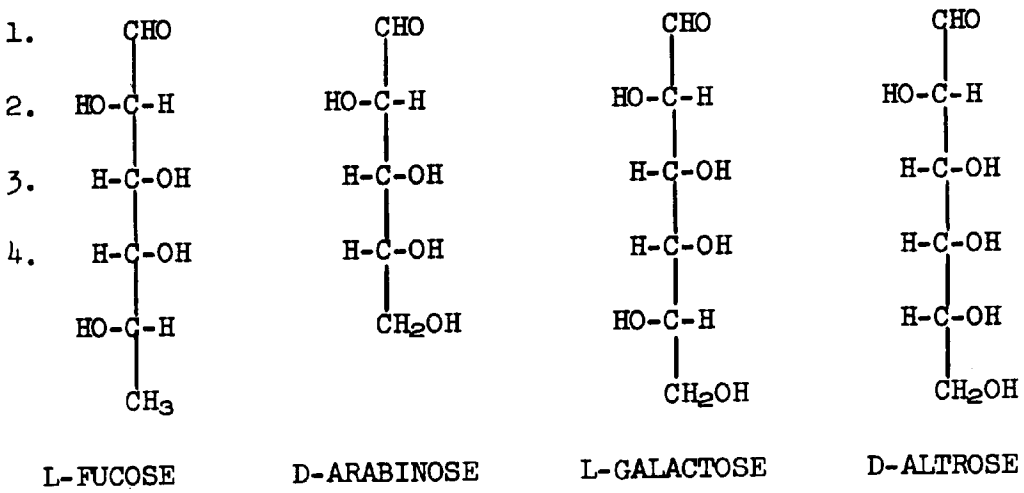
2. Conjugation of ♂ Z^+ (those which are able to synthesize β-galactosidase) with ♀ Z^- (those lacking the capacity to synthesize β-galactosidase) resulted in the acquisition in the recipient cells of the ability to synthesize β-galactosidase (284,285).

It is believed that the lack of capacity to synthesize certain enzymes (in detectable quantities) is caused by the presence of a factor referred to as the "repressor" in the prototrophs of Escherichia coli that inhibits the system which controls the biosynthesis of β-galactosidase and galactoside-permease, and that the function of the "inducer" is to antagonize the action of the "repressor." Since the "repressor" is absent in the lac^+ mutants, therefore, the presence of the inducer will not be necessary for these cells in order to be able to synthesize

the enzymes involved in lactose metabolism (284).

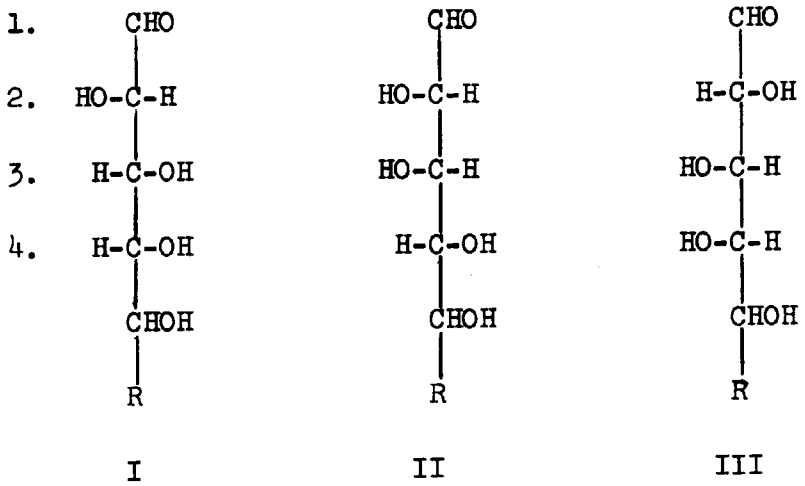
B. Fucose-Fuculose Isomerization

The initial step in the catabolism of L-fucose is its isomerization to the corresponding ketose, i.e., L-fuculose. This is a representative of a general type of reaction known to be involved in the catabolism of other methylpentoses (3,185,191,193), pentoses (261,2,274,286), and some hexoses (196). The fact that L-fucose-grown Escherichia coli can catalyze the isomerization of D-arabinose (2,3) and vice versa, may indicate that the presence of L-fucose or D-arabinose cause the induction of a single isomerase with broad specificity, or two separate isomerases (with limited substrate specificity). Green and Cohen (3) suggested that the isomerization of both of these compounds is catalyzed by a single enzyme which is specific for the stereochemical configurations of C-2 to C-4 of the molecule as shown in Scheme 2. This speculation was strengthened by the fact that substances such as L-galactose and D-altrose also served as substrates for L-fucose isomerase. The structural formulae of these sugars shown below illustrates their identical configurations at C-2, C-3,and C-4 (Scheme 1).



Scheme 1. Projection formulae of aldoses which can serve as substrates for L-fucose isomerase.

Similar observations have been made by Palleroni and Doudoroff (196) concerning the substrate specificity of D-mannose isomerase. As this enzyme was active only with D-mannose, D-rhamnose, and D-lyxose, these authors proposed that mannose isomerase is specific for the configurations of C-2 to C-4 of the molecule as depicted in Scheme 2. In addition, Simpson et al. have also demonstrated that the specificity of L-arabinose isomerase relates to the stereochemical configurations of the substrate at positions 2, 3, and 4 (Scheme 2).

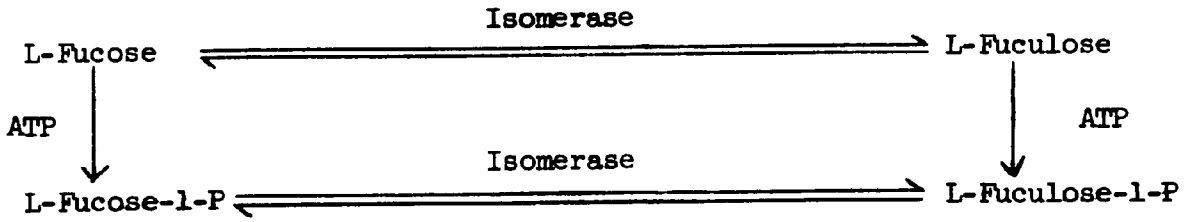


I = L-Fucose Isomerase (3).
II = D-Mannose Isomerase (196).
III = L-Arabinose Isomerase (273).

Scheme 2. Substrate specificity of various isomerases with regard to the stereochemical configurations of C-2, C-3, and C-4 of the molecule.

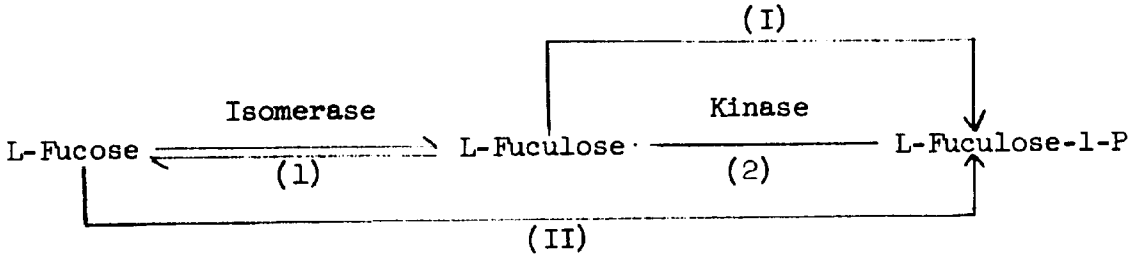
C. Specificity of L-Fuculose Kinase

The second reaction involved in the catabolism of L-fucose is the phosphorylation of L-fuculose to L-fuculose-1-P. Previously, Huang and Miller (6) reported that crude extracts of L-fucose-grown Escherichia coli Ba_{15} could catalyze the phosphorylation both of L-fucose and L-fuculose in the presence of ATP. Although no direct evidence was obtained regarding the chemical nature of the intermediates, these authors suggested the following sequence of reactions to account for their results:



The proposed pathway is not supported by the present studies on the basis of the following.

1. This interpretation was based solely on slight differences in the extent of phosphorylation of these two compounds. As these authors reported the presence of L-fucose isomerase in their crude extracts, the use of either L-fucose or L-fuculose as substrate in such extracts would rapidly yield an equilibrium mixture of both. This point can be further clarified by taking into consideration the effect of equilibria in multi-enzyme systems. In the system illustrated below both of the pathways designated as (I) and (II) may be operative, and lead to the same product.



The determination of the over-all equilibrium of the above multi-enzyme system requires at least a knowledge of the rates of the individual reactions (1) and (2) and the affinity of each of the enzymes for its respective substrate. The coexistence of L-fuculose kinase in crude extracts (in optimal quantities), L-fucose isomerase, and ATP, particularly

when long-term incubations are applied may result in almost quantitative conversion of L-fucose to the final product (i.e., L-fuculose-1-P). This may lead the observer to conclude that both L-fucose and L-fuculose could be phosphorylated. This type of interpretation of the results can be avoided by working with isolated enzyme systems and/or isolation and characterization of the intermediates and products of the reactions. However, the conclusion of Huang and Miller (6) concerning the phosphorylation of L-fucose and L-fuculose was based only on the partial disappearance of these sugars upon incubation with ATP and crude extracts followed by $Ba(OH)_2$-$ZnSO_4$ treatment. They made no attempt to isolate any of the proposed products, i.e., L-fucose-1-P or L-fuculose-1-P. An alternative way to test the validity of such interpretations, is to preincubate the enzyme with L-fucose (in the absence of ATP), then add ATP, and compare the rate of phosphorylation with an identical system in which the preincubation step has been excluded. As an example of a situation of this type, Wilson and Ajl (192) in their studies on the catabolism of L-rhamnose, observed that both L-rhamnose and L-rhamnulose could serve as substrate for phosphorylation by crude extracts of Escherichia coli; however, by the application of the above technique (preincubation experiment), they were able to suggest that L-rhamnulose was the true substrate of the kinase, not L-rhamnose.

2. The possibility that crude extracts of this organism contain separate enzymes (or a single enzyme with a broad substrate specificity) that can catalyze the phosphorylation of both L-fucose and L-fuculose

seems untenable on the basis of the following.

a. Partially purified preparations were obtained which contained as high as 90% of the oringal activity present in the crude extracts, and exhibited activity only with L-fuculose as substrate, but not with L-fucose, unless L-fucose isomerase was added.

b. The product of the phosphorylation of L-fuculose was isolated and characterized as L-fuculose-1-P; it was homogeneous by all the criteria applied and contained no detectable quantitites of L-fucose-1-P.

3. The possible conversion of L-fucose-1-P (as proposed by Haung and Miller (6) to exist as an intermediate in the formation of L-fu-1-P) to L-fuculose-1-P appears to be remote on the basis of the following.

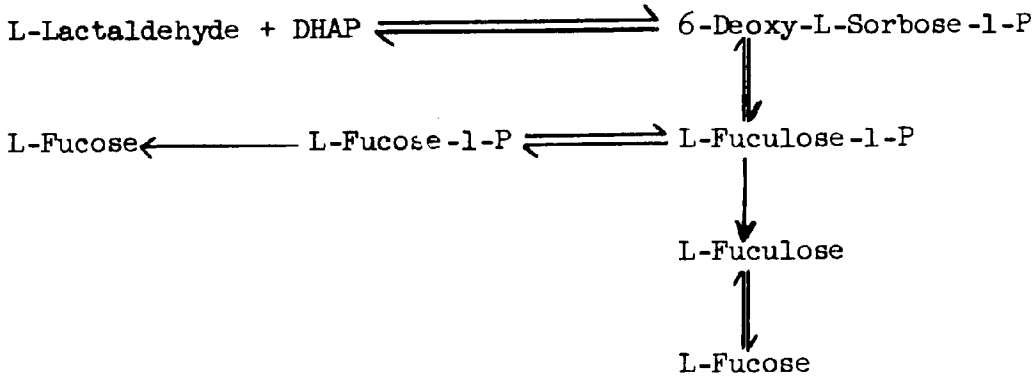
a. There is no evidence available for such a reaction; neither its reactant nor its product were isolated by these authors. Furthermore, it must be hypothesized that either L-fucose isomerase or another (specific) isomerase is responsible for the isomerization of L-fucose-1-P to L-fuculose-1-P. The first possibility is not supported by the data available in the literature concerning aldose-ketose isomerization. It is now established that the known isomerases exhibit certain substrate specificity requirements some of which are exemplified below.

Example 1. The known pentose isomerases (except phosphoribose isomerase) are specific for the free form of the sugars, they will not catalyze isomerization of pentose-phosphates.

Example 2. Phosphoribose isomerase catalyzes the isomerization of pentose-phosphates, not of free pentoses; furthermore, the phosphate group must be at C-5 of the molecule and pentose-3-phosphates have been found to be inactive (287). Thus there is no biochemical precedent for the isomerization of L-fucose-1-P by L-fucose isomerase. Since there is no experimental evidence for this reaction, it seems highly improbable that such a reaction exists.

The second hypothesis that assumes the occurrence of a specific isomerase (in fucose-grown Escherichia coli) which could catalyze the interconversion of L-fucose-1-P and L-fuculose-1-P, also seems improbable on the basis of the following.

Presumably, one of the reasons to speculate the existence of such an isomerase was based on the hypothesis concerned with biosynthesis of L-fucose. It was suggested (6) that L-fucose may possibly be biosynthesized via the following pathway:



Scheme 3. A speculative pathway for biosynthesis of L-fucose (6,198).

Now, that it is established that the biosynthesis of L-fucose from D-glucose involves no fragmentation of the carbon chain of the precursor (43,

44,46) and moreover, this conversion occurs at the nucleotide level (109, 110), the possibility of another biosynthetic pathway as depicted in Scheme 3 appears to be untenable; by consequence the existence of L-fucose-1-P isomerase seems to be unnecessary. This idea is strengthened by the data obtained in the present studies, which indicate that these enzymes (L-fucose isomerase, L-fuculose kinase and L-fuculose-1-P aldolase) operate in a catabolic pathway rather than a biosynthetic one. Moreover, since the significance of L-fucose isomerase in the catabolism of L-fucose has been established (3,4) the existence of another isomerase (L-fucose-1-P isomerase), that one of its required substrates, i.e., L-fucose-1-P has never been isolated and whose function seems to have no contribution to the over-all metabolism of L-fucose by Escherichia coli appears to be improbable with respect to the cell's biochemical economy.

