GR

Original Submission

D-Ribose GRAS Notification

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January 28, 2002

Letter

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January 28, 2002

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FOOD ADDITIVE SAFETY

Dr. Laura Tarantino
Acting Director
Office of Premarket Approval
Center for Food, Safety and Nutrition HFS 200
Food and Drug Administration
200 C St. SW
Washington, DC 20204

Dear Dr. Tarantino:

In accordance with the proposed rule for Substances Generally Recognized as Safe, which was published in the *Federal Register* at Vol. 62, No. 74 on April 17, 1997, Bioenergy, Inc. of Ham Lake, Minnesota would like to submit notice of a claim that the use of Bioenergy D-ribose as a food ingredient is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act, because such use is GRAS.

D-ribose (α-D-ribofuranose) is a naturally occurring 5-carbon sugar, that is found in most living organisms. It forms the backbone of genetic material in humans, and is used by the body to synthesize nucleotides, nucleic acids, glycogen, and other important metabolic products. Ribose is also integral to the production of adenosine triphosphate (ATP), the molecule used by the cell to supply energy.

A GRAS Report in support of the safe use of D-ribose in foods was prepared by Bioenergy, Inc., and was reviewed by a Panel of Experts qualified by training and experience to assess the safety of food ingredients. The Experts concurred with Bioenergy, Inc.'s determination that D-ribose is safe for general use in foods. The Panel relied upon the results of numerous animal toxicology studies, reports of human consumption, and a large body of published literature. A copy of the Expert Opinion is attached to this notice.

This Notification of a claim for premarket exemption is based on a GRAS determination under proposed §170.36. Bioenergy, Inc. has prepared a Notification document in triplicate, which accompanies this letter. The

company would appreciate notice of the receipt of this document, and looks forward to any comments the agency would care to make on the Notification. If you have any questions regarding the content of the Notification, you may reach either myself at the number listed above or Mr. Tim Hight at (763) 757-0032.

Sincerely,

Lee B. Dexter
Technical Consultant

CC: Mr. Tim Hight, Vice President, Bioenergy, Inc.
Dr. Linda Kahl, CFSAN
Dr. Michael DiNovi, CFSAN

Claim

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Bioenergy, Inc.
GRAS Notification

Introduction

D-ribose is a monosaccharide found in the cells of most living organisms. It forms the backbone of each cell's genetic material, and it is important in a number of complex metabolic events leading to the production of energy. Within the body D-ribose participates in the formation of nucleosides, ATP, vitamins, and nucleotides. In foods, it functions as a nutritive sweetener and it provides a number of beneficial technical effects. Bioenergy, Inc. (Bioenergy) of Ham Lake, Minnesota, USA has made D-ribose available to be used as a food ingredient. This Notification document contains the information required in proposed regulation 21 CFR 170.36 to allow the FDA to evaluate whether the submitted notice provides a sufficient basis for a generally recognized as safe (GRAS) determination.

In compliance with 21 CFR § 170.30, Bioenergy determined that Bioenergy D-ribose can be considered GRAS when used in accordance with current Good Manufacturing Practices. Bioenergy wishes to voluntarily notify the Center for Food Safety and Applied Nutrition (CFSAN) of that determination, and according to proposed 21 CFR § 170.36, the company is submitting the following GRAS exemption claim.

Bioenergy, Inc. has prepared a multi-volume GRAS Report, which forms the basis for the information found in this notification. The company also commissioned a panel of experts (Expert Panel), qualified by scientific training and experience to assess the safety of food ingredients, who critically evaluated the D-ribose GRAS Report as well as other data and information relevant to the use and safety of this ingredient. The panel concurred with the company's determination that Bioenergy D-ribose can be considered generally recognized as safe for general use in food. Based on the data and information contained in the Report and the opinion of the expert panel (which is attached to this notification), Bioenergy explicitly accepts responsibility for the GRAS determination of Bioenergy D-ribose.

GRAS Exemption Claim

Bioenergy, Inc. hereby notifies the U.S. Food and Drug Administration that the use of Bioenergy D-ribose as a food ingredient is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act, because Bioenergy has determined that such use is GRAS.

1. Notifier:

   Bioenergy, Inc.
   13840 Johnson St. N.E.
   Ham Lake, Minnesota 55304, USA
   Telephone: (763) 757-0032
   Fax: (763) 757-0588

2. Common or Usual Name:

   D-Ribose

3. Applicable Conditions of Use:

   Applications for D-ribose include general use in foods as a multiple-use direct additive. The ingredient should be used under the conditions of current Good Manufacturing Practice. In order to classify the various effects ingredients may have in food, FDA has published a list of 32 physical or technical functional effects for which direct food ingredients may be added to food. These are codified at 21 CFR §170.3 (o) (1-32). Applications for D-ribose are covered under several of the following terms as listed under 21 CFR §170.3 (o).

   (11) "Flavor enhancers": Substances added to supplement, enhance or modify the original taste and/or aroma of a food, without imparting a characteristic taste or aroma of its own.
   (16) "Humectants": Hygroscopic substances included in food to promote retention of moisture, including moisture-retention agents and antidusting agents.
   (20) "Nutrient supplements": Substances which are necessary for the body's nutritional and metabolic processes.

Section I Notification Claim (Continued)

(21) "Nutritive sweeteners": Substances having greater than 2 percent of the caloric value of sucrose per equivalent unit of sweetening capacity.
(28) "Stabilizers and thickeners": Substances used to produce viscous solutions or dispersions, to impart body, improve consistency, or stabilize emulsions, including suspending and bodying agents, setting agents, jellying agents, and bulking agents, etc.
(31) "Synergists": Substances used to act or react with another food ingredient to produce a total effect different or greater than the sum of the effects produced by the individual ingredients.
(32)"Texturizers": Substances, which affect the appearance or feel of the food.

4. Basis of the GRAS Determination

The basis of the GRAS determination for Bioenergy D-ribose was the use of scientific procedures.

5. Availability of Data and Information and Key to References

The data and information that are the basis of the GRAS determination for Bioenergy D-ribose will be available for FDA review and copying at the address of the notifier listed above. The notifier will also be pleased to provide the FDA with a copy of the GRAS Report, or any references contained therein, upon written request. Throughout this Notification, citations to the published literature, which were included in the multi-volume GRAS Report are denoted as follows: [Author (*et al*), Year, Tab (number) Volume (number)]. In order to facilitate review of this document a complete list of references from the D-ribose GRAS Report is included in Appendix 2 as a key. Recently identified references, which were not included in the GRAS Report are shown between parentheses ( ) within the text of this document and given in a standard bibliographic form.

Section I: GRAS Notification Claim

Section I Notification Claim (Continued)

6. Signature of an official for Bioenergy, Inc.

Official for Bioenergy, Inc. Date

1/28/02

Tim Hight
Vice President, Regulatory Affairs

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Notification

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Section II

Section II: Chemical Identity of D-Ribose

Introduction

Bioenergy, Inc., Ham Lake Minnesota (Bioenergy), has compiled the following GRAS Notification in support of the safety of D-ribose as an ingredient in foods in general. In compiling the Notification, Bioenergy has included relevant information and data that is publicly available, as well as the results of studies that have been commissioned by the company. The Notification presents a balanced discussion of the safety and use of D-ribose, and the company certifies that no adverse information has been knowingly omitted.

D-ribose (α-D-ribofuranose) is a naturally occurring 5-carbon sugar, that is associated with most living organisms. It is used by the human body to synthesize nucleosides, nucleotides, nucleic acids, glycogen, and other important metabolic products [Lehninger, 1982 Vol 8 Tab 94]. Ribose is also the starting point for the production of adenosine triphosphate (ATP), the molecule used by the human body to supply energy [Lehninger, 1982 Vol 8 Tab 94].

Ribose (in the form of ribose-5-phosphate) is formed in the body from the conversion of glucose via a metabolic system known as the pentose phosphate pathway (PPP) [Lehninger, 1982 Vol 8 Tab 94]. The pentose phosphate pathway is a conversion pathway that utilizes glucose in the form of glucose-6-phosphate to generate specialized molecules important to the cell, such as nicotinamide adenine dinucleotide phosphate (NADPH), which provides reducing power, and ribose-5-phosphate, which serves as a substrate for the formation of 5-phosphoribosyl-1-pyrophosphate (PRPP). PRPP which then enters the biochemical cycle leading to the formation of ATP, and the purine and pyrimidine nucleotides [Lehninger, 1982 Vol 8 Tab 94 and Pauly *et al.*, 2000 Vol 5 Tab 43]. It is also important to note that one other important function of the PPP is to allow the oxidative degradation of ingested five carbon sugars, which are generally present in the diet from plant sources [Pauly *et al.*, 2000 Vol 5 Tab 43].

Section II: Chemical Identity of D-Ribose (Continued)

Orally supplemented ribose allows the body to by-pass the rate-limiting steps of the PPP. Supplemented D-ribose enters the metabolic pathway, just prior to the biosynthesis of PRPP. D-ribose actually enters the pathway after having been phosphorylated to ribose-5-phosphate (R-5-P) by the enzyme ribokinase [Lehninger, 1982 Vol 8 Tab 94 and Pauly *et al.*, 2000 Vol 5 Tab 43]. As mentioned above R-5-P is an intermediate that is utilized in a number of physiological reactions to:

1. Form PRPP
2. Generate ATP
3. Synthesize nucleotides and nucleosides
4. Regenerate glucose via the PPP
   [Hiatt, 1957 Vol 3 Tab 28, Segal, *et al.*, 1958 Vol 5 Tab 50, Bloom, *et al.*, 1954 Vol 2 Tab 5 and Coffey, *et al.*, 1965 Vol 2 Tab 8].

Thus D-ribose is vital for the formation of each living cell's genetic material, and as a substrate for the energy cycle.

Bioenergy, Inc. manufactures D-ribose under current Good Manufacturing Conditions (cGMP), in a manner that produces a product identical to that found in nature. A high level of purity has been established in the company's specifications. The product is produced by non-pathogenic strains of the genus *Bacillus* that are eliminated in the Bioenergy process. Therefore, Bioenergy submits that its D-ribose product is safe and suitable for its intended use as a food ingredient.

Section II. Chemical Identity

A. Common or Usual Name and Identity

1. D-ribose
2. Ribose is the preferred common or usual name for Bioenergy's product.

B. Formal Names (IUPAC or Chemical Abstracts Names)

1. α-D(-) ribofuranose
2. D-ribose

C. Synonyms, Other Common Names, Tradenames

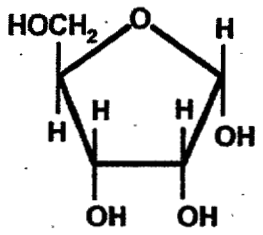
Trademark: Bioenergy Ribose™

D. Chemical Formulae, Structures and Molecular Weights

1. Empirical Formula

   $C_5H_{10}O_5$

2. Structural Formula



Section II: Chemical Identity of D-Ribose (Continued)

3. Molecular Weight

150.13

E. Chemical Abstracts Service Registry Number (CAS Registry No.)

1. CAS Number 50-69-1

F. Specifications for Food Grade Materials

1. Raw Materials

The following substances used in the manufacturing of D-ribose are food grade materials, or are used in accordance with current Good Manufacturing Practices (21 CFR 110.80(a)).

Activated Carbon
Ammonium Salts
Calcium Salts
Corn Steep Liquor
Corn Syrup
D-Fructose
D-Glucose
Diatomaceous Earth
Phosphates
Yeast
Ethanol
Ion Exchange Resin
Potassium Salts
Sodium Salts
Sorbitol
Vegetable Extracts
Amino Acids

G. Quantitative Composition of Bioenergy D-Ribose

1. Chemical Identity

D-ribose (α-D-ribofuranose) is a five (5) carbon sugar, which is classified as an aldopentose, since it contains an aldehyde group. (An aldehyde contains a carbonyl (C=0) group at the end of the carbon chain.) D-ribose is chemically related to other 5-carbon monosaccharides, such as arabinose and xylose [Morrison et al. 1968 Vol 8 Tab 108]. D-ribose has three chiral centers (the center three carbons). The pre-fixes D- or L- refer to the configuration of the carbon most distant from the carbonyl carbon atom. In the case of D-ribose the hydroxyl group of the most distant chiral carbon projects to the right [Lehninger, 1982 Vol 8 Tab 94]. D-ribose has been described as one of the most useful of the naturally occurring aldoses.

2. Chemical Reactions of D-Ribose

D-Ribose is most well known for its role as the backbone of the cell's hereditary material. It occurs in ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). The pre-fix deoxy indicates a lack of an -OH group at the 2nd carbon position. Attached to the C-1 of each sugar, through a β-linkage, is one of a number of heterocyclic bases (such as purines and pyrimidines) in which the ring is made up of more than one kind of atom. A base-sugar unit is called a nucleoside; a base-sugar-phosphoric acid unit is called a nucleotide [Morrison, et al., 1968 Vol 8 Tab 108]. Nearly all living organisms are able to synthesize purine and pyrimidine nucleotides [Lehninger, 1982 Vol 8 Tab 94].

The two parent purine nucleotides of nucleic acids are adenosine 5'-monophosphate (AMP), also called adenylate, and guanosine 5'-monophosphate (GMP), or guanylate. These nucleotides contain the purine bases adenine and guanine. The biological synthesis of

purine nucleotides begins with ribose-5-phosphate, and the purine ring is built on it step by step. Therefore, the first steps in this *de novo* pathway (new synthesis) yield an open-chain ribonucleotide, which subsequently undergoes ring closure to yield a purine nucleotide (Figure 1). Purine nucleotides can also be made by a salvage pathway [Lehninger, 1982 Vol 8 Tab 94].

The Pentose Phosphate Pathway (Hexose Monophosphate Shunt) and the Production of Ribose-5-phosphate

As stated above ribose-5-phosphate is the building block for purine and pyridimine synthesis. It is formed in the body via the pentose phosphate pathway (PPP) which constitutes part of the secondary metabolism of glucose.

The pentose phosphate pathway (PPP), also known as the hexose monophosphate shunt (HMP), the phosphogluconate pathway, or the phosphogluconate oxidation pathway leads to two special products in animal tissues: NADPH and ribose-5-phosphate [Lehninger, 1982 Vol 8 Tab 94]. Free pentoses, particularly D-ribose, are formed once again during the metabolism and recycling of nucleotides [Pauly, *et al.,* 2000 Vol 5 Tab 43].

The PPP is especially prominent in tissues actively carrying out the biosynthesis of fatty acids and steroids from small precursors, such as the mammary gland, adipose or fat tissue, the adrenal cortex, and the liver. However, other tissues less active in synthesizing fatty acids, such as skeletal muscle, are virtually lacking in the pentose phosphate pathway. In a classic experiment in rats, Bloom, *et al.,* 1954, showed that about 50% of the utilization of glucose in the liver occurs via the phosphogluconate oxidation pathway [Bloom, *et al.,* 1954 Vol 2 Tab 5]. Additionally, red blood cells utilize the reactions of the PPP to generate large amounts of NADPH used in the reduction of glutathione [Lehninger, 1982 Vol 8 Tab 94].

Section II: Chemical Identity of D-Ribose (Continued)

This pathway is in contrast to the main route by which most of the glucose is catabolized by animal tissues. The vast majority of the glucose catabolized proceeds down the glycolytic sequence with the formation of pyruvate and the generation of ATP. NADPH is also a carrier of chemical energy in the form of reducing power. The biosynthesis of fatty acids requires NADPH to reduce the double bonds of intermediates in this process [Lehninger, 1982 Vol 8 Tab 94].

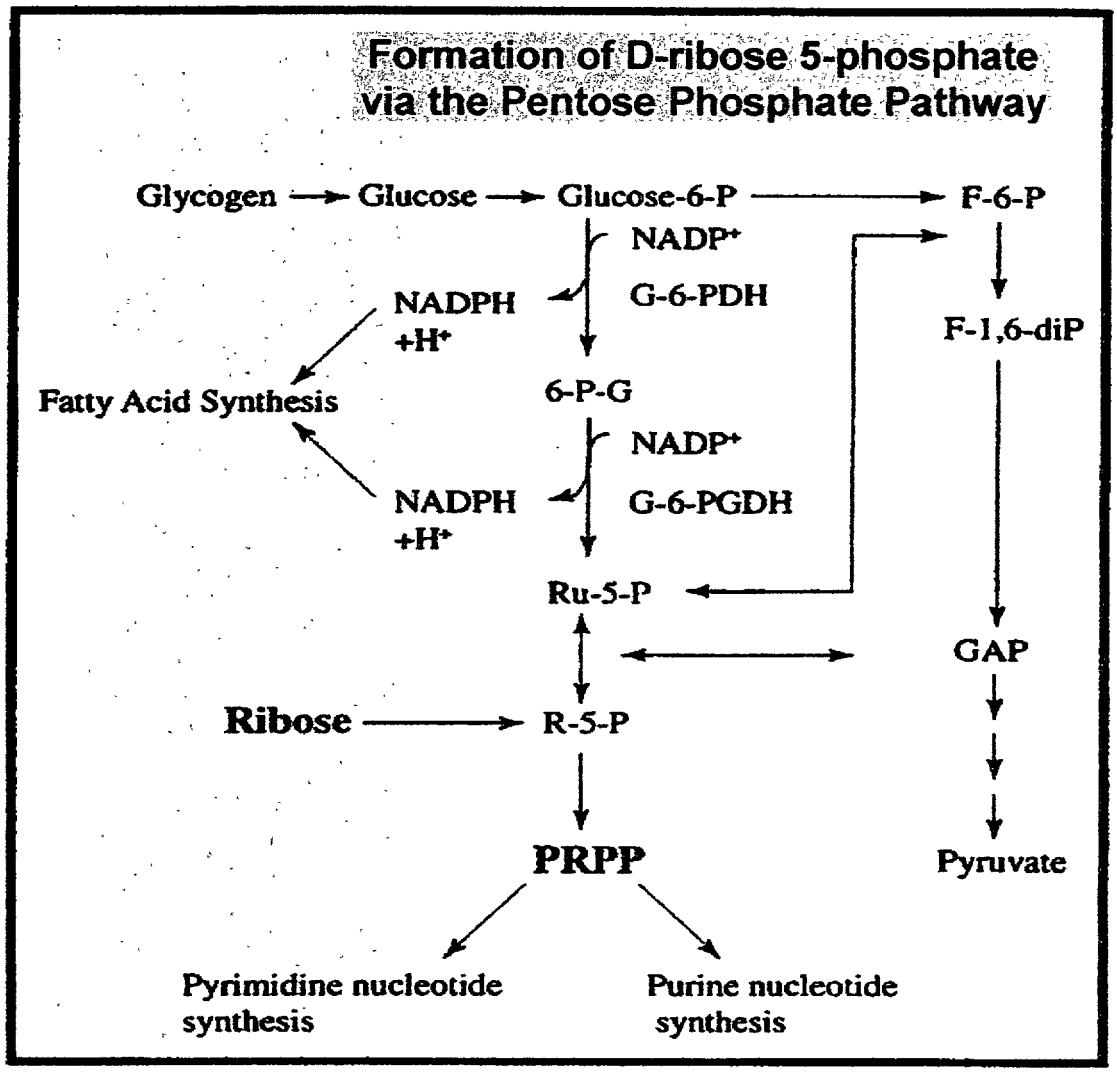
The first reaction of the pentose phosphate pathway is the enzymatic dehydrogenation of glucose-6-phosphate by glucose-6-phosphate dehydrogenase to form 6-phosphogluconate. $NADP^+$ is the electron acceptor. The first product is 6-phosphoglucono-α-lactone, which is hydrolyzed to the free acid by a specific lactonase. The overall equilibrium lies far in the direction of formation of NADPH. In the next step 6-phosphogluconate undergoes dehydrogenation and decarboxylation by 6-phosphogluconate dehydrogenase to form the ketopentose, D-ribulose-5-phosphate, a reaction that generates a second molecule of NADPH. Phosphopentose isomerase then converts D-ribulose-5-phosphate into its aldose isomer, D-ribose-5-phosphate, which can be used in the biosynthesis of ribonucleotides and deoxyribonucleotides (Figure 1). For clarity the names of many of the compounds discussed above have been abbreviated). In some cells the pentose phosphate pathway ends at this point, and its overall equation is then written

$$\text{Glucose 6-phosphate} + 2NADP^+ + H_2O \rightarrow \text{D-ribose 5-phosphate} + CO_2 + 2NADPH + 2H^+$$

The net result is the production of NADPH for reductive biosynthetic reactions in the extramitochondrial cytoplasm and the production of D-ribose-5-phosphate as a precursor for nucleotide synthesis [Lehninger, 1982 Vol 8 Tab 94].

Section II: Chemical Identity of D-Ribose (Continued)

Figure 1



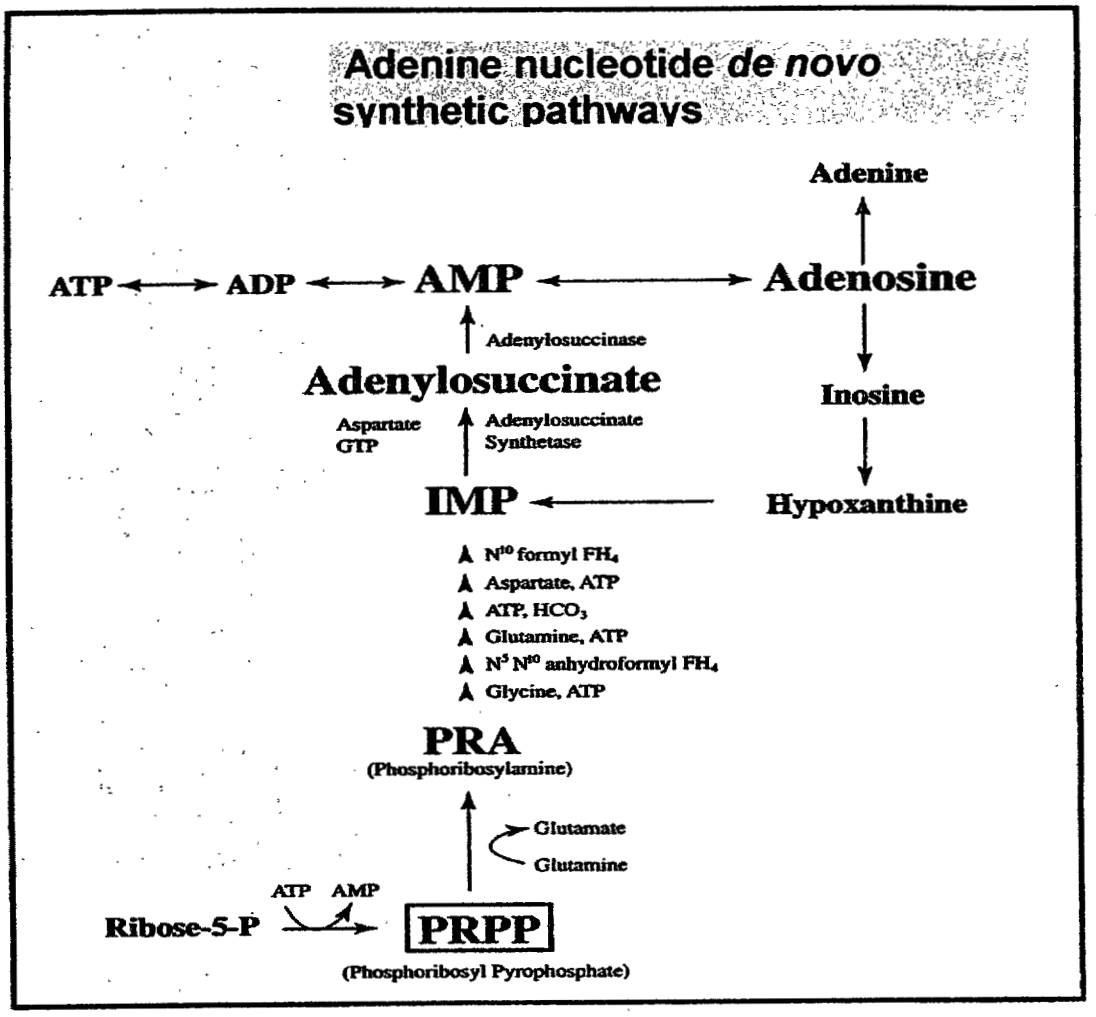
Section II: Chemical Identity of D-Ribose (Continued)

Regulating The Rate Of *De Novo* Purine Nucleotide Synthesis

Three major feedback mechanisms cooperate in regulating the overall rate of *de novo* purine nucleotide synthesis and the relative rates of formation of the two end products, adenylic acid and guanylic acid. The first of these control mechanisms is exerted on the first reaction that is unique to purine synthesis, i.e., the step leading to the transfer of an amino group to PRPP to form 5-phosphoribosylamine (See Figure 2). This reaction is catalyzed by an allosteric enzyme that is inhibited by the end products AMP and GMP. In the second control mechanism, which is exerted at a later stage, an excess of GMP in the cell brings about allosteric inhibition of its formation from inosinic acid, without affecting the formation of AMP. Conversely, an accumulation of adenylic acid results in inhibition of its formation without affecting the biosynthesis of GMP [Lehninger, 1982 Vol 8 Tab 94].

Section II: Chemical Identity of D-Ribose (Continued)

Figure 2



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Pyrimidine Nucleotide Synthesis

The common pyrimidine nucleotides are cytidine-5'-monophosphate (CMP), or cytidylate, and uridine-5'-monophosphate (UMP), or uridylate, which contain the pyrimidines cytosine and uracil. In the case of the pyrimidine nucleotides, the six-membered pyrimidine ring is made first and then attached to ribose phosphate. Another compound is required in the process, carbamoyl phosphate, which is made in the cytosol by carbamoyl phosphate synthase II. The cytosolic carbamoyl phosphate reacts with aspartate to yield *N*-carbamoylaspartate. By removal of water from *N*-carbamoylaspartate, the pyrimidine ring is closed with the formation of L-dihydroorotic acid. This compound is then oxidized to yield the pyrimidine derivative, orotic acid, a reaction in which NAD^+ is the ultimate electron acceptor [Lehninger, 1982 Vol 8 Tab 94].

It is at this point that the ribose-5-phosphate side-chain provided by PRPP is attached to orotate to yield orotidylic acid. Orotidylate is then decarboxylated to yield uridylate, which is phosphorylated to UTP and then accepts an amino group from glutamine to form cytidine triphosphate, or CTP. Pyrimidine synthesis is also regulated by cytidine triphosphate. An excess of this compound inhibits the enzyme, which catalyzes the first reaction in the sequence [Lehninger, 1982 Vol 8 Tab 94].

Formation of DNA

The ribonucleotides described above are the building blocks of DNA. DNA is formed by a reacton in which the 2' carbon atom of the D-ribose portion of the ribonucleotide is directly reduced to form the 2'-deoxy derivative. For example adenosine diphosphate (ADP) is reduced to form 2'- deoxyadenosine diphosphate (dADP). The reduction of the D-ribose portion of the ribonucleoside diphosphates to 2'-deoxy-D-ribose requires a pair of hydrogen atoms, which are ultimately donated by NADPH [Lehninger, 1982 Vol 8 Tab 94].

The Salvage Pathway

Free purine and pyrimidine bases are constantly formed in cells during the metabolic degradation of nucleotides. However, free purines are in large part salvaged and used over again to remake nucleotides. This occurs by a simpler pathway called the salvage pathway in which adenine reacts with PRPP to yield the corresponding adenine nucleotide:

$$\text{Adenine} + \text{PRPP} \rightarrow \text{AMP} + \text{PP}_i$$

This pathway also requires less ATP energy [Lehninger, 1982 Vol 8 Tab 94]. A later section of this report will discuss the effects of *de novo* synthesis and the salvage pathway in the human body. Figures 3 and 4 demonstrate the major reactions of the purine nucleotide salvage pathway.

Section II: Chemical Identity of D-Ribose (Continued)

Figure 3
Purine Nucleotide Salvage Pathway

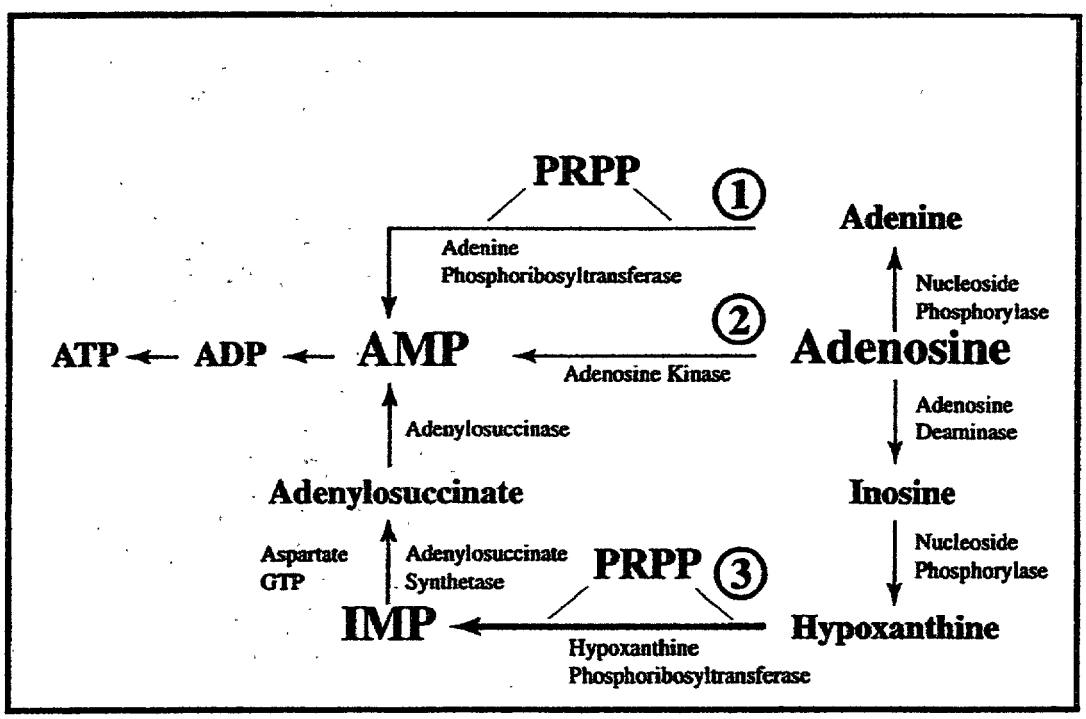

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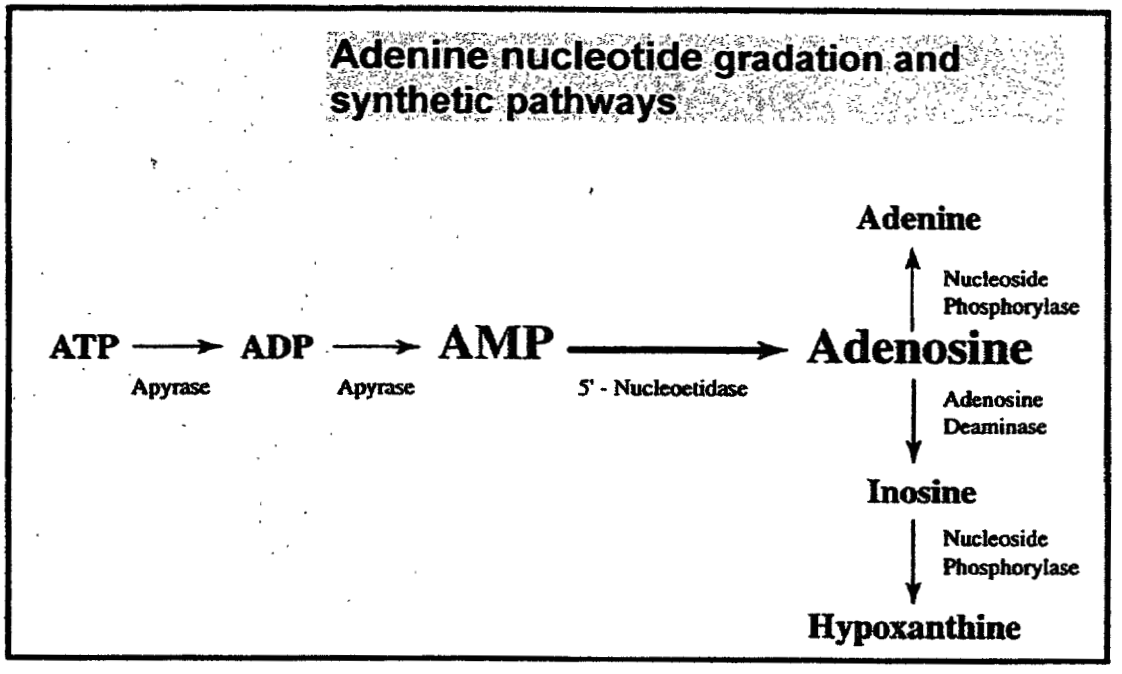
Figure 4



G. Quantitative Composition of D-Ribose (continued)

3. Product Identity

The Bioenergy D-ribose crystalline product is a reducing monosaccharide sugar produced by fermentation of a starch-derived carbohydrate. The D-ribose product is composed of ≥ 97% D-ribose, <2.0% moisture, and <0.2% ash. The Bioenergy production process results in D-ribose, which contains low levels of chloride, sulfate, heavy metals, and arsenic. The average lead level measured over four (4) lots was less than 0.1 ppm (Table 1) Residue on ignition was 0.0 % as measured on the same lots.

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Section II: Chemical Identity of D-Ribose (continued)

Table 1
Bioenergy D-Ribose Physicochemical Properties[1]

Type of Analysis	Method	Specifications For Dextrose	Lot No. 26293A	Lot No. 26343A	Lot No. 26191A	Lot No. 26241A
Identification		A copious red precipitate of cuprous oxide is formed	Meets Requirement (s)	Meets Requirement (s)	Meets Requirement (s)	Meets Requirement (s)
Specific rotation	USP 781S	+52.6° to +53.2°	-21.2°	-21.0°	-20.5°	-18.1°
Acidity		NMT 0.30mL of 0.02 *N* NaOH required	0.20 mL	0.28 mL	0.30 mL	0.30 mL
Residue on ignition	USP 281	NMT 0.1%	0.0%	0.0%	0.0%	0.0%
Chloride	USP 221	NMT 0.018%	< 0.018%	< 0.018%	< 0.018%	< 0.018%
Sulfate	USP 221	NMT 0.025%	< 0.025%	< 0.025%	< 0.025%	< 0.025%
Arsenic	USP Method	NMT 1 ppm	< 1 ppm	< 1 ppm	< 1 ppm	< 1 ppm
Heavy metals	USP Method I	NMT 5 ppm	< 5 ppm	< 5 ppm	< 5 ppm	< 5 ppm
Soluble starch sulfites	Iodine	The liquid is colored yellow by iodine TS.	Meets Requirement (s)	Meets Requirement (s)	Meets Requirement (s)	Meets Requirement (s)
Barium	EP[2]	Not more opalescent than the control	Meets Requirement (s)	Meets Requirement (s)	Meets Requirement (s)	Meets Requirement (s)
Calcium	EP[2] 2,4,3	NMT 200 ppm	< 200 ppm	< 200 ppm	< 200 ppm	< 200 ppm
Lead in sugars	EP[2] 2,4,10	NMT 0.5 ppm	0.0 ppm	0.1 ppm	0.0 ppm	0.0 ppm
Sulfated ash	EP[2] 2,4, 14	NMT 0.1%	0.0%	0.0%	0.0%	0.0%

[1]Samples were tested according to specifications for dextrose at the request of the manufacturer. All analyses were conducted by Lancaster Laboratories, Lancaster, Pennsylvania.
[2]European Pharmacopoeia, 3rd Edition, 1997

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Section II: Chemical Identity of D-Ribose (continued)

Microbial limits have been set to meet food grade specifications (Table 2).

Bioenergy D-ribose is described as a crystalline product or as an undried syrup. The syrup is further described as a clear syrupy liquid with a pale yellow – pale yellowish-brown appearance. The solids content of the syrup as is 62 – 68% on a weight basis. The minimum ribose content, as measured by HPLC is 92.0%, on the dry basis. The specific gravity is 1.28 – 1.35 (at 20° C), and the pH may range from 3.5 – 5.0. Specifications for this syrup product have been developed by Bioenergy, Inc., including appropriate microbiological limits (Table 3). [Bioenergy Raw Data, 1999-2000 Vol 7 Tab 80].

Section II: Chemical Identity of D-Ribose (continued)

TABLE 2
FINAL PRODUCT SPECIFICATIONS OF BIOENERGY D-RIBOSE

Food Grade Chemical Specifications	
D-ribose	97.0 – 103.0%
Loss on Drying (Moisture)	≤ 2.0 %
Ash	≤ 0.2%
Lead	≤ 0.1 ppm
Microbiological Specifications	
Aerobic Plate Count	≤ 100 CFU/g
Salmonella sp.	Negative/125g
Coliforms	≤ 10 CFU/g
Yeast & Mold	≤ 100 CFU/g
Product Form	
Consistency	Powder or Granular
Color	White to Slightly Yellow
Flavor	Slightly Sweet

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Section II: Chemical Identity of D-Ribose (continued)

TABLE 3
FINAL PRODUCT SPECIFICATIONS OF BIOENERGY D-RIBOSE SYRUP

Food Grade Chemical Specifications	
Solids	62 – 74% (w/w)
D-ribose	≥ 92.0%
pH	3.5 – 6.0
Specific Gravity	1.28 – 1.35 [d] [20]
Lead	< 0.1 ppm
Arsenic	< 1.0 ppm
Product Form	
Consistency	Syrup
Color	Pale Yellow – Yellowish Brown
Microbiological Specifications	
Aerobic Plate Count	≤ 100 CFU/g
Salmonella sp.	Negative/125g
Coliforms	≤ 10 CFU/g
Yeast & Mold	≤ 100 CFU/g

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4. Analysis of Four Lots of Bioenergy D-Ribose

An analysis of four lots of Bioenergy D-ribose was conducted by Lancaster Laboratories, Lancaster, Pennsylvania in December of 1999 (Table 1) [Bioenergy Raw Data, 1999-2000 Vol 7 Tab 80]. At the request of the manufacturer, the samples were analyzed against standards for dextrose as a model sugar.

A more recent analysis of four lots was performed between February and May, 2001 (Table 4). The purity of D-ribose ranged from 97.8 to 99.8%, and the ash content ranged from 0.0% to 0.08%. Loss on drying (moisture content) ranged from 0.09 to 0.18%. The product met the microbial limits set by Bioenergy, Inc., in that total plate counts were ≤ 10, and yeast and mold counts were also ≤ 10. Coliforms were below the level of detection, and *Salmonella* was negative per 125 grams. [Bioenergy Raw Data, 1999-2000 Vol 7 Tab 80].

Section II: Chemical Identity of D-Ribose (continued)

Table 4
Bioenergy D-Ribose Analyses of Four Lots

Type of Analysis	Methods	Lot No. 10216701	Lot No. 10206701	Lot No. 10116701	Lot No. 10096701
Date Analyzed		May 4, 2001	May 3, 2001	February 7, 2001	February 7, 2001
D-ribose (purity) (%)	Bioenergy	99.8	99.5	98.5	97.8
Loss on Drying (%)	Bioenergy	0.09	0.09	0.14	0.18
Ash (%)	Bioenergy	0.06	0.08	0.00	0.02
Total Plate Count (CFU/g)	Bioenergy	<10	<10	10	<10
Yeast and Mold (CFU/g)	Bioenergy	10	<10	<10	<10
Coliform (CFU/g)	Bioenergy	<10	<10	<10	<10
Salmonella/125g	Bioenergy	Negative	Negative	Negative	Negative

5. Food Grade Specifications

a. Crystalline Product

Based on its analysis of multiple lots of D-ribose, Bioenergy, Inc. has set food grade specifications for its products. (Table 2). The specifications define a very low moisture product, with a purity of 97.0 – 103.0%. The product contains no more than 0.2% ash and no more than 0.1 ppm lead. Microbiological specifications provide for the product to contain ≤ 100 colony forming units (CFU) of aerobic bacteria per gram. The product appears as a white to slightly yellow powder with a slightly sweet taste.

b. Syrup Product

Based on its analysis of multiple lots of Bioenergy D-ribose syrup, Bioenergy, Inc. has set food grade specifications for the product. (Table 3). A sample analysis of two lots shows that the Bioenergy D-ribose syrup product is a clear yellowish syrupy liquid, which contains an average of 64.10% solids. The product has a specific gravity of 1.32, and a pH of 3.7 (Table 5). The D-ribose content of the solids was 92.0%. Color according to the APHA scale (a spectroscopic standard) was 110 and 250, respectively for the two lots, indicating a yellowish color [Bioenergy Raw Data, 1999-2000 Vol 7 Tab 80].

TABLE 5
ANALYSIS OF TWO LOTS OF BIOENERGY D-RIBOSE SYRUP

Variables	Lot FCR988037BS[1]	Lot FCR98804B[2]
Solids	64.4%	63.8%
D-ribose **(Area Under Peak)**	92.0%	92.0%
pH	3.7	3.7
Specific Gravity	1.32 [d] ^{20}	1.32 [d] ^{20}
Product Form		
Consistency	Clear Syrupy Liquid	Clear Syrupy Liquid
Color (APHA)	110	250

[1] Manufactured June 18, 1998; Analyzed June 19, 1998 [Bioenergy Raw Data, 1999-2000 Vol 7 Tab 80].
[2] Manufactured September 1, 1998; Analyzed September 3, 1998 [Bioenergy Raw Data, 1999-2000 Vol 7 Tab 80].

H. Manufacturing

1. D-Ribose Production Summary

D-ribose is a constituent of nucleic acids, and hence occurs in all organisms. A reduction product of D-ribose, ribitol, is present in vitamin B_2 (riboflavin), and in ribitol-teichoic acid, which is a common component of cell walls [US Patent 3,919,046, 1975 Vol 8 Tab 105]. Early methods for the production of D-ribose involved extraction from natural substances, and synthesis from furan or D-glucose [US Patent 3,919,046, 1975 Vol 8 Tab 105].

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In the mid-1970s Takeda Chemical Industries of Osaka, Japan filed two patents describing the production of D-ribose via fermentation from a D-ribose producing strain of the genus

Bacillus [US Patent 3,919,046, 1975 Vol 8 Tab 105 and US Patent 3,970,522, 1976 Vol 8 Tab 106]. The company's interest in producing D-ribose was to provide an economical starting material for the manufacture of vitamin B_2. The same basic method continues to be used with modifications to produce Bioenergy, Inc. D-ribose. The process involves the cultivation of a D-ribose producing microorganism of the genus *Bacillus*, whose transketolase activity is very low. These organisms are non-pathogenic and are non-sporulating. When transketolase is limiting, certain strains accumulate D-ribose, which can then be recovered from the culture broth [US Patent 3,919,046, 1975 Vol 8 Tab 105]. The following section contains a discussion of the role of transketolase in two important physiological pathways found in living organisms. Its relationship to ribose as a core molecule can be discerned from the reaction sequences.

a. Transketolase

Transketolase, an enzyme of both the Calvin Cycle of photosynthesis in plants, and of red blood cells in animals, catalyzes the transfer of a 2-carbon fragment from a 5-carbon keto sugar (D-xyulose-5-phosphate) to a 5-carbon aldo sugar (D-ribose-5-phosphate) to form a 7-carbon keto sugar (sedoheptulose-7-phosphate) and a 3-carbon aldo sugar (glyceraldehyde-3-phosphate). These enzymes use thiamine pyrophosphate (vitamin B1) as a cofactor in the reaction mechanism [Lehninger, 1982 Vol 8 Tab 94].

Transketolase also participates in the pentose phosphate pathway, which takes place in the cytoplasm of all cells, but mainly in liver and adipose tissue. Red blood cells also use the oxidative section of this pathway to help maintain cellular integrity. The main function of the pentose phosphate pathway is to generate NADPH for biosynthetic pathways and to provide intermediates for biosynthesis of nucleotides [Lehninger, 1982 Vol 8 Tab 94].

There are two steps, which oxidize glucose-6-phosphate to 6-phosphogluconate, then to ribulose-5-phosphate and finally reducing 2 $NADP^+$ to NADPH. The enzymes involved are glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. CO_2 is given off in the second reaction and a 5-carbon ketose ribulose-5-phosphate results [Lehninger, 1982 Vol 8 Tab 94]. In the non-oxidative section of the pathway, the ribulose-5-phosphate is then either converted to ribose-5-phosphate or xylulose-5-phosphate, and then on to biosynthetic intermediates [Lehninger, 1982 Vol 8 Tab 94].

Both transketolase and ribose (as a core molecule) take part in photosynthesis. Thus, ribose has been selected by nature to play a vital role in the functioning of nearly all living cells. In the late 1940's Calvin and his colleagues at the University of California at Berkeley proposed a complex cyclic mechanism for the total biosynthesis of all six carbon atoms of glucose from carbon dioxide. In the Calvin cycle, one molecule of ribulose-1,5-diphosphate is consumed for each molecule of CO_2 fixed, but at the end of the cycle a molecule of ribulose diphosphate is regenerated. This type of reaction is used in the citric acid cycle and in the urea cycle, both of which end by regenerating a compound required in the first step. The individual reactions of the cyclic Calvin pathway are shown in the figure below. The cycle contains seven of the steps (reactions (2) to (8)) of the gluconeogenesis pathway found in animal tissues. However, in photosynthesis NADPH rather than NADH is the reductant of glyceraldehyde 3-phosphate. The remaining reactions are catalyzed by six additional enzymes [Lehninger, 1982 Vol 8 Tab 94].

The sequential reactions of the Calvin cycle are listed below. Reactions (1) to (8) describe the formation of glucose from

CO_2 and ribulose-1,5-diphosphate. Reactions (9) to (15) are concerned with the regeneration of ribulose-1,5-diphosphate, required to begin another round of the Calvin cycle. Reaction (9) is catalyzed by the enzyme transketolase. This enzyme contains thiamine pyrophosphate as its prosthetic group and requires Mg^{2+} for it's activity. Here, transketolase catalyzes the reversible transfer of a ketol (CH_2OH--CO--) group from a ketose phosphate, in this case fructose-6-phosphate, to an aldose phosphate, in this case glyceraldehyde 3-phosphate. Reaction (10) is promoted by aldolase, which catalyzes the reversible condensation of an aldehyde, in this case, erythrose 4-phosphate, with dihydroxyacetone phosphate, yielding the 7-carbon sedoheptulose-1,7-diphosphate. After the latter loses its 1-phosphate group, it undergoes another transketolase reaction to yield two different pentose phosphates, which are ultimately converted into ribulose-1,5-diphosphate. All the reactions listed above, except the first, catalyzed by ribose diphosphate carboxylase, also occur in animal tissues [Lehninger, 1982 Vol 8 Tab 94]. Thus, D-ribose has been selected by nature as an important component in the biochemical reactions of nearly all living cells, whether plant or animal.

b. The Calvin Cycle

1. $6CO_2$ + 6 ribulose 1,5-diphosphate + $6H_2O \rightarrow$ 12 3-phosphoglycerate

2. 12 3-Phosphoglycerate + 12 ATP $\rightarrow$ 12 3-phosphoglyceroyl phosphate + 12 ADP

3. 12 3-Phosphoglyceroyl phosphate + 12 NADPH + 12 $H^+ \rightarrow$ 12 glyceraldehyde 3-phosphate + 12 $NADP^+$ + 12 P_i

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Section II: Chemical Identity of D-Ribose (continued)

4. 5 Glyceraldehyde 3-phosphate → 5 dihydroxyacetone phosphate

5. 3 Glyceraldehyde 3-phosphate + 3 dihydroxyacetone phosphate → 3 fructose 1,6-diphosphate

6. 3 Fructose 1,6-diphosphate + $3H_2O$ → 3 fructose 6-phosphate + 3 P_i

7. Fructose 6-phosphate → glucose 6-phosphate

8. Glucose 6-phosphate + H_2O → glucose + P_i

9. 2 Fructose 6-phosphate + 2 glyceraldehyde 3-phosphate $\xrightarrow{\text{transketolase}}$ 2 xylulose 5-phosphate + 2 erythrose 4-phosphate

10. 2 Erythrose 4-phosphate + 2 dihydroxyacetone phosphate $\xrightarrow{\text{aldase}}$ 2 sedoheptulose 1,7-diphosphate

11. 2 Sedoheptulose 1,7-diphosphate + $2H_2O$ $\xrightarrow{\text{phosphatase}}$ 2 sedoheptulose 7-phosphate + $2P_i$

12. 2 Sedoheptulose 7-phosphate + 2 glyceraldehyde 3-phosphate $\xrightarrow{\text{transketolase}}$ 2 ribose 5-phosphate + 2 xylulose 5-phosphate

13. 2 Ribose 5-phosphate $\xrightarrow{\text{isomerase}}$ 2 ribulose 5-phosphate

14. 4 Xylulose 5-phosphate $\xrightarrow{\text{epimerase}}$ 4 ribulose 5-phosphate

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15. 6 Ribulose 5-phosphate + 6 ATP $\xrightarrow{\text{phosphoribulokinase}}$ 6 ribulose 1,5-diphosphate + 6 ADP

Section II: Chemical Identity of D-Ribose (continued)

Sum: $6CO_2 + 18\ ATP + 12\ H_2O + 12\ NADPH + 12\ H^+ \rightarrow C_6H_{12}O_6 + 18P_i + 18\ ADP + 12\ NADP^+$

One way of writing the overall equation of this complex cycle is:

6 Ribulose-1,5-diphosphate + $6CO_2$ + 18ATP + $12H_2O$ + 12NADPH + $12H^+ \rightarrow$ 6 ribulose 1,5-diphosphate +glucose + $18P_i$ +18ADP +12 $NADP^+$

Ribulose-1,5-diphosphate is written on both sides of the equation to show that it is a necessary component regenerated at the end of each cycle. After canceling out the ribulose diphosphate on both sides, the net reaction equation of the Calvin cycle is:

$6CO_2$ + 18ATP + $12H_20$ + 12NADPH + $12H^+ \rightarrow C_6H_{12}O_6 + 18P_i$ + 18ADP + $12NADP^+$

To synthesize each molecule of glucose from 6 molecules of CO_2, 18 ATPs and 12 NADPHs are consumed, which are replenished at the expense of the light reactions of photosynthesis.

Sedoheptulose-7-phosphate + 2-glyceraldehyde-3-phosphate $\xrightarrow{\text{transketolase}}$ 2 ribose 5-phosphate + xylulose 5-phosphate

Organisms that do not exhibit transketolase activity therefore accumulate energy pathway intermediates, such as D-ribose. It is these types of organisms that have been used commercially for the production of D-ribose.

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In humans, transketolase is a thiamine dependent enzyme, whose deficiency leads to neurological disorders such as the

Wernicke-Korsakoff syndrome often associated with alcoholics [Lehninger, 1982 Vol 8 Tab 94].

c. Production Sequence

A generalized production scheme for D-ribose is shown in Figure 5. A D-ribose producing strain of *Bacillus sp* must be cultivated in a nutrient rich medium containing certain amino acids, such as L-tyrosine, L-tryptophan, and L-phenylalanine, which are required for its growth [US Patent 3,919,046, 1975 Vol 8 Tab 105]. A nutrient rich medium, such as corn steep liquor can provide the carbon and nitrogen sources necessary for the growth of the D-ribose producing strain [US Patent 4,904,587, 1990 Vol 8 Tab 107]. Table 6 shows the production of D-ribose by several strains of *Bacillus sp.*

Section II: Chemical Identity of D-Ribose (continued)

Table 6

Relative D-Ribose Producing Abilities[1]	
Organism	**D-ribose accumulated mg/ml**
Bacillus pumilus	
Bacillus pumilus No. 503	29
Bacillus pumilus No. 537	28
Bacillus pumilus No. 716	45
Bacillus pumilus No. 735	49
Bacillus pumilus No. 783	47
Bacillus subtilis	
Bacillus subtilis No. 429	26
Bacillus subtilis No. 608	45
Bacillus subtilis No. 632	48
Bacillus species	
Bacillus species Shi 7	27

[1] US Patent 3,919,046 1975 Vol 8 Tab 105

Section II: Chemical Identity of D-Ribose (continued)

The cultural conditions for D-ribose production include a controlled temperature of 25 - 40°C, a preferred pH level of 5.5 - 8, and a cultivation time of between 36 - 120 hours [US Patent 3,919,046, 1975 Vol 8 Tab 105]. After incubation, the fermentation broth can be harvested by a conventional method for the recovery of D-ribose (Figure 5). The cells of the producing strain are removed through filtering or centrifugation. The filtrate or supernatant is then purified, decolorized and desalted by treatment with successive steps using activated carbon, ion exchange or similar purification techniques. These purification steps are followed by concentration through evaporation, or other appropriate means. An organic solvent, such as ethanol, is then added to facilitate crystallization. The crystals are recovered by centrifugation, and evaporation, and are finally, dried, milled, and packaged [US Patent 4,904,587, 1990 Vol 8 Tab 107]. Additionally, the form of the dry product may be altered by practices such as agglomeration. Figure 5 shows a typical production scheme for D-ribose. Similar production schemes and equipment may be used to produce a product, which conforms to the specifications listed in this Section.

Section II: Chemical Identity of D-Ribose (continued)

Figure 5
D-Ribose Production Scheme

Section II: Chemical Identity of D-Ribose (continued)

Figure 5 (Continued)
D-Ribose Production Scheme

Section II: Chemical Identity of D-Ribose (continued)

2. Critical Process Controls for Manufacturing

The manufacturers of D-ribose for Bioenergy, Inc. have developed a system of critical process controls for the manufacture of D-ribose. These controls assure that the product will consistently meet the company's specifications. The critical process controls are shown in Figure 6.

Section II: Chemical Identity of D-Ribose (continued)

Figure 6
D-Ribose Production Scheme

Section II: Chemical Identity of D-Ribose (continued)

Figure 6 (Continued)
D-Ribose Production Scheme

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I. Analytical Methods

Bioenergy has provided standardized analytical methods for the testing of its D-ribose product. These procedures assure that the product is tested consistently against specification parameters. These methods are either identical to or are based on those approved by AACC, AOAC, or FDA in its *Bacteriological Analytical Manual*. Additional methods may be used internally to provide more detailed insight into the quality of the product. A Panel of Experts qualified for determining the safety of foods by training and experience has reviewed all methods utilized by Bioenergy, Inc. to assess the quality of its product. A sample internal Certificate of Analysis has also been provided.

Section II: Chemical Identity of D-Ribose (continued)

Bioenergy CERTIFICATE OF ANALYSIS

6/20/01

Powdered D-Ribose **Lot No: 10276501**

TEST	RESULTS
Ribose (%)	99.81%
Loss on Drying (%)	0.08%
Ash (%)	<0.05%
Total Plate Count (%)	10 CFU/g
Yeast Count/gm	<10 CFU/g
Mold Count/gm	10 CFU/g
Coliform	<10 CFU/g
E. coli	<10 CFU/g
Salmonella	Neg/100 g

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J. D-Ribose Producing Strains

The following organisms are listed by The American Type Culture Collection (ATCC, U.S.A., 12301 Parklawn Rockville, Md. 20852 U.S.A), as producers of D-Ribose:

Listing of D-Ribose Producing organisms

Bacillus pumilus

Accession Numbers
ATCC 21356
ATCC 21357
ATCC 21358
ATCC 21951
ATCC 31095
ATCC 31098
ATCC 31093

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Bacillus subtilis

Accession Numbers
ATCC 21359
ATCC 21360
ATCC 21952
ATCC 31096
ATCC 31097
ATCC 31091
ATCC 31092
ATCC 31094

K. Recall and Tracking Program

Bioenergy's manufacturing process is organized batchwise into a series of fermentation and purification steps. Each batch produced is assigned a unique lot number. Each finished lot is assigned an 8-digit number. The first digit equates to the last digit of the year in which manufacturing was assigned. The next three digits are a consecutive assignment from a serial numbering log. In this log, numbers are assigned consecutively by date. The final four digits represent the Bioenergy product code.

The reference numbers and quantities of all materials and processing aids used in each unit of fermentation and purification can be recorded for various lot numbers. Thus, it is possible for Bioenergy to track not only the finished product, but also each raw material component that was used in the processing of that finished product. All finished products can be traced to a given day or two of production.

Retainer samples for each lot are maintained for 2 years post expiration date or 4 years past manufacturing date if no expiration date is assigned. All sales of Bioenergy D-ribose

Section II: Chemical Identity of D-Ribose (continued)

are recorded and kept in secure files, and duplicate copies of records are stored at a location separate from the production facility. Thus each lot may be tracked to a final destination, and recalled if a foodborne emergency should arise.

Section III

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Section III. Intended Uses, Functional Effects, Consumption Estimates, and Self Limiting Levels

Introduction

The use of D-ribose as a supplement to human nutrition has been investigated for several decades [Quraishi, *et al.*, 1999 Vol 5 Tab 47]. While the previous investigations focused on the pharmacokinetic effects of D-ribose, more recent research has shown that D-ribose can have a dual role in foods. That is, when D-ribose is incorporated into foods, it supplements the diet, and performs specific functions within the food matrix.

Bioenergy, Inc. and its associates have developed food formulations in which D-ribose may be utilized across a wide range of food categories. In many of these uses D-ribose is used as a substitute for a portion of the sucrose normally found in similar formulations. Its use provides the food formulator a route to modifying the sensation of sweetness in a given food, and it may also be used to modify the color of certain baked or cooked foods. This experience provides examples upon which to base physical and technical functional effects, the intended uses, consumption estimates and self-limiting aspects of this product.

Example formulations have been provided in this section for the use of D-ribose [shown as a weight percentage of the formulation] in baked goods (1.88 - 5%), crackers (7.14%), breakfast cereals (7.14%), vegetable and fruit juices (2%), dairy products (1.8 - 3%), meat dishes (0.44%), glazes (25%), gravies (2%), soft candies (5.6%), hard candies (11.1%), energy drinks (0.714%), and non-alcoholic beverages (2%). In these formulations D-ribose has a number of technical effects, which are listed below.

A. Intended Uses, Functional Effects, and Use Levels

Bioenergy, Inc. D-ribose has a variety of potential uses in the U.S. (Table 1). These potential products are based on company research and information from U.S. patents. This information provides realistic intended uses, functional effects and use levels that are associated with the use of D-ribose under current Good Manufacturing Practice.

Section III. Technical Effects, Intended Uses, Consumption Estimates, and Information on Self-Limiting Levels

1. Intended Uses

Bioenergy, Inc. D-ribose is a multiple-use direct food additive. It has a number of technical effects in food, which have been utilized to develop the example formulations shown in Table 1. The formulations have been developed by Bioenergy, Inc. and its associates to demonstrate the utility of D-ribose as a food ingredient.

In order to classify the various effects ingredients have in food, FDA has published a list of 32 physical or technical functional effects for which direct food ingredients may be added to food. These are codified at 21 CFR § 170.3 (o) (1 - 32). Applications for D-ribose are covered under several of the following terms as listed under 21 CFR 170.3 (o).

(11) "Flavor enhancers": Substances added to supplement, enhance, or modify the original taste and/or aroma of a food, without imparting a characteristic taste or aroma of its own.
(16) "Humectants ": Hygroscopic substances incorporated in food to promote retention of moisture, including moisture-retention agents and antidusting agents.
(20) "Nutritional supplement": Substances added to food to supplement the diet, or increase the nutritional value of a food.
(21) "Nutritive sweeteners": Substances having greater than 2 percent of the caloric value of sucrose per equivalent unit of sweetening capacity.
(28)"Stabilizers and thickeners": Substances used to produce viscous solutions or dispersions, to impart body, improve consistency, or stabilize emulsions, including suspending and bodying agents, setting agents, jellying agents, and bulking agents, etc.
(31) "Synergists": Substances used to act or react with another food ingredient to produce a total effect different or greater than the sum of the effects produced by the individual ingredients.
(32) "Texturizers": Substances, which affect the appearance or feel of the food.

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Food Ingredient--One primary category of intended use of D-ribose is as a multifunctional food ingredient that provides physical and technical effects that improve the quality of a variety of food products. Several of the food products provided in the following table would be likely

candidates for use in the US. It is proposed that the use levels in the formulations provide a reasonable guide for the use levels in the US.

B. D-Ribose Applications

Examples of foods, which can be formulated with D-ribose are shown in Table 1. Bioenergy, Inc. continues to conduct applications research to investigate the effects of D-ribose in other foods similar to those shown in this section.