Therefore, on the basis of the data presented, there appears to be no evidence for the direct phosphorylation of L-fucose. It is concluded that the extracts of L-fucose-grown Escherichia coli O111-B_4 contain an enzyme (L-fuculose kinase) that will catalyze the phosphorylation of L-fuculose, but not of L-fucose. Furthermore, other 6-deoxyhexoses tested (L-rhamnose, L-rhamnulose and 6-deoxy-L-sorbose) were also found to be inactive in this system.

D. Aldol Cleavage of L-Fuculose-1-P

At the beginning of the study concerning the further metabolism of

L-fuculose-1-P by Escherichia coli O111-B_4, several hypotheses were proposed which are presented below:

The cleavage of L-fuculose-1-P involves two steps; epimerization at C-3 to yield 6-deoxy-L-sorbose-1-P, followed by aldol cleavage of the latter by FDP-aldolase to trioses.

The aldol cleavage of L-fuculose-1-P is catalyzed by a nonspecific aldolase, i.e., one which exhibits broad substrate specificity and that can catalyze the cleavage of ketose-phosphates regardless of the stereochemical configurations of hydroxyl groups at positions 3 and 4 (i.e., one which cleaves the molecule between trans and cis C-3,4 hydroxyl groups).

The cleavage of L-fuculose-1-P is catalyzed by a specific aldolase with unique substrate specificity requirements, i.e., one that is specific for the cis configuration of hydroxyl groups at positions 3 and 4 of the molecule of ketose-phosphate. These three proposed hypotheses will be examined in the following sections.

1. The involvement of an epimerase in the catabolism of L-fuculose-1-P, as proposed by Huang and Miller (6), is not supported by the data obtained in the present studies. This conclusion was based primarily on small differences in the kinetic data obtained when crude extracts of fucose-grown Escherichia coli were incubated in the presence of ATP and L-fucose, followed by measurement of the formation of alkali-labile phosphates (presumably DHAP). No attempt was made to isolate the intermediates of the reactions; they neither used L-fuculose-1-P as substrate, nor did they isolate or demonstrate 6-deoxy-L-sorbose-1-P as an inter-

mediate in the cleavage of L-fu-1-P. That crude extracts of L-fucose-grown Escherichia coli contain FDP-aldolase is an undeniable fact. However, the data obtained in the present studies suggest that FDP-aldolase plays no role in the metabolism of L-fu-1-P by this organism. FDP-aldolase is a constitutive enzyme of this organism, and it has always been found to be present in varying quantities irrespective of the nature of the carbon source used in the culture medium. On the other hand, growth of Escherichia coli on L-fucose is accompanied by the appearance of another aldolase with unique substrate specificity, i.e., L-fuculose-1-P aldolase. The significance of this enzyme in the catabolism of L-fucose by this organism is apparent, since it catalyzes the cleavage of L-fuculose-1-P molecule to trioses that can be utilized further by pathways which have been established for other systems. In fact, DPNH-linked lactaldehyde reductase activity (223,288) has been detected in crude extracts and in partially purified preparations of L-fuculose-1-P aldolase. This reduction to 1,2-propanediol may represent a first step in the pathway for utilization of L-lactaldehyde by this organism. Several investigators (220,289-293) have proposed various pathways for further utilization of 1,2-propanediol by bacterial and mammalian systems.

The conclusions presented in this thesis do not agree with the hypothesis of the involvement of an epimerase in the cleavage of L-fuculose-1-P on the basis of the following evidence.

a. A specific aldolase has been purified from extracts of L-fucose-grown Escherichia coli O111-B_4 which exhibits specificity

for the cis configuration of hydroxyl groups at C-3,4 of the molecule of ketose-phosphate, in contrast to FDP-aldolase that preferentially catalyzes the cleavage of ketose-phosphates containing trans configuration at these positions (see Tables 24 and 26).

b. At no stage of purification of L-fuculose-1-P-aldolase, was there any indication of the involvement of more than one enzyme in the cleavage of L-fuculose-1-P. Purified preparations of this enzyme were obtained which contained 75% of the original activity after a minimum of 120-fold purification.

c. The possibility of the mediation of an epimerase in the catabolism of L-fuculose-1-P could not be demonstrated in the present studies. The conclusion of Huang and Miller (6) concerning the necessity of an epimerase in this pathway, was based primarily on the observation that the addition of crude extracts of Lactobacillus plantarum (as a source of D-xylulose-5-P, 3-epimerase) to their system (containing L-fucose, ADP, and crude extracts of Escherichia coli Ba_{15}) stimulated the formation of DHAP. As these authors suggested the involvement of an epimerization in the molecule of L-fuculose-1-P (which contains cis C-3,4 hydroxyl groups) to form ketose-phosphates with trans configuration of C-3,4 hydroxyl groups prior to cleavage by FDP-aldolase, the following experiment was designed to examine this possibility.

Such a proposed epimerization could take place via either of the following enzymes: (1) D-Xu-5-P, 3-epimerase, or (2) L-Ru-5-P,

4-epimerase. Chemically characterized L-fuculose-1-P was preincubated with purified preparations of either D-Xu-5-P, 3-epimerase[1], or L-Ru-5-P, 4-epimerase,[1] followed by incubation with crystalline FDP-aldolase, and the formation of DHAP was measured spectrophotometrically. The hypothetical basis of this experiment is illustrated in Figure 44. Using a very sensitive assay procedure (by which 0.01 μmole of DHAP could easily be determined), no detectable amounts of DHAP was formed in either of the two systems.

d. Further, the possibility of epimerization of L-fu-1-P by either of the two pentose-phosphate epimerases appears to be remote on the basis of the specificity requirements of these enzymes as reported in the literature. Hurwitz and Horecker (270), and Ashwell and Hickman (266) have determined the substrate specificity of D-Xu-5-P, 3-epimerase purified from various sources. This enzyme appears to be specific for D-Ru-5-P and D-Xu-5-P. Although L-fu-1-P and D-Ru-5-P have the same stereochemical configuration at C-2 to C-4, they differ with respect to the location of the phosphate group. On the other hand, the hypothetical action of L-Ru-5-P; 4-epimerase on L-fu-1-P cannot be explained on any theoretical basis, since L-fu-1-P molecule has no stereochemical similarity with L-Ru-5-P or D-xu-5-P. As has been reported, this enzyme appears to act only on L-Ru-5-P and D-xu-5-P (215).

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1. Purified preparations of D-xylulose-5-P, 3-epimerase (50-fold) and L-ribulose-5-P, 4-epimerase (240-fold) were kindly supplied by Dr. W. A. Wood, Michigan State University, East Lansing.

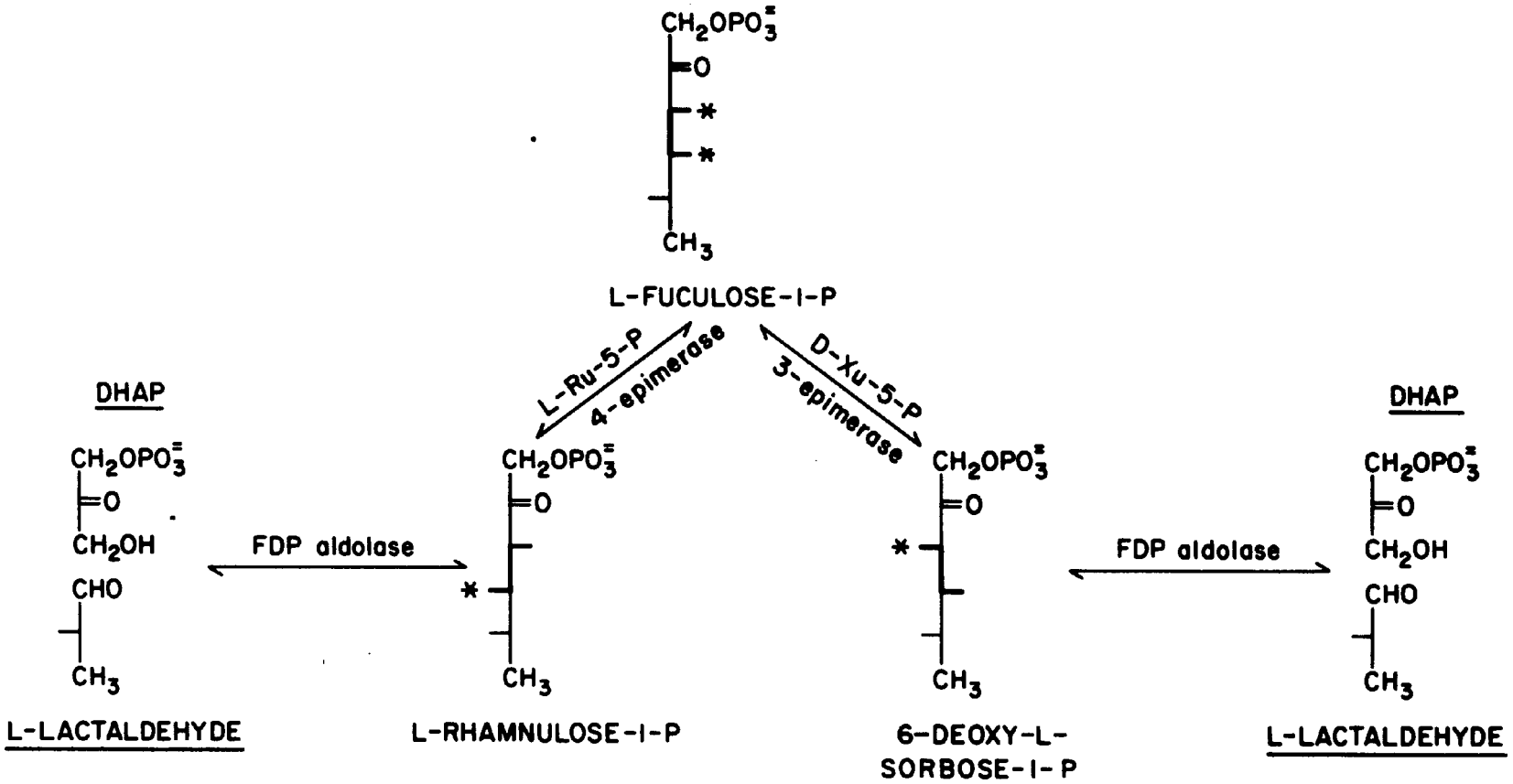


Figure 44. Theoretical basis for the possible epimerization of L-fuculose-1-P via L-Ru-5-P, 4-epimerase and D-Xu-5-P, 3-epimerase.

e. The conclusive evidence for the exclusion of the involvement of an epimerase in the catabolism of L-fuculose-1-P, and by consequence, the unique substrate specificity of L-fuculose-1-P aldolase was obtained by the following experiment. The proposed intermediate in the cleavage of L-fu-1-P, i.e., 6-deoxy-L-sorbose-1-P was tested with a purified preparation of L-fu-1-P aldolase. Incubation of L-fuculose-1-P aldolase with 6-deoxy-L-sorbose-1-P resulted in no significant formation of DHAP, while the addition of L-fuculose-1-P to this incubation mixture resulted in rapid formation of DHAP (see Figure 45). In order to further contrast the substrate specificity of L-fuculose-1-P aldolase and FDP-aldolase, to an identical incubation mixture containing 6-deoxy-L-sorbose-1-P, was added FDP-aldolase which resulted in the rapid appearance of DHAP. The small activity observed with L-fuculose-1-P aldolase and 6-deoxy-L-sorbose-1-P is believed to be due to the presence of residual amounts of FDP-aldolase in the L-fuculose-1-P aldolase preparations.

2. The second possibility assumes the existence of a nonspecific aldolase, one which could catalyze the cleavage of the carbon-carbon bond between C-3 and C-4 of the molecule of ketose-phosphate irrespective of the stereochemical configuration of hydroxyl groups at these positions. This possibility seems untenable both on theoretical and experimental grounds.

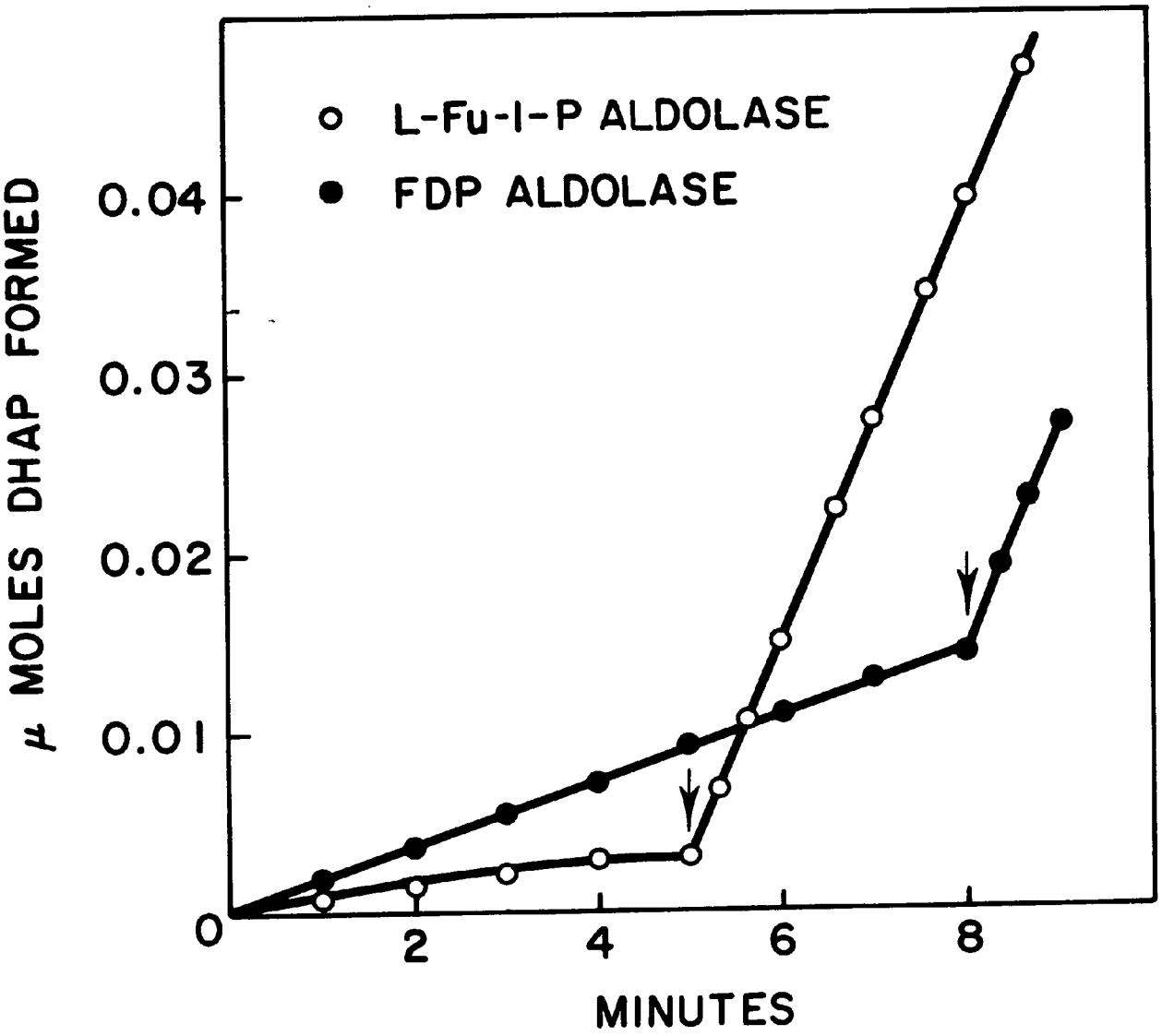


Figure 45. Specificity distinction between L-fu-1-P aldolase and FDP aldolase on the basis of their reactivity with 6-deoxy-L-sorbose-1-P. The incubation mixture contained the following (in μmoles): 6-deoxy-L-sorbose-1-P, 1.25, DPNH, 0.40; Tris buffer, pH 7.2, 10; an excess of α-GPD, and either L-fu-1-P aldolase (o—o—o) or FDP aldolase (·—·—·) in a final volume of 0.35 ml. A substrate-less control was incubated for each of the enzymes. At the times indicated by the arrows the following additions were made: 1.25 μmoles of L-fuculose-1-P were added to the cuvette containing L-fu-1-P aldolase (o—o—o), and 1.25 μmoles of FDP were added to the cuvette containing FDP aldolase (·—·—·). This experiment was conducted by the one-step assay procedure.