Section III. Technical Effects, Intended Uses, Consumption Estimates, and Information on Self-Limiting Levels

Table 1
Example Formulations With D-Ribose

Product	Ingredients	Weight (g)
Yeast Bread		
(1 serving)	Flour	60
	Yeast	0.3125
	Water	30
	Sugar	5
	Ribose	5
Total Weight		**100.31**
% Ribose of the Total Weight		**4.98%**
Muffins		
	White flour	60
	Salt	0.3125
	Sugar	15
	Baking Powder	0.625
	Eggs, beaten	10
	Butter	5
	Milk	45
	Ribose	5
Total Weight		**140.94**
% Ribose of the Total Weight		**3.55%**
Cookies		
(1 serving, 2 cookies)	Butter	30
	Sugar	15
	Ribose	5
	Egg White	5
	White Flour	45
	Vanilla	0.3125
Total Weight		**100.31**
% Ribose of the Total Weight		**4.98%**

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Table 1 (Continued)
Example Formulations with D-Ribose

Product	Ingredients	Weight (g)
Cake		
(1 serving)	White Flour	80
	Baking Powder, sodium bicarbonate	1.25
	Canola Oil	45
	Water	60
	Milk	15
	Egg Whites	30
	Sugar	30
	Ribose	5
Total Weight		**266.25**
% Ribose of the Total Weight		**1.88%**
Mixed Vegetable Juice		
	Tomato juice	180
	Celery Juice	30
	Carrot juice	30
	Salt	0.625
	Ribose	5
Total Weight		**245.625**
% Ribose of the Total Weight		**2.04%**
Fortified Orange Juice		
	Reconstituted Orange Juice	240
	Ribose	5
Total Weight		**245**
% Ribose of the Total Weight		**2.04%**
Fortified Pear Juice		
	Pear Juice	240
	Sugar	10
	Ribose	5
Total Weight		**255**
% Ribose of the Total Weight		**1.96%**

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Table 1 (Continued)
Example Formulations with D-Ribose

Product	Ingredients	Weight (g)
Yogurt		
	Milk	240
	Cultures	1.25
	Sugar	15
	Ribose	5
	Berries	15
Total Weight		**276.25**
% Ribose of the Total Weight		**1.81%**
Ice Cream		
	Cream	120
	Sugar	45
	Ribose	5
	Vanilla	0.3125
Total Weight		**170.31**
% Ribose of the Total Weight		**2.94%**
Chocolate Milk		
	Milk	240
	Chocolate	15
	Sugar	15
	Ribose	5
Total Weight		**275**
% Ribose of the Total Weight		**1.8182%**
Honey Ham		
(1 serving)	Ham slice	
	Honey	15
	Ribose	5
Total Weight		**20**
% Ribose of the Total Weight		**25%**

Table 1 (Continued)
Example Formulations with D-Ribose

Product	Ingredients	Weight (g)
Stew		
	Beef cubes	56.6
	Garlic	0.3125
	Onion	2.5
	Tomato Sauce	30
	Water	120
	Parsley	0.3125
	Potato Cubes	45
	Carrot Cubes	15
	Celery, Chopped	15
	Ribose	1.25
Total Weight		**285.98**
% Ribose of the Total Weight		**0.44%**
Beef Gravy		
(1 serving)	Juice from Beef Roast	30
	Ribose	0.625
Total Weight		**30.63**
% Ribose of the Total Weight		**2.04%**
Caramels		
(1 serving)	White Corn Syrup	30
	White Sugar	30
	Butter	2.5
	Evaporated Milk	22.5
	Ribose	5
Total Weight		**90.0**
% Ribose of the Total Weight		**5.56%**
Hard Candy		
(1 Serving)	Sugar	30
	Dark Corn Syrup	5
	Water	2.5
	Cream	2.5
	Ribose	5
Total Weight		**45**
% Ribose of the Total Weight		**11.11%**

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Table 1 (Continued)
Example Formulations with D-Ribose

Product	Ingredients	Weight (g)
Flavored Coffee		
	Coffee	240
	Almond Flavoring	2.5
	Sugar	5
	Ribose	5
Total Weight		**252.50**
% Ribose of the Total Weight		**1.98%**
Sweetened Tea		
	Tea	240
	Sugar	5
	Ribose	5
Total Weight		**250**
% Ribose of the Total Weight		**2%**
Low Cal Fruit Drinks		
	Fruit Juice	120
	Water	120
	Aspartame	0.625
	Ribose	2.5
Total Weight		**243.13**
% Ribose of the Total Weight		**1.03%**

Table 1 (Continued)
Example Formulations with D-Ribose

Product	Ingredients	Weight (g)
Low Cal Carbonated Drinks		
	Water	240
	Sugar	10
	Ribose	2.5
	Flavoring	2.5
Total Weight		**255.0**
% Ribose of the Total Weight		**0.98%**
Energy Bar		
	High Fructose Corn Syrup	22.5
	Soy Protein	12.5
	Maltitol Syrup	5.4
	Glycerin	4
	Oat Bran	4
	D-Ribose	2
	Vitamin/Mineral Pre-blend (optional)	0.75
	Natural & Artificial Flavors	0.6
Total Weight		**51.75**
% Ribose of the Total Weight		**3.86%**

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Section III. Technical Effects, Intended Uses, Consumption Estimates, and Information on Self-Limiting Levels

Table 1 (Continued)
Example Formulations with D-Ribose

Product	Ingredients	Weight (g)
Breakfast Cereal		
	Wheat Flour	10.0
	Oat Flour	5.0
	Sugar	5.0
	High Fructose Corn Syrup	3.0
	Dextrose	2.0
	Ribose	2.0
	Salt	0.4
	Vitamin Mix	0.2
	Flavorings	0.2
	Colors	0.2
Total Weight		**28.0**
% Ribose of the Total Weight		**7.14%**
Readv-To-Eat Crackers		
	Wheat Flour	12.0
	Hydrogenated Vegetable Oil	7.0
	Sugar	5.0
	Ribose	2.0
	Soy Lecithin	1.0
	Salt	0.7
	Flavorings	0.2
	Color	0.1
Total Weight		**28.0**
% Ribose of the Total Weight		**7.14%**
Energy Drink		
	Water	320.0
	Sucrose	12.0
	Dextrose	8.0
	Fructose	5.0
	Ribose	2.5
	Citric Acid	1.0
	Flavoring	0.5
	Salt	0.5
	Potassium	0.3
	Color	0.2
Total Weight		**350.0**
% Ribose of the Total Weight		**0.714%**

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C. Current Maximum Use Levels

A summary of the maximum levels of Bioenergy, Inc. D-ribose in the food categories listed by the US FDA at 21 CFR §170.3 (n) is shown in Table 2. The maximum usage levels were derived from applications trials using D-ribose as a food ingredient. Future applications development for D-ribose is not expected to impact the total exposure to the product, which is discussed in the next section.

Table 3 shows the maximum use level of D-ribose considered to represent current Good Manufacturing Practices, given the applications data available. The food categories shown correspond to the major food groups surveyed in USDA's Continuing Survey of Food Intakes by Individuals [Enns, *et al.*, 1997 Vol 7 Tab 78].

Section III. Technical Effects, Intended Uses, Consumption Estimates, and Information on Self-Limiting Levels

Table 2: Maximum Use of Bioenergy D-Ribose In the Food Categories Listed by the US FDA At 21 CFR §170.3 (n)

	Food Categories	Realistic Percentage of Use in the US Market
01	Baked Goods, Baking Mixes	5.0%
02	Beverages alcoholic	0
03	Beverages and beverage bases, non-alcoholic	2.0%
04	Breakfast Cereals	7.14%
05	Cheeses	0
06	Chewing Gum	2.0%
07	Coffee and tea	2.0%
08	Condiments and relishes	5.0%
09	Confections and frostings	5.0%
10	Dairy product analogs	0
11	Egg Products	0
12	Fats and oils	0
13	Fish Products	0
14	Fresh eggs	0
15	Fresh fish	0
16	Fresh fruits and fruit juices	2.0%
17	Fresh Meats	0
18	Fresh poultry	0
19	Fresh vegetables	0
20	Frozen dairy desserts and mixes	3.0%
21	Fruit and water ices	2.0%
22	Gelatins, puddings and fillings	2.0%
23	Grain products and pastas	0
24	Gravies and Sauces	2.0%
25	Hard candy	11.0%
26	Herbs, seeds, spices, seasonings, blends	0
27	Jams and jellies, home prepared	0
28	Jams and jellies commercial	5.0%
29	Meat products	0.2%
30	Milk, whole and skim	0
31	Milk products	1.8%
32	Nuts and Nut products	0
33	Plant protein products	0
34	Poultry products	0.2%
35	Processed fruits and fruit juices	2.0%
36	Processed vegetables and vegetable juices	2.0%
37	Snack foods	0.5%
38	Soft Candy	5.6%
39	Soups, home prepared	0
40	Soups and soup mixes	1.0%
41	Sugar, white granulated	0
42	Sugar substitutes	16.0%
43	Sweet Sauces, toppings, and syrups	25.0%

Table 3
Current Good Manufacturing Use Levels For Bioenergy D- ribose in Foods

Food Category[1]	Use Level[2] (maximum)
Baked Goods	5%
Rice and Other Grain-Based Dishes	5%
Vegetables and Vegetable Dishes	2%
Fruits and Fruit Preparations	2%
Milk Desserts/Cheese Products	3%
Meat, Poultry and Fish	0.2%
Eggs and Egg Dishes	0%
Legumes and Legume Dishes	0%
Sugars and Sweets	25%
Coffee	2%
Fruit Drinks and Ades	2%

1. Major food categories are based on those used in USDA's *Continuing* Survey of Food Intake by Individuals with rice and other grain-based dishes shown as a separate category [Enns, *et al.*, 1997 Vol 7 Tab 78].
2. Maximum use levels are those which have been formulated into test applications, or have been prepared for commercial test markets.

D. D-ribose Technical Effects in Food-Sample Formulations

Bioenergy has provided sample formulations for the use of its D-ribose food ingredient [Section B]. The sample formulations utilized between 0.2% and 25.0% D-ribose on a total weight basis. Lower levels were used to enhance the flavor and color (0.44% to 2.04%) of meat products, and higher levels (25.0%) were used in syrups to provide both sweetness and viscosity. Other uses for the product were in yeast bread to lower sweetness, in energy bars to maintain product shape and integrity, and act as a supplement, in coffee and tea to adjust sweetness and mask bitterness, and in hard candy to provide bulk and texture. Use levels of D-ribose as a percentage of the total formulation were 5% in the yeast bread, 2% in the coffee and tea formulations, 11.11% in hard candies, 0.44% in stew, 2.04% in beef gravy, 7.14% in cereals, and 4% in energy bars. The product was also used at a 2% level to alter the sweetness of chocolate milk. These sample formulations are provided to show the range of technical effects provided by the D-ribose product, and the contribution of D-ribose as a percent of the weight of the total formulation. The technical effects of D-ribose in foods are summarized in Tables 4 and 5.

Table 4: Technical Effect of D-Ribose

Product	D-ribose Use Level (%)	Technical Effect of D-ribose
Yeast Bread	4.98%	Reduce sweetness
Energy Bar	3.86%	Maintain product shape and integrity, Supplement the diet
Low-cal Fruit Drink	1.03%	Mask bitterness
Flavored Coffee	1.98%	Mask bitterness, Sweeten
Caramels	5.56%	Texturizer, Sweetener
Stew	0.44%	Flavor Enhancer Increase Browning
Ice Cream	2.9%	Reduce Sweetness

Color, texture, and/or flavor are all characteristics in which sugar plays some role in most foods. The Flavor and Extract Manufacturers Association of the United States (FEMA) has considered D-ribose generally recognized as safe (GRAS) as a flavor enhancer in certain foods. Those foods and the level considered GRAS is shown in Table 5 [Smiseth, *et al.*, 1983 Vol 7 Tab 81].

Table 5

FEMA GRAS USES for D-Ribose (No. 3793)

Food	Use Level (ppm)
Baked Foods	200 - 1,000
Meat Products	600 - 1,000
Soups	500 - 1,000
Snack Foods	200 - 1,000

E. Exposure

A basic estimate of exposure to D-ribose was calculated based on the data reported in the 1994-96 Continuing Survey of Food Intakes by Individuals [Enns, *et al.*, 1997 Vol 7 Tab 78]. Data covering the intakes of men and women 20 years and over was averaged across the major food categories in which D-ribose is expected to be used. The maximum use level expected for D-ribose in those categories, the average food intake for the category in grams per day, and the estimated D-ribose intake from each of the food categories is shown in Table 6.

Section III. Technical Effects, Intended Uses, Consumption Estimates, and Information on Self-Limiting Levels

Table 6
Estimated Intake of D-Ribose in Selected Food Categories

Major Food Codes	Food Category	Use level (%)	Food Intake (g)	D-Ribose Intake (g)	CSFII
	Baked goods, baking mixes				
51	Yeast Breads and Rolls	5.0%	50	2.5	1996
52, 55	Quick breads, pancakes, etc.	5.0%	20	1	1996
53	*Cakes, cookies, pastries, pies	5.0%	38	1.9	1996
58	Mixtures mainly grain		107		1996
	Breakfast cereals				
571 - 574, 578	Ready-to-eat cereals	7.1%	17	1.2	1996
	Grain products and pastas				
562	Rice	0.0%	19		1996
561	Pasta	0.0%	21		1996
54	Snack foods(crackers, chips)	0.5%	12	0.6	1996
	Total Vegetables		132		1996
	Fresh vegetables	0.0%	0		1996
	Processed vegetables, juices	2.0%	99	1.98	1996
	Total Fruits		162		1996
612	Citrus juices	2.0%	59	1.18	1996
621	Dried fruits		1		1996
641,642	Non-citrus juices and nectars	2.0%	26	0.52	1996
	Fruits and mixtures		76		1996
	Milk products				
114	Yogurt	1.8%	8	0.144	1996
131,132, 133, 134	Milk desserts	3.0%	24	0.72	1996
140-147	Cheese		16		1996
116, 118	Milk-Based Beverages	1.8%	34	0.612	1966
	Meat Products				
252	Sausages, processed meats	0.2%	21	0.042	1996
271,272,273,274	Mixtures mainly meat	0.2%	99	0.198	1996
	Eggs				
321,323,324, 33,34	Egg Products	0.0%	18		1996
	Legumes				
	Legumes	0.0%	28		1996
	Nuts and Nut Products				
	Nuts and Nut Products	0.0%	3		1996
	Fats and Oils				
832	Salad dressings	0.0%	8		1996
	Total sugars and sweets				
917,918	Candy (Soft and Hard)	8.3%	7	0.58	1996
911 - 916	Other Sugar Products**	15.3%	15	2.3	1996
	Beverages Non-Alcoholic				
	Coffee	2.0%	254	5.1	1996
923	Teas	2.0%	128	2.56	1996
925	Low Cal.Fruit drinks and ades	2.0%	18	0.36	1996
92400100	Low Cal. Carbonated drinks	2.0%	74	1.48	1996
	Total Intake (grams)		**1594**	**24.98**	
	Dietary Supplement			**20**	
	* Includes dietetic bars				
	** Includes Jams, Jellies, etc.				

The maximum use level was determined from food applications generated by Bioenergy, Inc., and its associates. The Office of Premarket Approval, FDA has provided a guidance document for estimating exposure to direct food additives. In this document the agency has indicated that a number of conservative assumptions are initially used to determine exposure. These are:

1. That a direct additive will be used in the broadest possible food groups that can be considered appropriate for the ingredient;

2. That the maximum possible concentration in the food category is presented;

3. That the direct additive will be contained in all foods within a category.

It is also suggested in the guidance document that a crude estimate of the 90th percentile exposure can be made by multiplying the mean by a factor of 2. The estimated mean and 90th percentile exposure levels for Bioenergy D-ribose, given the conservative assumptions listed above, are 24.98 grams and 49.96 grams, respectively. The use of Bioenergy D-ribose as a dietary supplement with a recommended dosage of 20 grams per day has also been noted in Table 6.

Using the intakes reported for adults, and averaged for the sexes, the mean intake of D-ribose per day was estimated to be 24.98 grams per day. The 90th percentile exposure level was then calculated based on doubling the intake levels in Table 4. The total shown in Table 6 suggests that the 90th percentile exposure level is 49.96 grams per day. Using these data would suggest that D-ribose would constitute about 5% of the total amount of solid food eaten (812 grams average). The agency advises that when the use of these conservatisms leads to an estimated daily intake (EDI) that is very high, the petitioner can submit additional data and information to allow for refinement of the EDI.

F. Self-Limiting Levels and Other Limiting Factors

The Sponsor asserts that the use of Bioenergy, Inc. D-Ribose will be self-limiting on the basis of the nature of the product itself. Bioenergy, Inc. submits that D-ribose consumption will not reach the levels suggested by the conservative assumptions shown above. Several logical reasons can be suggested for this assumption:

1. Bioenergy, Inc. is the only supplier of D-ribose as a direct food additive in the United States.

2. In the U.S., the total consumption of all sugars in the diet, exclusive of lactose, is estimated to be 80 grams/day, with the 90th percentile being 139 grams/day, according to the FDA [Glinsman, *et al.,* 1986 Vol 7, Tab 79]. From these data it was shown that of the 80 grams/day, approximately 53 grams/day represented all sugars (except lactose) that are added to the diet. Therefore, D-ribose could only be used in a maximum mean amount of 53 grams/day (104 grams/day for 90th percentile) if it was substituted for all added sugar in the diet. If it was used to substitute for 50% of all sugars added to the diet, this would constitute 26.5 grams/day, with the 90th percentile being 52 grams/day. However, the use of even this amount of D-ribose seems highly unlikely for the following reasons.

3. The calculation for the theoretical use of D-ribose equaled 24.98 grams/day, Table 6, which correlates to approximately 25% of all sugars added to the diet [80 grams/day]. It does not seem likely that a product with D-ribose's profile could be used at an amount that equals that of all sugars consumed.

4. D-ribose is relatively more expensive than the other carbohydrate ingredients for which it might be substituted. It is well known that the food industry in the US is one of the most cost sensitive sectors of the economy. Therefore, unless the

technical merits of D-ribose are sufficient to offset the increased cost there is no advantage in its use.

5. Bioenergy D-ribose is not expected to have technical advantages that would result in the replacement of all added sugars. Many of the functional applications are similar or marginally advantageous to other more common, less costly sugars. Additionally, the functional properties of D-ribose may not allow for the total substitution of a common sweetener with D-ribose. For example, D-ribose could be used in place of sucrose to reduce the sweetness in a baked product. Since D-ribose caramelizes extensively, its use in a baked product where limited browning is desired, would restrict the amount of its use. In this case (where browning must be controlled), sucrose or maltose would be better product choices. Several other examples can be provided where the use of D-ribose may be restricted by mutually exclusive properties in specific applications.

6. D-ribose has no advantage of being less caloric than sucrose. Further, because of its reduced sweetness compared to sucrose, high fructose corn syrup and glucose, use of D-ribose for other technical properties may necessitate the addition of a high intensity sweetener, which would add both to the technical complexity of product development and cost.

Given these factors, Bioenergy feels it is reasonable to assume that the daily exposure level for D-ribose would be substantially less than the consumption of 25% of all sugars added to the diet, as reported by the FDA [Glinsman, *et al.,* 1986 Vol 7, Tab 79]. However, to provide a conservative estimate, if D-ribose was substituted for 25% of all sugars added to the diet, consumption would equal 20 grams/day, with a 90th percentile of 40 grams/day. It is felt that these levels would still represent the maximum consumption that could be expected from even the most brand loyal consumer.

Bioenergy, Inc. submits that there is a sufficient margin of safety to support this very conservative maximum consumption level. All feeding studies conducted in rodents and in humans have demonstrated a no-adverse-effect-level (NOAEL) in gram quantities of D-ribose per kilogram per day. Additionally, there have been several reports in which 60 grams of D-ribose dissolved in water; has been supplied to subjects. In none of these instances was it reported that the patient experienced adverse reactions. These data are discussed in detail in Section IV, Safety.

Section IV

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Section IV: Basis for the Determination that Bioenergy, Inc. D-Ribose Is Safe when Consumed as an Ingredient in Processed Foods

Introduction

This section contains a comprehensive discussion of generally available scientific data, and information that the Notifier has relied upon to reach its conclusion that Bioenergy, Inc. D-Ribose is safe, when used under current Good Manufacturing Practice as a direct additive to food. Citations to the published scientific literature appear in brackets, followed by a reference to a volume and tab number. The volume and tab numbers appeared in a GRAS Report, that was prepared by the Notifier for the review of an Expert Panel. A complete listing of each citation can be found in Appendix II. The safety of Bioenergy, Inc. D-Ribose was considered under scientific procedures.

A. Description of D-Ribose

A balanced review of the published literature and unpublished information contained in this document concerning D-ribose (α-D-ribofuranose), supports the conclusion that it is safe to be consumed by humans. The basic absorption and metabolism of D-ribose is such that it is easily assimilated into the body and all the molecules associated with the process are common to other dietary monosaccharides. In addition to the basic chemistry and metabolism of D-ribose, several pre-clinical studies have been preformed to examine the safety of D-ribose ingestion in various species (see below). This section also includes several published studies in which humans were given relatively large doses of D-ribose. None of the animal or human studies reviewed suggested any significant or consistent untoward effects attributable to D-ribose.

Because of the relatively high cost to produce D-ribose it has not been historically used as a food or food ingredient. Knowledge of ancient diets suggests that the amount of D-ribose consumed by our ancestors was much greater than at present, mainly because of a greater consumption of organ meats, known to contain relatively high levels of D-ribose [Diamond, 1992 Vol 8 Tab 102 and Eaton, *et al.*, 1988 Vol 8 Tab 103]. However, the

presence in the human intestinal system of a known mechanism for the absorption and metabolism of D-ribose strongly suggests that humans have the capacity to safely consume D-ribose at much greater concentrations [Hiatt, 1958 Vol 8 Tab 104]. This, together with several recent animal toxicology studies, and reports of high levels of human consumption, convincingly support the position that the consumption of D-ribose as a macronutrient would pose no risk to the consumer.

D-ribose (α-D-ribofuranose) is a naturally occurring 5-carbon sugar that is associated with all living organisms. It is used by the human body to synthesize nucleotides, nucleosides, nucleic acids, glycogen, and other important metabolic products [Burke, 1999 Vol 8 Tab 98]. Ribose is also the starting point for the production of adenosine triphosphate (ATP), the molecule used by the human body to supply [Lehninger, 1982 Vol 8 Tab 94] Thus D-ribose is vital for the formation of most living cells' genetic material, and for the proper functioning of the energy cycle.

B. Natural Sources of D-Ribose

D-ribose occurs in nearly every living organism. While it is formed in the body from glucose, it does not occur in the free form in most foods. Humans are most likely to encounter D-ribose in red meats or organ meats. Therefore, it has been a continuous part of the human diet in relatively small quantities. Over the last 50 years scientists around the world have experimented with D-ribose as a supplement to the diet to provide a substrate for the energy cycle of the cell, and to assist in the recovery of skeletal muscle following exertion.

C. Nutritional Analysis of Bioenergy D-ribose

1. Summary

The D-Ribose to which consumers will be exposed was analyzed for its nutritional components by Bioenergy, Inc. A Nutritional analysis of 7 lots of Bioenergy's D-ribose showed a minimum carbohydrate content of 99% (Table 1). [Bioenergy Raw Data, 1999-2000 Vol 7 Tab 80]

Total calories conformed to the range of 4.5 - 5.0 kcal/g or 450 – 500 kcal/100g [Bioenergy Raw Data, 1999-2000 Vol 7 Tab 80]. Cholesterol, total fat, standard fat, calories from fat, dietary fiber, protein, sugars, vitamin a and vitamin c were non-detectable. Iron, calcium and sodium were present at levels less than 10 ppm maximum.

Table 1
Test Results on Nutritional Components Per 100 g of D-ribose (7 lots)[1]

Variables	Lot No.	Lot No.	Lot No.	Lot No.	Lot No.	Lot No.	Lot No.	Mean
Lot Number	9912016P	9912020P	9912022P	9912024P	9912025P	9912026P	9912028P	
Total Calories (kcal/100 g)	450 – 500	450 – 500	450 – 500	450 – 500	450 – 500	450 – 500	450 - 500	475
Total Carbohydrates	99% min	99% min	99% min	99% min	99% min	99% min	99% min	99% min
Cholesterol	ND	ND	ND	ND	ND	ND	ND	ND
Total Fat	ND	ND	ND	ND	ND	ND	ND	ND
Standard Fat	ND	ND	ND	ND	ND	ND	ND	ND
Calories from Fat	ND	ND	ND	ND	ND	ND	ND	ND
Dietary Fiber	ND	ND	ND	ND	ND	ND	ND	ND
Protein	ND	ND	ND	ND	ND	ND	ND	ND
Sugars	ND	ND	ND	ND	ND	ND	ND	ND
Iron	10 ppm max	10 ppm max	10 ppm max	10 ppm max	10 ppm max	10 ppm max	10 ppm max	10 ppm max
Calcium	10 ppm max	10 ppm max	10 ppm max	10 ppm max	10 ppm max	10 ppm max	10 ppm max	10 ppm max
Sodium	10 ppm max	10 ppm max	10 ppm max	10 ppm max	10 ppm max	10 ppm max	10 ppm max	10 ppm max
Vitamin A	ND	ND	ND	ND	ND	ND	ND	ND
Vitamin C	ND	ND	ND	ND	ND	ND	ND	ND

ND = Not Detected
[1]Analyses were conducted by AIDP, Inc., 2570 Corporate Place, #E102, Monterey Park, California, USA.

2. Methods for Nutritional Analyses

Test methods for the various nutritional variables were the methods published in the United States Pharmacopoeia 20th Edition, or the European Pharmacopoeia, 3rd Edition, 1997. Other methods have been published by the American Association of Cereal Chemists (AACC), or the American Organization of Analytical Chemists (AOAC).

D. Biochemistry

Beginning in 1942 published papers began to appear that reported on the biochemical role of D-ribose in humans and other mammals. The research covering the biochemical role of D-ribose has continued to the present day.

Y. Naitô published one of the earliest biochemical studies in 1942. This paper discussed the mechanism of absorption of D-ribose from the digestive tract [Naitô, 1942 Vol 5 Tab 38]. The author explained that D-ribose is one of the important constituents of ribonucleic acid, as well as of various co-enzymes, giving this pentose a special biochemical significance. However, at the time, the knowledge on the metabolism of this sugar was still very limited especially when compared with the information available on the chemical properties of the sugar. The author cited earlier studies in which Hamburger (1929) observed that a portion of the ribose that perfused through the frog kidney was retained by the tissue; Raymond and Blanco (1928), and Dickens (1938) found that ribose was not fermented by living yeast. Ribose-5-phosphoric acid was found by Dickens to be fermentable by the Lebedew extract, one molecule of the substrate producing one molecule of ethyl alcohol, carbon dioxide and phosphoric acid, respectively, along with a substance of unknown nature [Naitô, 1942 Vol 5 Tab 38].

The increasing interest in the metabolism of ribose prompted further investigation, and Naitô published the results of his work in a very detailed paper [Naitô, 1942 Vol 5 Tab 38]. The D-Ribose employed in the present experiment was synthesized from gluconic acid. In the first stage of the synthesis, calcium gluconate was converted into D-arabinose by the Hockett

and Hudson method (1934). The yield was 100gm of D-arabinose from 350 gm of calcium gluconate dihydrate. D-Arabinose melted at 155-156°C. Arabonate was epimelized to ribonate and the acid was then converted into cadmium salt. It was found advisable to put the salt through a rigorous process of purification, so as to perform the following operations more easily, and to increase the yield of the products. The yield was 24 gm of pure cadmium ribonate from 100 gm. of calcium arabonate. Crystalline lactone of ribonic acid was also used. The yield was 12gm. of syrupy ribose, or 8 gr. of ribose from 12 gm of lactone [Naitô, 1942 Vol 5 Tab 38].

1. Physical Properties Of D-Ribose

Naitô, 1942 reviewed the physical properties published for the sugar:

- Levene and Jacobs (1909): Freezing Point (FP)= 86-87°C (corrected)
- vanEkenstein and Blanksama (1913): FP= 95°C
- Phelps *et al.* (1934): FP= 87°C; Optical Rotation= $[\alpha]^{18}_{D}$ -21.4° (H20, 2.57% 2 dm)
- Levene and Jacobs: Optical Rotation= $[\alpha]^{18}_{D}$ -19.5° (H_2O, 4%)
- van Ekenstein and Blanksama: Optical Rotation= $[\alpha]^{18}_{D}$ -21.4°
- Kuhn *et al* (1935): Optical Rotation= $[\alpha]^{22}_{D}$ -22.5° (H_2O, 0.9%)

  [Naitô, 1942 Vol 5 Tab 38].

2. Biochemical Studies

Naitô, 1942 reported that ribose was more sensitive to alkaline conditions than other pentoses, as seen in Table 2.

Table 2
Decrease of Reducing Power[1] of Pentoses in Alkaline Medium
5 cc of 1% pentose solution + 5 cc of n NaOH; 20° C

Time of incubation (Hrs.)	Decrease Of Reducing Power (%)			
	D-Ribose	L-Arabinose	D- Arabinose	D-Xylose
3	5.6	4.1	1.8	4.6
6	11.6	8.1	5.4	8.4
9	17.9	10.5	7.0	12.6

[1]Determination of reducing power: Somogyi's Method.

Table 3 shows the conversion of pentoses into furfural, and the yield of furfural from the various pentoses. The almost quantitative production of furfural was achieved after one and one half hours' distillation, while the conversion over the same period was about 90% for xylose, and about 50% for D- and L-arabinose. Ribose in purine nucleosides and purine nucleotide of yeast origin was found by Hoffman and Ishikawa (1935) to be also quantitatively determinable by the furfural method, though the rate of the production of furfural conversion was more or less retarded by the presence of purines and phosphoric acid combined with the sugar. Younburg (1927), and Andrews and Milroy (1933) were unable to carry out the 100% conversion of free and combined ribose into furfural. The author speculated that this might have been due to the incomplete formation of furfural from ribose under different conditions from that of the Hoffman method.

TABLE 3

Production of Furfural from 10 mg of -Pentose (Hoffman's method)

Sugar		Time of distillation (min.)					
		15	30	60	90	120	180
D-ribose	mg	2.93	4.92	6.18	6.30		6.28
	%	45.7	76.9	96.7	98.4		98.0
D-xylose	mg	-	4.21	5.40		5.99	6.36
	%		65.8	84.4		93.6	99.4
D-arabinose	mg			2.82		3.78	4.24
	%			44.1		59.1	66.3
L-Arabinose	mg			2.71		3.76	4.19
	%			42.3		58.7	65.5

Naitô, 1942, also presented data showing the fate of D-ribose in the digestive tract after oral administration and following intravenous injection [Naitô, 1942 Vol 5 Tab 38]. The influence of the intravenous injection and the oral administration of ribose on the blood sugar in rabbits that had been starved for 24 hours was examined. Tables 4 and 5 summarize the protocol that reported the appearance of reducing substances in blood [Naitô, 1942 Vol 5 Tab 38]. The values of non-fermentable sugar, which increased after the introduction of ribose, fell to a normal level more rapidly than those of a fermentable sugar, such as glucose [Naitô, 1942 Vol 5 Tab 38].

TABLE 4
Intravenous Injection of D-Ribose to Rabbit
250mg/ kg Rabbit: 2450 gm.

Time	Reducing Substance In Blood		
	Total (mg%)	Fermentable (mg%)	Non-Fermentable (mg%)
Before	88	82	6
After 10 min.	113	92	21
30	123	105	18
1 hr	130	120	10
2 hr	107	98	9
3 hr	95	88	7
4 hr	90	84	6
5 hr	88	83	5
7 hr	88	83	5

Extra-excretion of non-fermentable reducing substance in urine during 7 hours: 48 mg glucose equivalent.

TABLE 5
Oral Administration of D-Ribose to Rabbit
D-ribose: 2.0 gm/per kg.
Rabbit: 2,300 gm ♂

Time	Reducing Substance in Blood		
	Total (mg%)	Fermentable (mg%)	Non-Fermentable (mg%)
Before	92	88	4
After 10 min.	119	106	13
30 min.	125	114	11
1 hr.	136	129	7
2 hr.	124	119	5
4 hr.	105	100	5
6 hr.	96	91	5
8hr.	90	86	4

Extra-excretion of non-fermentable reducing substance in urine during: 8 hrs: 5 mg glucose equivalent.

3. Biological Properties of D-Ribose

The excretion of sugars was examined by determining the increase of the non-fermentable reducing power of the total urine, which was collected by combining the portion excreted during the experimental period with the portion catheterized from the bladder at the end of the period. The minimum quantity of ribose causing pentosuria after an intravenous injection into the marginal veins of rabbits that had been starved for 24 hours was 250 mg per kilogram of body weight. The oral administration of 2 gm of ribose per kilogram of body weight via stomach tube did not produce definite pentosuria. The tolerance for other pentoses was much lower than that of ribose; less than 50 mg per kilogram of body weight with an intravenous injection of D-xylose and D-arabinose, and less than 0.5 gm in the case of an oral administration. The far higher tolerance of ribose suggests a greater utilization of this sugar than other pentoses in the mammalian body [Naitô, 1942 Vol 5 Tab 38].

In another study the absorption of ribose from the intestinal tract of the rat was studied. Rats weighing 100 – 150 grams received 2 cc of a 25% sugar solution after having been starved for 48 hours. The sugar was administered via stomach tube. The amount of sugar absorbed was calculated from the difference between the quantity of the sugar given and that remaining in the stomach and the entire small intestine. The average reducing substance present in the stomach and the small intestine of fasting rats was 0.80 mg and 3.78 mg, respectively, based on glucose equivalents (n = 5 experiments) [Naitô, 1942 Vol 5 Tab 38]. The results as shown in Table 6 indicated that D-ribose was absorbed at a much higher rate in comparison to other pentoses, and that absorption exceeded that of fructose [Naitô, 1942 Vol 5 Tab 38].

TABLE 6
Absorption of D-Ribose from Intestinal Tract of Rat

Period of absorption (hr)	Sugar	Body weight (gm)	Amount of Sugar Absorbed (gm)	Absorption Coefficient	Mean	Relative rate of absorption (glucose = 100)
1	D-Ribose	136	0.205	0.151		
		128	0.192	0.150		
		123	0.156	0.153	0.143	74
		119	0.159	0.127		
		132	0.201	0.134		
	D-Glucose	136	0.265	0.195		
		102	0.204	0.200		
		127	0.234	0.184	0.193	100
		114	0.201	0.176		
		135	0.284	0.210		
	D-Xylose	119	0.081	0.068		29
		138	0.063	0.046		
		147	0.079	0.054	0.056	
		126	0.062	0.049		
		133	0.084	0.063		
2	D-Ribose	136	0.205	0.151		67
		128	0.192	0.150		
		123	0.156	0.153	0.114	
		119	0.159	0.127		
		132	0.201	0.134		
	D-Glucose	136	0.265	0.195		100
		102	0.204	0.200		
		127	0.234	0.184	0.171	
		114	0.201	0.176		
		135	0.284	0.210		
	D-Xylose	119	0.081	0.068		15
		138	0.063	0.046		
		147	0.079	0.054	0.025	
		126	0.062	0.049		
		133	0.084	0.063		

The author also indicated that the possible formation of glycogen from natural pentoses had often been discussed, but other workers in the field had been unable to demonstrate it. In this study isolated livers of rabbits were perfused with 60 ml of physiological saline containing one of the sugars (at 0.2% concentration) shown in Table 7. The results of Table 7 depict the first published evidence that ribose can be converted to glycogen [Naitô, 1942 Vol 5 Tab 38].

Table 7
Conversion of Sugars to Glycogen

Sugar	Wt. Of Liver (gms)	Liver glycogen (mg%)			
		Before Perfusion	After Perfusion	Increase	
D-Glucose	75	113	204	91	Avg. 64
	63	124	152	28	
	40	175	226	51	
	61	104	161	57	
	50	143	236	93	
D-Ribose	30	113	131	18	Avg. 22
	43	105	133	28	
	36	131	154	23	
	40	120	144	24	
	52	107	125	16	
D-Xylose	55	124	117	-7	Avg.. -2
	50	111	116	5	
	47	140	138	-2	
	42	105	103	-2	
	38	126	125	-1	

Ben Bloom *et al.*, from the Division of Nutrition and Physiology, The Public Health Research Institute of The City of New York, Inc., and the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland studied glucose catabolism in liver slices via the phosphogluconate oxidation pathway. Earlier experiments involved liver slices that were incubated with glucose, gluconate, or lactate, labeled in specific positions with C^{14}. The recoveries of isotope in CO_2 and in fatty acids had been measured, and on the basis of these measurements it was concluded that a pathway other than the Embden-Meyerhof route was operating in liver tissue. It has further been estimated that approximately half of the glucose catabolized by rat liver slices enters the Embden-Meyerhof pathway, whereas the remaining half is degraded by steps which resemble those of the phosphogluconate oxidation pathway [Bloom, *et al.*, 1954 Vol 2 Tab 5].

In a 1954 study, in addition to CO_2 and fatty acids, glycogen was recovered from liver slices after incubation with labeled substrates. The glucose derived from this glycogen was subjected to chemical degradation to determine the quantity of isotope in certain positions (Table 8). On the basis of the known reactions, which together comprise the phosphogluconate oxidation pathway, certain predictions relating to the redistribution of carbon atoms within the sugar molecule were made. [Bloom, *et al.*, 1954 Vol 2 Tab 5].

At the suggestion of Dr. B. Horecker, the name phosphogluconate oxidation pathway, was given to a pathway that had variously been termed the hexose monophosphate shunt, the Warburg-DickensLipmann pathway and the direct oxidative route. The authors explained that the terms glycolysis, glycolytic, glycolyzed, etc., had been used in reports from their laboratory, as well as by others, to designate the Embden-Meyerhof pathway, and that such usage was incorrect. In this report, the term Embden-Meyerhof was adhered to for designation of that pathway by which 1 mole of glucose, via glucose-6-phosphate, fructose-6-phosphate, fructose-1-6-diphosphate, etc., can give rise to 2 moles of lactate [Bloom, *et al.*, 1954 Vol 2 Tab 5].

Liver slices were incubated with 4.5 ml. of buffer containing 50 μmoles of glucose or gluconate. Radiochemical yields were calculated per 500 mg of

tissue for a 2 hour incubation period. Each flask actually contained from 998 to 1010 mg of tissue and from 1 X 10^4 to 2 X 10^4 cpm of substrate C^{14}. The values recorded below were normalized to 2 X 10^4 cpm per flask. The authors pooled three experiments for all determinations except the CO_2 values, which were the averages of the three experiments [Bloom, *et al.*, 1954 Vol 2 Tab 5].

TABLE 8
Incorporation of Isotope into Glycogen, Fatty Acids, and CO_2

Substrate	Per cent radio-chemical yield		Specific activity* of glycogen carbons			
	CO_2	Fatty acid	1-6 (a)	1 (b)	6 (c)	2-5 ** (d)
Glucose-1-C^{14}	4.74	0.23	1750	7400	215	720
Glucose-2-C^{14}	1.53	0.31	2350	1080	55	3240
Glucose-6-C^{14}	1.46	0.69	2220	94	11,110	530
Gluconate-1-C^{14}	2.64	0	0	-	-	-
Gluconate-6-C^{14}	0.28	0.19	65	57	242	23

* Counts per minute per milliatom of carbon.
** Calculated from the expression d = (6a - b - c)/4.

The distribution of carbon atoms of gluconate in glycogen supported the author's earlier assumption that gluconic acid as it is catabolized, enters the phosphogluconate oxidation pathway, after phosphorylation at position 6 [Bloom, *et al.*, 1954 Vol 2 Tab 5].

The recoveries of C^{14} in CO_2 and in fatty acids (Table 8) after incubation of rat liver slices with glucose-1-C^{14}, glucose-2-C^{14}, glucose-6-C^{14}, gluconate-1-C^{14}, and gluconate-6-C^{14} were in good agreement with values secured earlier in comparable experiments. Probably as a consequence of the direct incorporation of glucose from the medium into glycogen, the specific activity of glycogen is relatively independent to the location of the labeling in glucose. This is borne out by finding the bulk of the isotope in carbon 1 of glycogen-glucose after incubation of tissue with glucose-1-C^{14}; in carbon 6 after incubation with glucose 6-C^{14}. The investigators found that carbon 1 of nutrient glucose made a small contribution to position 6 of glycogen, and likewise carbon 6 of glucose of the medium made a small contribution to position 1. Such randomization between positions 1 and 6 of glucose, apparently of limited extent under their experimental conditions, were thought to result from the reversible operation of the Embden-Meyerhof pathway down to the level of the triosephosphate isomerase reaction. This limited extent of randomization between carbons 1 and 6 of glucose has been observed in rats yeast, and perfused mammary gland [Bloom, *et al.*, 1954 Vol 2 Tab 5].

Incubation of liver slices with glucose-2-C^{14} yielded glycogen which contained about 20 times as much C^{14} in position 1 as in position 6 (Table 8).

4. Phosphogluconate Oxidation Pathway

In contrast to carbon 1, carbon 6 of gluconate was found to be readily recoverable in glycogen. These results could not have been due to the direct reduction of gluconate, since this possibility had been ruled out in the experiment with gluconate-1-C^{14}. The authors indicated a mechanism by which gluconate 6-C^{14} could give rise to glucose labeled in position 6. Another possibility the investigators suggested involved the transfer of "active" glycolaldehyde to tetrose-4-phosphate-4-C^{14} under the influence of transketolase. Labeling of carbon 1 of glucose after incubation with gluconate-6-C^{14} might result from a randomization incident involving the reversal of the Embden-Meyerhof sequence, commencing with triose-3-phosphate-3-C^{14} [Bloom, *et al.*, 1954 Vol 2 Tab 5].

Bloom *et al.*, presented the results of two experiments conducted with liver slices. It was the intent of the study to compare the incorporation of C^{14} initially present in position 2 of glucose and in position 1 of ribose into carbons 1 and 6 of glycogen-glucose. As in an earlier experiment, carbon 2 of glucose contributed to a far greater extent to position 1 than to position 6. Similarly, carbon 1 of ribose was recovered in far higher yields in position 1 than in position 6 of glucose. These findings were again in accord with expectations based upon the phosphogluconate oxidation pathway, in which carbon 1 of ribose, assuming a preliminary phosphorylation at position 5, is derived from carbon 2 of 6-phosphogluconate or glucose-6-phosphate [Bloom, *et al.*, 1954 Vol 2 Tab 5].

It has been suggested that ribose-5-phosphate is degraded by rat liver acetone powder over the steps of the phosphogluconate oxidation pathway. This might indicate, with a high degree of probability, that this same pathway was operating in the intact cells within the rat liver. The available evidence supported the contention that the alternative pathway, previously shown to account for about half of the glucose catabolized by liver slices, was the phosphogluconate oxidation pathway .

Rat liver slices have been incubated with glucose-1-C^{14}, glucose-2-C^{14}, glucose-6-C^{14}, gluconate-1-C^{14}, gluconate-6-C^{14}, and ribose-1-C^{14}. The radioactivity of CO_2, fatty acids, and glycogen was determined, and glycogen samples were degraded to permit separate measurement of isotope in positions 1 and 6 of glucose.

Glucose-1-C^{14} yielded glycogen labeled predominantly in position 1, whereas glucose-6-C^{14} gave glycogen containing most of its isotope in position 6. Glucose-2-C^{14}, as well as ribose-1-C^{14}, yielded glycogen far more heavily labeled in position 1 of glucose than in position 6. Gluconate-1-C^{14} failed to introduce detectable amounts of isotope into glycogen, whereas gluconate-6-C^{14} resulted in glycogen labeled predominantly in position 6. All of these findings were in accord with predictions based on the occurrence of the phosphogluconate oxidation pathway of glucose utilization in liver slices [Bloom *et al.*, 1954 Vol 2 Tab 5].

5. Glycogen Formation via the Pentose Phosphate Pathway

In 1957 Hiatt described glycogen formation via the pentose phosphate pathway in mice. The author concluded that D-ribose appeared to be as efficient a precursor of mouse liver glycogen as glucose under the conditions of his study. The proportion of administered hexose and pentose carbon converted to glycogen was comparable to that found by Cook and Lorber in the rat. The conversion of D-xylose carbon to liver glycogen was of considerable interest, for the preponderance of reported studies with non-isotopic xylose suggested that it was not glycogenic. D-xylose, however, was poorly assimilated, with approximately 10 per cent as much of the isotope from xylose-C^{14} appearing in liver glycogen as was the case with D-glucose and D-ribose. This very small transformation of xylose to glycogen may explain the apparently negative results with unlabeled sugar [Hiatt, 1957 Vol 3 Tab 28].

The presence of the isotope predominantly in C-1 and C-3 of liver glycogen of animals given ribose-1-C^{14} is consistent with transformation of the pentose to hexose via the transketolase and transaldolase reactions of the pentose phosphate pathway. Horecker *et al.* suggested that 3 molecules of ribose-1-C^{14} would be converted by these reactions to a molecule of unlabeled triose and to 2 molecules of glucose containing two thirds of the isotope in C-1 and one-third in C-3. Their results with rat liver enzymes were consistent with this scheme. In glucose formed by rat liver slices from ribose-1-C^{14}, however, Katz *et al.* found C-1 and C-3 to be labeled approximately equally. They interpreted their results as indicating that the tetrose formed after the transaldolase cleavage of sedoheptulose was not used as a glycolaldehyde acceptor. The initial studies of Hiatt in vivo with liver glycogen formed from ribose-1-C^{14} appeared to widen the discrepancy even further, in that more isotope was found in C-3 than in C-1 (Table 9). An explanation for the findings of Hiatt and Katz *et al.* may lie in a consideration of the difference in the conditions of the studies with purified enzymes as compared with those in vivo and with the observations with liver slices. In the experiments of Horecker *et al.*, the only hexose precursor present was the added pentose phosphate. In most of the Hiatt studies and in those of Katz *et al.*, on the other hand, the added pentose was present in small quantity as

compared with the amount of added or endogenous hexose present. It has been shown that fructose phosphate as well as ketopentose phosphate may be a glycolaldehyde donor in the transketolase reaction [Hiatt, 1957 Vol 3 Tab 28].

TABLE 9
Distribution of C^{14} in Mouse Liver Glycogen after Oral Administration of D-Ribose-1-C^{14}

Mouse No.	Percent Total Activity in Liver Glycogen Carbon Atoms					
	C-1	C-2	C-3	C-4	C-5	C-6
24	34.0	3.5	52.0	4.5	3.0	3.0
29	36.0	4.0	48.0	12 (sum C-4-C-6)[1]		
30	39.7	2.7	47.6	10 (sum C-4-C-6)[1]		
51	36.0	5.0	41.0	6.0	5.0	7.0

[1] Lactate oxidized to CO_2.

Coffey, *et al.*, reported on the synthesis of nucleic acid constituents in the early chick embryo in 1966 [Coffey *et al.*, 1965 Vol 2 Tab 8]. Earlier studies indicated that the pentose phosphate pathway of carbohydrate metabolism was very active in early sea urchin embryos', mammalian-fetal-heart tissues and the chick embryo. The authors stated that since the rate of synthesis of DNA and RNA decreases during development and the pentose phosphate pathway is related to the synthesis of the carbohydrate moiety of these compounds, it was of interest to examine these relations further. Interestingly, the studies supported the possibility of separate pools of RNA and DNA precursors in the early chick embryo. In particular, it was suggested that the deoxyribose of DNA may be formed in part by reactions different from the direct reduction of ribose.

The authors confirmed this finding and postulated that the acetaldehyde plus glyceraldehyde 3-phosphate mechanism may account for the ^{14}C distribution found in deoxyribose labeled by [^{14}C] glucose. Evidence was found to support the concept of separate pools of DNA and RNA precursors. Specific activities of deoxyribose samples derived from all substrates tested except

[1-^{14}C] ribose and [^{14}C] ribose were higher than corresponding samples of ribose [Coffey *et al.*, 1965 Vol 2 Tab 8].

The labeling of purines by radioactive ribose, pyruvate, and acetate was noticeably different in RNA than DNA. These differences were interpreted as due in part to different extents of dilution of radioactive nucleic acids by the unlabeled nucleic acids and nucleotides present in the growth medium [Coffey *et al.*, 1965 Vol 2 Tab 8].

Evidence was found for differences in the nucleic acid synthesizing mechanisms between the embryo area and the membrane area of the chick embryo explant. Many of these differences were associated with changes in metabolism from the one- to the two-day explants [Coffey *et al.*, 1965 Vol 2 Tab 8].

In the embryo of one-day explants, the specific activity of deoxyribose was greater than ribose when [^{14}C] glucose or [^{14}C] acetate was the substrate. The specific activities of ribose associated with pyrimidine bases were higher than those associated with purines, and ribose taken from puridine was labeled more heavily. The specific activities of pyrimidine bases were greater than those of purine bases, and bases were labeled by [2-^{14}C] glucose less, in general, than by [1-^{14}C] glucose or [6-^{14} C] glucose [Coffey *et al.*, 1965 Vol 2 Tab 8].

In the embryo of two-day explants, the specific activity of deoxyribose was similar to that of ribose when [^{14} C] glucose or [^{14}C] ribose was the substrate. In the presence of [^{14}C] pyruvate or [^{14}C] acetate, the specific activity of deoxyribose $^{14}CO_2$ is greater than that of ribose. The specific activities of DNA purines were greater than RNA purines. The specific activity of ribose nearly doubled over the one-day value, whereas that of deoxyribose increased only slightly in the presence of [^{14}C] glucose. In the presence of [1-^{14}C] acetate, the specific activity of both sugars increased in the two-day period [Coffey *et al.*, 1965 Vol 2 Tab 8].

In the extraembryonic area of one-day explants, the specific activity of deoxyribose was similar to that of ribose in the presence of [^{14}C] glucose (Table 10).

Table 10

Percent ^{14}C Found in Various Fractions From Labeled Substrates In Two-Day Chick Embryo Explants

Percent of original glucose added to medium (radiochemical basis)

Fraction	[1- C^{14}] Glucose		[2-^{14}C] Glucose		[6-C^{14}] Glucose	
	Embryo	Extra-Embryonic area	Embryo	Extra-Embryonic area	Embryo	Extra-Embryonic area
Trichloroacetic acid extract	0.69	0.85	0.62	0.90	0.60	0.82
Chloroform-methanol (2:1) extract	0.87	0.81	0.99	0.77	0.85	0.77
Protein	2.36	1.09	1.67	0.71	2.54	1.20
RNA	0.61	0.28	0.97	0.24	0.50	0.26
DNA	0.27	0.09	0.27	0.08	0.18	0.08
Total recovery from homogenate	4.83	3.21	4.38	2.93	4.65	3.22
Total in original homogenate (determined prior to fractionation)	4.94	3.37	4.59	3.71	4.15	3.13
$^{14}CO_2$ from whole explant	12.7		11.8		10.1	

Table 10 (Continued)

Percent ^{14}C Found in Various Fractions From Labeled Substrates In Two-Day Chick Embryo Explants

Percent of original glucose added to medium (radiochemical basis)

Fraction	[1-^{14}C] Ribose		[^{14}C] Ribose		[1-^{14}C] Pyruvate	
	Embryo	Extra-Embryonic area	Embryo	Extra-Embryonic area	Embryo	Extra-Embryonic area
Trichloroacetic acid extract	0.27	0.42	0.29	0.55	0.17	0.37
Chloroform-methanol (2:1) extract	0.13	0.14	0.14	0.23	0.11	0.25
Protein	0.40	0.20	0.46	0.20	0.36	0.30
RNA	0.40	0.45	0.43	0.37	0.11	0.06
DNA	0.09	0.06	0.10	0.06	0.02	0.01
Total recovery from homogenate	1.29	1.27	1.42	1.41	0.77	0.99
Total in original homogenate (determined prior to fractionation)	1.64	1.56	1.39	1.44	0.70	0.91
$^{14}CO_2$ from whole explant	1.12		2.16		16.0	

Ribose from puridine was again more heavily labeled than from other bases. Specific activities of pyrimidines were higher than purines, and were generally lower from [2-^{14}C] glucose than from [1-^{14}C] glucose and [6-^{14}C] glucose. In the presence of [1-^{14}C] acetate, the specific activity of deoxyribose was greater than that of ribose. In the extraembryonic area of two-day explants, the specific activity of deoxribose was slightly greater than that of ribose in the presence of [^{14}C] glucose, [^{14}C] pyruvate or [1-^{14}C] acetate. The specific activities of deoxyribose and ribose were similar to the one day values or slightly lower, whereas the bases were much less radioactive than the corresponding one day values. The bases of DNA were slightly more radioactive than those of RNA [Coffey *et al.*, 1965 Vol 2 Tab 8].

The incorporation of ^{14}C from [1-^{14}C] ribose and [^{14}C] ribose was greater in ribose than deoxyribose in both the embryo and the extraembryonic area. One of the interesting questions raised from the author's results concerned the possibility that the deoxyribose of DNA is synthesized in part by pathways other than the direct reduction of ribonucleotides. With all the substrates used except [^{14}C] ribose, deoxyribose samples had a higher specific activity than the corresponding ribose samples. The authors explained that the specific activity was calculated on the basis of the pentose actually synthesized during the time intervals studied. One possible explanation of the differences in RNA and DNA specific activities could involve different pools of nucleotides or polynucleotides [Coffey *et al.*, 1965 Vol 2 Tab 8].

The goal of research by Quraishi and Illsley, 1999, was to investigate movement of sugars across placental plasma membranes [Quraishi, *et al.*, 1999 Vol 5 Tab 47]. The investigators showed that D-ribose along with mannitol, fructose, and 2-deoxyribose were transported solely by lipid-diffusional processes and not by a protein carrier. According to the authors, sugar transport across placental membranes had been proposed by Reid and Boyd (1994), who obtained kinetic evidence for a fructose transporter. The transport of ribose has been observed in other organisms and tissues, but has not been investigated in the human placenta.

Studies of sugar transport require the capability to measure not only alterations in transporter expression, but also those in transporter fluxes, since changes in rates may not always be accompanied by complementary changes in transporter expression. This is especially true for epithelial cell membranes such as the placental microvillous and basal membranes that have been shown to have differing properties including the proportions of lipid components. Although the mRNA and protein can be measured for transporters such as the GLUT family, the need for an efficient, accurate and rapid method of assessing transporter kinetics has remained undeveloped. For example, in pregnancies complicated by maternal diabetes, there are significant changes in the expression of placental glucose transporters, but complementary measurements of glucose transport activity are also

necessary. Furthermore, while the ability of placental membranes to transport glucose and galactose had long been recognized, the question of whether other sugars can also be transported has yet to be answered [Quraishi, *et al.*, 1999 Vol 5 Tab 47].

Current techniques for measuring sugar transport utilizing radioactive uptake were said to be sub-optimal for several reasons. First, because carrier-mediated transport of sugars across placental membranes is extremely rapid, radioisotopic methods are limited in their time resolution. Second, the poor time resolution limits the temperature range which can be used, since transport above room temperature makes measurements using the uptake of radiolabel, an extremely rapid and difficult procedure requiring high levels of radioisotope. Third, the use of radioactive methods in measuring sugar uptake into membrane vesicles requires multiple washing and filtration steps which are time consuming and limit both the number and precision of those measurements. For these reasons the investigators developed an alternate method for measuring sugar fluxes, based upon the changes in light scattering by human placental membrane vesicles, that take place as a result of osmotically induced changes in volume. This method was originally used to examine the lipid diffusional movement of solutes across placental membranes [Quraishi, *et al.*, 1999 Vol 5 Tab 47].

Changes in vesicle volume produced by solute uptake were measured in this study by light scattering. Analysis, performed by fitting of the light scattering data to exponentials, revealed that for certain sugars such as glucose, a rapid component and a second, slower transport process were present. Measurements in the presence of the glucose transport inhibitor, phloretin, have been compared with the transport of mannitol. The analysis of the concentration dependence of the two transport components demonstrated that these two processes are consistent with protein-mediated and lipid-diffusional transport of glucose [Quraishi, *et al.*, 1999 Vol 5 Tab 47].

Calculation of glucose flux rates using the time constants which define these processes, provided values similar to those determined by radioisotopic methods. Glucose, 2-deoxyglucose and galactose were transported both by carrier-mediated and diffusional processes. The rate of glucose transport

across the syncytiotrophoblast basal membrane was slightly greater than that across the microvillous membrane, in contrast to that predicted previously by immunoblotting. In addition, measurements of hexose transmembrane diffusion showed that microvillous and basal transport rates were similar and lower than previously determined. The authors concluded that this new technique represented a simple and rapid method for investigating sugar transport across placental membranes [Quraishi, *et al.*, 1999 Vol 5 Tab 47].

6. Other sugars

In addition to glucose, the transport of a number of other sugars into MVM and BM was tested in a manner identical to that described for glucose, using a 40 mm inwardly directed gradient. The sugars tested were glucose, 2-deoxyglucose, galactose, mannitol, fructose, ribose and 2-deoxyribose. Exponential analysis revealed that while glucose, 2-deoxyglucose and galactose uptake curves were best fitted to double exponentials, the curves for mannitol, fructose, ribose and 2-deoxyribose were best fitted to a single exponential (Table 11). These data demonstrate that the latter group of sugars cross syncytial membranes solely by the lipid diffusional pathway, while the former have a carrier-mediated component. The flux rates for those substances transported only by lipid diffusion are consistently higher than the flux rates for the slow component of those substances, similar in molecular weight, which move both via transporter and by lipid diffusion. This was again probably due to the greater counter-flux of raffinose which develops over the longer-time course necessary for lipid diffusion, decreasing the osmotic gradient, shortening the transport process and generating an apparently faster flux rate [Quraishi, *et al.*, 1999 Vol 5 Tab 47].

Table 11

Exponential Analysis of Sugar Intake By Human Placental Membrane Vesicles

VesicleType	Sugar	n	J_s* (nmol/sec/mg)	F	D_s (nmol/sec/mg)
Microvillious	Glucose	3	13.7 ± 1.2	0.50 ± 0.03	2.1 ± 0.1
	2-Deoxyglucose	3	10.1 ± 0.5	0.43 ± 0.03	2.1 ± 0.1
	Galactose	3	15.2 ± 1.1	0.60 ± 0.03	1.5 ± 0.1
	Mannitol	3		1	3.7 ± 0.2
	Fructose	3		1	2.8 ± 0.3
	Ribose	3		1	3.1 ± 0.1
	2-Deoxyribose	3		1	6.1 ± 0.2
Basal	Glucose	5	15.2 ± 1.2	0.41 ± 0.01	2.2 ± 0.1
	2-Deoxyglucose	3	16.3 ± 1.8	0.42 ± 0.02	2.2 ± 0.1
	Galactose	5	17.1 ± 1.0	0.43 ± 0.01	2.3 ± 0.1
	Mannitol	5		1	5.6 ± 0.4
	Fructose	5		1	5.8 ± 0.4
	Ribose	5		1	6.2 ± 0.3
	2-Deoxyribose	3		1	7.1 ± 0.3

*J_s is transporter-mediated uptake; F is the fraction of transporter-mediated uptake [=A,/(A_1 + A_2)]; D_s is uptake occurring via simple lipid diffusion.

The results of the study by Quraishi, *et al.*, demonstrated that the light-scattering method can be used to examine sugar transport rapidly and accurately in human placental vesicles. The light-scattering technique resolves vesicular glucose uptake into two processes, which the authors hypothesized were representative of a protein-mediated transporter and a lipid-diffusional component. A 40 mm gradient was used rather than a more physiological one, since use of this gradient provided data on both the transporter-mediated and diffusional processes.

This hypothesis was confirmed by several pieces of evidence. First, the uptake of mannitol, which is not a substrate for the glucose transporter, was best represented by a single exponential with a time constant significantly greater than that of the fast process and much closer to that of the slow process (τ_2) for glucose. This suggests that the slow glucose component is due to trans-membrane lipid diffusion, while the faster component of the exponential (τ_1) represents a more rapid transport pathway. Second, when an inhibitor of glucose transport, phloretin, was added to the vesicles, the transport curve was reduced to a single exponential with a long time constant, demonstrating elimination of the fast process, as might be expected if this process was a glucose transporter.

Third, as predicted, the fraction of glucose uptake occurring via the fast process decreased with increasing gradient size. If the two transport processes had both been due to non-saturable diffusion pathways with differing permeabilities, the fractional uptake due to the fast process would be expected to be constant over the range of gradients. On the other hand, if the fast process was a transporter, then the fractional uptake would be expected to decrease as substrate concentration increased, approaching transporter saturation. It was concluded, therefore, that the two glucose uptake processes observed represent a glucose transporter and a lipid-diffusional component. The values of Jglc, calculated from the time constants and gradient concentrations are of a similar magnitude to those obtained from radioactive uptake studies using zero-trans-flux measurement techniques [Quraishi, *et al.*, 1999 Vol 5 Tab 47].

7. D-Ribose Metabolism in Man

The classic research on the metabolism of D-ribose in man was carried out by Stanton Segal and Joseph Foley from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md. The work was published in 1958 [Segal, *et al.*, 1958 Vol 5 Tab 50].

The authors stated that the pentose, D-ribose, in the form of the ester, ribose-5-phosphate, was previously thought to be primarily a structural component of nucleic acids, but had been shown to be an important intermediate in the pentose phosphate pathway of glucose metabolism. The sequence of the reactions of this pathway had previously been summarized by Horecker and Mehler and Wood, and surveys of its significance in various mammalian tissues have been reported [Segal, *et al.*, 1958 Vol 5 Tab 50].

They acknowledged the work of Naitô, discussed above, who investigated the glycogenic effect of ribose after its injection into mice and rabbits, and stated that Herrmann and Hickman had described the utilization of ribose in beef cornea. Horecker, Gibbs, Klenow, and Smyrniatis had demonstrated the conversion of ribose to hexose with rat liver homogenates and Katz, Abraham, Hill, and Chaikoff, incubating ribose 1-C^{14} with rat liver slices, observed the formation of glucose with a pattern of labeling that was predictable from the reactions of the pentose phosphate pathway [Segal, *et al.*, 1958 Vol 5 Tab 50].

The authors stated that similar findings had been reported by Hiatt, studying the *in vivo* conversion of labeled ribose to glucose in the mouse. Just prior to the authors' publication Agranoff and Brady had isolated and purified an enzyme, ribokinase, from calf liver which was capable of phosphorylating ribose to ribose-5-phosphate, thus permitting the sugar to enter the pentose phosphate pathway [Segal, *et al.*, 1958 Vol 5 Tab 50].

Since ribose appeared to be of importance in mammalian metabolism, the authors undertook a study of certain phases of ribose metabolism in man. Their publication presented data concerning the fate of ribose after its

intravenous administration in both the unlabeled and ^{14}C labeled form to both normal and diabetic subjects [Segal, *et al.*, 1958 Vol 5 Tab 50].