a. Theoretically, in the case of aldolases previously studied, it has been shown that these enzymes are stereospecific for the trans configuration of hydroxyl groups at positions 3 and 4. Thus, the existence of an aldolase which can act on ketose-phosphates irrespective of the configuration of hydroxyl groups at these positions seems improbable.

b. Experimentally, as indicated in the foregoing pages, L-fu-1-P aldolase specifically catalyzes the cleavage and formation of ketose-phosphate with cis C-3,4 hydroxyl groups.

3. The last possibility to consider, is one which assumes the occurrence of an aldolase with stereospecificity for the cis C-3,4 configuration of hydroxyl groups; the confirmation of this suggestion requires the establishment of the existence and distinctness of a new type of aldolase. The fact that L-fu-1-P aldolase exhibits unique substrate specificity requirements appears to be acceptable on the basis of the data presented. However, the answer to the problem of whether the protein molecule of this enzyme bears any structural resemblances to other known aldolases, requires elaborate studies which are beyond the scope of the intended investigation. In relation to this subject, Peanosky et al. (257), Swenson et al. (295), Velick (296), and Taylor and Lowry (297) have demonstrated that bovine liver aldolase and rabbit muscle aldolase are similar with respect to the following molecular properties:

Number of sulfhydryl groups per mole (257,295).

Estimated molecular weights by sedimentation and diffusion (257,297).

Electrophoretic mobilities and absorption spectra (257,296).

The preliminary experiments conducted in the present studies, indicate that L-fuculose-1-P aldolase appears to be distinct from previously described aldolases of animal and microbial origin. These results are summarized as follows.

a. As far as metal ion requirement, L-fuculose-1-P aldolase seems to be distinct from the known aldolases of microbial origin. While the bacterial (Clostridium perfringens and Aspergillus niger) and yeast aldolases are markedly (at least 7-fold) stimulated by K^+ (Rutter, et al., 298,299), L-fuculose-1-P aldolase appears to be indifferent. Also yeast aldolase is inhibited by cysteine (0.05M resulted in 50% inhibition),and Clostridium perfringens aldolase is enhanced by cysteine (253), whereas, L-fuculose-1-P aldolase is not affected by this substance. On the contrary, L-fuculose-1-P aldolas activity is remarkably stimulated (at least 10-fold) by Mg^{++} or Mn^{++}, whereas other microbial aldolases (Clostridium perfringens and yeast) not only are not stimulated, but are inhibited by 0.01M concentrations of either of these cations (253,301). In contrast to Lactobacillus bifidus (111), yeast (302),and Aspergillus niger (303) aldolases the loss of activity of which upon treatment with chelating agent is recoverable by the addition of Zn^{++}, the activity of L-fuculose-1-P aldolase could not be recovered when treated under

similar conditions. Therefore, on the basis of these criteria, L-fuculose-1-P aldolase cannot be included in the so-called class II aldolases described by Rutter (300).

b. Aldolases of animal origin, and FDP-aldolase of pea, are not inactivated by chelating agents and appear to be indifferent to the action of metal ions (257,245,304), and in some cases are stimulated by sulfhydryl-containing compounds, whereas, L-fu-1-P aldolase is inactivated at least 90% by EDTA and exhibits an absolute requirement for metal ions. Furthermore, it seems to be indifferent to the effect of cysteine. A summary of the comparison of various aldolases with L-fu-1-P aldolase is shown in Table 32.

c. FDP-aldolase and L-fu-1-P aldolase of Escherichia coli 0111 have been separated by calcium phosphate gel and DEAE-cellulose fractionation; indicating that these two enzymes are indeed distinuishable not only with regard to substrate specificity, but also on the basis of their physicochemical properties.

Therefore, on the basis of the data presented, the contention of the involvement of an epimerase and/or FDP-aldolase in the metabolism of L-fucose by Escherichia coli 0111-B_4 is not supported, and it is the conclusion of the present study that the cleavage of L-fuculose-1-P is a direct cleavage, which is catalyzed by a specific enzyme, L-fuculose-1-P aldolase.

TABLE 32

COMPARISON OF VARIOUS ALDOLASES WITH L-FU-1-P ALDOLASE

	Aldolases[a]	Inhibition by Chelating Agents	Reactivation by Divalent Cations	Stimulation by K^+	Activation by Cysteine	Optimum pH for Reaction
Class I[b]	Muscle	No	-	-		6.5-8.5
	Pea	No	-	-		6.5-8.5
	Tetrahymena	No	-	-		6.5-8.5
Liver	(Fructose-1-P)	No	-	-		
Class II[b]	Yeast	Yes	Fe^{++}, Co^{++}, Zn^{++}			7.0-7.5
	Clostridium perfrigens	Yes	Fe^{++}, Co^{++}, Zn^{++}	+	+ (253)[c]	7.0-7.5
	Aspergillus niger	Yes	Fe^{++}, Co^{++}	+		7.0-7.5
	Escherichia coli	Yes	Mn^{++}	+	+ (252)	7.0-7.5
L-Fuculose-1-P Aldolase		Yes	Mg^{++}, Mn^{++}, Ca^{++}	-	-	7.1-7.3

[a]All of the aldolases listed are FDP-aldolase except liver aldolase and L-Fu-1-P aldolase of Escherichia coli O111-B_4.

[b]Rutter, (300), and Grove and Rutter (299).

[c]Numbers in parentheses are references.

E. Mechanism of Aldol Condensations

A discussion of the mechanism of aldol condensation in general seems to be pertinent to this subject. In general, there are two main types of aldol condensations: (a) non-enzymatic and (b) enzymatic. These subjects will be briefly discussed in the following section.

1. NON-ENZYMATIC ALDOL CONDENSATIONS

Aldol condensation may result in the formation of new C-C bonds between two molecules of substances containing a hydrogen in α position to the carbonyl group (or capable of yielding an active hydrogen under appropriate conditions). Non-enzymatic aldol condensations are catalyzed either by base or by acid. Regardless of the catalyst, the condensations of the aldol type can be explained on the basis of ionic displacement mechanisms (305), and are considered to proceed via two steps: an activation phase, followed by condensation. For the simplicity of illustration, the base-catalyzed condensation of DHA and D-glyceraldehyde will be discussed as amodel system.

a. Activation Phase

The labilization of an α hydrogen (a hydrogen located on a carbon atom adjacent to a carbonyl group) by the attack of a nucleophilic reagent (i.e., a base) results in the liberation of the hydrogen as a proton in the medium, with concommitant formation of a carbanion (or activated DHA). Since the activation phase in non-enzymatic aldol condensations

proceed in a random fashion non-stereospecifically, an enantiomorphic pair of carbanions will be formed (see "Step 1, Figure 46"). The consequence of the activation phase is the formation of a resonance system consisting of the enantiomorphic carbanions and their common enol.

b. Condensation Phase

The subsequent reaction of the carbanions with electrophilic carbonyl carbon of the aldehyde establishes a new C-C bond. The condensation step is stereospecific, since only a single diastereoisomer is formed by condensation of each carbanion with the polarized carbonyl group of aldehyde. Had this step been nonspecific for the stereochemical configuration of the newly formed C-C bond, one would expect the formation of two diastereoisomers from each carbanion (one possessing cis configuration of hydroxyl groups at the new C-C bond, the other containing trans configuration) and by consequence, a total of four diastereoisomers will be expected upon condensation of DHA and D-glyceraldehyde (these are: D-sorbose, D-tagatose, D-fructose, and D-psicose).

Fischer and Baer (306) using DHA and D-glyceraldehyde, were able to recover a mixture of 50% D-fructose and 50% D-sorbose as the condensation products, with no detectable amounts of the other two diastereoisomers, namely, D-tagatose and D-psicose. Although the results of Fischer and Baer suggest that the condensation phase in base-catalyzed aldol condensations is specific for the formation of trans adducts (as evident by the fact that D-sorbose and D-fructose have the same configuration (trans)

MECHANISM OF BASE-CATALYZED ALDOL CONDENSATION

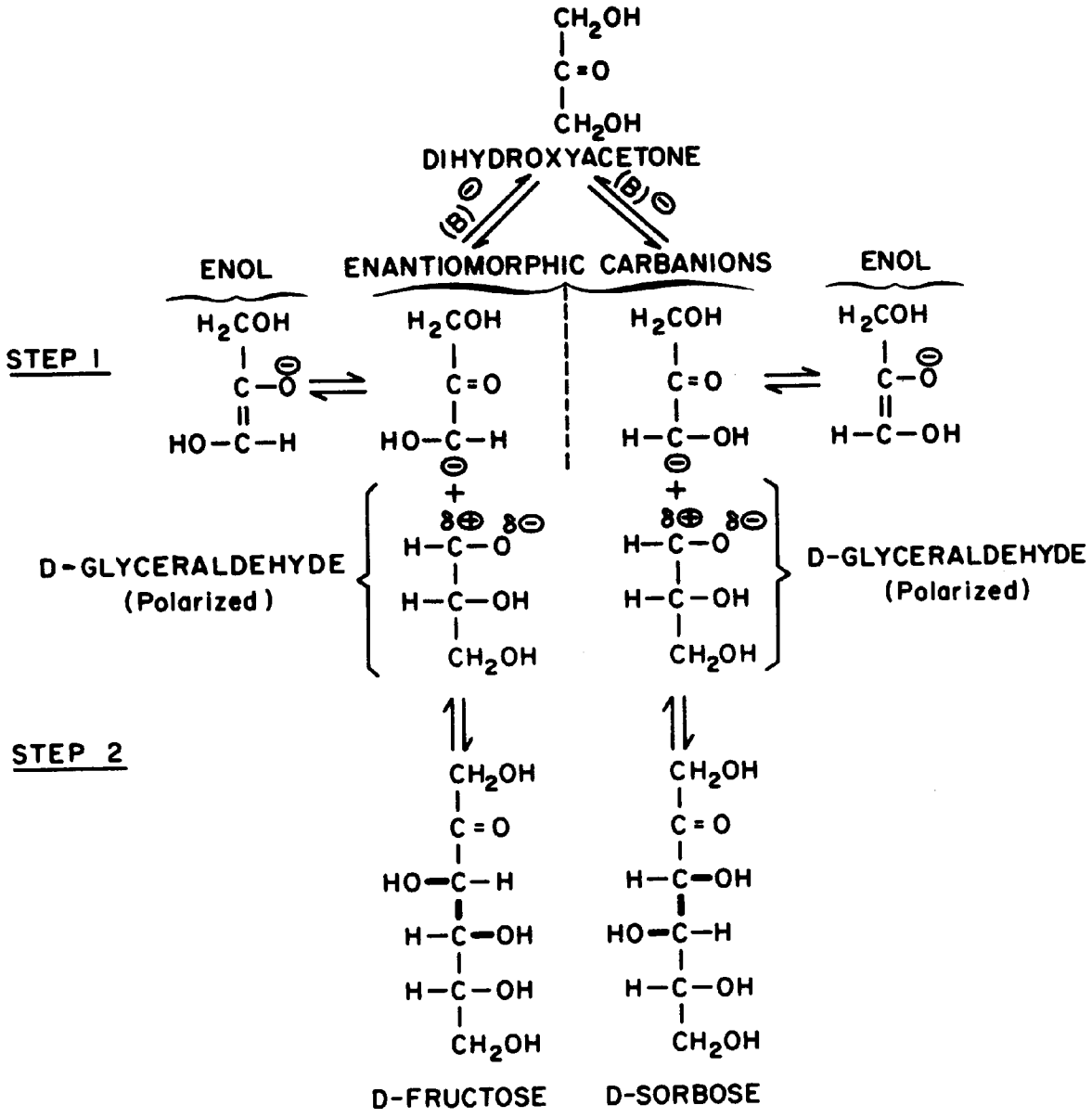


Figure 46. The mechanism of base-catalyzed aldol condensation.

at C-3 and C-4 of their molecules). However, it must be emphasized that the reported analyses were based on the composition of the products of condensation under equilibrium conditions. Furthermore, due to the lack of availability (in 1936) of specific and sensitive methods to definitively and quantitatively determine the degree of stereospecificity of the reaction, the findings of Fischer and Baer would seem questionable (300). Particularly, since a study of the rates of formation and conversion of each of the possible diastereoisomers, their stability under the conditions of the experiments applied, and further studies with model systems using various base catalysts appears to be necessary in order to establish a decisive conclusion. A representation of base-catalyzed aldol condensation is illustrated in Figure 46.