The sugar used was pure, by the criteria of both optical rotation and paper chromatography. A seven and a half per cent sterile pyrogen-free solution of the sugar was used for infusion. Ribose 1-C^{14}, specific activity 2.01 μc per mg and 0.89 μc per mg was obtained from the National Bureau of Standards and was found to be solely ribose by chromatography and radioautography [Segal, *et al.*, 1958 Vol 5 Tab 50].

Twenty-seven intravenous infusions of various amounts of D-ribose were given to six fasting normal male and one female volunteers, 19 to 24 years of age, and to three subjects with diabetes mellitus. All of the normal subjects were maintained on a 250 gm carbohydrate diet prior to the study. Diabetics received their last dose of crystalline insulin 24 hours before each experiment. Administration of the ribose solution was carried out either over a 15 minute period or, after a small priming dose, by constant infusions using a Bowman pump [Segal, *et al.*, 1958 Vol 5 Tab 50].

Blood specimens were obtained at 3 to 10 minute intervals for glucose and ribose determinations, and at 20 minute intervals for phosphate and pyruvate analyses. In eleven experiments, 0.1 U per Kg body weight of crystalline insulin was given intravenously, in order to ascertain the effects of insulin on blood levels and urinary excretion of the pentose. Fractional purines were obtained for 24 hours and preserved by freezing [Segal, *et al.*, 1958 Vol 5 Tab 50].

Results of the disappearance from blood of ribose administered intravenously showed that an entirely linear curve was obtained when 3 gm amounts were injected, and that doses larger than 3 gm produced a lag phase before the onset of a linear disappearance. The deviation from a straight line of the blood ribose values in the lag phase did not represent a distribution gradient between blood and the total ribose compartment, for deviations to this phenomenon lead to initial values higher than the linear curve. Likewise, the deviation was not due to a distribution gradient of ribose between plasma and red blood cells [Segal, *et al.*, 1958 Vol 5 Tab 50].

The authors found that in the normal subject, the rate constant ranged from 3.8 to 5.8 per cent per minute after a 20 gm dose. As the dose decreased, the rate constant increased to as high as 13.8%/min for 3 gm. The researchers found it interesting to note that as the dose was halved, in Subject L. C., the K value was increased by a constant factor of 1.5 [Segal, *et al.*, 1958 Vol 5 Tab 50].

The blood glucose values in the three diabetics were 470, 310 and 293 mg per 100 ml, respectively. Despite the obvious lack of adequate ability to metabolize glucose, the ribose disappearance curve was identical in pattern to the normal, with a K_i of 4.6 to 5.1. A normal disappearance curve of the pentose D-xylose had similarly been noted in a previous study [Segal, *et al.*, 1958 Vol 5 Tab 50].

8. The Effect Of Insulin On Blood Ribose Levels

The previously reported enhancement by insulin on the disappearance from blood of D-xylose, L-arabinose and D-xylose had also been observed with D-ribose. The authors concluded that the response of ribose to insulin was independent of hypoglycemia.

9. The Distribution Of Ribose in Body Fluids

The volume of distribution of ribose calculated from C^{14} values of the linear phase of the ribose disappearance curve to t_o averaged 16.1 L in 12 studies on five normal subjects. The authors noted a wide range in the volume of distribution, when calculated by this method, depending on the dose of ribose due to the kinetics of disappearance from blood. The fact that the C^{14} obtained by extrapolation of the decay curve after a 3 gm dose was essentially equal to the observed C^{14} may indicate that complete mixing had occurred. Assuming that equilibrium of distribution had been achieved during the 15 minute infusion in the other experiments, the observed C^{14} appeared suitable for use in the calculation. Employing the latter value, the average value of distribution was 25.8 L, with very little dose variation. The diabetics appeared to have a normal ribose "space," as determined by both of the methods of estimation [Segal, *et al.*, 1958 Vol 5 Tab 50].

Calculations of the above type were said to present an inherent difficulty in estimating the amount present in the body at time zero, the theoretical moment of mixing, as well as the concentration at that time, due mainly to the fact that ribose had a metabolic fate other than simple distribution and excretion [Segal, *et al.*, 1958 Vol 5 Tab 50].

10. The Urinary Excretion Of Ribose

The urinary excretion of ribose as an orcinol reactive material was determined by the researchers. An average of 21 per cent of the dose was excreted by a normal subject after a 15 minute ribose infusion, nearly all of this appearing in a 90 minute postinfusion period. The diabetic and the normal subject who received insulin excreted amounts of ribose within the control range. Data on a subject who received various ribose doses suggested that the urinary excretion decreased as the dose was diminished. When 20 gm of ribose was given at a constant slow rate, only 5 to 12 per cent was found in the urine. According to the authors this appeared to be a consequence of a slow infusion rate, and was not related to the insulin given during the study. In one such experiment, urine collected prior to insulin injection revealed a urinary loss of 8 mg per minute, while 146 mg per minute was being infused. This excretion rate of 5.5 per cent per minute approximated the total excretion of 5 per cent of the dose. These results were in contrast to those obtained with D-xylose. The total urinary loss of this pentose was approximately the same following the rapid injection of various doses, as well as after slow constant infusion [Segal, *et al.*, 1958 Vol 5 Tab 50].

11. The Effect Of Ribose On Blood Levels Of Glucose, Pyruvate and Inorganic Phosphate

It had been reported that the infusion of ribose into the normal and some diabetic individuals caused a lowering of blood glucose of about 30 mg per cent. A typical response of blood glucose to ribose was cited in one subject. Immediately after ribose injection, only a slight fluctuation in glucose levels occurred. However, about 30 minutes after the end of the infusion the blood glucose began to decline, reached a nadir in about 30 minutes, and then

returned to approximately the control level. The effect was observed when blood ribose values had decreased to low levels [Segal, *et al.*, 1958 Vol 5 Tab 50].

The lowest blood glucose value after insulin in this study was about equal to the lowest value seen after ribose in the same subject, here little change occurred in phosphate or pyruvate values [Segal, *et al.*, 1958 Vol 5 Tab 50].

12. The Urinary C^{14} Excretion After Labeled Ribose Injection

A total of 10 per cent of the C^{14} administered to subjects in tracer dosage appeared in the urine, whereas 40 per cent of the 5 μc dose given as a load (on 20 gm) was excreted via this route. During the first hour after injection, about four times as much label tracer appeared in urine in the latter study as in the former. The authors stated that this difference probably explained the greater rate constant for C^{14} disappearance from blood after the 20 gm dose [Segal, *et al.*, 1958 Vol 5 Tab 50].

The concomitant administration of carrier ribose with C^{14} ribose permitted comparison of urinary excretion of C^{14} and orcinol reactive material. Whereas the cumulative urinary excretion of C^{14} was 40 per cent, only 20 per cent of administered ribose, as measured by orcinol, was excreted by this route. The data showed that with time, an increase in the proportion of total counts to ribose counts took place, a phenomenon which paralleled increasing non-ribose counts in blood [Segal, *et al.*, 1958 Vol 5 Tab 50].

Urine from these studies was chromatographed on paper, and the ribose area was eluted and counted. Urine collected during the first hour after the tracer dose was chosen for study, since it contained nearly all of the C^{14} excreted. It was found that 92 per cent of the C^{14} eliminated after the tracer dose was unaltered ribose. A similar study of urine from the load experiment (20 to 42 minute period) revealed that only 42 per cent of the C^{14} applied to the paper could be located the within ribose band. This gave a ratio of total counts to ribose counts of 2.4, corroborating the ratio calculated (2.24) from urine counting and chemical data [Segal, *et al.*, 1958 Vol 5 Tab 50].

13. The metabolism of ribose-1-C^{14} in diabetes mellitus and evidence for the existence of the pentose phosphate pathway in man

Five μc of ribose-1-C^{14} was given intravenously to a 53 year old, 60 Kg, unstable diabetic, whose last dose of crystalline insulin was given 24 hours prior to the study. Blood glucose, during the period of observation, was 475 mg per cent and acetone was present in the urine [Segal, *et al.*, 1958 Vol 5 Tab 50].

The specific activity of the expired $C^{14}O_2$ in this subject compared to results obtained in the tracer study in the normal subject was much lower, and the shape of the curve greatly altered. In the diabetic, the labeling of CO_2 reaches a low maximum and then remains constant throughout the period of observation [Segal, *et al.*, 1958 Vol 5 Tab 50].

The low specific activity of expired $C^{14}O_2$, and decreased excretion of C^{14} in CO_2 seen in the diabetic may be interpreted as being due not only to the low specific activity in an expanded glucose pool, but also to the defect in glucose metabolism known to exist in the diabetic [Segal, *et al.*, 1958 Vol 5 Tab 50].

According to the reactions of the pentose phosphate pathway, radioactive glucose derived from ribose-1-C^{14} should be labeled predominantly in carbons one and three. Indeed, in the intact mouse, Hiatt observed that ribose-1-C^{14} gave rise to C^{14} glucose with 34 per cent of the activity in carbon one, 56 per cent in carbon three and a small quantity of activity in the other carbons. In order to determine whether ribose-1-C^{14} was converted to glucose by this pathway in man, glucose, from urine obtained during the fourth hour after ribose injection, was isolated as crystalline potassium gluconate. An aliquot was converted to CO_2, to obtain the total radioactivity in carbon one to carbon six collectively. Another portion was degraded so as to obtain carbon one and carbon six, individually, and carbon two to carbon five, collectively, as CO_2. Thirty-one per cent of the activity in carbon one to carbon six resided in carbon one, 6 per cent in carbon six and the remainder in the carbon two to carbon five fraction, presumably the activity being in carbon three. These figures correspond with Hiatt's results in the mouse, and

give evidence that ribose was metabolized in man by conversion to glucose via the pentose phosphate pathway [Segal, *et al.*, 1958 Vol 5 Tab 50].

The authors concluded that the kinetics of the disappearance of D-ribose from blood differ from those of D-glucose or D-xylose by being dose dependent. Their kinetic data indicated that the system for clearing ribose from blood is saturated after the infusion of small amounts of the sugar. That this assumption has some validity may be derived from the expired $C^{14}O_2$ data after tracer and 20 gm doses of labeled-ribose were given [Segal, *et al.*, 1958 Vol 5 Tab 50].

With the larger dose, the maximum specific activity of the $C^{14}O_2$ appeared at a later time and reached a much lower value. The rate constants of disappearance of ribose from blood ranged from 3.8 to 13.8-per cent per minute, depending on the dose. Rate constants observed after the infusion of small amounts of ribose were larger than those observed after the infusion of various hexoses and other pentoses. Only D-galactose disappeared from blood at a rate similar to that of ribose. At comparable dose levels, the rate constant of ribose removal from blood is four to five times as large as that of other pentoses. Helmreich and Cori had similarly reported that injected ribose disappears from the body of the nephrectomized rat at rates much greater than those of other pentoses [Segal, *et al.*, 1958 Vol 5 Tab 50].

The average total urinary excretion of ribose after a 15 minute infusion was 21 per cent of the dose. A much smaller amount was excreted after constant infusion of the sugar at a slow rate. None of the author's studies was designed to determine accurately the renal mechanism for excreting ribose. One difference between the renal handling of ribose and xylose was suggested from another study. In that study plasma glucose levels of about 300 mg per cent caused a saturation of the xylose tubular reabsorbing mechanism and increased the renal clearance of xylose. No such effect was observed in the ribose studies in diabetics, for with blood glucose levels of about 300 mg per cent these subjects excreted no greater amounts of ribose in urine than the subjects with normal blood glucose levels [Segal, *et al.*, 1958 Vol 5 Tab 50].

Normally, small amounts of ribose can be detected in urine. Prior to 1958 there were several reports of a ribosuria associated with muscular dystrophy and neoplastic disease. Whether this ribosuria is a primary defect in these diseases, or is merely a consequence of tissue breakdown, was not established definitely at that time [Segal, *et al.*, 1958 Vol 5 Tab 50].

The present studies with ribose-1-C^{14} in the normal subject clearly demonstrated that the ribose escaping urinary excretion was rapidly metabolized, with prompt excretion of a portion of the first labeled carbon as CO_2. Since $C^{14}O_2$ appeared so soon after the injection of the labeled sugar, it was not likely that bacterial degradation of ribose that may have entered the gastrointestinal tract could be held responsible. Furthermore, the C^{14} ribose experiment in the diabetic showed that the ribose not excreted in urine is largely converted to glucose by the sequence of reactions of the pentose phosphate pathway [Segal, *et al.*, 1958 Vol 5 Tab 50].

E. Exercise

Adenine nucleotide metabolism in skeletal muscle is essential to the role of muscle as a transducer of chemical energy to mechanical work. Precise regulation of muscle adenine nucleotide metabolism is imperative to enable the performance of high-intensity exercise. During intense exercise, the rate of ATP hydrolysis exceeds its synthesis, resulting in an increased production of ADP. Subsequently, a series of reactions catabolize ADP, resulting in the formation of inosine-5'-monophosphate (IMP) and ammonia. Muscle IMP and ammonia accumulation are closely matched to the magnitude of ATP degradation. A proportion of the IMP produced may be degraded to inosine, which can be further catabolized to hypoxanthine. Both inosine and hypoxanthine can diffuse across the sarcolemma and may represent a loss of purine base from the muscle. Nucleosides and bases lost from the muscle cell are replenished by de novo synthesis, which is a slow energy-consuming process in comparison with the purine salvage pathway. The purine salvage pathway provides a mechanism for the conversion of hypoxanthine to IMP, and the latter metabolite can be reaminated via the reactions of the purine nucleotide cycle, eventually resulting in ATP resynthesis. Thus the purine salvage pathway in conjunction with the purine nucleotide cycle act to reduce

the potential loss of ATP from contracting and recovering muscles [Stathis, *et al.*, 1994 Vol 5 Tab 55].

Stathis, *et al.*, 1994, published a study on the influence of sprint training on human skeletal muscle purine nucleotide metabolism [Stathis, *et al.*, 1994 Vol 5 Tab 55]. In order to examine the effect of sprint training on human skeletal muscle purine nucleotide metabolism, eight active untrained subjects completed a maximal 30 second sprint bout on a cycle ergometer before and after 7 weeks of sprint training.

Sprint training involves repeated bouts of intense exercise that induce a large stress on skeletal muscle purine nucleotide metabolism. A number of studies have examined the effects of sprint training on various components of purine nucleotide metabolism in humans. Prior to this research, no study has simultaneously examined the major purine nucleotide degradation pathways utilized by human skeletal muscle during, and in recovery from, a maximal exercise bout before and after a sprint training regime. Information from such a study would provide greater insight into the possible adaptation of purine nucleotide metabolism to an intermittent metabolic stress. Therefore, the purpose of that study was to examine the effects of sprint training on the concentrations of muscle adenine nucleotides and their major degradation products before, immediately after, and during recovery from a 30 second maximal cycling bout [Stathis, *et al.*, 1994 Vol 5 Tab 55].

Resting muscle ATP and total adenine nucleotide content were reduced ($P < 0.05$) by 19 and 18%, respectively, after training. Training resulted in a 52% attenuation ($P < 0.05$) in the magnitude of ATP depletion after exercise and a similar reduction ($P < 0.00$) in the accumulation of inosine-5'-monophosphate and ammonia. During recovery, muscle inosine-5'-monophosphate ($P < 0.05$) and inosine ($P < 0.01$) content were reduced after training, as was the accumulation of inosine ($P < 0.05$). Plasma ammonia was higher ($P < 0.05$) after training early in recovery. In contrast, plasma hypoxanthine concentrations were reduced ($P < 0.05$) during the latter stages of recovery. According to the authors, the attenuated resting ATP and total adenine nucleotide contents after training probably resulted from the acute effects of prior training sessions. The reduction in the magnitude of ATP depletion

during a 30 second sprint bout after training must reflect an improved balance between ATP hydrolysis and resynthesis. The authors concluded that it was unclear which mechanism(s) was responsible for the reduction in the magnitude of ATP degradation after training [Stathis, *et al.*, 1994 Vol 5 Tab 55].

Tullson, *et al.*, 1995 addressed whether AMP deaminase (AMPD):myosin binding occurs with deamination during intense exercise in humans and the extent of purine loss from muscle during the initial minutes of recovery. Skeletal muscle is capable of profound changes in the turnover rate of high-energy phosphates that enables a high work output and may eventually lead to contractile failure when the means of maintaining a favorable intracellular milieu is exhausted. During periods of high-energy turnover, a high muscle phosphorylation potential is maintained in part by tempering the rise in free ADP (ADPf). This occurs by oxidative and substrate-level phosphorylations as well as the near-equilibrium. phosphotransferases, including creatine (Cr) kinase and adenylate kinase. For adenylate kinase (ADPf + ADPf - AMPf + ATP, where AMPf is free AMP) to have the most advantageous effect on the ATP/ADP ratio, AMP must be consumed. The deamination of AMP to inosine-5'-monophosphate (IMP) and ammonia, via AMP deaminase (AMPD) probably serves this purpose among others. IMP formation can be substantial, especially when the capacity to replenish ATP via oxidative phosphorylation is low relative to the rate of ATP utilization, and thus should contribute to moderating increases in the ADPf concentration [Tullson, *et al.*, 1995 Vol 6 Tab 61].

In addition to several modulators of AMPD that are known to change as a consequence of exercise, reversible AMPD:myosin binding has recently been identified as a potential mechanism that contributes to the control of deamination in rat muscle. The substrate affinity of skeletal muscle AMPD has been reported in the millimolar range, 1,000-fold higher than levels of AMPf estimated in skeletal muscle. Myosin binding specifically alters the kinetic characteristics of rodent muscle AMPD such that a much higher affinity for AMP is observed (affinity constant of -0.05 for bound AMP vs. affinity constant of -1.7 mM for free AMP). Furthermore, it has been shown in rats that AMPD:myosin binding increases in mixed muscle and in discrete

fiber sections during demanding in situ muscle contractions and in fast twitch white fibers during high-speed running. It is not known whether AMPD binding to myosin helps to control IMP formation during intense exercise in humans [Tullson, *et al.*, 1995 Vol 6 Tab 61].

Intense exercise increases plasma levels of inosine and hypoxanthine, most likely due to purine efflux from active muscle. Purine efflux continues after the cessation of exercise even at a time when phosphocreatine (PCr) has substantially recovered. Results from recent experiments have shown that intense sprint training can lead to a decline in resting muscle adenine nucleotides, which suggests that exercise induced purine loss may be substantial and not fully restored by the activity of the purine salvage or de novo synthesis pathways. The potential dephosphorylation of IMP to inosine had not been thoroughly investigated despite evidence that intense exercise leads to purine loss from skeletal muscle. The purpose of this study was to further investigate the control of AMPD during intense dynamic exercise and the metabolic fate of IMP (i.e., degradation vs. reamination) during the early recovery phase after exercise in humans [Tullson, *et al.*, 1995 Vol 6 Tab 61].

Male subjects in this study performed cycle exercise (265 ± 2 W for 4.39 ± 0.04 minutes) to stimulate muscle inosine-5'-monophosphate (IMP) formation. After exercise, blood flow to one leg was occluded. Muscle biopsies (vastus lateralis) were taken before and 3.6 ± 6.2 minutes after exercise from the occluded leg and 0.7 ± 0.0, 1.1 ± 0.0, and 2.9 ± 0.1 minutes post-exercise in the non-occluded leg. Exercise activated AMPD; at exhaustion IMP was 3.5 ± 0.4 mmol/kg dry muscle. Before exercise, 16.0 ± 1.6% of AMPD cosedimented with the myosin fraction; the extent of AMPD:myosin binding was unchanged by exercise. Inosine amount increased about threefold during exercise and twofold more during recovery; by 2.9 min post-exercise it was 0.43 ± 0.02 mmol/kg dry muscle. IMP decreased 2.1 ± 0.3 mmol/kg dry muscle with no change in total adenylates. Total purines declined significantly ($P < 0.05$) during the recovery period in the non-occluded leg, consistent with a loss of purines to the circulation, whereas total purines were unchanged in the occluded leg. The authors concluded that regulation of muscle purine content is a dynamic process that

must accommodate rapid changes due to degradation and efflux [Tullson, *et al.*, 1995 Vol 6 Tab 61].

In an earlier study, Tullson, *et al.*, 1991, studied adenine nucleotide synthesis in exercising and endurance-trained skeletal muscle. The authors explained that strenuous exercise leads to increased efflux of purine nucleoside and base that should necessitate recovery of adenine nucleotides by either the de novo synthesis or salvage pathway. De novo synthesis of adenine nucleotide was measured in quiescent and contracting muscle of sedentary and exercise trained rats using an isolated perfused hindquarter preparation. Previously, it was not known if exercise had any effect on adenine nucleotide synthesis. Flux through the de novo synthetic pathway could be increased during muscle contraction to compensate for accelerated loss of nucleosides and bases. On the other hand accelerated energy demands of contraction could compromise de novo synthesis because the first reaction of the de novo synthesis pathway, 5-phosphoribosyl-1-pyrophosphate synthetase was subject to energy charge regulation [Tullson, *et al.*, 1991a Vol 6 Tab 62].

The purpose of the Tullson, *et al.* study was to examine how de novo synthesis of adenine nucleotides in skeletal muscle may be affected by an increased rate of energy turnover. The authors chose experimental conditions that produced different energy demands and assessed the response in fiber sections of widely differing oxidative capacities among muscle fiber types, which displayed a fourfold range of mitochondrial contents, and between muscles of sedimentary and endurance-trained rats, where muscle oxidative capacity was increased. The team measured de novo synthesis rates in a perfused rat hindquarter preparation during concurrent contractions with mild (1-Hz) twitch and intense (60 tetani/min) stimulation conditions and found that de novo synthesis was generally reduced in fast-twitch muscle during a sustained increase in energy turnover. This observation led to further experiments designed to examine one aspect of the control of de novo synthesis during exercise [Tullson, *et al.*, 1991a Vol 6 Tab 62].

The activity of the pentose phosphate pathway is apparently low in normal skeletal muscle. Consequently, the formation of ribose-5-phosphate by this pathway is thought to set an upper limit on the de novo synthesis rate. According to the authors, this limitation can be overcome via ribose supplementation, which leads to increased provision of 5-phosphoribosyl-1-pyrophosphate (PRPP) and stimulation of de novo synthesis in vitro and in vivo skeletal muscle. The team tested the hypothesis that exercise-induced reduction in de novo synthesis is due to PRPP depletion and by supplementing the perfusate with ribose a measured increase in de novo synthesis occurs [Tullson, *et al.*, 1991a Vol 6 Tab 62].

Synthesis rates were assessed by measuring the incorporation of [1-^{14}C]glycine into adenine nucleotide in muscles of both resting and stimulated hindlimbs after 1 hour of either low- or high-energy demand isometric contractions. In non-stimulated sedentary and trained muscles, rates of de novo synthesis were similar. The effect of muscle contractions on de novo synthesis varied among muscle fiber types. Contracting, non-fatigued fast--twitch muscle sections showed significant declines in de novo synthesis in both sedentary and trained groups. Rates in slow-twitch red fibers and fatigued fast-twitch white fiber sections were not different from rest. Supplementing the perfusate with 5 mM ribose caused de novo synthesis to rise three- to fourfold in each of the fiber sections. However, the response in synthesis rates due to exercise was similar with or without ribose supplementation. De novo synthesis does not increase during exercise but exhibits an unchanged or reduced rate depending on the expected energy balance within the cell. This would occur if the energy state of muscle exerts significant control over de novo synthesis of adenine nucleotide [Tullson *et al.*, 1991a Vol 6 Tab 62].

Quiescent skeletal muscle synthesizes adenine nucleotide from non-purine precursors at rates that scale with increasing muscle oxidative capacity. Under conditions in which the muscle purine nucleotide content is constant, the rate of de novo synthesis must equal the rate of net loss of purine nucleotides and their degradation products from muscle. Thus it appears that more oxidative muscle fibers lose purine metabolites at a greater rate than do less oxidative fibers. The positive correlation between the rate of adenine

nucleotide synthesis and oxidative capacity is consistent with the activity of 5'-nucleotidase [5'-AMP] (or 5'-IMP) + $H_2O \rightarrow$ adenosine (or inosine) + P_i found in muscles of different oxidative capacities. The activity of this degradative enzyme is reported to be considerably higher in oxidative red muscle than in low-oxidative white muscle. This could contribute to a greater production and loss of nucleosides and bases in red muscle which can be balanced by a greater rate of de novo synthesis [Tullson, *et al.*, 1991a Vol 6 Tab 62].

Gross, *et al.*, studied D-ribose administration during-exercise and its effects on end products of energy metabolism in healthy subjects and a patient with myoadenylate deaminase deficiency [Gross, *et al.*, 1991b Vol 2 Tab 19].

The deficiency of the muscle isoform of adenylate deaminase (myoadenylate deaminase deficiency) was first described in 1978 as a new disease of skeletal muscle. It is now considered to be the most common known enzyme defect in muscle, found in about 2% of all muscle biopsies. The spectrum of symptoms ranges from asymptomatic carriers of the disease to patients that suffer from exercise-induced muscle pain and cramps and includes cases of rhabdomyolysis [Gross, *et al.*, 1991b Vol 2 Tab 19].

It was previously suggested that in the myocytes of these patients AMP is degraded during exercise to adenosine which can then easily diffuse out of the cell. It had also been shown that ribose accelerates de novo adenine nucleotide synthesis in cardiac muscle. For that reason ribose was administered in these patients to increase the adenine nucleotide synthesis. It has been shown that continuous administration of D-ribose during exercise (10-20 g per hour) can in some patients prevent or decrease the symptoms [Gross, *et al.*, 1991b Vol 2 Tab 19].

At the same time, several studies have demonstrated that the myocytes of myoadenylate deaminase deficient patients are not depleted of adenine nucleotides. Thus, the mechanism behind the reported salutatory effects of ribose administration in these patients is unclear [Gross, *et al.*, 1991b Vol 2 Tab 19].

Previous studies had demonstrated an almost complete absorption of orally administered ribose. Ribose given to healthy subjects at rest can lower serum glucose levels as much as 25%. The mechanism of this effect was not understood at the time of publication. The authors stated that ribose administration was not associated with side effects in doses under 200 mg/kg per hour [Gross, *et al.*, 1991b Vol 2 Tab 19].

The study investigated the effects of oral ribose administration on muscle metabolism in healthy subjects and a patient with myoadenylate deaminase deficiency by measuring: 1) plasma glucose and free fatty acids, two main substrates of muscle energy metabolism, 2) lactate, an end product of the glycolytic pathway, and 3) ammonia and hypoxanthine, degradation products of adenine nucleotides [Gross, *et al.*, 1991b Vol 2 Tab 19].

Nine healthy men and a patient with myoadenylate deaminase deficiency were exercised on a bicycle ergometer (30 minutes, 125 Watts) with and without oral ribose administration at a dose of 2 g every 5 minutes of exercise. Plasma or serum levels of glucose, free fatty acids, lactate, ammonia and hypoxanthine and urinary hypoxanthine excretion were determined. After 30 minutes of exercise without ribose, the healthy subjects showed significant increases in plasma lactate ($p<0.05$), ammonia ($p<0.01$) and hypoxanthine ($p<0.05$) concentrations and a decrease in serum glucose concentration ($p<0.05$). When ribose was administered, the plasma lactate concentration increased significantly higher ($p < 0.05$) and the increase in plasma hypoxanthine concentration was no longer significant. The patient showed the same pattern of changes in serum or plasma concentrations with exercise with the exception of hypoxanthine in plasma, which increased higher when ribose was administered [Gross, *et al.*, 1991b Vol 2 Tab 19].

After 30 minutes of exercise without ribose intake, the healthy subjects showed a significant decrease in serum glucose and significant increases in plasma lactate, ammonia and hypoxanthine. When ribose was given, the increase in plasma hypoxanthine concentration was no longer significant, and the increase in plasma lactate concentrations was significantly higher.

Glucose and free fatty acids were measured as two main energy sources of skeletal muscle. Except for the relatively small increase in FFA concentrations in the patient in the second half of the experiment, there were no differences found in glucose and FFA concentrations between the patient and controls. The possibility of an increased turnover could not be excluded by the study. Although the first possibility cannot be excluded, an increased carbohydrate degradation during ribose intake seems to be more likely to the investigators. Earlier studies found no evidence for an increased glucose utilization during ribose administration. The authors speculated that ribose itself could be degraded to lactate via the pentose-phosphate pathway in the muscle cells providing the cells with additional ATP. This might cause or contribute to the beneficial effect of ribose in the patient. Since considerable amounts of plasma lactate are taken up by liver, kidney and heart, the small difference in increase in plasma lactate with and without ribose intake is consistent with the proposed pathway [Gross, *et al.*, 1991b Vol 2 Tab 19].

In both experiments the patient showed an increase in serum lactate level at the upper range of the values in healthy subjects. This supported the hypothesis that the patients activate the citric acid cycle less during exercise than healthy controls. Fumarate, produced by the purine nucleotide cycle, is very important for the activation of the citric acid cycle. In myoadenylate deaminase deficiency this cycle is disrupted. The finding that the plasma lactate concentration increased even higher during ribose intake in both healthy subjects and the patient excludes a significant activation of the citric acid cycle by ribose. Thus the beneficial effect of ribose in myoadenylate deaminase deficiency could not be explained by an increase in oxidative energy metabolism [Gross, *et al.*, 1991b Vol 2 Tab 19].

Hypoxanthine (plasma level and urinary excretion) was measured as the principal extracellular metabolite of ATP breakdown in man. Both the plasma level and the urinary excretion increased less in the healthy subjects when ribose was taken during exercise. Although the differences were not statistically significant, they might represent much larger changes in muscle metabolism since only 0.5% of the purine nucleotide loss from muscle could be recovered in urine. Reduced hypoxanthine levels could result from an enhanced salvage by the hypoxanthine guanine phosphoribosyltransferase,

if ribose is transformed to the limiting factor 5-phosphoribosyl-1-pyrophosphate. A reduced production could also result from a degradation of ribose to lactate in muscle cells. The gain in ATP would help to stabilize the energy charge and thereby reduce the activity of the purine nucleotide cycle and the resultant adenine nucleotide degradation. In the patient the hypoxanthine plasma concentration increased higher when ribose was taken. Studies with more patients are needed to investigate whether this is a reproducible difference compared with healthy subjects [Gross, *et al.*, 1991b Vol 2 Tab 19].

Since the ATP degradation to hypoxanthine involves deamination, the plasma ammonia levels were measured as another parameter of ATP breakdown. If ribose serves as an additional energy source, the release of ammonia should be reduced. The data showed no effect of ribose on the ammonia level, as the variation was too high to answer that question [Gross, *et al.*, 1991b Vol 2 Tab 19].

One advantage of the experimental design was that the use of a bicycle ergonometer involved a much greater amount of skeletal muscle than a forearm test. However, blood samples were taken from an antecubital vein to reduce the burden for the subjects. This design certainly reduced the changes in plasma concentrations due to dilution of the femoral vein blood and contributed to the wide range of the data. Studies with blood taken from a femoral vein or forearm test might result in higher significant data [Gross, *et al.*, 1991b Vol 2 Tab 19].