2. ENZYME-CATALYZED ALDOL CONDENSATIONS

Enzyme-catalyzed aldol condensations resemble base-catalyzed aldol condensations in that they proceed via two steps, i.e., an activation phase, followed by condensation; however, the enzymatic aldol condensations are distinct from non-enzymatic type with regard to their stereospecificity for both of the phases of the reaction (307). The aldolase-catalyzed condensations have received extensive attentions from various laboratories; a brief discussion of some interesting aspects of this problem concerned with the two phases of the reaction will be presented in the following section.

a. Activation Phase

The reaction of DHAP with aldolase labilizes one of the α hydrogens of the carbinol group of DHAP, which results in the exchange of this α hydrogen with the protons of the medium. In this case, the aldolase performs the function of the base, except that the enzyme acts stereospecifically. The following interesting studies have led to the conclusion that the activation phase involves a stereospecific complex formation between the enzyme and DHAP.

The formation of such a complex was established by the following facts.

(1) Exchange reaction occurred only in the presence of an active enzyme; heat-inactivated enzyme could not form a complex with DHAP.

(2) The complex formation was inhibited by Ag^{+}, Cu^{++}, and ethanol; agents which inhibit the aldolase activity.

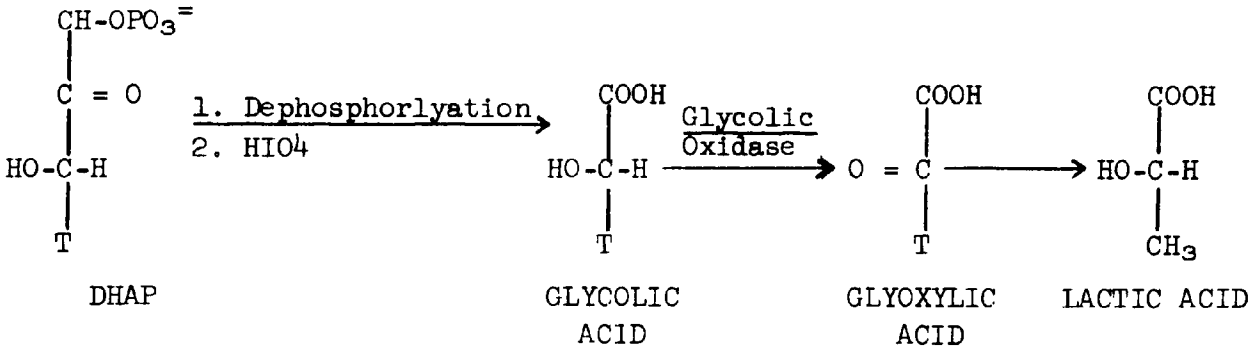
(3) The exchange reaction obeyed the limited kinetic requirements.

(4) DHAP-aldolase complex formation was further established by direct spectrophotometric evidences presented by Topper et al. (308). Thus, incubation of DHAP with aldolase resulted in the formation of a complex with strong ultra-violet absorption at 230-250 mμ.

The exchange of one atom of carbon bound hydrogen with the aqueous solvent was established by isotopic techniques (302,308,310-312). The stereospecificity of activation of DHAP with aldolase was deduced from the observation that only one atom of tritium was exchanged per mole of DHAP, when the latter was incubated with aldolase (muscle or yeast) in the presence of T_2O. This idea was further strengthened by the fact

that triose phosphate isomerase and aldolase activated different H atoms of DHAP. Thus when tritium labeled DHAP (produced by incubation of DHAP and aldolase in T_2O) was incubated with triose phosphate isomerase in H_2O, no tritium was liberated in the medium and vice versa.

In their studies on the nature of the condensation products of DHAP with glyceraldehyde-3-P in the presence of aldolase, Bloom and Topper (307) deduced the absolute configuration of the DHAP carbanion formed upon its reaction with aldolase in the activation phase. However, this observation was confirmed by the elegant technique of Rose (313). Tritiated DHAP (prepared by incubation of DHAP and aldolase in the presence of T_2O) was dephophorylated, followed by periodate oxidation to yield tritium-labeled glycolic acid; the latter was oxidized to glyoxylic acid in the presence of glycolic oxidase. This treatment resulted in no loss of tritium. Since glycolic oxidase is specific for L-lactic acid and will not act on the D-isomer, Rose concluded that the glycolic acid formed from DHAP, and therefore the tritiated DHAP formed by the action of aldolase, carried the label in the same relative position occupied by the proton in D-lactic acid, as illustrated in the following scheme:



Degradation of tritiated DHAP to glyoxylic acid (313,314).

The activation phase in the aldolase-catalyzed reactions is depicted in Figure 47.

b. Condensation Phase

The subsequent reaction of the DHAP-E complex with the polarized carbonyl group of aldehyde appears to be governed by the same mechanisms as described for the condensation phase of base-catalyzed reactions; this suggests why the aldolases appear to be nonspecific for the aldehydes. Consequently, the sole activating function of aldolases relates to DHAP, which clarifies the psecificity of all known aldolases for this compound. However, the over-all rate of the reaction of aldolase may not be solely dependent on DHAP, on the basis of the great discrepancies found in relation with the reactivity of phosphorylated aldehydes vs. non-phosphorylated aldehydes; a point which will be discussed in the following section.

As illustrated in Figure 47, since both steps involved in the aldolase-catalyzed condensations are stereospecific, only one diastereoisomer is expected to be formed; a fact that has been established. These results may explain the stereospecificity of the known aldolases for the trans C-3,4 hydroxyl groups of the molecule of ketose-phosphates, either cleaved or formed. In fact, in the cases of the aldolases studied to date, this specificity holds true, and has been attributed to the stereospecific complex formed between enzyme and DHAP. However, Bloom and

PROPOSED MECHANISM OF ENZYME-CATALYZED ALDOL CONDENSATION

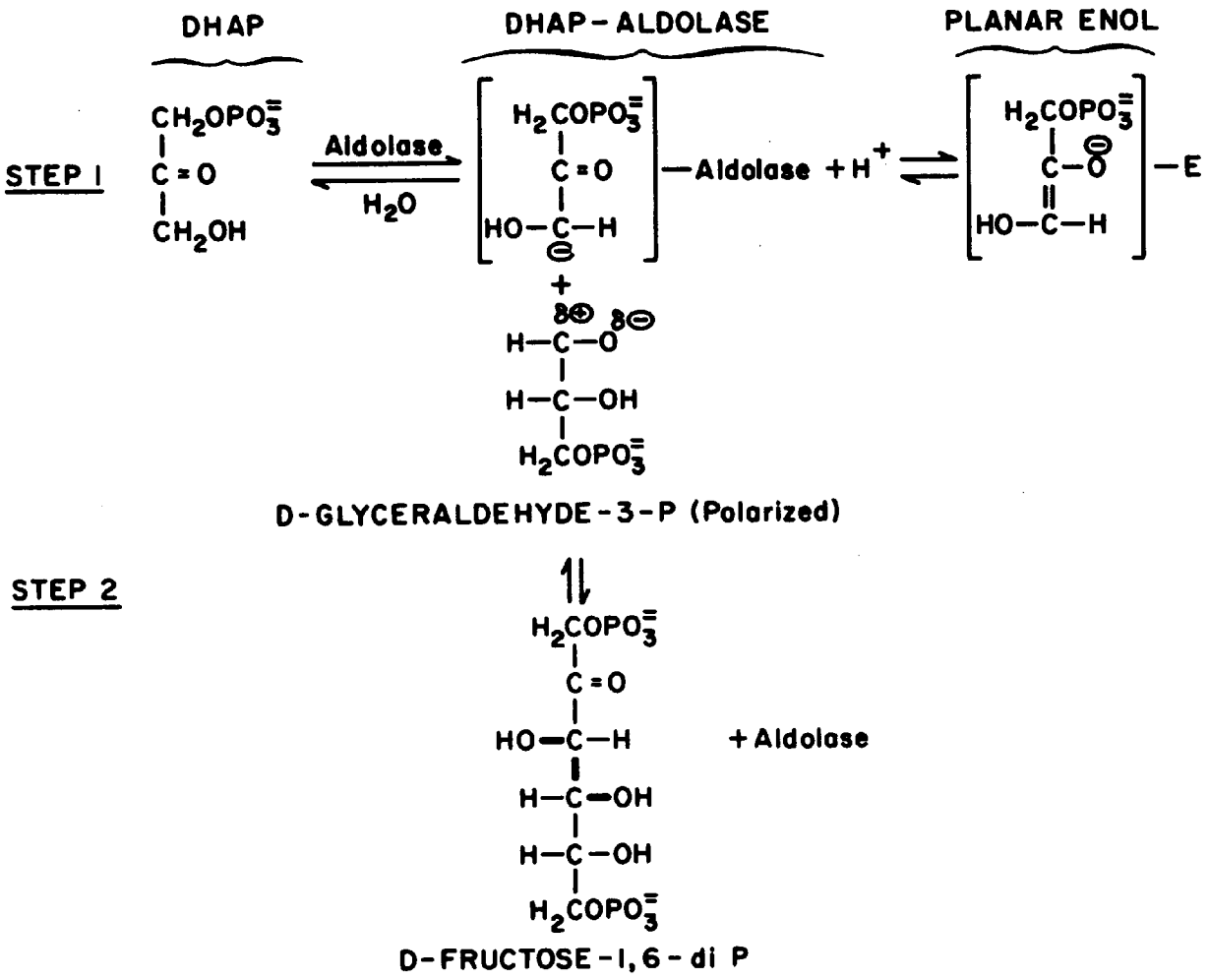


Figure 47. The proposed mechanism of reaction of FDP-aldolases.

Topper (307) on the basis of their studies on the nature of the DHAP-aldolase complex, attributed the substrate specificity of aldolases to the following factors.

(1) These authors, speculated a three-point attachment of the aldolase to the molecule of DHAP to yield a stereospecific carbanion. Therefore, only two of the possible products can be formed (by condensation of DHAP with GAP), namely, either D-fructose-di-P or D-tagatose-di-P; in other words, the enzyme exhibits specificity only with respect to the stereochemical configuration at C-3 of the molecule of ketose-phosphates (L-configuration of OH groups at this carbon is required). Rose and Rieder (311) have also investigated this possibility,and have suggested that the stereospecificity of aldolase may be related to C-3 configuration of the molecule of ketose-P, rather than C-3 and C-4. This suggestion is based on the fact that all of the substrates of aldolases studied so far (D-fructose-di-P, D-fructose-1-P, D-xylulose-P, D-tagatose-di-P,and D-sedoheptulose-di-P) contain identical configuration at C-3 of the molecule, i.e., they bear a hydroxyl group at C-3 which is located to the left in the Fischer projection formulae.

(2) The stereospecificity of DHAP-aldolase complex has been further considered by Rose and Rieder (311); these authors indicated that the fact that aldolases are specific for only one of the α hydrogens (of the carbinol group) neither proves nor denies the possibility of formation of either <u>cis</u> or <u>trans</u> substituents on the newly linked carbon atoms. Since the nature of the product may be governed by the configuration of

the attacking aldehydes in the transition state; a fact which has also been studied by Bloom and Topper (307,310). On the basis of the observation of greater tendency of the FDP-aldolases toward the phosphorylated aldehydes as compared with non-phosphorylated aldehydes, these authors suggested that the binding of the aldehyde moiety to enzyme may also be an important factor. This is even more apparent in cases where pentoses are used to condense with DHAP. Dische (315) reported the biosynthesis of octulose-di-P by condensation of DHAP with ribose-5-P, whereas, this reaction has never been observed with the free pentose.

F. Specificity of Enzyme-Catalyzed Aldol Condensations

On the basis of the extensive investigations conducted independently in several laboratories, the specificity of enzyme catalyzed aldol condensation may be summarized as follows.

1. EXISTENCE OF TWO INDEPENDENT PHASES

The fact that the over-all reaction of aldolase involves two distinct phases (exchange phase and condensation phase) was definitively demonstrated by Rose and Rieder (311). Using yeast aldolase, these authors observed that the rate of exchange is markedly pH-dependent. The pH optimum for exchange reaction was about 6.0, whereas the pH optimum for the over-all aldol cleavage was 7.2. Similar results have been obtained by Richards and Rutter (298). These authors demonstrated that the pH optimum for aldol synthesis is 7.0-7.5, whereas the exchange

occurred at pH values below 6.5 even at a rate higher than the aldol cleavage. At pH values higher than 6.5 the rate of aldol cleavage exceeded that of the hydrogen exchange. Furthermore, the exchange rate of yeast aldolase is stimulated specifically by potassium ion (298).

2. SPECIFICITY FOR DHAP IN THE EXCHANGE PHASE

The following results have led to the conclusion that the FDP-aldolases studied so far exhibit a strict specificity for DHAP.

a. Exchange of an α hydrogen (attached to the carbinol group) of DHAP and medium tritium or deuterium in the presence of aldolase.

b. Specificity of the exchange reaction for one of the carbon-bound hydrogens.

c. Formation of a stereospecific complex between DHAP and aldolase.

d. Absolute requirement of a phosphate group at C-1 of DHAP.

e. A ketone group on C-2 and a hydroxyl group on C-3 appear to be also required (298,300); chemical properties which characterize the chemical structure of DHAP.

3. IDENTICAL STEREOSPECIFICITY IN THE EXCHANGE AND CONDENSATION

The involvement of only one α hydrogen in the exchange phase and condensation was shown by the fact that condensation of tritiated DHAP (prepared by conducting the exchange reaction in T_2O) with cold GAP resulted in the synthesis of unlabeled FDP. This indicates that the C-H bond which is involved in the exchange phase is also involved in the

condensation. Furthermore, since the exchange reaction is inhibited by FDP and GAP, it is suggested that both the exchange reaction and the condensation occur at the same site of enzyme.

4. THE ROLE OF THE ALDEHYDE MOIETY

The specificity of the known FDP aldolases toward the DHAP moiety is an established fact, however, the nonspecificity of these enzymes toward aldehydes as suggested by some authors seems to be questionable. For example the Km values of DHAP and GAP are not very far from each other, a fact which indicates that the affinity of binding sites of aldolase is similar for the phosphate groups of these two compounds (300). Although the exchange experiments demonstrate that no aldolase-catalyzed exchange reaction takes place with aldehydes (310), the possibility exists that this might be due to the induced polarization of the aldehydes in the presence of DHAP.