The data showed that the oxidative metabolism that seems to be reduced in the patient due to the lack of fumarate production is not significantly influenced by ribose administration. The changes in plasma lactate and hypoxanthine level and urine hypoxanthine excretion might indicate, but did not prove, to the authors, that ribose was degraded to lactate thus supplying the muscle with ATP. The authors stated that further studies were necessary to verify this hypothesis [Gross, *et al.*, 1991b Vol 2 Tab 19].

D.R. Wagner, *et al.* also investigated the effects of oral ribose on muscle metabolism during bicycle ergometer in AMPD-deficient patients [Wagner, *et*

al., 1991a Vol 7 Tab 65]. Three patients with AMP deaminase deficiency (AMPD deficiency) performed exercise on a bicycle ergometer with increasing work load with and without administration of D-ribose (3 g orally every 10 min, beginning 1 hour before exercise until the end). The patients performed exercise until their heart rate was 200 minus age (Table 12). Maximum capacity was not increased by administration of ribose, but post-exertional muscle stiffness and cramps disappeared almost completely in 2 of 3 AMPD-deficient patients. Plasma concentrations of lactate and inosine were increased in AMPD-deficient patients after oral administration of ribose. The researchers data suggested that ribose may both serve as an energy source and enhance the de novo synthesis of purine nucleotides [Wagner, *et al.*, 1991b Vol 7 Tab 65].

AMP deaminase deficiency (AMPD deficiency) is a relatively frequent enzyme defect of skeletal muscle (2% of muscle biopsies). Clinically, it is characterized by post-exertional muscle stiffness and cramps. AMP deaminase (myoadenylate deaminase) is one component of the purine nucleotide cycle. The disruption of the purine nucleotide cycle reduced by AMPD deficiency has been postulated to be the cause of the muscular disorder. At the time of publication the only successful therapeutic approach was the oral administration of ribose. The beneficial effect of ribose in AMPD deficiency has only been achieved by high oral doses (3-4 g) administered continuously (every 10 min) during exercise, as first described by Zollner *et al.* Lower and non-exercise-related doses of 500 mg ribose 4 times a day were not successful. At this level the authors noted that diarrhea occurred occasionally although no other side effects were recorded. The beneficial effect of ribose has mainly been attributed to an additional energy source and possibly also to enhanced de novo synthesis of purine nucleotides [Wagner *et al.*, 1991b Vol 7 Tab 65].

In this study the authors measured plasma concentrations of ammonia, lactate, inosine and hypoxanthine in AMPD-deficient patients during exercise, without oral ribose on day 1 and with oral ribose on day 2, in order to investigate changes in muscular metabolism during ribose therapy. Imitating physiological conditions, muscular work was performed on a bicycle ergometer with increasing work load [Wagner, *et al.*, 1991b Vol 7 Tab 65].

On day 1, after exercise without oral ribose, patients 1 and 2 complained of stiffness and cramps. The muscular performance was normal in comparison with the muscular performance of control subjects obtained by Wagner *et al.* On day 2, after exercise with oral ribose, patient 1 had no more muscular symptoms, whereas patient 2 still complained of muscle stiffness and cramps. On day 1 (without ribose), patient 3, who suffered from severe muscular dystrophy, was not able to perform a normal muscular exercise compared with the control subjects. His maximum capacity was 80 W (controls of the same age: 100- 160 W). After exercise, he complained of muscle fatigue and cramps. On day 2 (with ribose), his maximum capacity did not increase. After exercise, patient 3 had no more muscle cramps, but continued to complain of fatigue [Wagner, *et al.*, 1991b Vol 7 Tab 65].

Table 12
Individual Data Of 3 AMPD-Deficient Patients Participating In The Study

Patient Number	Sex	Age (years)	Height (cm)	Weight (kg)	Additional Clinical Diagnosis
1	M	58	181	84	
2	M	33	177	83	Polyneuropathy
3	M	34	180	70	Muscular Dystrophy[1]

[1]Muscular dystrophy type Becker-Kiener.

Plasma ammonia concentrations increased during exercise on day 1 (without ribose) from 13.0 (range 10-16) to 24.0 µmol (range 21-26). On day 2 (with ribose), plasma ammonia concentrations were 5.0 µmol (range 12-23) at rest and 22.0 µmol (range 15-28) at the end of exercise [Wagner, *et al.*, 1991b Vol 7 Tab 65].

Plasma lactate concentrations increased during exercise on day 1(without ribose) from 0.7 mmol/l (range 0.7 - 0.8) to 4.1 mmol/l (range 1.5 - 5.5). On day 2 (with ribose), plasma lactate concentrations were 0.8 mmol/l (range 0.6-1.0) at rest and 5.2 mmol/l range (3.5 - 6.9) at the end of exercise [Wagner, *et al.*, 1991b Vol 7 Tab 65].

The authors stated that the effect of ribose on AMPD-deficient patients may be due to an additional energy source. However, this hypothesis is in part contradicted by the fact that plasma lactate concentrations of the patient with the best therapeutic effect of ribose (patient 1) remained unchanged after oral administration of ribose, whereas plasma lactate concentrations of the patient with no apparent therapeutic effect at all (patient 2) were most increased after oral administration of ribose [Wagner, *et al.*, 1991b Vol 7 Tab 65].

Inosine and hypoxanthine plasma concentrations are reduced in AMPD-deficient patients during exercise. The increase of inosine and hypoxanthine plasma concentrations in all AMPD-deficient patients during muscular exercise after oral administration of ribose may be explained by enhanced de novo synthesis of purine nucleotides via ribose-5-P and PRPP. The enhancement of de novo synthesis of purine nucleotides is a valuable explanation for the beneficial effect of ribose only in case of purine losses in AMPD deficiency during muscular exercise. However, it was demonstrated by Sabina *et al.*, that the total amount of adenine nucleotides in AMPD-deficient muscle cells remains unchanged after exercise. A close examination of the results of Sabina *et al.* revealed that AMPD-deficient muscles had a 6% decrease in ATP content (normal subjects 34% decrease) and a 16-fold increase of adenosine concentrations (normal subjects 2-fold increase). Inosine and hypoxanthine levels were similar in all groups. From these experiments, it may be concluded that in AMPD deficiency, large amounts of adenosine may be lost by myocytes into the plasma during exercise, with phosphorylated compounds remaining in the cells. This conclusion is consistent with the data of Wagner *et al.*, which showed a higher ATP concentration in red cells of AMPD-deficient patients during exercise, probably due to higher plasma adenosine concentrations. In that case, ribose could enhance the salvage of purine nucleotides before they are lost in the form of adenosine [Wagner, *et al.*, 1991b Vol 7 Tab 65].

McAnulty *et al.* argued that adenine and ribose may stimulate ATP synthesis during hypothermic preservation of kidneys, Swain *et al.* were able to accelerate the repletion of ATP and GTP pools in post-ischemic myocardium

using a precursor of purine de novo synthesis and Vincent *et al.* found that the increase of ribose-5-P is an important factor in PRPP production in hepatocytes. Zimmer and Gerlach, Zimmer and Ibel reported an increase in the available pool of PRPP and adenine nucleotides in myocardial cells by continuous intravenous infusion of ribose. The results of the Wagner study suggested that the enhanced de novo synthesis of purine nucleotides by administration of ribose found in the kidney, heart and liver may also occur in muscles, and therefore may be of therapeutic value in AMPD-deficient patients [Wagner, *et al.*, 1991b Vol 7 Tab 65].

Zollner *et al.*, first demonstrated that the oral administration of high doses of ribose may be a successful therapy in AMPD deficiency. Gross *et al.* from the same laboratory found that orally administered ribose was 88-100% absorbed intestinally in both healthy subjects and AMPD-deficient patients. Since the studies of Segal and Foley (1958) it was known that after absorption ribose is converted either to glucose via the pentose phosphate pathway or to nucleotides via ribose-5-P and PRPP. As the response of ribose to insulin is small, it may be metabolized more rapidly than glucose. The rapid utilization of ribose seems to be insufficient to account for the beneficial effect of ribose in AMPD deficiency for two reasons: first, the amount of energy contained in the dose of ribose (3 g every 10 min) is insufficient to account for the muscular performance; second, there is no explanation why other sugars are not successful. [Wagner, *et al.*, 1991a Vol 7 Tab 65].

The effect of high-intensity intermittent training on the adenine nucleotide content of skeletal muscle was studied by Hellsten-Westing, *et al.*, 1993. Eleven male subjects (group A) performed high-intensity intermittent training on a cycle ergometer three times per week for 6 weeks, followed by 1 week of the same kind of training with two sessions per day. Nine males (group B) exclusively performed 1 week of training with two sessions per day. In group A, skeletal muscle total adenine nucleotide (TAN) levels decreased from 25.1 ± 0.7 (SE) to 22.0 ± 0.6 mmol/kg dry weight over the 6-wk period ($P < 0.01$). The subsequent intensive week did not further alter TAN levels. In group B, the intensive week of training reduced TAN levels from 25.1 ± 0.5 to 19.4 ± 0.6 mmol/kg dry wt ($P < 0.001$). The decrease was sustained 72 hours

after training (P <0.001). During the intensive week, there was no change in plasma creatine kinase activity in either group A or group B [Hellsten-Westing, *et al.*, 1993b Vol 3 Tab 25].

The plasma activity was, however, higher in group B than in group A on days 4 and 7 of the intensive week (P < 0.05). The results from this study indicate that high-intensity intermittent exercise causes a decrease in resting levels of skeletal muscle adenine nucleotides without a concomitant indication of muscle damage. A training-induced adaptation appears to occur with training by which a further loss of adenine nucleotides is prevented despite an increased training dose [Hellsten-Westing, *et al.*, 1993b Vol 3 Tab 25].

The authors explained the fate of the various energy molecules during exercise [Hellsten-Westing, *et al.*, 1993b Vol 3 Tab 25]. A majority of adenine nucleotides lost through generation of IMP during exercise are however, rapidly restored at the onset of recovery via the purine nucleotide cycle. ATP levels are observed to be back to pre-exercise values shortly after termination of exercise. Nevertheless, a fraction of IMP is dephosphorylated to inosine via 5'nucleotidase. Inosine is in turn oxidized to hypoxanthine, xanthine, and uric acid. Hypoxanthine can be converted back to IMP and subsequently to AMP, thereby partially restoring the adenine nucleotide pool. However, because hypoxanthine can diffuse through cell membranes to the bloodstream, the salvage of this purine may not necessarily occur in the previously exercised muscle. Moreover, the oxidation of hypoxanthine to xanthine and uric acid is irreversible. Thus the accumulation of these purines in the bloodstream indicates a net loss of adenine nucleotides [Hellsten-Westing, *et al.*, 1993b Vol 3 Tab 25].

A marked accumulation of plasma hypoxanthine and uric acid can be observed after high-intensity exercise. Although a large part of the exercise-induced loss in nucleotides may be restored, it is possible that a frequent loss of purines will exceed the rate of purine salvage and de novo synthesis of adenine nucleotides. Thus, frequently repeated exercise may affect resting adenine nucleotide levels [Hellsten-Westing, *et al.*, 1993b Vol 3 Tab 25].

F. Blood Glucose Levels

Segal and Foley showed in 1958 that generous infusions of the pentose sugar D-ribose into normal human subjects produced a clear and sometimes profound drop in blood glucose concentration, rather than the rise in blood glucose which might have been expected. Infusion of ribose into the pancreatic artery of dogs by Pozza and others also led to a drop in blood glucose, which was interpreted by them as due to increased release of insulin from pancreatic beta cells [Goodman, *et al.*, 1970 Vol 2 Tab 18].

D-ribose has been reported to decrease the concentration of glucose in the plasma in humans in spite of the fact that a portion of the available ribose is converted into glucose. Since this effect was not observed in diabetics, the mobilization of insulin has been accepted as one mechanism contributing to hypoglycemia, and the mobilization of insulin has been demonstrated by Stein *et al.* However, the amount of insulin mobilized by that investigator was found inadequate to induce the degree of hypoglycemia which was found to persist for more than 1 hour after the insulin level in peripheral venous blood had returned to normal. "Insulin release", therefore, does not appear to adequately explain the late occurring hypoglycemia produced by D-ribose. A possible explanation put forth by Hetenyi, *et al.*, 1968 for this effect of ribose could be a combination of its induced release of insulin plus its interference with a homeostatic mechanism that otherwise maintains or restores normal glycemic leveled after the disappearance of insulin [Hetenyi, *et al.*, 1968 Vol 3 Tab 27].

An intravenous infusion of D-ribose given to normal non-anesthetized dogs was found to decrease the concentration of glucose significantly in the blood plasma. This confirmed the reports of others concerning this effect in humans. The dogs were given infusions at the rate of 1.9 – 3.0 mg/kg/min for 100 minutes [Hetenyi, *et al.*, 1968 Vol 3 Tab 27]. The rate of endogenous glucose production, calculated by an infusion method, decreased during the first 45 min of the infusion.

After 45-100 minutes it returned to its pre-infusion level. The metabolic clearance of glucose increased, indicating enhanced glucose utilization. Effects of a ribose infusion on turnover could be stimulated by an intraportal infusion of insulin at 1-3 mU/kg per min. Ribose effectively prevented or counteracted, at least transiently, the increase in the rate of endogenous glucose production evoked by the infusion of phlorizin. The importance of the release of insulin as a causative factor in ribose-induced hypoglycemia was confirmed, but other factors affecting rates of hepatic glucose production were recognized by the authors [Hetenyi, *et al.*, 1968 Vol 3 Tab 27].

More recently, Fenstad, *et al* designed a blinded counterbalanced study to investigate the effects of ribose ingestion on blood glucose (BG), blood lactate (LA), and estimated glucose oxidation rates. Six non-diabetic subjects (24-30 years old) ingested either 0 (PL), 2, 5, or 10 g of D-ribose in 300 ml of water following a 12 h fast. Glucose oxidation rates were calculated from the Lusk Table using R and $V0_2$ values. Data were collected over a 2 hr period. Results showed that no significant difference was seen for LA between treatments. Mean LA concentrations for PL, 2, 5, and 10 g were 1.1 (.03), 1.15 (.09), 1.15 (.08), and 1.16 (0.13) mmol/L, respectively. A significant difference (p=0.00) was observed between treatments for BG. Mean BG concentrations- for PL, 2, 5, and 10 g were 3.98 (.17), 3.38 (.07), 3.08 (.23), and 2.77 (.5) mmol/L, respectively. Two sample t-tests revealed significant differences between 0 and 2 g and 2 and 10 g treatments. No significant differences were seen for carbohydrate oxidation rates between treatments. Mean carbohydrate oxidation rates for PL, 2, 5, and 10 were .958 (.09), .973 (.08), 1.08 (.17), and 1.19 (.05) kcal/min, respectively. In conclusion, ingestion of 2 and 5 g of ribose maintained blood glucose over 120 min. There was a strong trend (p= 0.07) for oxidation rates to be greater with 10 g and 5 g than with 0 g and 2 g [Fenstad, *et al.*, 2000 Vol 2 Tab 12].

Goodman and Goetz, 1970, studied the effects of administration routes of oral and intravenous D-ribose and plasma insulin in healthy humans. The authors were aware that the pentose sugar, D-ribose

was known to cause a prompt release of insulin when administered to human beings and other mammals. To gain further understanding of the mechanism by which ribose leads to insulin release, a study was carried out on the acute changes in peripheral vein plasma insulin levels in ten healthy human subjects when equal doses of ribose were given by mouth or by vein. The effects of preliminary administration of epinephrine or propranolol on the insulin response to ribose were observed. The results showed that the route of administration made no difference in the rapid, brief insulin response to ribose, and was unrelated to the ribose level in venous blood. Epinephrine infusion suppressed the insulin response both to oral and to intravenous ribose [Goodman, *et al.*, 1970 Vol 2 Tab 18]. Preliminary infusion of propranolol did not alter the magnitude or the promptness of the acute insulin response to ribose given by vein. The authors concluded that it is unlikely that an insulin-releasing factor from the gut was involved in the response to ribose.

1. Intravenous Ribose

Ribose concentration rose steadily during the infusion (0.5 gm/kg body weight as a 7.5% solution) reaching a peak level of about 80 mg per 100 ml at 30 minutes, the last sample taken before the end of the infusion. In contrast, the mean peripheral insulin level at the first sample following ribose administration (5 minutes) had approximately doubled, but then dropped back toward baseline at the 10 minute sample and was at base line by 15 minutes. Glucose concentration dropped gradually after about 15 minutes of infusion of ribose, the lowest mean level being at 45 minutes, at about 85 per cent of the control levels.

2. Oral Ribose

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Ribose concentration for the 10 subjects was not measurable until the 15 minute sample. There was then a steady rise to a peak level of about 30 mg/per 100 ml at 60 minutes. Despite this prolonged rise in blood ribose, the mean insulin level was first noted to rise slightly

at 15 minutes, peaked at 20 minutes at about double the base line values, and then fell off rapidly. Thus the peak insulin level was delayed in comparison to that seen with intravenous D-ribose and plasma insulin.

The change in peripheral insulin levels after ribose administration was modest, contrasting with the large and persisting increases observed when pancreatic insulin output was measured directly in dogs during ribose infusion. This suggests the possibility that the rate of disposition of insulin in the liver may be increased by the metabolism of carbohydrates, such as ribose [Goodman, *et al.*, 1970 Vol 2 Tab 18].

The authors felt their data lent support to the latter suggestion by direct measurement of insulin output from the pancreases of dogs receiving intraportal vein infusions of ribose. In their experiments, the response to ribose was more striking than that to glucose, and it was sustained throughout a 30-minute infusion [Goodman, *et al.*, 1970 Vol 2 Tab 18].

In a study by Steinberg, *et al.*, nine normal subjects received D-ribose intravenously at a rate of 750 mg/min for 20 minutes and 3 normal subjects received a similar infusion at the same rate for a 5 minute period. D-ribose, 7 ½ per cent in water, was infused for 20 minutes in 9 subjects and for 5 minutes in 3 subjects. Blood specimens were obtained at frequent intervals for 105 minutes following the beginning of the infusion. At a later date, 2 of the subjects received 1 unit of crystalline insulin and 2 others received 0.25 unit of crystalline insulin as a rapid intravenous injection. Blood specimens were drawn as previously described for 105 minutes following insulin administration.

Infusions resulted in a total dose of 15 and 3.75 gm of D-ribose, respectively. Although similar transient elevations in concentration of serum immuno-reactive insulin occurred with all infusions, hypoglycemia appeared only with the 15 gm infusion. When the

serum insulin concentration was subsequently elevated to similar levels as those attained during the D-ribose infusions in 2 subjects by the intravenous injection of crystalline insulin, no hypoglycemia occurred [Steinberg, *et al.*, 1967 Vol 6 Tab 58].

It had been shown that in infusion of D-ribose would consistently produce hypoglycemia in human subjects. The mechanism by which hypoglycemia was produced had not been defined. It had been suggested that it was not the result of increased insulin secretion, because the decrease in blood sugar was not accompanied by a significant decrease in inorganic phosphate or a significant increase in pyruvate as might be expected with insulin secretion. On the other hand, it had been proposed from the results of studies in which D-ribose was injected into the pancreatic artery of dogs that the hypoglycemia was mediated by an increased secretion of insulin. The authors pointed out that in none of the studies involving the hypoglycemic effects of D-ribose, had insulin been measured [Steinberg, *et al.*, 1967 Vol 6 Tab 58].

The investigators demonstrated that the intravenous infusion of D-ribose into normal human subjects consistently produced a slight and transient elevation in serum insulin, which was probably not responsible for the noted hypoglycemia. [Steinberg, *et al.*, 1967 Vol 6 Tab 58].

The authors concluded that D-ribose-induced hypoglycemia has a dose/response relationship. Although the concentration of serum insulin was transiently elevated during the infusion of D-ribose, the level of insulin attained was probably insufficient in itself to account for the hypoglycemia. At the time, the mechanism of D-ribose induced hypoglycemia remained unexplained [Steinberg, *et al.*, 1967 Vol 6 Tab 58].

Goetz, *et al.*, 1974, researched the possible action of D-Ribose on pancreatic, beta cells. Acute experiments were carried out: 1) ribose infusions into hepatic artery and right atrium were as effective as

those into portal vein in stimulating directly measured pancreatic insulin output; pancreatic artery infusion was ineffective. 2) 11 cross-circulation experiments, arterial blood from donor dogs receiving a dilute intra-atrial infusion of ribose was transfused into pancreatic arteries of test dogs. Insulin output of test dogs regularly increased within 1 min. All infusions were carried out with a Harvard constant-infusion apparatus.

Recoveries of ribose and glucose were considered satisfactory in the range 5-100 mg/100 ml and concentrations of ribose as low as 2 mg/100 ml were regularly detected in experiments using standard solutions of ribose. Ribose at 3.75% was infused directly into the hepatic artery, after a control period of isotonic saline infusion. The perfusing solution consisted of the dog's blood mixed with half its volume of Ringers solution, with or without added amounts of ribose or glucose, as calculated to raise the concentration of sugar to about 300 mg/100 ml. In three successive 15-min periods, the organs were perfused with solutions containing 1) glucose, 40 mg/100 ml, 2) glucose, 40 mg/100 ml, plus ribose, 300 mg/100 ml and 3) glucose 300 mg/100 ml, without D-ribose. The perfusion rate was held at 15 ± 2 ml/min. Samples of the effluent were taken at 1 - to 5-min intervals.

In 14 experiments, the isolated dog's pancreas-duodenal section responded to the perfusion of glucose but not ribose. These results indicated that the portal vein was essential for the ribose effect. After acute hepatectomy a slight rise in peripheral vein insulin was seen during infusion of ribose it was concluded that ribose-induced insulin release in dogs depends on an indirect humoral mechanism. It was suggested that the metabolism of ribose in various tissues, including red blood cells, yields substances such as reduced pyridine nucleotides with direct insulin-releasing properties. The authors hypothesized that glucose may share such a mechanism [Goetz, *et al.*, 1974 Vol 2 Tab 17].

The probability that an insulin release is not the direct stimulation of the beta cell with D-ribose was indicated by both *in vivo* and *in vitro*

experiments. Direct pancreatic arterial infusion, *in vivo*, was not effective in causing a release of insulin. Also, when the isolated dog pancreas was directly exposed to rising ribose concentrations (up to 250 mg/100 ml or more), there was no increase in insulin secretion [Goetz, *et al.*, 1974 Vol 2 Tab 17].

The final effect on the pancreas itself was shown to be a humoral rather than a neural effect. In cross-circulation experiments where there was no neural connection between the donor dog and the responding pancreas of the test dog there was immediate insulin release within 1 minute after the beginning of the intravenous infusion of ribose. (A humoral mechanism, involving rapid release of a cell stimulator from the hypothalamus or other neural mechanism was postulated). [Goetz, *et al.*, 1974 Vol 2 Tab 17].

It was not clear to the investigators why ribose leaving the pancreas in the pancreatic artery infusions did not lead to an insulin release. One theoretical possibility considered was that the concentrations of ribose above a critical level may actually directly inhibit insulin release from the beta cells, but no evidence was collected for this. The authors stated that the liver was not necessary for the insulin-release mechanism to be activated, though it may still be considered one of the sites of activation. This was demonstrated in two studies. Infusion into the right atrium of the heart proved to be as effective as portal vein infusion in producing insulin release. In preliminary experiments with acutely hepatectomized dogs, the ribose effect was still present [Goetz, *et al.*, 1974 Vol 2 Tab 17].

G. Cardiac Metabolism

D-ribose has been investigated for a number of decades as a supplement for those with various heart conditions, such as coronary heart disease, patients with ischemic left ventricular dysfunction, and stunned or hibernating myocardium. In hibernating myocardium, the blood flow is chronically reduced as opposed to the stunned segment

where the dysfunction persists after an ischemic event despite normal or near normal blood flow.

Myocardial stunning and hibernation represent different phenomena, both of which result in contractile dysfunction. Myocardial stunning is defined as the mechanical dysfunction that persists after reperfusion, despite the absence of irreversible damage and restoration of normal or near normal coronary flow [Bolli, *et al.*, 1990 Vol 7 Tab 82]. Myocardial stunning may also result from repetitive or protracted episodes of ischemia that may occur with stress testing or coronary vasospasm [Bolli, *et al.*, 1990 Vol 7 Tab 82]. Hibernating myocardium was first noted in the clinical arena, when left ventricular dysfunction was observed to be reversible after revascularization [Braunwald, 1988 Vol 7 Tab 83, and Rahimtoola, 1989 Vol 7 Tab 84]. Many authors believe that it is likely that these phenomena combine to create the left ventricular dysfunction noted in ischemic coronary artery disease.

1. D-Ribose in the Detection of Viable Myocardium

The differentiation between the two problems has been described as less important than the necessity to distinguish non-viable from viable tissue. Various techniques have been used in an attempt to help distinguish non-viable from viable myocardium including perfusion imaging using thallium-201 technetium-99m sestamibi, low dose dobutamine echocardiography, or positron emission topographic (PET) scanning [Sinusas, *et al.*, 1989 Vol 8 Tab 85; Verani, *et al.*, 1988 Vol 8 Tab 86; Smart, *et al.*, 1993 Vol 8 Tab 87, and Piérard, *et al.*, 1990 Vol 8 Tab 88]. The goal is observation of functional recovery of myocardial segments after revascularization.

Ribose has also been shown to accelerate thallium-201 redistribution in the heart [Hegewald, *et al.*, 1991 Vol 3 Tab 23]. Thus, ribose has the potential ability to unmask viable myocardium by promoting thallium-201 redistribution in regions that would ordinarily demonstrate fixed image defects acquired three to four hours after stress.

Various studies have suggested that ribose may be a useful therapeutic agent improving contractile dysfunction. D-Ribose used together with low dose dobutamine may provide the greatest sensitivity for detection of viable myocardium. These agents work by different mechanisms to enhance contractility of viable but poorly contracting myocardium.

Angello, *et al.*, investigated the effect of ribose on thallium-201 myocardial redistribution, reasoning that myocardial 201-Tl redistribution after transient ischemia may be too slow to allow identification of a reversible myocardial defect within the routine 201-Tl imaging period. To determine whether 201-Tl redistribution could be affected by a metabolic intervention, intravenous ribose was administered post-ischemia.

Myocardial thallium-201 (^{201}Tl) uptake is dependent upon coronary blood flow, cellular extraction of ^{201}Tl from the blood, and ^{201}Tl myocardial clearance. It is well known that the initial distribution of ^{201}Tl is a function of coronary blood flow. The normalization of ^{201}Tl myocardial content over time between transiently ischemic and normally perfused myocardium is often referred to as ^{201}Tl redistribution.

It is likely that factors other than coronary flow are more important in determining the extent of ^{201}Tl redistribution. Cellular extraction of ^{201}Tl, like potassium, may depend on active, energy-requiring, membrane transport processes. Prolonged post-ischemic derangements in mechanical function, metabolism, cellular volume, and electrolyte regulation have been reported in myocardium "stunned" by even brief periods of ischemia. Acidosis and decreased ATP levels, which result from transient ischemia, could be factors in post-ischemic depression of cellular ^{201}Tl transport and delayed ^{201}Tl redistribution. In patients with coronary artery disease and in experimental animals with regional ischemia, ^{201}Tl redistribution has been demonstrated to occur slowly over several hours to days after

coronary blood flow has been restored [Angello, *et al.*, 1988b Vol 2 Tab 1].

Infusion of adenine nucleotide precursors, such as ribose, have been shown to favorably affect myocardial high energy phosphate stores and function post-ischemia. The effect of such metabolic interventions on ^{201}Tl kinetics had not been previously described prior to the Angello, *et al.*, 1988 publication [Angello, *et al.*, 1988b Vol 2 Tab 1].

In this study seventeen domestic swine were subjected to a 10-min ischemic period followed by either a 30-min i.v. of ribose (n = 8) or saline (n = 9) infusion. Thallium-201 was injected during ischemia and myocardial 201-Tl activity was continuously monitored in ischemic and non-ischemic regions with miniature CdTe radiation detection probes. Coronary flow in the ischemic region was reduced to 25% of that in the non-ischemic regions in both saline and ribose groups. The 201-Tl time-activity curves demonstrated a significant enhancement in the % 201-Tl redistribution in the ribose-treated animals at the end of ribose infusion: ribose (48 ± 110/6), saline (20 ± 40/6), $p < 0.06$. Alteration of 201-Tl kinetics by ribose may permit earlier recognition of 201-Tl myocardial redistribution after transient ischemia [Angello, *et al.*, 1988b Vol 2 Tab 1].

2. Animal Models of Ischemia: the Role of D-Ribose in Recovery and Prevention

Multiple animal models have shown that in both acute and chronic ischemia, a depletion of high energy phosphate levels, such as from adenosine triphosphate (ATP), may be at least partially responsible for the contractile dysfunction seen in ischemic states [Ibel *et al.*, 1986 Vol 8 Tab 89; Mahoney, *et al.*, 1990 Vol 8 Tab 90; Ward, *et al.*, 1984 Vol 7 Tab 67; Mauser, *et al.*, 1985 Vol 8 Tab 91; and St Cyr, *et al.*, 1989 Vol 5 Tab 49]. Depleted myocardial ATP can be resynthesized via two separate metabolic pathways; the de novo biosynthetic pathway and the salvage pathway [Zimmer, *et al.*, 1984 Vol 7 Tab 74]. A key limiting step in both of these pathways is the availability of

PRPP [Ibel *et al.*, 1986 Vol 8 Tab 89; Mahoney, *et al.*, 1990 Vol 8 Tab 90; Ward, *et al.*, 1984 Vol 7 Tab 67; Mauser, *et al.*, 1985 Vol 8 Tab 91; St Cyr, *et al.*, 1989 Vol 5 Tab 49 and Zimmer, *et al.*, 1984 Vol 7 Tab 74]. The salvage pathway is apparently much faster than the de novo pathway, which may take days to weeks to replenish diminished ATP levels provided adequate substrate is available. Several precursors to these biosynthetic pathways have been studied including 5-amino-4-imidazolcarboximide (AICAR), inosine and hypoxanthine, all of which have been shown to moderately increase depleted ATP levels in dysfunctional myocardium [Ibel *et al.*, 1986 Vol 8 Tab 89; Mahoney, *et al.*, 1990 Vol 8 Tab 90; Ward, *et al.*, 1984 Vol 7 Tab 67; Mauser, *et al.*, 1985 Vol 8 Tab 91; St Cyr, *et al.*, 1989 Vol 5 Tab 49 and Sami, *et al.*, 1987 Vol 8 Tab 92].

D-ribose, a pentose sugar, has also been shown to expedite the repletion of high energy phosphate stores in experimental models of ischemia by acting as a precursor in both the de novo and salvage synthetic pathways. In addition, researchers have shown that D-ribose improves functional recovery of myocardium subjected to various ischemic events and possibly increases the ischemic threshold [Pliml, *et al.*, 1992 Vol 5 Tab 45; Pasque, *et al.*, 1982 Vol 5 Tab 41; Sami, *et al.*, 1987 Vol 8 Tab 92 and Clay, *et al.*, 1988 Vol 8 Tab 93]. In several animal models, ribose has been shown to facilitate functional recovery of contractility when compared to placebo [Pasque, *et al.*, 1982 Vol 5 Tab 41; Sami, *et al.*, 1987 Vol 8 Tab 92, and Clay, *et al.*, 1988 Vol 8 Tab 93]. It has been postulated that the mechanism of this functional recovery is related to restoring diminished ATP reserves. The following paragraphs review the publications of several researchers who have investigated the role of D-ribose in animal models of ischemia.