It is of interest to point out that the substrate specificity of the aldolases which have been intensively studied to date is no longer believed to be stringently restricted to DHAP alone. In fact there are several lines of evidence to suggest that various aldolases do exhibit some kind of a specificity towards the aldehydes. This fact had remained unrecognized for a long time, due to the lack of information concerning the actual mechanism of action of these enzymes and more so, because of disregarding the significance of the quantitative aspects of this type of condensation reactions, i.e., the rates of activation and the magnitude

of the tendency of various aldolases toward the aldehyde moiety in the course of the reaction. The greater affinity of some FDP-aldolases towards the phosphorylated aldehydes as compared with the non-phosphorylated aldehydes, has been indicated by Horecker (314). This fact was strengthened by Dische (315), who reported that in the case of enzyme-catalyzed condensation of DHAP with pentoses, the latter must possess a phosphate group in the 5 position; furthermore, the aldolase-catalyzed condensation of the non-phosphorylated pentoses with DHAP has never been observed. In summary, on the basis of the suggestion of Rutter (300), a phosphate group in the 1 position of DHAP is required for the enzymatic aldol reaction, although, this does not exclude the possibility of such a role being played by an aldehyde possessing a phosphate group. In fact there is at least one actual case, where the presence of a phosphate group in the molecule of dihydroxyacetone is not required for the formation of a complex between this compound and the enzyme. However, the presence of a phosphate group in the aldehyde moiety was necessary in order to condense with the activated DHA. This case is the unequivocal evidence presented by Horecker et al. (317) and Racker et al. (318) concerning the formation of a stable (transaldolase-DHA) complex in the course of transaldolase reaction. Transaldolase does not catalyze a reaction identical to that of FDP-aldolase. However, due to the fact that in both cases the end result of the reaction is the establishment of a new C-C bond with trans hydroxyl groups at C-3,4 of the molecule, the mention of the results obtained in studies with this enzyme does not seem impertinent to the sub-

ject of this discussion. Therefore, the role of various aldehydes played in the catalytic process of enzymatic aldol condensations remains to be established

G. Comparison of the Substrate Specificity of Aldolases

The substrate specificity of L-fuculose-1-P aldolase shown in Tables 24 and 26, illustrates that this enzyme specifically catalyzes the cleavage and synthesis of ketose-phosphates with cis C-3,4 hydroxyl groups. This property would automatically place L-fuculose-1-P aldolase in a separate class distinct from other known aldolases, which by far are considered to be stereospecific for the trans configuration at positions 3 and 4. Although this classification is valid qualitatively, however, from a quantitative standpoint there are some major differences among aldolases isolated from various sources (muscle, yeast, pea and liver). A comparison of the substrate specificity of various aldolases is shown in Table 33. As indicated, there are some major differences among the substrate specificity of aldolases isolated from various sources. In the following section, it is intended to compare and contrast some of the properties of these enzymes, and explain the reasons for some of the observed discrepancies on the basis of the recent studies available in the literature.

1. As indicated, FDP-aldolase of muscle is not strictly specific for trans C-3,4 hydroxyl groups, since it cleaves D-tagatose-diphosphate

TABLE 33

COMPARISON OF SUBSTRATE SPECIFICITY OF VARIOUS ALDOLASES[a]

1. IN THE DIRECTION OF ALDOL CLEAVAGE

Reaction		Sources of Aldolases[b]			
Substrates	Products	Muscle	Liver	Yeast	Pea
F-1,6-diP	DHAP + D-GAP	100	100	100	100
S-1,6-diP	DHAP + L-GAP	80 (268)	-	+ (298)	-
T-1,6-diP	DHAP + D-GAP	10 (268)	-	-	-
F-1-P	DHAP + D-GA	16 (268)	40 (257)	.04 (298)	-
S-1-P	DHAP + L-GA	12 (268)	-	+ (298)	-
F-6-P	0	0 (268)	-	0 (298)	0 (304)

[a]The following abbreviations are used in this portion of the table: F-1,6-diP, D-fructose 1,6-diphosphate; DHAP, dihydroxyacetone phosphate; D-GAP, D-glyceraldehyde 3-phosphate; S-1,6-diP, L-sorbose 1,6-diphosphate; L-GAP, L-glyceraldehyde 3-phosphate; T-1,6-diP, D-tagatose 1,6-diphosphate; F-1-P, D-fructose 1-phosphate; S-1-P, L-sorbose 1-phosphate; F-6-P, D-fructose 6-phosphate.

[b]Numbers in parentheses are literature references. Numbers outside parentheses represent the relative rates.

The following symbols are used in this table:

0 = no reaction.
- = the reaction has not been studied to date.
+ = the reaction has been investigated qualitatively; no rate studies have been reported.
{ = a racemic mixture of reactants used, or product consisted of a mixture of two diastereoisomers.

TABLE 33 (Concluded)

2. IN THE DIRECTION OF ALDOL CONDENSATION[a]

Reaction		Sources of Aldolases			
Substrates	Products	Muscle	Liver	Yeast	Pea
DHAP[b] + D-GAP	F-1,6-diP	280 (298)	-	300 (298)	-
DHAP + L-GAP	S-1,6-diP	+ (268)	-	-	-
DHAP + D-GA	F-1-P	2 (268)	-	.03 (298)	-
DHAP + L-GA	S-1-P	+ (268)	-	-	-
DHAP + L-LAD	DS-1-P	+	-	-	+
DHAP + D-LAD	Rhu-1-P	+ (198)	-	-	+ (221)
DHAP + D-Eryth-P	Sed-1,7-diP	+ (319-320)	-	-	-
DHAP + Acetald.	5-Deoxy-Xu-1-P	+ (260)	-	-	+ (321)
DHAP + Glycolald.	Xu-1-P	+ (263-322)	-	-	+ (216)
DHAP + Glycolald-P	Xu-1,5-diP	+ (263)	-	-	-
DHAP + Formald.	Erythul-1-P	+ (257)	+ (257-258)	+ (259)	-
DHA + D-GAP	0	0 (298)	-	0 (297)	-
α-GP + D-GAP	0	0 (302)	-	-	-

[a]The following abbreviations are used in this portion of the table: DS-1-P, 6-deoxy-L-sorbose-phosphate; L-LAD, L-actaldehyde; D-LAD, D-lactaldehyde; Rhu-1-P, D-rhamnulose 1-phosphate; D-Eryth-P, D-erythrose 4-phosphate; Sed-1,7-diP, sedoheptulose 1,7-diphosphate; Acetald., acetaldehyde; 5-deoxy-xu-1-P, 5-deoxy-xylulose 1-phosphate; Glycolald., glycolaldehyde; Xu-1-P, D-xylulose 1-phosphate; Glycolald-P, glycolaldehyde 2-phosphate; Formald., formaldehyde, Erythul-1-P; D-erythrulose 1-phosphate.

[b]In some of these experiments D-fructose 1,6-diphosphate was used as a source of DHAP; furthermore, most of the enzyme systems employed contained triose phosphate isomerase.

which contains cis configuration at these positions (268). This may be explained by the hypothesis of Rose and Rieder (310), who suggested that the specificity of FDP-aldolase is attributed to the stereochemical configuration at C-3 of the molecule of ketose-phosphate (D-tagatose and D-fructose have the L-configuration at this position). However, there is an exceptional case where the ketose-phosphate possesses the D-configuration at C-3 and yet it is acted upon by muscle aldolase; that is, D-erythrulose-1-phosphate (257-259).

2. The substrate specificity of the known aldolases is not directed solely to the trans C-3,4 hydroxyl groups; other substituents of the substrates such as phosphate groups also play an important role in certain cases. For example, the rate of the reaction of liver aldolase with D-fructose-1-P is three times greater than muscle aldolase (268), and 1000 times greater than that of yeast aldolase (298). This may indicate that the stereospecificity of liver aldolase requires the presence of a single phosphate group at C-1. Furthermore, the reported rate studies are affected to a great extent by the purity of the enzyme preparations applied in the experiments. Korkes (323) indicated that the highly purified FDP-aldolase of muscle did not catalyze the cleavage of D-fructose-1-P. This observation was strengthened by Richards and Rutter (298), who reported that the rate of cleavage of FDP by muscle aldolase was 75 times higher than D-fructose-1-P, and 60 times higher than L-sorbose-1-P. These results clearly establish that the specificity of muscle aldolase requires the presence of two phosphate groups at C-1 and C-6 of the molecule of

ketose-phosphate in addition to the presence of trans C-3,4 hydroxyl groups. Similar results have been obtained with regard to this subject in the direction of condensation. Bloom and Topper (307) reported that the rate of condensation of phosphorylated aldehydes with DHAP is much greater than that of DHAP with non-phosphorylated aldehydes.

3. Recent investigations have elucidated some interesting aspects of substrate specificity of various aldolases on the basis of the nature of the chemical groups at or close to the catalytically active site of the molecule of these enzymes. Drechsler et al. (324), have shown that treatment of muscle aldolase with carboxypeptidase (CP) results in an aldolase which has lost 93% of its original FDP-cleavage activity. Furthermore, they demonstrated that this loss of activity was accompanied by the release of 3 tyrosyl residues in carboxyl-terminal position of the peptide chain. These authors subsequently demonstrated that while carboxypeptidase treatment of muscle aldolase resulted in 93% loss of its original FDP cleavage activity, its reactivity toward fructose-1-P cleavage was fully retained or even enhanced. Hence it was suggested that if the tyrosyl groups determine the efficacy of the catalytic activity, their release from the enzyme may diminish the FDP cleavage activity. Richards and Rutter (298) have conducted some interesting studies with regard to the effect of carboxypeptidase on aldolases. These authors demonstrated that while carboxypeptidase treatment of class I aldolases i.e., muscle, pea,and Tetrahymena (298,299) resulted in 93% loss of their original activity toward FDP cleavage accompanied by altered substrate

specificity, the activity of class II aldolases, i.e., Aspergillus niger, Clostridium perfringens, and yeast (298,299) remained unaffected by carboxypeptidase treatment. Moreover, the substrate specificity of class II aldolases is restricted to FDP. The higher reactivity of liver aldolases with fructose-1-P than class I aldolases with fructose-1-P, may be explained by the observation that the carboxypeptidase-treated class I aldolases acquire the properties of the liver aldolases, since the treatment of the former results in a great loss of FDP cleavage activity, with full retention of the reactivity toward fructose-1-P (298,325). On the basis of these experiments, Rutter (325,326) suggested a close structural resemblance between muscle and liver aldolases; particularly, since it has also been established that these enzymes have similar physicochemical properties (molecular weight, number of SH groups per mole, electrophoretic mobilities and absorption spectra).

H. Biosynthesis of New Ketose Phosphates

The study of L-fuculose-1-P catabolism by Escherichia coli O111-B_4 has developed into a consequence of broader biochemical significance, i.e., the discovery of a method for biosynthesis of a variety of new ketose-1-phosphates. The preparation of such compounds has not been achieved to date, either by non-enzymatic or by enzyme-catalyzed aldol condensations. In fact, the synthesis of the three new compounds (D-ribulose-1-P, D-psicose-1-P and L-tagatose-1-P) reported in this thesis

has never been mentioned in the literature. The most important asset of L-fuculose-1-P aldolase, is its distinct and unique substrate specificity requirements, which makes it possible to synthesize ketose-1-phosphates containing cis configuration of hydroxyl groups at positions 3 and 4 of the molecule; this goal can be achieved through condensation of DHAP with the appropriate aldehydes.

The fact that no information is available concerning the role of such ketose-phosphates in the over-all metabolism of living organisms does not preclude the possible importance of these compounds in the biological activities of at least certain forms of life. In fact, it has been indicated (3) that crude extracts of Escherichia coli are capable of biosynthesis of D-ribulose, L-tagatose, and D-psicose (exactly the three compounds whose 1-phosphate esters have been biosynthesized in these studies) through isomerization of their respective aldoses. If these compounds are to be incorporated into some constituents of this organism, it is conceivable that perhaps their corresponding 1-phosphate esters would be more likely than the free sugars to participate in certain biochemical reactions leading to biosynthesis of substances possessing higher molecular weights. This may provide a basis for the significance of the synthesis of these ketose-phosphates via the aldolase reaction, unless some other mechanism(s) is available in the organsim to convert these free sugars to their corresponding phosphorylated forms prior to their incorporation into the desired substances. Although this alternative hypothesis seems possible, it does not appear to be

practical on the basis of the cell's biochemical economy. Since at least two of the aldoses (L-galactose and D-allose or D-altrose) required for the synthesis of L-tagatose and D-psicose, respectively, are considered among the rare naturally occurring sugars. Furthermore, L-galactose and D-altrose (or D-allose) have never been found to exist together in the polysaccharides of bacterial sources which have been analyzed to date (3). According to the suggestion of Green and Cohen (3),the above mentioned sugars may follow the phenomenon of mutual exclusion, i.e., the presence of one excludes the incorporation of the other in the same polymer. The observation that these ketose-1-phosphates are biosynthesized upon condensation of smaller units, would suggest that this mechanism is more likely an anabolic pathway rather than a catabolic one; since it is conceivable that the further dissimilation of the smaller fragments (DHAP and aldehydes) by this organism would be more probable than the ketose-1-phosphates biosynthesized.

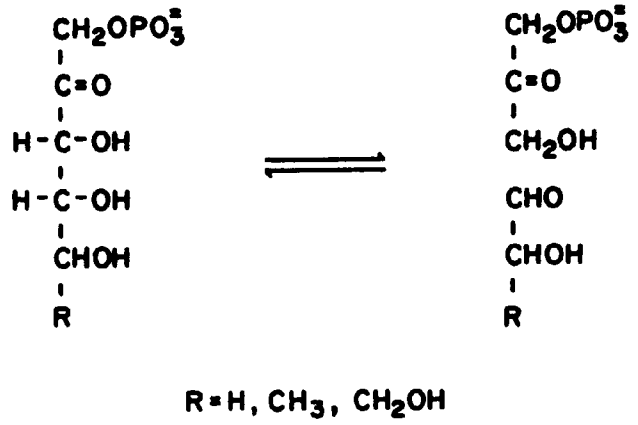
However, the fact that D-ribulose, D-psicose,and L-tagatose have not been detected among the degradation products of Escherichia coli cells would render the problem of the possible role(s) of these ketose-1-phosphates in the biologic activity of the organism a complicated one, whose solution necessitates some intensive and elaborate investigations which are beyond the subject of the present studies. Furthermore, it must be emphasized that these results reported here are based solely on in vitro experiments that may or may not be the same as those obtained under in vivo conditions. Therefore, the general biochemical significance of this new series of ketose-1-phosphates remains to be established.