Zimmer and Gerlach studied whether pentoses and pentitols could stimulate myocardial adenine nucleotide biosynthesis [Zimmer, *et al.*, 1978 Vol 7 Tab 72]. In rats, pentoses and pentitols, intravenously injected in a single dose of 100 mg/kg, induced a considerable enhancement of the available pool of 5-phosphoribosyl-1-

pyrophosphate and increased the rate of adenine nucleotide biosynthesis in the heart, but not in liver and kidney. De novo synthesis of adenine nucleotides, which were not detectable in the skeletal muscle of normal rats became measurable after application of ribose. The scientists found that the stimulatory effect of isoproterenol on myocardial adenine nucleotide biosynthesis could be further potentiated by ribose and xylitol, but not by glucose. The isoproterenol-induced decrease of cardiac adenine nucleotide concentrations could be almost completely prevented by repeated administrations of ribose (100 mg/kg). Thus, pentoses and pentitols in combination with β-receptor stimulation markedly and specifically enhanced adenine nucleotide biosynthesis in the rat heart. The results indicated that the increase in the available pool of 5-phosphoribosyl-1-pyrophosphate is an important factor for the enhancement of cardiac adenine nucleotide biosynthesis. Moreover, the availability of 5-phosphoribosyl-1-pyrophosphate and the rate of de novo synthesis of adenine nucleotides in the heart seemed to be limited by the flow through the hexose monophosphate shunt [Zimmer, *et al.*, 1978 Vol 7 Tab 72].

In a similar study, Foker, *et al.*, 1980 discussed adenosine metabolism and myocardial preservation [Foker, *et al.*, 1980 Vol 2 Tab 15]. The authors explained that an important part of the continuing improvement in the results of cardiac surgery has been the progress made in myocardial protection during cardiopulmonary bypass (CPB). Nevertheless, the consequences of myocardial ischemia during the operation remained a significant problem. Although the biochemical changes of myocardial ischemia had received a great deal of attention, the changes which convert reversible myocardial insult to irreversible damage were not known in 1980.

It was well established that the levels of the high energy compounds, adenosine triphosphate (ATP) and creatine phosphate (CP), fall with ischemia and do not recover if the ischemic period is too long. Certainly, cell integrity and function, in turn, are dependent on

adequate ATP levels. Foker and others tested the hypothesis that catabolism of adenosine triphosphate precursors limits the regeneration of this high-energy compound following ischemic insult. Dogs on cardiopulmonary bypass (CPB) had the aorta cross-clamped for 20 minutes under normothermic conditions and 30 minutes later CPB was discontinued. Mean pre-CPB values (μmoles/gm) in left ventricular biopsies for ATP, creatine phosphate (CP), ADP, and AMP were 5.30, 6.18, 1.32 and 0.43, respectively. Adenosine (Ad), inosine, and hypoxanthine/xanthine were not detectable. At the end of cross-clamping, ATP had fallen to 2.80 and CP to 0.57; however, the predicted rise in the levels of ADP, AMP, and Ad was not found. The levels of inosine (1.23) and hypoxanthine/xanthine (0.24) increased, an indication that ADP, AMP, and Ad had been further catabolized. Following release of the cross-clamp, ATP levels did not increase. Even 60 minutes after termination of CPB this level was only 3.07. CP levels rose to 10.2 within 5 minutes after cross-clamp release, indicating high-energy bonds could be formed. EHNA (10 mg/kg), an inhibitor of adenosine deaminase, was used to test the effect of blocking Ad catabolism. At the end of cross-clamping, tissue Ad, previously unmeasurable, was 1.30 μmoles/gm. Nevertheless, ATP levels did not rebound after release of the cross-clamp; the reason was found to be cellular loss, and the coronary sinus blood contained high levels of Ad. Ad (20 mg/kg) infusion alone increased myocardial blood flow five-fold compared to flow in control dogs, but did not alter subsequent ATP levels. Combined EHNA /Ad treatment similarly increased flow but, more importantly, resulted in recovery of ATP levels to 88% of pre-CPB values. The group concluded that ATP regeneration after ischemia was limited by the availability of ADP, AMP, and Ad. Inhibition of Ad catabolism and infusion of Ad would enhance the ATP return from ischemia. Current preservation methods, chiefly hypothermia and cardioplegia, were designed to decrease ATP utilization. Their approach, by providing precursors for ATP recovery, was thought to improve myocardial preservation [Foker, *et al.*, 1980 Vol 2 Tab 15].

Pasque *et al.*, 1982 stated that recovery of myocardial adenosine triphosphate (ATP) following moderate periods of ischemia was dependent upon the availability of adenosine monophosphate (AMP) and diphosphate (ADP) for rephosphorylation. Recovery of AMP and ADP levels following ischemia was, in turn, determined by the rates of salvage and de novo adenine nucleotide synthesis. 5-phosphoribosyl-1-pyrophosphate (PRPP) availability was rate limiting in both salvage and de novo adenine nucleotide synthesis. The authors noted that parenteral ribose infusions in rats had been documented to elevate myocardial PRPP levels with resultant enhancement of adenine nucleotide synthesis. They designed a study to investigate post-ischemic recovery of myocardial function and ATP levels in isolated, working rat hearts given ribose infusions before and after ischemia. These data were compared with recovery in control hearts subjected to the same protocol without ribose administration. The mean percent of functional recovery in control hearts following 15 minutes of warm ischemia reached values of 56.7% ± 4.1%, 63.5% ± 4.3%, 65.9% ± 4.6%, and 70.5% ± 4.7% at 2, 5, 10, and 15 minutes of work following ischemia. Hearts perfused with ribose demonstrated improved mean percent return of function at similar intervals of post-ischemic work with values of 67.9% ± 4.2%, 73.7% ± 3.7%, 81.0% ± 3.5%, and 85.4% ± 3.3%, respectively. Determinations of myocardial ATP levels (μmoles/gm of dry weight) made at the end of 15 minutes of post-ischemic work were significantly higher ($p < 0.02$) in the ribose-treated hearts (18.9 ± 0.7) than in controls (16.3 ± 0.6). Infusion of ribose before and after ischemia is a biochemically logical method of improving post-ischemic myocardial ATP and functional recovery by manipulation of adenine nucleotide synthetic pathways [Pasque *et al.*, 1982 Vol 5 Tab 41].

Ward and others, 1984, looked at recovery of adenine nucleotide levels after global myocardial ischemia in dogs [Ward, *et al.*, 1984 Vol 7 Tab 67]. To determine the long-term dynamics of ATP recovery and evaluate the effect of providing ATP precursors, the team devised a method of obtaining sequential ventricular biopsies in dogs

after 20 minutes of normothermic global ischemia on cardiopulmonary bypass. Their kinetic data showed that adenine was metabolically favored over adenosine to regenerate ATP levels when adequate ribose (R) was present to produce 5-phosphoribosyl-1-pyrophosphate. Therefore adenine (20 mM) plus R (80 mM) or saline (NS) was infused (1.0 ml – min^{-1}) into the right atrium of dogs for 48 hours after ischemia. During adenine infusion myocardial tissue adenine was 0.19 ± 0.07 nmol - mg^{-1}, arterial adenine was 18.3 ± 1.3 μM, coronary sinus adenine was 11.0 ± 1.6, μM, and extraction of adenine by the myocardium was 38% ± 10%. The group found that while the decrease in ATP levels during ischemia was at least 50% in both groups, the post-ischemic ATP recovery rate in A/R dogs was more than eightfold greater than de novo synthesis (2.8 ± 0.59 versus 0.34 ± 0.06 nmoles - mg^{-1}- day). ATP levels in NS dogs were only 54% ± 8% of pre-ischemic values by 48 hours and required 9.9 ± 1.4 days for full recovery. Recovery in A/R dogs required 1.2 ± 0.2 days. The results revealed that ATP recovery after a significant ischemic insult was slow, precursor availability was an important limiting factor in ATP recovery, and recovery time could be greatly shortened with precursor infusion, even when started after the ischemic insult [Ward, *et al.*, 1984 Vol 7 Tab 67].

D-Ribose has been found to be protective in a number of species in addition to humans. For example it is cardioprotective in the rat in a variety of pathophysiological conditions [Zimmer *et al.*, 1984 Vol 7 Tab 74]. The metabolic basis for this effect is the low capacity of the oxidative pentose phosphate pathway in the myocardium. Ribose bypasses this pathway, elevates the available pool of 5-phosphoribosyl-1-pyrophosphate, and thus stimulates the biosynthesis of adenine nucleotides. In a study reported in *Science* by Zimmer *et al.* in 1984 the activity of glucose-6-phosphate dehydrogenase, the first and rate-limiting enzyme of the oxidative pentose phosphate shunt, was very low in the human heart and was of the same order of magnitude in the myocardium of various animal species. Furthermore, ribose was shown to have a similar stimulating effect on myocardial adenine nucleotide biosynthesis in the guinea

pig, in which hemodynamic parameters are different from those in the rat. The authors concluded that the metabolic basis for the effectiveness of ribose is similar in all species.

Zimmer *et al.*, explained that a special metabolic feature of myocardium is the low capacity of the pentose phosphate pathway. In the oxidative branch of this shunt ribose-5-phosphate is produced and converted into 5-phosphoribosyl-1-pyrophosphate, an essential substrate for the synthesis of purine and pyrimidine nucleotides. As a consequence, the rates of these synthetic processes are very low in the myocardium. In searching for the rate-limiting step, Eggleston and Krebs found that glucose-6-phosphate dehydrogenase (G6PDH), the first enzyme of the oxidative pentose phosphate pathway, exerts a tight control in the liver. In the myocardium, the activity of this enzyme is lower than in the liver and in most other organs, and the available pool of 5-phosphoribosyl-1-pyrophosphate is smaller than in the liver and kidney [Zimmer *et al.*, 1984 Vol 7 Tab 74].

The limited pool of 5-phosphoribosyl-1-pyrophosphate and the low rate of adenine nucleotide biosynthesis can be overcome in the heart by administration of supplemental ribose, both in the control state and in various pathophysiological conditions. According to the authors, ribose enhances cardiac adenine nucleotide biosynthesis in catecholamine-treated rats and in animals recovering from oxygen deficiency and experimental surgery [Zimmer *et al.*, 1984 Vol 7 Tab 74].

Table 13 depicts the activities of the first two enzymes of the oxidative pentose phosphate pathway in the myocardium of different species. Heart tissue was homogenized in ice-cold 0.15M KCl containing 8 ml of 0.02M $KHCO_3$ per liter. Centrifugation, dialysis of the supernatant, and measurements of enzyme activities at 25°C were done in accordance with the methods of Glock and McLean, and protein concentration in the dialyzate was determined with the biuret reaction. The human papillary muscles, which were kept in the ice-cold solution, were homogenized within 2 hours after the samples

were obtained during cardiac surgery. When rat hearts were maintained in the same solution for this period of time after excision, enzyme activities were not altered. Values are means ± standard errors.

Table 13
Enzymes of the Oxidative Pentose Phosphate Pathway

Species	Enzyme (units per gram of protein)		
	G6PDH	6-Phosphogluconate dehydrogenase	P <
Guinea Pig	6.0 ± 0.46	11.6 ± 0.36	< 0.0005
Rat	4.3 ± 0.15	11.2 ± 0.25	< 0.0005
Rabbit	3.4 ± 0.77	12.2 ± 1.21	< 0.0005
Dog	1.5 ± 0.19	8.8 ± 0.43	< 0.0005
Calf	1.6 ± 0.35	9.3 ± 0.50	< 0.0005
Monkey	2.4 ± 0.59	5.0 ± 0.56	<0.0250
Human	3.4 ± 0.15	5.9 ± 0.27	< 0.0005

'Determined with Student's t-test for unpaired data

Chatham, *et al.*, studied the protective effect of ribose in myocardial ischemia by using ^{31}P-nuclearmagnetic-resonance spectroscopy [Chatham, *et al.*, 1985 Vol 2 Tab 7]. The authors stated that interventions to prevent nucleotide breakdown during ischemia were complicated by the potentially beneficial effect of adenosine and inosine. The emphasis was, therefore, on the acceleration of post-ischemic adenine nucleotide synthesis. In this context administration of ribose accelerated cardiac adenine nucleotide synthesis [Chatham, *et al.*, 1985 Vol 2 Tab 7].

Adenine nucleotide de novo biosynthesis in the heart occurs at approximately 10nmol/h per gram, which is increased 8-fold by constant ribose infusion. However, it has been reported that ribose (1.7mM) enhanced both ATP and functional recovery after just 15 minutes of re-perfusion following 15 min of ischemia. This suggests that ribose is acting by a mechanism other than stimulation of adenine nucleotide synthesis de novo. Therefore, the investigators

studied the effect of ribose on short-term re-perfusion (up to 3 hours) using ^{31}P-NMR [Chatham, *et al.*, 1985 Vol 2 Tab 7].

Wistar rat hearts were excised and perfused in the Langendorff model with oxygenated Krebs Henseleit bicarbonate buffer containing 11 mM glucose with a pressure of 70 cm of water. Hearts were perfused for more than 70 min before 14 min of total global ischemia (TGI). This was followed by 210 minutes of re-perfusion. There were four different experimental groups (1) no ribose (n = 5); (2) 1.7 mM ribose present only during re-perfusion (n = 7); (3) 1.7 mM ribose present only before ischemia (n=6) and (4) 1.7 mM ribose present throughout (n = 5).

The changes in ATP concentration during ischemia and re-perfusion revealed that at the start of re-perfusion there was little difference between the three groups. After 30 minutes of perfusion, the group with ribose present throughout the experiment exhibited significantly higher levels of ATP than the control group ($P<0.05$). This difference remained relatively constant for the remainder of the re-perfusion period. The addition of ribose during re-perfusion only resulted in ATP levels within one standard deviation of the mean of the control group. It has been shown that the presence of ribose both throughout ischemia and during re-perfusion significantly increases the level of ATP in cardiac tissue after ischemia. As new steady state levels of ATP are established within 30 minutes of re-perfusion it is unlikely that this is due to an increase in de novo synthesis of adenine nucleotides. In the authors' interpretation, the mechanism underlying the action of ribose does not alter the development of acidosis during ischemia, and therefore, appeared to be independent of any effect on anaerobic glycolysis [Chatham, *et al.*, 1985 Vol 2 Tab 7].

The authors speculated that ribose may be functioning in several ways to maintain ATP. In combination with glucose, ribose may improve the energetic state of the heart. Furthermore, ribose can be converted through ribose 5-phosphate to 5-phosphoribosyl-1-pyrophosphate, which may stimulate salvage of hypoxathine and

permit rapid re-synthesis of adenine nucleotides Alternatively, ribose may perform an inhibitory role in the breakdown of adenine nucleotides. Finally, ribose may stabilize cardiac membranes via an osmotic process, and thus prevent washout of components involved in adenine nucleotide maintenance [Chatham, *et al.*, 1985 Vol 2 Tab 7].

Jennings and Steenbergen, 1985, reviewed nucleotide damage involving myocardial ischemia [Jennings, *et al.*, 1985 Vol 3 Tab 33]. They stated that the heart is dependent on aerobic metabolism for energy production and when coronary arterial flow is reduced to the point that insufficient O_2 is available for oxidative phosphorylation, the myocardium becomes ischemic. Although most of the acute biologic changes induced by ischemia occur because of the absolute deficiency of O_2, ischemia cannot be equated with high-flow anoxia or hypoxia, where oxygen deficiency is produced by removing O_2 from the perfusate without reducing the coronary flow rate. The absence of arterial flow during ischemia stops substrate delivery and prevents efflux of metabolic end products. Substrate delivery during high-flow hypoxia can be curtailed by removing substrate from the perfusate, but washout of products of metabolism occurs continuously [Jennings, *et al.*, 1985 Vol 3 Tab 33].

Because the maximum rate of ATP production from anaerobic glycolysis is less than 10% of the rate of the oxidative phosphorylation necessary to sustain cardiac function, the onset of ischemia or anoxia will be associated with a marked imbalance between energy production and energy utilization. The concentration of high-energy phosphate compounds in aerobic myocardium is small compared with the rate of ATP utilization, therefore reserve stores of high-energy phosphates, principally creatine phosphate (CP), are consumed after the onset of anoxia. Contractile work is reduced shortly after aerobic metabolism ceases. As a result, the rate of ATP consumption is slowed, Nevertheless, there is a persistent deficit between ADP phosphorylation and ATP hydrolysis that causes the

net ATP content to decrease as the duration of ischemia or anoxia is prolonged [Jennings, *et al.*, 1985 Vol 3 Tab 33].

Zimmer, 1996, researched and described the regulation of the oxidative pentose phosphate pathway and the metabolism of adenine nucleotide in the rat heart [Zimmer, 1996, Vol 7 Tab 73]. The capacity of the oxidative pentose phosphate pathway (PPP) in the heart has been shown to be limited since the activity of glucose-6-phosphate dehydrogenase (G-6-PD), the first and regulating enzyme of this pathway, is very low. Two mechanisms are involved in the regulation of this pathway. Under normal conditions G-6-PD is inhibited by NADPH. This can be overcome in the isolated perfused rat heart by increasing the oxidized glutathione and by elevating the NADP/NADPH ratio. Besides this rapid control mechanism there is a long-term regulation which involves the synthesis of G-6-PD [Zimmer, 1996, Vol 7 Tab 73].

Under experimental conditions the activity of G-6-PD was elevated in the rat heart during the development of cardiac hypertrophy due to constriction of the abdominal aorta and in the non-ischemic part of the rat heart subsequent to myocardial infarction. The catecholamines, isoproterenol and norepinephrine, were shown to stimulate the activity of myocardial G-6-PD in a time- and dose-dependent manner. The isoproterenol-induced stimulation was cAMP-dependent and resulted from increased new synthesis of enzyme protein. The G-6-PD mRNA was elevated by norepinephrine. As a consequence of the stimulation of the oxidative PPP, the available pool of 5-phosphoribosyl-1-pyrophosphate (PRPP) was expanded. Zimmer described the PRPP as an important precursor substrate for purine and pyrimidine nucleotide synthesis. The limiting step in the oxidative PPP, the G-6-PD reaction, can be bypassed with ribose. This leads to an elevation of the cardiac PRPP pool. In the investigators' laboratory, the decline in ATP that is induced in many pathophysiological conditions was attenuated or even entirely prevented by i.v. infusion of ribose. In two in vivo rat models, the overloaded and catecholamine-stimulated heart and the infarcted

heart, the normalization of the cardiac adenine nucleotide pool by ribose was accompanied by an improvement in global heart function. The combination of ribose with adenine or inosine in isoproterenol-treated rats was more effective in completely restoring the cardiac ATP level within a short period of time than either intervention alone [Zimmer, 1996, Vol 7 Tab 73].

It is typical for the heart to restore its adenine-nucleotide pool very slowly once it has been depleted by a brief period of ischemia. Upon reperfusion it takes several days both in the dog and rat heart until normal ATP levels are regained. This phenomenon is related to the fact that the degradation products of ATP such as adenosine, inosine, and hypoxanthine that are produced during ischemia can permeate the cell membrane, and are subsequently washed out and lost from the heart. Thus they are not available for reutilization via the 'salvage pathways'. The repletion of the ATP pool can therefore be attained only via the de novo synthesis (biosynthesis) of adenine nucleotides. This process, however, is very slow since the available pool of 5-phosphoribosyl-1-pyrophosphate (PRPP), an important precursor substrate for the synthesis of purine and pyrimidine nucleotides, is limited in the heart. PRPP originates from ribose-5-phosphate, which is generated in the oxidative pentose phosphate pathway (PPP). The oxidative PPP is the link between carbohydrate and fatty acid as well as purine and pyrimidine nucleotide metabolism [Zimmer, 1996, Vol 7 Tab 73].

3. D-Ribose in Human Myocardial Recovery

According to Pauly, *et al.*, metabolic support for the heart has been an attractive concept since the pioneering work of Sodi-Pallares *et al.* in the 1960s. Recently, interest has increased in the use of over-the-counter supplements and naturally occurring nutraceuticals for enhancement of cardiac and skeletal muscle performance [Pauly, *et al.*, 2000 Vol 5 Tab 43]. These include amino acids such as creatine, L-carnitine, and L-arginine, as well as vitamins and cofactors such as α-tocopherol and coenzyme Q_{10}. In a similar fashion, D-ribose is a

naturally occurring compound. It is the sugar moiety of ATP and has also received interest as a metabolic supplement for the heart. In this article, the authors reviewed the biochemical basis for using supplemental D-ribose as metabolic support for the heart and discussed the experimental evidence for its benefit [Pauly, *et al.*, 2000 Vol 5 Tab 43].

The general hypothesis was that under certain pathologic cardiac conditions, nucleotides (particularly ATP, ADP, and AMP) are degraded and lost from the heart. The heart's ability to resynthesize ATP is then limited by the supply of D-ribose, which is a necessary component of the adenine nucleotide structure. In support of this hypothesis, recent reports have used D-ribose to increase tolerance to myocardial ischemia. Its use in patients with stable coronary artery disease improves time to exercise-induced angina and electrocardiographic changes. In conjunction with thallium imaging or dobutamine stress echocardiography, D-ribose supplementation has been used to enhance detection of hibernating myocardium [Pauly, *et al.*, 2000 Vol 5 Tab 43].

Medical researchers report that ribose has been shown to be safe with very few side effects when infused in humans at dosages as high as 222 mg/kg/hr for up to five hours [Gross, *et al*, 1989 Vol 3 Tab 21]. Ribose has been shown to transiently lower serum glucose levels in humans both during and for several hours after infusion [Gross, *et al*, 1989 Vol 3 Tab 21 and Segal, *et al.*, 1958 Vol 5 Tab 50]. All subjects have been asymptomatic despite glucose levels as low as 15 mg/100 ml [Segal, *et al.*, 1958 Vol 5 Tab 50]. Ribose infusion has also been shown to decrease serum phosphate levels, again without causing any adverse symptoms or long term sequelae [Segal, *et al.*, 1958 Vol 5 Tab 50]. According to Gross *et al*, 1989, there is no evidence that ribose infusion causes any adverse hemodynamic effects. However, ribose has been shown to cause diarrhea when given as an oral preparation in doses exceeding 200 mg/kg/hr [Gross, *et al*, 1989 Vol 3 Tab 21]. Nonetheless, Gross, *et al*, 1989, concluded that ribose has

been shown to be a safe compound given either intravenously or orally.

In studies of stable coronary artery disease in humans, ribose, when given orally three days prior to treadmill exercise testing, was shown to prolong the exercise time to ischemia as noted on the ECG, when compared to baseline [Pliml, *et al.*, 1992 Vol 5 Tab 45].

H. D-ribose and High Energy Phosphates

Energy compounds are mandatory to maintain cellular integrity and function. There is a perpetual turnover rate of these entities, which must be consistently present to provide the necessary energy levels to maintain a healthy existence. Adenosine triphosphate, a high energy phosphate molecule, provides the fuel for all cellular processes. Various situations can lower ATP levels, which, therefore, can directly compromise function, and viability.

Adenosine triphosphate, the energy currency of the cell, transfers energy from chemical bonds to endergonic (energy absorbing) reactions within the cell. Structurally, ATP consists of the adenine nucleotide (ribose sugar, adenine base, and phosphate group, PO_4^{-2}) plus two other phosphate groups. The following compounds comprise ATP:

1. An adenine base molecule
2. A ribose sugar molecule attached to the adenine on one of the nitrogen atoms of the adenosine heterocyclic structure
3. Three phosphates (PO_4) in a linear sequence with the first phosphate attached to the exterior carbon of ribose (the carbon on the outside of the five member ring for ribose)
   [Lehninger, 1982 Vol 8 Tab 94].

These phosphates are held together by high energy phosphate bonds. The extra energy is as a result of energy released by other biochemical processes occurring within the cell. This energy is stored

in these phosphate bonds, making them high energy. The whole function of ATP is as an "energy storage molecule" where energy is retained until it becomes necessary to release this energy to perform some function within the cell [Lehninger, 1982 Vol 8 Tab 94].

The storage mechanism is as follows:

$ADP + PO_4 + energy \rightarrow ATP + H_2O$

The above process stores the energy and requires a specific enzyme catalyst, ADPase. It is also classified as a "condensation" process since water is given off as a product as a result of the combination of the ADP, the phosphate, and energy.

Another energy route starts with AMP (adenosine mono-phosphate)

$AMP + PO_4 + energy \rightarrow ADP + H_2O$
$ADP + PO_4 + more\ energy \rightarrow ATP + H_2O$

The reverse process will release the energy to perform a biochemical function that requires an input of energy:

$ATP + H_2O \rightarrow ADP + PO_4 + energy$
$ADP + H_2O \rightarrow AMP + PO_4 + energy$

These energy releasing processes require enzymes known as ATPase. These processes are classified as hydrolysis processes since water is involved in the splitting off of the phosphate and the releasing of energy. All of this energy storage and energy releasing activity occurs in the mitochondria of the cell [Lehninger, 1982 Vol 8 Tab 94].

Myocardial ischemia, a condition which decreases blood flow to myocytes, can decrease ATP levels. It has been hypothesized that this lowering of ATP is caused by anoxic inhibition of oxidative

phosphorylation. ATP is eventually degraded to purine base precursors, which can diffuse across the sarcolemmal membrane, and, therefore, are lost for any potential regeneration of ATP. The recovery of this depressed ATP state is dependent on two pathways: the purine nucleotide salvage pathway and the de novo biosynthetic pathway. The salvage pathway, the faster of the two, relies on precursor availability, which may have limitations due to potential low precursor levels. Therefore, when substrate levels are limiting, de novo biosynthesis for energy enhancement plays an important role [Lehninger, 1982 Vol 8 Tab 94].

I. Supplementation of D-Ribose as a Precursor

It has been determined that the recovery of myocardial ATP levels after a moderate period of ischemia is slow, requiring days to approach normal, pre-ischemic levels. This limitation in recovery appears to be due to a lack of availability of the adenine nucleotide precursor, PRPP. This precursor is produced by the phosphorylation of ribose in the (Table 1) hexose monophosphate shunt (HMP) [Zimmer, *et al.*, 1984 Vol 7 Tab 74]. The lack of adequate PRPP levels necessary to provide for optimal energy recovery is likely due to the slow response of the HMP in heart tissue. It has been postulated that the decreased activity of the HMP in the heart is due to the low tissue levels of two required enzymes: glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Ribose enters the HMP at a site past these enzymatic steps, and, therefore, is not limited by HMP activity. Ribose can aid in regenerating ATP molecules through this route [Zimmer, *et al.*, 1984 Vol 7 Tab 74 and Zimmer, *et al.*, 1980 Vol 7 Tab 75].

J. Historical Research Regarding The Ability of D-ribose to Replenish Depressed ATP Levels

Beginning in the 1980s numerous studies began reporting the benefits of replenishing ATP levels with a supplement of D-ribose following ischemia. The heart is unable to rapidly replenish

depressed adenine nucleotides following ischemia. The biosynthesis of these nucleotides is very slow in the normal heart and is increased only moderately during a postischemic recovery state. In 1980, research by Zimmer revealed a marked enhancement in adenine nucleotide biosynthesis in the rat heart when using ribose [Zimmer, *et al.*, 1980 Vol 7 Tab 75]. Zimmer and Ibel, 1980 proposed using ribose in a state of reversible myocardial ischemia in the rat. When 15 minutes of myocardial ischemia was induced in rats, a continuous infusion of ribose during the recovery period produced a restoration of the cardiac ATP pool within 12 hours, whereas 72 hours was required for normalization of ATP levels when no substrate intervention was utilized [Zimmer, *et al.*, 1980 Vol 7 Tab 75]. Similarly, St. Cyr *et al.* reported in 1986 that depressed myocardial ATP levels produced by 20 minutes of global ischemia rebounded to 85% of control levels after 25 hours of a continuous D-ribose infusion [St. Cyr, *et al.*, 1986 Vol 5 Tab 48].

Pasque, *et al.*, 1982 demonstrated that in isolated working rat hearts, D-ribose enhanced myocardial recovery following 15 minutes of warm ischemia. D-Ribose perfused hearts demonstrated improved mean percent return of function when compared to control hearts (not receiving ribose infusion). Myocardial ATP levels measured at the end of 15 minutes of post-ischemic work were significantly higher in the ribose-treated hearts than in controls [Pasque, *et al.*, 1982 Vol 5 Tab 41].

Zimmer, *et al.*, 1983, produced a state of depressed ATP levels, total adenine nucleotides and myocardial function in rats by severely constricting the abdominal aorta and simultaneously injecting isoproterenol. A continuous infusion of ribose for 24 hours stimulated the production of cardiac adenine nucleotides, which prevented the reduction of ATP levels and total adenine nucleotide levels. Furthermore, these ribose treated hearts demonstrated normalization in left ventricular hemodynamic parameters. Zimmer postulated that ribose was a cardioprotective agent [Zimmer, *et al.*, 1983 Vol 7 Tab 71].

K. The Role of D-ribose In Humans With Coronary Artery Disease

Clinical studies in exercising coronary artery disease patients have demonstrated a benefit with supplemental D-ribose. Perlmutter, *et al.* subjected patients to two exercise thallium-201 stress tests. After immediate post-exercise planar imaging, patients were randomized to either a ribose or saline infusion. Additional imaging was performed at 1 and 4 hours post-exercise . Perlmutter, *et al.*, 1991 Vol 5 Tab 44].

Reversible defects were identified by count-profile analysis. Significantly more, reversible thallium-201 defects were identified at 1 and 4 hours post-exercise in the D-ribose treated patients than in the controls. In 13 patients, quantitative analyses using a coronary arteriogram confirmed that the additional reversible defects were in regions of the myocardium served by stenotic arteries. They concluded "that ribose appeared to facilitate thallium-201 redistribution in patients with coronary artery disease and enhanced identification of ischemic myocardium" [Perlmutter, *et al.*, 1991 Vol 5 Tab 44].

Pliml, *et al.*, 1992, investigated the role in which ribose may play in patients with stable coronary artery disease who underwent exercise-induced ischemia. Patients were subjected to two baseline treadmill studies (Bruce protocol) to determine eligibility. To qualify for this study, each patient had to demonstrate two positive baseline treadmill tests, as defined as ischemic ST-segment depression (>1 mm horizontal or down sloping, persisting for at least 80 ms after the J point in the electrocardiogram) and the development of angina during the first 9 minutes of exercise. Following eligibility, patients were randomized into two groups: one receiving oral D-ribose (60 grams/day in four divided doses), or oral glucose (60 grams/day in four divided doses) for three days. On day 5, each patient underwent another exercise treadmill test. The results demonstrated that the D-ribose group of patients had a mean walking time until the

development of 1 mm ST-segment depression significantly greater at day 5 than at baseline. The control group showed no significant change from baseline. The time to the onset of moderate angina was also increased significantly on day 5 from baseline in the D-ribose treated patients. The control group showed an increase in this variable, however this change did not achieve significance. The authors concluded that the addition of oral ribose improved the heart's tolerance to ischemia [Pliml, *et al.*, 1992 Vol 5 Tab 45].