VI. CONCLUSIONS

On the basis of the data presented, the substrate specificity requirements of L-fuculose-1-P aldolase may be formulated as illustrated in Figure 48. This enzyme appears to be specific for the cis configuration of hydroxyl groups at positions 3 and 4 of the molecule of ketose-phosphates. Thus either in the direction of cleavage, or condensation, the enzyme will act only on ketose-phosphates possessing the phosphate group in the one position, and cis hydroxyl groups at carbon atoms 3 and 4 of the molecule. Furthermore, only one of the two possible diastereoisomers is acted upon, namely, the one which has the cis C-3,4 hydroxyl groups situated to the right side (D-configuration) in the Fishcer projection formula.

With regard to the mechanism of L-fuculose-1-P aldolase reaction, no information is available. Whether a similar type of complex could be formed between DHAP and L-fuculose-1-P aldolase that favors the synthesis of cis C-3,4 compounds remains to be established. Regardless of its mechanism, and the fact that L-fuculose-1-P aldolase exhibits a different specificity from FDP-aldolase, all these enzymes are analogous in the fact that they each yield only one of the four possible diastereoisomers in the condensation reactions. It seems reasonable to suppose that two additional aldolases may exist in nature showing similar specificities toward the two remaining types of stereochemical configuration at C-3,4.

SPECIFICITY OF L-FU-1-P ALDOLASE



R = H, CH_3, CH_2OH

Figure 48. Substrate specificity of L-fuculose-1-P aldolase.

Concerning the aldehyde specificity of L-fuculose-1-P aldolase, the available data indicate some type of specificity exhibited by this enzyme toward the aldehydes. It appears that aldehydes containing at least one secondary alcoholic group can serve as substrate to condense with DHAP. However, this observation neither establishes nor denies the possibility of lack of specificity of this enzyme toward the aldehyde moiety; i.e., it may be possible that aldehydes lacking a secondary alcoholic group can condense with DHAP in this system under specific experimental conditions which would favor their reactivity in the transition state. That no conclusion may be drawn with regard to the aldehyde specificity of this enzyme is strengthened by the fact that only a few aldehydes lacking secondary hydroxyl groups have been tested in this system. Furthermore, no attempt was made to modify the experimental conditions to ascertain whether these aldehydes were indeed inactive with this enzyme. Therefore, further investigations will be necessary to establish whether or not L-fuculose-1-P aldolase exhibits any specificity toward the aldehyde moiety in the condensation reaction.

VII. SUMMARY

In the course of investigation of the catabolism of L-fucose by L-fucose-grown Escherichia coli O111-B_4, a pathway has been established which involves three distinctive enzymes. These results are summarized as follows.

1. L-Fucose isomerase activity was detected in the crude extracts of this organism and the product of the reaction was characterized as L-fuculose.

2. L-Fuculose kinase has been purified 35-80 fold. This enzyme catalyzes the phosphorylation of L-fuculose in the presence of ATP and either Mg^{++} or Mn^{++}. The following conclusions are drawn with regard to this enzyme.

a. The purified preparation of this enzyme has been used for preparation of its product; this product was characterized as L-fuculose-1-P by colorimetric reactions, paper chromatographic technique, paper electrophoresis,and periodate oxidation studies. The product was homogeneous by all the criteria applied.

b. Of the various 6-deoxyhexoses tested (L-fucose, L-rhamnose, L-rhamnulose, 6-deoxy-L-sorbose and L-fuculose) with a purified preparation of this enzyme, L-fuculose was the only compound which was phosphorylated. Thus, on the basis of these data, there appears to be no evidence for the direct phosphorylation of L-fucose in this system.

3. A specific type of aldolase, L-fuculose-1-P aldolase has been purified (at least 120-fold) which catalyzes the reversible aldol cleavage of L-fuculose-1-P to trioses which have been characterized as DHAP and L-lactaldehyde.

The following facts have been established with regard to this enzyme.

a. L-Fuculose-1-P aldolase exhibits a unique stereospecificity for the cis configuration of hydroxyl groups at C-3,4 of the molecule of ketose-phosphate. This characteristic distinguishes this enzyme from the other known aldelases which are relatively specific for the trans configuration of hydroxyl groups at these positions.

b. L-Fuculose-1-P aldolase is distinct from the other aldolases not only on the basis of its substrate specificity, but also on the basis of its responses toward metal ions and sulfhydryl-containing compounds. Moreover, L-fu-1-P and FDP aldolases of Escherichia coli O111-B_4 have been almost completely separated from crude preparations containing both of these enzymes by calcium phosphate gel and DEAE-cellulose fractionation.

c. The cleavage of L-fuculose-1-P is a single-step reaction catalyzed by L-fuculose-1-P aldolase, and does not involve an epimerization and/or FDP aldolase; since L-fu-1-P is not acted upon by either D-Xu-5-P, 3 spimerase or L-Ru-5-P, 4-epimerase to give rise to a ketose-phosphate with trans C-3,4 hydroxyl groups which could be subsequently cleaved by FDP aldolase. Furthermore, the proposed product of epimerization of L-fu-1-P by D-Xu-5-P, 3-epimerase,

6-deoxy-L-sorbose-1-P, is not acted upon by L-fuculose-1-P aldolase. Further evidence for the involvement of a single enzyme in the cleavage of L-fu-1-P was obtained from the fact that at no stage of purification of L-fu-1-P aldolase, was there any indication that more than a single protein was involved in the cleavage of L-fu-1-P. Purified preparations of this enzyme were obtained in high yields (70-75%) with a minimum of 120-fold purification.

Subsequent substrate specificity studies established that either in the direction of cleavage, or in the direction of synthesis, this enzyme will act only on ketose-monophosphates containing the phosphate group in the 1 position and cis configuration of hydroxyl groups at C-3,4. Furthermore, a single diastereoisomer is acted upon by this enzyme, namely,one which possesses the cis C-3,4 hydroxyl groups located on the right side in the Fischer projection formula. As far as the aldehyde specificity, it appears that L-fu-1-P aldolase exhibits some specificity toward aldehydes containing at least one secondary hydroxyl group. However, lack of sufficient data does not permit any speculation in this regard. Therefore, the aldehyde specificity of this enzyme remains to be established.

d. The unique specificity of this enzyme was established by the enzymatic synthesis of ketose-1-phosphates containing cis C-3,4 hydroxyl groups by condensation of DHAP with appropriate aldehydes in the presence of this enzyme. Three new ketose-1-phosphates with

C-3,4 stereochemical configurations specified above, were prepared by condensation of DHAP with either of D-glyceraldehyde, L-glyceraldehyde, or glycolaldehyde; the products of condensation were isolated and definitively characterized (by paper chromatographic techniques and preparation of appropriate derivatives) as D-psicose-1-P, L-tagatose-1-P, and D-ribulose-1-P, respectively.

4. In addition to the enzymes described above, another enzyme, lactaldehyde reductase, has also been detected in the crude extracts of this organism and in the crude preparations of L-fuculose-1-P aldolase, which in the presence of DPNH catalyzes the reduction of lactaldehyde. This enzyme also catalyzes the oxidation of 1,2-propanediol in the presence of DPN, which indicates that 1,2-propanediol might be the product of the reduction of lactaldehyde (in the presence of DPNH and lactaldehyde reductase). However, due to the lack of sufficient data, no conclusion can be drawn with regard to the chemical nature of the product of this reaction.

BIBLIOGRAPHY

1. Tadokoro, T., J. Agr. Chem. Soc. (Japan), 11, 365 (1935).

2. Cohen, S. S., J. Biol. Chem., 201, 71 (1953).

3. Green, M., and Cohen, S. S., J. Biol. Chem., 219, 557 (1956).

4. Heath, E. C., Federation Proc., 17, 239 (1958).

5. Segal, S., and Topper, Y. J., Biochim. et Biophys. Acta, 42, 147 (1960).

6. Huang, P. C., and Miller, O. N., J. Biol. Chem., 231, 201 (1958).

7. Ghalambor, M. A., and Heath, E. C., Bacteriol. Proc., 191 (1961).

8. Sowden, J. C., in Pigman, W. (Editor), The Carbohydrates, Chemistry, Biochemistry, Physiology, Academic Press, Inc., New York, 1957, p. 98-100.

9. Meyer, W., and Tollens, B., Ber., 40, 2434 (1907).

10. Tollens, B., and Rorive, F., Ber., 42, 2009 (1909).

11. Hudson, C. S., J. Am. Chem. Soc., 33, 405 (1911).

12. Clark, E. P., J. Biol. Chem., 54, 65 (1922).

13. Hoagland, D. R., and Lieb, L. L., J. Biol. Chem., 23, 287 (1915).

14. Nelson, W. L., and Cretcher, L. H., J. Biol. Chem., 94, 147 (1931).

15. O'Neill, A. N., J. Am. Chem. Soc., 76, 5074 (1954).

16. Black, W.A.P., Dewar, E. T., and Woodward, F. N., J. Sci. Food Agr., 3, 122 (1952).

17. Percival, E.G.V., Intern. Congr. Biochem., Abstrs. of Communs., 1st Congr., 222 (1949).

18. Dewar, E. T., Chemistry and Industry, 785 (1954).

19. Mori, T., in C. S. Hudson and M. L. Wolfrom (Editors), Advances in Carbohydrate Chemistry, Vol. 8, Academic Press, Inc., New York, 1953, p. 240-243.

20. Kylin, H., Z. Physiol. Chem., 83, 171 (1913).

21. Kylin, H., Z. Physiol. Chem., 94, 337 (1915).

22. Bird, G. M., and Hass, P., Biochem. J., 25, 403 (1931).

23. Percival, E.G.V., and Ross, A. G., J. Chem. Soc., 67, 402 (1948).

24. Percival, E.G.V., and Ross, A. G., J. Chem. Soc., 717 (1950).

25. Conchie, J., and Percival, E.G.V., J. Chem. Soc., 827 (1950).

26. Black, W.A.P., J. Sci. Food Agr., 5, 445 (1954).

27. Oshima, K., Report of Hokkaido Fisheries, 3, 302 (1902).

28. Tadokoro, T., and Saito, T., J. Soc. Chem. Ind. (Japan), 38, Suppl. binding, 270 (1935).

29. Tsukunaga, K., Graduation Thesis, Coll. Agr., Tohoku Imp. Univ. (1916).

30. Suzuki, Y., Report of Sapporo Nat. History, 1, 119 (1904).

31. Lasniewsky, V., Ber., 33, 141 (1900).

32. Bieler, K., and Tollens, B., Ber., 23, 1751 (1890).

33. Gunther, A., and Tollens, B., Ber., 23, 2585 (1890).

34. Mayer, W., and Tollens, B., Ber., 38, 3021 (1905).

35. Black, W.A.P., Cornhill, W. J., Dewar, E. T., and Woodward, F. N., J. Sci. Food Agr., 4, 85 (1953).

36. Tadokoro, T., and Nakamura, Y., J. Biochem. (Japan), 2, 461 (1923).

37. Kando, K., J. Chem. Soc. (Japan), 42, 259 (1921).

38. Colin, H., and Richard, P., Compt. Rend., 194, 643 (1932).

39. Hocket, R. C., Phelps, F. P., and Hudson, C. S. in R. M. Hann and N. K. Richtmyer (Editors), Claude S. Hudson Collected Papers, Vol. I, Academic Press, Inc., 1946, p. 832.

40. Akiya, S., and Suzuki, S., J. Pharm. Soc. (Japan), 74, 1296 (1954).

41. Lewin, R. A., Can. J. Microbiol., 2, 665 (1956).

42. Wilkinson, J. F., Dudman, W. F., Aspinall, G. O., Biochem. J., 59, 446 (1955).

43. Segal, S., and Topper, Y. J., Biochim et Biophys Acta, 25, 419 (1957).

44. Heath, E. C., and Roseman, S., J. Biol. Chem., 230, 511 (1958).

45. Misaki, A., and Hori, I., J. Ferment. Technol. (Japan), 32, 311 (1934).

46. Wilkinson, J. F., Nature, 180, 995 (1957).

47. Eagon, R. G., and Dedonder, R., Compt. Rend., 241, 527, 579 (1955).

48. Oshima, K., Hoppe-Seyler, Zeits, I., Z. Physiol. Chem., 36, 42 (1902).

49. Frommhagen, L. H., and Knight, C. A., Virology, 2, 430 (1956).

50. Andrews, P., Hough, L., Powell, D. B., and Woods, B. M., J. Chem. Soc., 774 (1959).

51. Kawmura, S., and Narasaki, T., Bull. Agr. Chem. Soc. (Japan), 22, 436 (1958).

52. Kawmura, S., and Narasaki, T., Bull. Agr. Chem. Soc. (Japan), 23, A, 71 (1959).

53. James, S. P., and Smith, F., J. Chem. Soc., 739 (1945).

54. Widtsoe, J. A., and Tollens, B., Ber., 33, 132 (1900).

55. Widtsoe, J. A., and Tollens, B., Ber., 32, 147 (1899).

56. Andrews, P., and Hough, L., J. Chem. Soc., 4476 (1958).

57. Jermstad, A., and Wall, T., Pharm. Acta Helv., 28, 120 (1953).

58. Winzler, R. J., in Glick, D. (Editor) Methods of Biochemical Analysis, New York, Vol. II, 294 (1955).

59. Waldron, D. M., Nature, 170, 461 (1952).

60. Hamerman, D., Hatch, F. T., and Reife, A., Proc. Soc. Exptl. Biol. Med., 89, 279 (1955).

61. Raboz, E., Murphy, J. B., Hess, W. C., and Froster, F. M., Proc. Soc. Exptl. Biol. Med., 89, 691 (1955).

62. Butt, W. R., J. Endocrinol., 3, 167 (1956).

63. Micheel, F., and Suthaus, F., Naturwissenschaften, 43, 109 (1956).

64. Glegg, R. E., Eidinger, D., and Leblond, C. P., Science, 118, 614 (1953).

65. Windrum, G. M., Kent, P. W., and Eastoe, J. E., Brit. J. Exptl. Path. 36, 49 (1955).

66. White, A. A., and Hess, W. C., Arch. Biochem. Biophys., 64,57 (1956).

67. Kuhn, R., Baer, H. H., and Gauhe, A., Chem. Ber., 88, 1135 (1955).

68. Kuhn, R., Baer, H. H., and Gauhe, A., Chem. Ber., 89, 2513 (1956).

69. Montreuil, J., Bull. Soc. Chim. Biol. (Paris), 39, 395 (1957).

70. Malpress, F. H., and Hytten, F. E., Biochem. J., 68, 708 (1958).

71. Montreuil, J., and Holleman, J., Compt. Rend., 234, 1069 (1956).

72. Denamur, R., Fauconeau, G., and Guntz, G., Compt. Rend., 246, 2820 (1958).

73. Carlson, D. M., and Hansen, R. G., Federation Proc., 20, 84 (1961).

74. Turumi, K., Fukushima J. Med. Sci., 3, 31 (1956).

75. Muller-Eberhardt, H. J., and Kukel, H. G., J. Exptl. Med., 104, 235 (1956).

76. Masamune, H., and Ogawa, M., Tohoku J. Exptl. Med., 56, 269 (1952).

77. Minganti, A., Ricerca Sci., 24, 1658 (1954).

78. Minganti, A., Exptl. Cell Research, Suppl. 3, 287 (1955).

79. Clermont, Y., Glegg, R. E., and Leblond, C. P., Exptl. Cell Research, 8, 453 (1955).

80. Messina, L., and Monroy, A., Pubbl. Staz. Zool. Napoli, 28, 226 (1956).

81. Akiya, S., and Nakazawa, Y., J. Pharm. Soc. (Japan), 75, 1332 (1955).

82. Akiya, S., and Nakazawa, Y., J. Pharm. Soc. (Japan), 75, 1335 (1955).

83. Maki, M., and Hiyama, N., Hirosaki Med. J., 7, 142 (1956).

84. Morgan, W.T.J., and King, H. K., Biochem. J., 37, 640 (1943).

85. Bray, H. G., Henry, H., and Stacey, M., Biochem. J., 40, 124 (1946).

86. Kabat, E. A., Baer, H., Bezer, A. E., and Knaub, V., J. Exptl. Med., 88, 43 (1948).

87. Baer, H., Dische, Z., and Kabat, E. A., J. Exptl. Med., 88, 59 (1958).

88. Kabat, E. A., Baer, H., and Knaub, V., J. Exptl. Med., 89, 1 (1949).

89. Partridge, S. M., Biochem. J., 42, 251 (1948).

90. Baer, H., Kabat, E. A., and Knaub, V., J. Exptl. Med., 91, 105 (1950).

91. Aminoff, D., Morgan, W.T.J., and Watkins, W. M., Biochem. J., 48, 426 (1950).

92. Annison, E. F., and Morgan, W.T.J., Biochem. J., 52, 247 (1952).

93. Masamune, H., and Yohizawa, Z., Tohoku J. Exptl. Med., 60, 146 (1954).

94. Winifred, M., Watkins, W. M., and Morgan, W.T.J., Nature, 169, 825 (1952).

95. Morgan, W.T.J., Nature, 180, 1038 (1957).

96. Kabat, E. A., Blood group substances, their chemistry and immunochemistry, Academic Press, Inc., New York, 1956, p. 100, 122.

97. Schiffman, G., Kabat, E. A., and Leskuitz, J. Am. Chem. Soc., 82, 1122 (1960).

98. Landsteiner, K., and Harte, R. A., J. Biol. Chem., 140, 673 (1941).

99. Landsteiner, K., and Harte, R. A., J. Exptl. Med., 71, 551 (1940).

100. Watkins, W. M., and Morgan, W.T.J., Nature, 175, 676 (1955).

101. Morgan, W.T.J., and Watkins, W. M., Brit. J. Exptl. Path., 34, 94 (1953).

102. Watkins, W. M., Proc. of Biochem. Soc., Biochem. J., xxxiii, 54 (1953).

103. Crumpton, M. J., and Morgan, W.T.J., Proc. of Biochem. Soc., Biochem. J., xxxiii, 54 (1953).

104. Westphal, O., and Feier, H., Chem. Ber., 89, 582 (1956).

105. Ginsburg, V., and Kirkman, H. N., J. Am. Chem. Soc., 80, 3481 (1958).

106. Ginsburg, V., J. Am. Chem. Soc., 80, 4426 (1958).

107. Ginsburg, V., Federation Proc., 19, 85 (1960).

108. Ginsburg, V., J. Biol. Chem., 235, 2196 (1960).

109. Ginsburg, V., J. Biol. Chem., 236, 2389 (1961).

110. Foster, D. W., and Ginsburg, V., Biochim. et Biophys. Acta, 54, 376 (1961).

111. Kuhn, R., and Tiedeman, H., Z. Naturforsch, 8b, 428 (1953).

112. Freudenberg, K., and Raschig, K., Ber., 60, 1633 (1927).

113. Power, F. B., and Rogerson, H., J. Chem. Soc., 101, 1 (1912).

114. Power, F. B., and Rogerson, H., J. Chem. Soc., 101, 398 (1912).

115. Davies, L. A., and Adams, R., J. Am. Chem. Soc., 50, 1749 (1928).

116. Leach, B. E., Calhoun, K. M., Johnson, L. E., Teeters, C. M., and Jackson, W. G., J. Am. Chem. Soc., 75, 4011 (1953).

117. Berger, J., Sternbach, L. H., Pollock, R. G., LaSala, E. R., Kaiser, S., and Goldberg, M. W., J. Am. Chem. Soc., 80, 1636 (1958).

118. Sternbach, L. H., Kaiser, S., and Goldberg, M. W., J. Am. Chem. Soc., 80, 1639 (1958).

119. Field, J. T., and Poe, C. F., J. Biol. Chem., 132, 473 (1940).

120. Hegedus, W., Tamm, C. H., and Reichstein, T., Helv. Chim. Acta, 36, 357 (1953).

121. Reichstein, R., Schindler, O., and Reichstein, T., Helv. Chim. Acta, 37, 67 (1954).

122. Schindler, O., and Reichstein, T., Helv. Chim. Acta, 37, 103 (1954).

123. Hegedus, H., and Reichstein, T., Helv. Chim. Acta, 38, 1133 (1955).

124. Rittel, W., Hunger, A., and Reichstein, T., Helv. Chim. Acta, 36, 434 (1953).

125. Okada, M., J. Pharm. Soc. (Japan), 73, 1118 (1953).

126. Yunsten, A. H., and Hiroshi, Y., Bull. Agr. Chem. Soc. (Japan), 21, 261 (1957).

127. Yunsten, H., J. Antibiotics (Japan), ser. A, 11, 77 (1958).

128. Yunsten, H., J. Antibiotics (Japan), ser. A, 11, 233 (1958).

129. Yunsten, H., and Yonehara, H., and Ui, H., J. Antibiotics (Japan), ser. A, 7, 113, 116 (1954).

130. Yunsten, H., Okhuma, K., and Ishii, Y., J. Antibiotics (Japan), ser. A, 9, 195 (1956).

131. Smith, D. W., Randall, H. M., MacLennan, A. P., and Lederer, E., Nature, 186, 887 (1950).

132. MacLennan, A. P., Smith, D. W., and Randall, H. M., Biochem. J., 74, 3P (1960).

133. Butler, K., Lloyd, P. F., and Stacey, M., J. Chem. Soc., 1531 (1955).

134. Butler, K., and Stacey, M., J. Chem. Soc., 1537 (1955).

135. Smith, D. W., Randall, H. M., MacLennan, A. P., Putney, R. K., and Rao, S. V., J. Bacteriol., 79, 217 (1960).

136. Mann, R. L., Gale, R. M., and Van Abeel, F. R., Antibiotics and Chemotherapy, 3, 1279 (1953).

137. Pittenger, R. C., Wolfe, R. N., Hoehn, M. M., Marks, P. N., Daily, W. H., and McGuire, J. M., Antibiotics and Chemotherapy, 3, 1268 (1953).

138. Elbein, A. D., Koffler, H., and Garner, H. R., Biochim. et Biophys. Acta, 56, 165 (1962).

139. Mann, R. L., and Wolf, O. D., J. Am. Chem. Soc., 79, 120 (1957).

140. Elbein, A. D., Mann, R. L., Renis, H. E., Stark, W. M., Koffler, H., and Garner, H. R., J. Biol. Chem., 236, 289 (1961).

141. Votocek, E., Ber., 43, 476 (1910).

142. Freundenberg, K., Ber., 62, 373 (1929).

143. Aspinal, G. O., Hirst, E. L., and Nicolson, A., J. Chem. Soc., 1697 (1959).

144. Stephen, A. M., J. Chem. Soc., 646 (1951).

145. Sinha, A., J. Proc. Inst. Chemists, 32, 70 (1960).

146. Jones, J.K.N., and Smith, F., in W. W. Pigman and M. L. Wolfrom (Editors), Advances in Carbohydrate Chemistry, Vol. 4, Academic Press, Inc., New York, 1949, p. 268, 270.

147. Aspinal, G. O., and Canas-Rodriques, J. Chem. Soc., 4020 (1958).

148. Gill, R. E., Hirst, E. L., and Jones, J.K.N., J. Chem. Soc., 1469 (1939).

149. Anderson, E., J. Biol. Chem., 104, 163 (1934).

150. Tipson, R. S., Christman, C. C., and Levene, P. A., J. Biol. Chem., 128, 609 (1939).

151. Neville, A., J. Agr. Soc., 5, 113 (1913).

152. Takei, H., Mokuzai Gokkaishi, 4, 211 (1958).

153. Acree, S. F., and Syme, W. A., Am. Chem. J., 36, 309 (1906).

154. Charaux, M. C., Bull. Soc. Chim. Biol., 6, 631, 641 (1934).

155. Horhammer, L., Wagner, H., and Probst, W., Naturwissenschaften, 48, 63 (1960).

156. Shils, M. E., and Goodhart, R. S. (Editors), The Flavonoids in Biology and Medicine, The National Vitamine Foundation, Inc., New York (1956).

157. Pulley, G. N., and VonLoeseke, H. W., J. Am. Chem. Soc., 61, 175 (1935).

158. Walton, C. F. in R. M. Hann and N. K. Richtmyer (Editors), Claude S. Hudson's Collected Papers, Vol. I, Academic Press, Inc., 1946, p. 453.

159. Clark, E. P., J. Biol. Chem., 38, 225 (1919).

160. Bates, F. J., and Associates, Natl. Bur. Standards, Circ., C 440 (1942).

161. Anderson, E., and Crowder, J. A., J. Am. Chem. Soc., 52, 3711 (1930).

162. Howarth, N., Kent, P. W., and Stacey, M., J. Chem. Soc., 1211 (1948).

163. Heidelberger, M., and Rebers, P. A., J. Bacteriol., 80, 145 (1960).

164. Tyler, J. M., and Heidelberger, M., Federation Proc., 21, 90 (1962).

165. Iwahara, S., and Ishizeki, R., Eisei shikenjo Hokoku, 76, 193 (1958).

166. Salton, M. R., Biochim. et Biophys. Acta, 10, 512 (1952).

167. Barkulis, S. S., and Jones, M. F., J. Bacteriol., 74, 207 (1957).

168. Southard, W. H., Hayashi, J. A., and Barkulis, S. S., J. Bacteriol., 78, 79 (1959).

169. Elliott, S. D., J. Exptl. Med., 111, 621 (1950).

170. Okazaki, R., Okazaki, T., and Kurki, Y., Biochim. et Biophys. Acta, 38, 384 (1959).

171. Okazaki, R., Biochim. et Biophys. Acta, 44, 478 (1960).

172. Kornfeld, S., and Glaser, L., Biochim. et Biophys. Acta, 42, 548 (1960).

173. Pazur, J. H., and Shuey, E. W., J. Biol. Chem., 236, 1780 (1961).

174. Pazur, J. H., and Shuey, E. W., J. Am. Chem. Soc., 82, 5009 (1960).

175. Smith, E.E.B., Galloway, B., and Mills, G. T., Biochim. et Biophys. Acta, 33, 276 (1959).

176. Jarvis, F. G., and Johnson, M. J., J. Am. Chem. Soc., 71, 4142 (1949).

177. Hauser, G., and Karnovsky, M. L., J. Biol. Chem., 233, 287 (1958).

178. Burger, M., Glaser, L., and Burton, R. M., Biochim. et Biophys. Acta, 56, 172 (1962).

179. Burger, M., Glaser, L., and Burton, R. M., Federation Proc., 21, 82 (1962).

180. Kornfeld, S., and Glaser, L., Federation Proc., 20, 84 (1961).

181. Kornfeld, S., and Glaser, L., J. Biol. Chem., 236, 1791 (1961).

182. Okazaki, R., Okazaki, T., and Strominger, J. L., Federation Proc., 20, 85 (1961).

183. Glaser, L., and Kornfeld, S., J. Biol. Chem., 236, 1795 (1961).

184. Kluyver, A. J., and Schneller, C., Enzymologia, 4, 7 (1937).

185. Englesberg, E., J. Bacteriol., 74, 8 (1957).

186. Ajl, S., and Wilson, D. M., Biochim. et Biophys. Acta, 17, 289 (1955).

187. Tecce, G. M., DiGirolano, M., and Lazzari, G. F., Proc. 3rd Intern. Congr. Biochem., Brussels, 96 (1955).

188. Englesberg, E., Federation Proc., 15, 586 (1956).

189. Englesberg, E., Arch. Biochem. Biophys., 71, 179 (1957).

190. Englesberg, E., and Baron, L. S., J. Bacteriol., 78, 675 (1959).

191. Wilson, D. M., and Ajl, S., J. Bacteriol., 73, 410 (1957).

192. Wilson, D. M., and Ajl, S., J. Bacteriol., 73, 415 (1957).

193. Eagon, R. G., J. Bacteriol., 82, 548 (1961).

194. Markovitz, A., Bacteriol. Proc., 193 (1961).

195. Markovitz, A., Biochem. and Biophys. Research Communs., 6, 250 (1961).

196. Palleroni, N. J., and Doudoroff, M., J. Biol. Chem., 218, 535 (1956).

197. Palleroni, N. J., and Doudoroff, M., J. Biol. Chem., 219, 957 (1956).

198. Huang, P. C., and Miller, O. N., J. Biol. Chem., 230, 805 (1958).

199. Dische, Z., and Shettles, L. B., J. Biol. Chem., 175, 595 (1948).

200. Somogyi, M., J. Biol. Chem., 160, 69 (1945).

201. Barnett, J., and Reichstein, T., Helv. Chim. Acta, 20, 1529 (1937).

202. Dische, Z., and Borenfreund, E., J. Biol. Chem., 192, 583 (1951).

203. Bevenue, A., and Williams, K. T., Arch. Biochem. Biophys., 34, 225 (1951).

204. Schroeder, W., and Hoeksema, H., J. Am. Chem. Soc., 81, 1767 (1959).

205. Wolfrom, M. L., Thompson, A., and Evans, E. F., J. Am. Chem. Soc., 67, 1793 (1945).

206. Mitsuhashi, S., and Lapman, J. O., J. Biol. Chem., 204, 1011 (1953).

207. Glatthaar, C., and Reichstein, T., Helv. Chim. Acta, 18, 80 (1935).

208. Shriner, R. L., and Fuson, R. S. (Editors), The Systematic Identification of Organic Compounds, 3rd edition, John Wiley and Son, Inc., New York, 1948, p. 116.

209. Pigman, W. (Editor), The Carbohydrates, Chemistry, Biochemistry, Physiology, Academic Press, Inc., New York, 1957, p. 455.

210. Bloink, G. J., and Pausacker, K. H., J. Chem. Soc., 622 (1951).

211. Bischler, A., Ber., 22, 2801 (1889).

212. Honeyman, J., in R. L. Whistler, and M. L. Wolfrom (Editors), Methods in Carbohydrate Chemistry, Vol. I, Academic Press, Inc., New York, 1962, p. 117.

213. Henseke, G., and Kohler, H., Ann., 614, 105 (1958).

214. Totton, E. A., and Lardy, H. A., J. Am. Chem. Soc., 71, 3076 (1949).

215. Wolin, M. G., Simpson, F. J., and Wood, W. A., J. Biol. Chem., 232, 559 (1958).

216. Hough, L., and Jones, J.K.N., J. Chem. Soc., 4047 (1952).

217. Reichstein, T., Helv. Chim. Acta, 17, 996 (1934).

218. Horecker, B. L., Hurwitz, J., and Weissbach, A., J. Biol. Chem., 218, 785 (1956).

219. Heath, E. C., Horecker, B. L., Smyrniotis, P. Z., and Y. Takagi, J. Biol. Chem., 231, 1031 (1958).

220. Huff, E., and Rudney, H., J. Biol. Chem., 234, 1060 (1959).

221. Hough, L., and Jones, J.K.N., J. Chem. Soc., 4052 (1952).

222. Boggs, L., Cuendet, L. S., Ehrenthat, I., Koch, R., and Smith, F., Nature, 166, 520 (1950).

223. Huff, E., Biochim. et Biophys. Acta, 48, 506 (1961).

224. Neuberg, C., and Vercellone, A., Biochem. Z., 279, 140 (1935).

225. Perline, A. S., and Brice, C., Can. J. Chem., 34, 85 (1956).

226. Neuberg, C., and Kobel, M., Biochem. Z., 203, 461 (1928).

227. Beck, W. S., in S. P. Colowick and N. O. Kaplan (Editors), Methods in Enzymology, Vol. III, Academic Press, Inc., New York, 1957, p. 205.

228. Dische, Z., and Devi, A., Biochim. et Biophys. Acta, 39, 140 (1960).

229. Chen, P. S., Toribara, T. Y., and Warner, H., Anal. Chem., 28, 1756 (1956).

230. Dische, Z. in R. L. Whistler, and M. L. Wolfrom (Editors), Methods in Carbohydrate Chemistry, Vol. I, Academic Press, Inc., New York, 1962, p. 482.

231. Scott, T. A., and Melvin, E. H., Anal. Chem., 25, 1650 (1953).

232. Mejbaum, W., Z. Physiol. Chem., 258, 117 (1939).

233. Dische, Z., and Dische, M. R., Biochim. et Biophys. Acta, 27, 184 (1958).

234. Barker, S. B., and Summerson, W. H., J. Biol. Chem., 138, 535 (1941).

235. Park, J. T., and Johnson, M. J., J. Biol. Chem., 181, 149 (1949).

236. Calkins, V. P., Anal. Chem., 15, 762 (1943).

237. Jones, L. R., and Riddick, J. A., Anal. Chem., 29, 1214 (1957).

238. Fiske, C. H., and SubbaRow, Y., J. Biol. Chem., 66, 375 (1925).

239. Waddel, J. J., J. Lab. and Clin. Med., 48, 311 (1956).

240. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem., 193, 265 (1951).

241. Horecker, B. L., and Kornberg, A., J. Biol. Chem., 175, 385 (1948).

242. Kornberg, A., and Pricer, W. E., Jr., J. Biol. Chem., 193, 481 (1951).

243. Racker, E., J. Biol. Chem., 167, 843 (1947).

244. Baranowski, T., and Niederland, T. R., J. Biol. Chem., 180, 543 (1949).

245. Warburg, O., and Christian, W., Biochem. Z., 314, 149 (1943).

246. Anet, E.F.L.J., and Reynolds, T. M., Nature (London) 174, 930 (1954).

247. Bandurski, R. S., and Axelrod, B., J. Biol. Chem., 193, 405 (1951).

248. Cassidy, H. G. (Editor), Fundamentals of Chromatography, Interscience Publishers, Inc., New York, 1957, p. 167.

249. Hough, L. in D. Glick (Editor), Methods of Biochemical Analysis, Vol. I, Interscience Publishers, Inc., New York, 1954, p. 231.

250. Lineweaver, H., and Burk, D., J. Am. Chem. Soc., 56, 658 (1934).

251. Tanko, B., and Robinson, R., Biochem. J., 29, 961 (1935).

252. Knox, W. E., Stumpf, P. K., Green, D. E., and Auerbach, H. E., J. Bacteriol., 55, 451 (1948).

253. Bard, R. C., and Gansalus, I. C., J. Bacteriol., 59, 387 (1950).

254. Gray, N. D., Kupferberg, L. L., and Graf, L. H., J. Bacteriol., 69, 478 (1955).

255. Baur, E., Z. Physiol. Chem., 242, 15 (1936).

256. Sibley, J. A., and Lehninger, A. L., J. Biol. Chem., 177, 859 (1949).

257. Peanasky, R. J., and Lardy, H. A., J. Biol. Chem., 233, 365, 371 (1958).

258. Charalampous, F. C., and Mueller, G. C., J. Biol. Chem., 201, 161 (1953).

259. Charalampous, F. C., J. Biol. Chem., 211, 249 (1954).

260. Meyerhof, O., Lohmann, K., and Schuster, Ph., Biochem. Z., 286, 301 (1936).

261. Anderson, R. L., and Wood, W. A., J. Biol. Chem., 237, 296 (1962).

262. Stumpf, P. K., and Horecker, B. L., J. Biol. Chem., 218, 753 (1956).

263. Byrne, W. L., and Lardy, H. A., Biochim. et Biophys. Acta, 14, 495 (1954).

264. Glock, G. E., Biochem. J., 52, 575 (1952).

265. Simpson, F. J., Wood, W. A., and Wolin, M. G., J. Biol. Chem., 230, 473 (1958).

266. Ashwell, G., and Hickman, J., J. Biol. Chem., 226, 65 (1957).

267. Meyerhof, O., Lohmann, K., and Schuster, Ph., Biochem. Z., 286, 319 (1936).

268. Tung, T. C., Ling, K. H., Byrne, W. L., and Lardy, H. A., Biochim. et Biophys. Acta, 14, 488 (1954).

269. Wolfrom, M. L., Olin, S. M., and Evans, E. F., J. Am. Chem. Soc., 66, 1204 (1944).

270. Steiger, M., and Reichstein, T., Helv. Chim. Acta, 18, 790 (1935).

271. Fischer, H.O.L., and Baer, E., Helv. Chim. Acta, 19, 579 (1939).

272. Lockwood, L. B., in R. L. Whistler and M. L. Wolfrom (Editors), Methods in Carbohydrate Chemistry, Vol. I, Academic Press, Inc., New York, 1961, p. 1953.

273. Simpson, F. J., Wolin, M. G., and Wood, W. A., J. Biol. Chem., 230, 457 (1958).

274. Lampen, J. O., Abstracts of papers of the 126th meeting of the Am. Chem. Soc., New York (1954), p. 44c.

275. Heath, E. C., Hurwitz, J., Horecker, B. L., and Ginsburg, A., J. Biol. Chem., 231, 1009 (1958).

276. Marshall, R. O., and Kooi, E. R., Science, 125, 648 (1957).

277. Hochster, R. M., Can. J. Microbiol., 1, 346 (1954-55).

278. Slein, M. W., J. Am. Chem. Soc., 77, 1663 (1955).

279. Monod, J., and Cohn, M., Advances in Enzymology, 13, 67 (1952).

280. Monod, J., in O. H. Gebler, Enzymes: "Units of Bacterial Structure and Functions," Academic Press, New York (1956).

281. Cohn, M., Bacteriological Reviews, 21, 140 (1957).

282. Revel, H. R., Luria, S. E., and Rotman, B., Proc. Nat. Ac. Sci., 47, 1956 (1961).

283. Luria, S. E., Adams, J. N., and Ting, R. C., Virology, 12, 348 (1960).

284. Pardee, A. B., Jacob, F., and Monod, J., J. Molecular Biol., 1, 165 (1959).

285. Jacob, F., and Monod, J., J. Molecular Biol., 3, 318 (1961).

286. Hochster, R. M., and Watson, R. W., J. Am. Chem. Soc., 75, 3284 (1953).

287. Axelrod, B., and Jang, R., J. Biol. Chem., 209, 847 (1954).

288. Sandman, R. T., and Miller, O. N., J. Biol. Chem., 230, 791 (1958).

289. Rudney, H., J. Biol. Chem., 210, 361 (1954).

290. Abeles, R. H., and Lee, H. A., Jr., J. Biol. Chem., 236, 2347 (1961).

291. Taylor, M. B., and Juni, E., Bacteriol. Proc., 98 (1958).

292. Sellinger, O. Z., and Miller, O. N., J. Biol. Chem., 234, 1641 (1959).

293. Miller, O. N., Huggins, C. G., and Arai, K., J. Biol. Chem., 202, 263 (1953).

294. Hurwitz, J., and Horecker, B. L., J. Biol. Chem., 223, 993 (1956).

295. Swenson, A. D., and Bayer, P. D., J. Am. Chem. Soc., 79, 2174 (1957).

296. Velick, S. F., J. Phys. Colloid Chem., 53, 135 (1949).

297. Taylor, J. F., and Lowry, C., Biochim. et Biophys. Acta, 20, 109 (1956).

298. Richards, O. C., and Rutter, W. J., J. Biol. Chem., 236, 3185 (1961).

299. Groves, W. E., and Rutter, W. J., Federation Proc., 21, 254 (1962).

300. Rutter, W. J., in P. D. Boyer, H. Lardy, and K. Myrback (Editors), The Enzymes, Vol. V, Ed. 2, Academic Press, Inc., New York, 1961, p. 350, 358, 366.

301. Richards, O. C., and Rutter, W. J., J. Biol. Chem., 236, 3177 (1961).

302. Rutter, W. J., and Ling, K. H., Biochim. et Biophys. Acta, 30, 71 (1958).

303. Jagrannthan, V., Singh, K., and Domodaran, M., Biochem. J., 63, 94 (1956).

304. Stumpf, P. K., J. Biol. Chem., 176, 223 (1948).

305. Swain, C. G., and Brown, J. F., J. Am. Chem. Soc., 74, 2534, 2538 (1952).

306. Fischer, H.O.L., and Baer, E., Helv. Chem. Acta, 19, 519 (1936).

307. Bloom, B., and Topper, Y. J., Nature, 181, 1128 (1958).

308. Topper, Y. J., Mehler, A., and Bloom, B., Science, 126, 1287 (1957).

309. Rose, I. A., and Rieder, S. V., J. Am. Chem. Soc., 77, 5764 (1955).

310. Bloom, B., and Topper, Y. J., Science, 124, 982 (1956).

311. Rose, I. A., and Rieder, S. V., J. Biol. Chem., 231, 315 (1958).

312. Rose, I. A., Proc. Nat. Acad. Sci., 44, 10 (1958).

313. Rose, I. A., J. Am. Chem. Soc., 80, 5835 (1958).

314. Horecker, B. L., Symposium on Enzyme Reaction Mechanisms, J. Cellular Comp. Physiol., 54 (Suppl. 1), 89 (1959).

315. Dische, Z., Ann. N. Y. Acad. Sci., 75, 129 (1958).

316. Herbert, D., Gordon, H., Sabrahmanyan, F., and Green, D. E., Biochem. J., 34, 1108 (1940).

317. Horecker, B. L., Pontremoli, S., Rieci, C., and Cheng, T., Proc. Natl. Acad. Sci., 47, 1949 (1961).

318. Venkataraman, R., and Racker, E., J. Biol. Chem., 236, 1883 (1961).

319. Smyrniotis, P. Z., and Horecker, B. L., J. Biol. Chem., 218, 745 (1956).

320. Ballou, C. E., Fischer, H.O.L., and MacDonald, D. L., J. Am. Chem. Soc., 77, 5967 (1955).

321. Gorin, P.A.J., Hough, L., and Jones, J.K.N., J. Chem. Soc., 2140 (1953).

322. Forrest, R. S., Hough, L., and Jones, J.K.N., Chem. and Ind. (London), 1063 (1951).

323. Korkes, S., in J. M. Luck (Editor), Annual Review of Biochemistry, Vol. 25, Annual Reviews, Inc., Palo Alto, Calif., 1956, p. 701.

324. Drechsler, E. R., Boyer, P. D., and Kowalsky, A. G., J. Biol. Chem., 234, 2627 (1959).

325. Rutter, W. J., Richards, O. C., and Woodfin, B. M., J. Biol. Chem., 236, 3193 (1961).

326. Richards, O. C., and Rutter, W. J., Federation Proc., 19, 86 (1960).

327. Singer, T. P., and Kearny, E. G., Arch. Biochem. Biophys., 29, 190 (1950).

328. Gupta, N. K., and Robinson, W. G., J. Biol. Chem., 235, 1609 (1960).