L. Effect of D-Ribose on Human Skeletal Muscle

Similar to results with experimental animals and heart patients, researchers have found comparable results when examining the dynamics of ATP in healthy human subjects. In 1986 M. E. Cheetham reported that after 30 seconds of maximal sprinting, female subjects had decreased muscle glycogen, phosphocreatine, and ATP levels (as determined by biopsy examination) [Cheetham, *et al.*, 1986 Vol 8 Tab 95]. N. McCartney reported similar findings based on research using human male subjects [McCartney, *et al.*, 1986 Vol 8 Tab 96]. After performing four 30 second bouts of maximal isokinetic cycling at 100 rpm, with 4 minute recovery intervals, ATP, glycogen and phosphocreatine muscle tissue levels were greatly reduced in the subjects.

More recently in 1993, Y. Hellsten-Westing, *et al.*, reported that in human male subjects, intermittent exercise caused a reduction in ATP tissue levels [Hellsten-Westing, *et al.*, 1993b Vol 3 Tab 25]. These studies demonstrated that ATP tissue levels could be reduced from exercise.

M. Gross and coworkers investigated the effect of D-ribose, administered orally or intravenously, on healthy volunteers and patients with myoadenylate deaminase deficiency. Intravenous administration rates of ribose at 83, 167 and 222 mg/kg/hr were tolerated well, but an oral dose of 200 mg/kg/hr caused diarrhea. The average steady state serum ribose level was 4.8 mg/100 ml

(oral) and 81.7 mg/100 ml (i.v.). The intestinal absorption rate of oral ribose was 87.8 – 99.8% of the intake at doses up to 200 mg/kg/hr. Serum glucose levels decreased during ribose administration. However, a decrease in concentration down to 15 mg/100 ml without clinical symptoms has been reported [Gross, *et al*, 1989 Vol 3 Tab 21].

In 1991 Gross, *et al* also reported that in nine healthy men and a patient with Myoadenylate Deaminase Deficiency (MADD) that after 30 minutes of bicycle ergometric exercise, the nine men showed significant increases in plasma lactate, ammonia, and hypoxanthine levels and a decrease in serum glucose concentration. When oral ribose was provided at 2 gm/5 min during exercise, their plasma lactate levels increased significantly higher and the prior noted increase in plasma hypoxanthine was no longer significant. The MADD patient showed similar patterns of change in plasma concentrations with exercise, except for plasma hypoxanthine levels which were higher with ribose supplementation [Gross, *et al.*, 1991b Vol 2 Tab 19].

In 1991 D. R. Wagner and coworkers researched the effects of oral ribose administration to three patients with AMP deaminase deficiency. Three grams of ribose was given to subjects orally, every 10 minutes, beginning 1 hour before exercising and continued until the end of the exercise periods. Exercise was performed on a bicycle ergometer. The researchers found that ribose administration did not improve maximum exercise capacity in these three patients, but found that post exercise muscle stiffness and cramps disappeared almost completely in 2 of the 3 patients tested. They concluded that ribose may both serve as an energy source and enhance the synthesis of ATP [Wagner, *et al.*, 1991b Vol 7 Tab 65].

Two key studies have verified the safety and effectiveness of ribose in human subjects. In 1986, N. Zollner found that D-ribose taken orally improved tolerance to exercise in a 55 year old patient suffering from exercise-induced muscle pain and stiffness, due to

primary myoadenylate deaminase deficiency. Over a two-year period the subject took 4 grams of D-ribose before exercising to prevent the symptoms. This dosage was taken every 10 to 30 minutes of exercise. A total dose of 50 to 60 grams per day was tolerated without side-effects [Zollner, *et al.*, 1986 Vol 7 Tab 76].

In 1992, W. Pliml and coworkers reported in the *Lancet*, the results of their research on the effects of ribose on exercise-induced ischemia in stable coronary artery disease. The purpose of the study was to develop a strategy to protect or restore cardiac energy metabolism, which had been impaired by ischemia. The existing scientific evidence suggested that D-ribose stimulated ATP synthesis and improved cardiac function. This information led them to test the notion that D-ribose increased tolerance to myocardial ischemia in male patients with documented severe coronary artery disease. The researchers selected male subjects who took either 60 grams of D-ribose daily (divided into four doses per day) for 3 days, or a placebo (60 grams/day in four divided doses). They found that the ribose test group was more tolerant of a treadmill walking exercise than the control [Pliml, *et al.*, 1992 Vol 5 Tab 45].

M. Supplemental D-Ribose in Animal Models

The authors summarized the use of D-ribose as a supplement in their review. In isolated perfused rat hearts, moderate, periods (10-30 minutes) of ischemia generally resulted in 50% to 70% decreases in myocardial ATP levels. In spite of reperfusion, these levels remain depressed for hours to days before fully recovering. Supplemental D-ribose administration can ameliorate this delay. In one study, infusion of supplemental D-ribose during reperfusion restored ATP pools to normal within 12 hours, whereas 72 hours were required without D-ribose administration. In another study, isolated perfused rat hearts exposed to transient ischemia-reperfusion showed double the recovery of ATP levels when treated with D-ribose. These measurements were made in situ with ^{31}P magnetic resonance spectroscopy. This effect of D-ribose on ATP levels translates into

functional improvement as well. Minute-work in isolated perfused rat hearts exposed to 15 minutes of ischemia and 10 to 15 minutes of reflow reached only 66% to 69% of baseline in control hearts, but reached 89% to 96% in the D-ribose-treated hearts [Pauly, *et al.*, 2000 Vol 5 Tab 43].

Mechanistically, similar results have been obtained in open-chested dog models of ischemia. Following 12 to 30 minutes of ischemia, ATP levels dropped by 43% to 62%, and total adenine nucleotides decreased by 35% to 50%. Despite reperfusion, these levels remained depressed for 1 to 7 days. Their recovery lagged well behind recovery of mitochondrial respiratory function and adenine nucleotide translocase activity. This was consistent with the mechanism of washout of the adenine nucleotide pools and delayed resynthesis of ATP [Pauly, *et al.*, 2000 Vol 5 Tab 43].

Supplemental D-ribose enhanced recovery of ATP levels in the post-ischemic dog heart. Following 20 minutes of global ischemia and 24 hours of reflow ATP levels did not improve in control dogs, but rebounded to 85% of baseline in the D-ribose-treated group. Cardiac function improved as well. D-Ribose infusion following a 15 minute ischemic interval improved contractility to 50% of baseline at 5 minutes and 95% of baseline at 60 minutes. In contrast, saline-treated control dogs had only 10% to 20% recovery of their contractility during the 5- to 60-minute post-ischemic period [Pauly, *et al.*, 2000 Vol 5 Tab 43].

Complete recovery of systolic function following an ischemic insult, however, did not require complete recovery of ATP levels. In fact, complete restoration of ATP levels actually lagged behind normalization of many indices of systolic cardiac function. Oxygen consumption, mitochondrial respiration, phosphocreatine levels, and myocardial contractility all recovered more quickly than ATP levels in several models of transient experimental ischemia. This has led to speculation that the partially restored ATP levels that are present after systolic function had recovered may have consequences on

post-ischemic diastolic cardiac function [Pauly, *et al.*, 2000 Vol 5 Tab 43].

The reviewers concluded that the available data suggested that D-ribose supplementation, either by oral or intravenous administration, may have clinical utility in diverse cardiovascular conditions, including ischemia, hibernation, hypertrophy, and cardiomyopathy. The group noted that the enzymatic pathways necessary to synthesize D-ribose in the intact heart were limited, and multiple lines of investigation indicated that supplemental D-ribose may provide a benefit by increasing the speed of ATP repletion and enhancing functional recovery after injury. Further clinical studies in humans are likely to be forthcoming in this area, which may provide further insight into the role of this compound in improving cardiac energy metabolism in pathologic cardiac conditions [Pauly, *et al.*, 2000 Vol 5 Tab 43].

N. Toxicological Studies

Bioenergy, Inc. has commissioned several classical toxicological studies in order to provide further support for the safety of its specific D-ribose product. These studies are summarized in Table 14. A discussion of each study follows the Table.

Table 14 Classical Non-Human Toxicity Assessments of D-Ribose

Species	Dosage of D-Ribose	Duration	Effect	Reference
Bacterial Reverse Mutation Test with Salmonella typhimurium strains TA 1535, TA 1537, TA 98, TA 100, and Eschericia coli strain WP2 uvrA	62-5000μg/plate in the first assay and 313-5000μg/plate in the second assay		Slight decrease in the mean number of revertant colonies observed in some strains in one test indicated possible toxicity to these strains by the test substance; however, in both the absence and the presence of a metabolic activator, and in all strains, D-ribose did not cause a reproducible increase in the mean number of revertant colonies appearing in the test plates; Investigators concluded that D-ribose was not mutagenic in this study.	van Ommen, 1999 Vol 7 Tab 64

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Table 14 (Continued)
Classical Non-Human Toxicity Assessments of D-Ribose

Species	Dosage of D-Ribose	Duration	Effect	Reference
Chromosomal Aberration Test - Chinese hamster ovary cells, in both the absence and presence of a metabolic activation system (S9-mix); Two independent chromosome aberration assays were conducted.	Based on the toxicity of the test substance to the cells	Maximum exposure times were 18 hours without, and 32 hours with metabolic activation	D-Ribose did not induce a biologically relevant or statistically significant increase in the number of cells with structural chromosome aberrations at any of the concentrations and time points analyzed; it was concluded that D-Ribose was not clastogenic under the conditions used in the study.	De Vogel, 2000b Vol 6 Tab 9
Gene Mutation Test At The TK-locus Of Mouse Lymphoma L5178Y Cells	10 mM D-ribose (highest concentration tested)		D-Ribose did not induce a reproducible significant increase in mutant frequency and there was no dose related increase in mutant frequency in the absence or presence of metabolic activation; D-ribose was not mutagenic under the study conditions.	Steenwinkel, 2000 Vol 6 Tab 57

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Table 14 (Continued)
Classical Non-Human Toxicity Assessments of D-Ribose

Species	Dosage of D-Ribose	Duration	Effect	Reference
Micronucleus Test In Mice	2000mg/kg-bw	One dose via gavage; erythrocytes examined at 24 and 48 hours	The study indicates that treatment with D-ribose did not result in genotoxicity or cytotoxicity to bone marrow cells. The study concluded that D-ribose did not produce micronuclei in polychromatic erythrocytes in either male or female mice.	De Vogel, 2000a Vol 2 Tab 10
Oral Embryotoxicity/Terato-genicity In Wistar Rats	0,5, and 10% of diet; 1 group was fed 10% from 0-3 days of gestation and 20% from days 3-21 of gestation	Daily in feed	No mortalities were observed; No untoward clinical observations; Body weights were reduced in certain test groups; food consumption was decreased in all groups during the first 2 weeks of gestation, and in all weeks in the high dose group; Maximum intake ranged from 8.13-11.61 g D-ribose/kg/bw/day; No significant differences in mean fetal body weight; Wavy ribs and incompletely ossified interparietals were treatment related for the mid and high dose groups; The NOAEL was set at 5% for developmental toxicity, due to impaired nutritional status of the dams.	Wolterbeek, and Waalkens-Berendsen, 200(Vol 7 Tab 69

Table 14 (Continued)
Classical Non-Human Toxicity Assessments of Ribose

Species	Dosage of D-Ribose	Duration	Effect	Reference
Sub-Chronic (13 Week) Oral Toxicity Study in Wistar Rats	5, 10 and 20% of diet; Highest intake was 15.0 g/kg/bw in males and 15.7 g/kg bw in females	Daily in feed	No treatment related mortalities were observed; Several changes, mainly in the mid- and/or high-dose group, e.g., decreased body weights accompanied by transiently lower food consumption and food efficiency, increased water intake paralleled by increased urinary volume and decreased urinary density, decreased glucose excretion, decreased sodium excretion, decreased urinary pH, changes in certain clinical chemistry variables decreased weight of the thymus and increased relative weights of the kidneys, liver, spleen and caecum; The investigator speculated that it was likely that the high amounts of D-ribose fed in this study were not completely absorbed and that unabsorbed D-ribose was fermented by the gut flora; A number of the above changes were attributed to unspecific effects that are generally observed in rodent feeding studies with incompletely absorbed yet fermentable carbohydrates. The NOAEL was set at less than 5% of the diet (4.0 g/kg/bw average).	Jonker, 2000 Vol 4 Tab 34

1. Bacterial Reverse Mutation Test with D-Ribose

D-Ribose was examined for mutagenic activity in a bacterial reverse mutation test using the histidine-requiring Salmonella typhimurium strains TA 1535, TA 1537, TA 98 and TA 100, the tryptophan-requiring Escherichia coli strain WP2 uvrA. A metabolic activator (Aroclor 1254) derived from a liver fraction was used to induce rates for metabolic activation (S9-mix) [van Ommen, 1999 Vol 7 Tab 64].

Two tests were performed with all strains in both the absence and the presence of S9-mix. The test substance was dissolved in water, and negative controls (solvent) and positive controls were run simultaneously with the test substance. Five different concentrations of the test substance were used per test, ranging from 62 – 5,000 µg/plate in the first assay and 313 – 5,000 µg/plate in the second assay.

A slight decrease in the mean number of revertant colonies observed in some strains in the second test indicated possible toxicity to these strains by the test substance D-Ribose. However, D-Ribose did not cause a reproducible increase in the mean number of revertant colonies appearing in the test plates compared to the negative control. This was the case across all strains both in the presence and absence of the metabolic activator system [van Ommen, 1999 Vol 7 Tab 64].

The positive controls gave the expected increase in the number of his^+ and trp^+ revertants both with and without the S9-mix. The study investigator concluded that the test substance D-Ribose was not mutagenic under the conditions employed in the study [van Ommen, 1999 Vol 7 Tab 64].

2. Chromosomal Aberration Test With D-Ribose In Cultured Chinese Hamster Ovary Cells

D-Ribose was examined for its potential to induce structural chromosome aberrations in Chinese hamster ovary cells, in both the absence and presence of a metabolic activation system (S9-mix) [De Vogel, 2000b Vol 2 Tab 9].

D-ribose was dissolved in Ham's F-12 medium prior to testing, and two independent chromosome aberration assays were conducted. In the first assay, in the absence of S9-mix, the treatment/harvest times were 18/18 hours and 4/18 hours and in the presence of S9-mix the treatment/harvest times were 4/18 hours. In the second assay, in the absence of S9-mix, the treatment/harvest times were 18/18 and 32/32 hours and in the presence of S9-mix, the treatment/harvest times were 4/18 and 4/32 hours. The highest concentration tested was based on the toxicity of the test substance to the cells.

D-Ribose did not induce a biologically relevant and statistically significant increase in the number of cells with structural chromosome aberrations at any of the concentrations and time points analyzed, when compared to the vehicle control values [De Vogel, 2000b Vol 2 Tab 9].

The positive control substances mitomycin C, which was used in the absence of the S9-mix, and cyclophosphamide, used in the presence of the S9-mix induced the expected increases in the incidence of structural chromosome aberrations [De Vogel, 2000b Vol 2 Tab 9]. The investigator concluded that D-Ribose was not clastogenic under the conditions used in the study [De Vogel, 2000b Vol 2 Tab 9].

3. Gene Mutation Test At The TK-locus of L5178Y Cells With D-Ribose

D-Ribose was tested for its potential to induce gene mutations at the TK-locus of cultured mouse lymphoma L5178Y cells, in both the absence and the presence of a metabolic activation system (S9-mix). The material was dissolved in the culture medium without serum prior to testing, and two assays were subsequently conducted [Steenwinkel, 2000 Vol 6 Tab 57].

Methyl methanesulphonate (MMS) and 3-methylcholanthrene (MCA) were used as positive control substances in the absence and in the presence of the S9-mix, respectively. Culture medium without serum served as the negative control. The treatment with the positive controls yielded the expected significant increase in mutant frequency compared to the negative controls.

In the absence or presence of S9-Mix, D-Ribose did not induce a significant reproducible increase in mutant frequency. Also no dose related increase in mutant frequency was observed. D-Ribose was not cytotoxic to the L5178Y cells both in the absence and in the presence of S9-mix. The highest concentration tested and evaluated was 10 mM D-Ribose. It was concluded that under the conditions used in this study D-Ribose was not mutagenic at the TK-locus of mouse lymphoma L5178Y cells [Steenwinkel, 2000 Vol 6 Tab 57].

4. Micronucleus Test With D-Ribose In Mice

D-Ribose was examined in a bone marrow micronucleus test in both male and female mice. 10 male mice and 10 female mice were treated once by gavage with D-Ribose (2,000 mg/kg-bw), after a fasting period of 2 hours. The negative control group, consisting of 10 males and 10 females, were treated in a similar way with the vehicle only (maize-oil). A positive control group, consisting of 5 males, was given a single intraperitoneal injection with the mutagen mitomycin C (0.75 mg/kg-bw) [De Vogel, 2000a Vol 2 Tab 10].

At 24 hours after treatment, 10 animals of the negative control group (5/sex), 10 animals of the test group (5/sex) and all 5 animals (5 males) of the positive control group were sacrificed. At 48 hours after treatment, 10 animals of the negative control group (5/sex) and 10 animals of the test group (5/sex) were sacrificed [De Vogel, 2000a Vol 2 Tab 10].

The number of PE per 1,000 erythrocytes (E) in male and female mice treated with the test substance D-Ribose did not differ significantly at either time point (24h and 48H) analyzed when compared with the negative controls. This indicated that treatment with D-Ribose did not result in cytotoxicity to bone marrow cells of male and female mice [De Vogel, 2000a Vol 2 Tab 10].

The incidences of micronucleated polychromatic erythrocytes (MPE) per 1,000 polychromatic erythrocytes (PE) in male and female mice, treated with D-Ribose, were not statistically significantly higher than those found in the negative controls. Therefore, the study indicated that treatment with the test substance D-Ribose did not result in genotoxicity to bone marrow cells [De Vogel, 2000a Vol 2 Tab 10].

The sensitivity of the test system was demonstrated by the fact that the positive control group differed significantly from the negative control [De Vogel, 2000a Vol 2 Tab 10].

It was concluded that the test substance D-Ribose did not induce micronuclei in polychromatic erythrocytes in either male or female mice under the conditions used in the study [De Vogel, 2000a Vol 2 Tab 10].

5. Thirteen (13) Week Sub-Chronic Study

Bioenergy, Inc. commissioned TNO Nutrition and Food Research (TNO), The Netherlands, to conduct a thirteen-week sub-chronic toxicology study in rats [Jonker, 2000 Vol 4 Tab 34]. The objective of

the study was to examine the sub-chronic oral toxicity of D-ribose. This substance was administered in the diet for 13 consecutive weeks to groups of 20 male and 20 female Wistar rats at dietary levels of 5, 10 or 20%. A control group consisting of 20 rats per sex was kept on a diet containing 20% potato starch, but no D-ribose.

Clinical observations, neurobehavioral observations, growth, food and water consumption, food conversion efficiency, ophthalmoscopy, hematology, clinical chemistry, urinalysis, organ weights, gross examination at necropsy and microscopic examination of a large number of organs and tissues were used as criteria for disclosing possible harmful effects [Jonker, 2000 Vol 4 Tab 34].

The feasibility of feeding the dose levels listed above was examined in a 14-day range-finding study [Jonker, 2000 Vol 4 Tab 34]. The overall mean daily intake of D-ribose in the low-, mid- and high-dose group was, respectively, 3.6, 7.6 and 15.0 g/kg body weight in males and 4.4, 8.5 and 15.7g/kg body weight in females.

This 13-week study was conducted with albino rats. The rat was selected because this species is considered suitable for this type of study [Jonker, 2000 Vol 4 Tab 34]. Eighty five male and 85 female Wistar rats (Crl:(WI) WU BR) were obtained from a colony maintained under SPF-conditions at Charles River Deutschland, Sulzfeld, Germany. The animals arrived on 27 October 1999 when they were about three weeks old.

The study was comprised of four groups of 20 males and 20 females each. The treatment period was started on 8 November 1999 (nominal day 0) and terminated with the necropsy of the rats on 7-9 February (males) and 9-11 February (females) 2000. Table 15 shows the experimental protocol group codes, color codes, dietary levels of D-ribose/potato starch, and numbers of animals.

Table 15
Experimental Protocol

Group Code	Color Code	Dietary supplement[1] (%, w/w)		Number of rats M/F
		D-ribose[2]	Potato Starch[3]	
A (control)	white	0	20	20/20
B (low-dose)	blue	5	15	20/20
C (mid-dose)	green	10	10	20/20
D (high-dose)	red	20[4]	0[4]	20/20

[1]Basal diet was a modified RM3 diet.

[2]The feasibility of these dietary levels was examined in a 14-day range-finding study. The details on the conduct and results of this study are presented in Vol 5 Tab 46 [Jonker, 2000 Vol 4 Tab 34].

[3]Pregelatinized potato starch, Paselli WA-4.

[4]To adapt the rats to high dietary levels of D-ribose (to lessen initial growth retardation) all rats of the high-dose group were fed a diet containing 10% D-ribose (and 10% potato starch) during the first three days of the treatment period. Thereafter they were kept on a diet containing 20% D-ribose.

The D-ribose used in the study was supplied by Bioenergy Inc. Table 16 below lists the characteristics of the substance as received by TNO.

Table 16
Characteristics of Bioenergy D-Ribose

CAS Reg. number	50-69-1
Batch number	B00599-13
Appearance	white to yellow crystalline powder
Purity	98%+
Molecular weight	150.13
Molecular formula	$C_5H_{10}O_5$
TNO internal reference numbers	990254 (sample received 9-14-1999); 990315 (sample received 11-2-1999)
Packaging	Plastic bag
Quantity received (net weight)	4 x 10 kg (sample received 9-14-1999); 2 x 10 kg (sample received 11-2-1999)
Storage Conditions (Temperature)	Ambient
Expiry date	1 September 2001

The test substance was administered at constant concentrations in the diet 7 days per week, for 13 consecutive weeks. This route was used to simulate human consumption. Potato starch was added to the basal diet (modified RM3 diet) at a level of 20% (w/w) and the test substance was incorporated at the expense of potato starch. The experimental diets were prepared by mixing in a mechanical blender. Immediately after mixing, each diet was divided into portions sufficient for one day (in plastic bags) and stored in a freezer (< -18°C). The experimental diets were replaced daily by a fresh portion from the freezer.

Results showed that there were no treatment-related mortalities. The daily clinical observations revealed yellow fur on the abdomen of all animals of the mid-dose group (after the 12th week) and on the high-dose group (after the 8th week). Mean body weights were approximately 10% lower in the high-dose group and were also reduced, although to a lesser extent (< 5%) in the low- and mid-dose groups. The growth depression was most obvious at initiation of treatment [Jonker, 2000 Vol 4 Tab 34].

During the first week of treatment, food consumption decreased by about 25% (in comparison to the control group) in the high-dose group and to a lesser extent in the low- and mid-dose groups. Over the course of the study, mean food consumption in the test and control groups was comparable. Food conversion efficiency was reduced in mid-dose females and high-dose rats of both sexes in the first week of the study and in the mid- and high dose-males in week 12.

Water consumption increased in all test groups. The increases were dose-dependent and reached approximately 40-50% at the high-dose level.

Hematology results revealed that absolute and relative neutrophil counts increased, and that the relative lymphocyte count in the high-dose males decreased. Clinical chemistry values showed a number of changes. High-dose rats of both sexes experienced increased alkaline phosphatase activity. Increased aspartate aminotransferase activity was also found in the mid- and high-dose females, but this effect was not dose-related. The investigators noted a reduction in the total protein in high-dose rats of both sexes, and decreased albumin in high-dose males. Mid-dose females and high-dose rats of both sexes showed an increase in the ratio of albumin to globulin, and high-dose males were lower in cholesterol, triglycerides and phospholipids [Jonker, 2000 Vol 4 Tab 34].

Changes were also noted in quantitative urinary analysis. The alterations occurred in non-fasted and fasted rats, and water was available free choice. The high-dose group, and to a lesser extent the low and mid-dose groups, produced an increase volume (about 2-fold) of urine, although the difference was not statistically significant at the lower dosage levels. A decrease in urine density was noted in the high-dose group and in the low-dose males. In general the low- and mid dose groups also had urine of lower density (density was measured in fasted rats only), although again this difference was not statistically different from the controls. A decrease in urinary pH was observed in non-fasted mid- and high-dose rats of both sexes and in fasted high-dose females. Reduced glucose excretion was detected in the high-dose group, especially in fasted rats, and in non-fasted mid-dose females. Low and mid-dose groups also showed reduced glucose excretion, however in those instances it was not statistically different from the control [Jonker, 2000 Vol 4 Tab 34].

Sodium excretion was also reduced in high-dose females. However, the investigator stated that sodium was measured only in fasted rats. Microscopic observations of urine showed fewer crystals in the urinary sediment in males of all test groups and in high-dose females [Jonker, 2000 Vol 4 Tab 34].

Relative organ weights showed the following changes:

- Kidney: increased weight (by 6-7%) in males of all test groups.
- Thymus: decreased weight (by about 10-20%) in males of all test groups.
- Spleen: increased weight in males of all test groups (9-18%, dose-dependent) and mid-and high-dose females (9%).
- Liver: increased weight in mid-dose males (10%) and low-, mid- and high-dose females (17, 27 and 14%, respectively).
- Caecum: increased weight (full and empty) in all test groups; dose-dependent, up to approximately 2-fold in the high-dose group.

No abnormalities attributable to the ingestion of D-ribose were revealed by gross or histopathological examination, and ophthalmoscopic examination reveled no treatment-related ocular changes [Jonker, 2000 Vol 4 Tab 34]. The investigators found no changes indicative of neurotoxic effects from the consumption of D-ribose.

The author explained that the feeding of D-ribose resulted in several changes, mainly in the mid- and/or high-dose group, e.g., decreased body weights accompanied by transiently lower food consumption and food efficiency, increased water intake paralleled by increased urinary volume and reduced urinary density, reduced glucose excretion, reduced sodium excretion, reduced urinary pH, changes in clinical chemistry variables (increased alkaline phosphatase and aspartate aminotransferase activity, decreased plasma levels of proteins and lipids), decreased weight of the thymus and increased relative weights of the kidneys, liver, spleen and caecum.

While data on the intestinal absorption of D-ribose in rats has not been available, the investigator hypothesized that it was likely that the high amounts of D-ribose fed in this study were not completely absorbed and that unabsorbed D-ribose was fermented by the gut flora. Hence, a number of the above changes (e.g. growth reduction,

decreased urinary pH, increased plasma alkaline phosphatase activity and increased caecum weight) might represent non-specific effects that are generally observed in rodent feeding studies with incompletely absorbed yet fermentable carbohydrates (Newberne *et al.*, 1988; Bär *et al.*, 1995). Also increases in water intake and urinary volume in response to the feeding of high doses of a specific polyol have been reported and discussed previously [Lina, 1996 Vol 8 Tab 99].

The investigator stated that although some of the above effects might represent non-specific effects related to the feeding of incompletely absorbed carbohydrates, other effects found in the present study could not be related to this phenomenon. Some of these effects (e.g., a tendency toward reduced glucose excretion, reduced thymus weight and increased kidney, liver and spleen weights) were still observed at the lowest dose level. It was noted that these findings were not accompanied by any histopathological changes and that their toxicological relevance might be in doubt. However, the author stated that they could not be completely disregarded. Therefore, it was concluded that under the conditions of this study the No-Observed-Effect Level (NOEL) of D-ribose was lower than 5% in the diet (equivalent to 3.6 and 4.4 g/kg body weight/day in males and females, respectively) [Jonker, 2000 Vol 4 Tab 34].

Further research by the manufacturer revealed that two issues may be relevant to the results discussed above. First, according to the manufacturer, Bioenergy, Inc., the yellow fur noted by the investigator is consistent with the rats rolling in their spilled feed, as D-ribose turns yellow upon exposure to moisture. More importantly, a study published in *Psychological Reports* reported on the results of taste preference studies in mice and rats. The authors noted that rats were immediately adverse to the taste of D-ribose, and that a related sugar, xylose was toxic to some rodents, including rats. A simple dislike of D-ribose as a food substance may account for lack of weight gain at the higher levels fed in this study [Wagner, 1971 Vol 8 Tab 100].

6. Oral Embryotoxicity/Teratogenicity Study With D-Ribose In Rats

D-Ribose was fed in the diet to mated female Wistar rats (28 animals per dose group) from days 0-21 of gestation at concentrations of 0, 5 and 10%. Furthermore, one group was fed D-Ribose at 10% from gestation days 0-3 and at 20% from gestation days 3-21. On gestation day 21 the dams were killed and macroscopically examined. Fetuses, placentas, reproductive organs and the liver and full empty caecum were weighed and fetuses were examined after Caesarian Section. The viscera of the fetuses of the control and high-dose groups and the skeletons of the fetuses of all groups were examined [Wolterbeek, *et al.*, 2000 Vol 7 Tab 69].

The test substance was homogeneously distributed in the diets, and the content of the test substance measured in the diets prepared for this study was close to intended at all dose levels. The diets were stable upon storage at room temperature for 1 day and in the freezer (<-18°C) for 5 weeks.

Daily clinical observations during the gestation period did not reveal any remarkable findings in the animals' appearance, general condition or behavior among the treatment or control groups. No mortalities were observed [Wolterbeek, *et al.*, 2000 Vol 7 Tab 69].

For all dose groups 28 females were mated. At gestation day 21 (day of Caesarian section), 26, 25, 26, and 23 females in the control, low-, mid- and high-dose group were pregnant (in the low-and mid-dose groups one dam delivered just before Caesarian section). There were no statistically significant differences between the control group and the groups fed D-Ribose in the number of corpora lutea, implantations, live and dead fetuses and early and late resorptions nor in the pre- and post-implantation loss. Furthermore, no effect was observed on sex ratio. No remarkable differences in gravid and empty uterus weight, ovary weight, carcass weight and net weight

change (body weight gain from day 0 to day 21 of gestation minus gravid uterine weight) were observed between the control group and the groups fed D-Ribose.

Mean body weights of the pregnant females of the mid- and high-dose groups were statistically significantly lower on gestation days 7 and 14. Weekly body weight gains were lower in the mid- and high-dose groups during the first week, and all treatment groups were lower during the second week of gestation. Body weight increased during the last week of gestation in the mid- and high dose groups when compared with the control group [Wolterbeek, *et al.*, 2000 Vol 7 Tab 69].

Food consumption was lower in all treatment groups during the first two weeks of gestation and in the high-dose group during the last week of gestation. D-ribose intake during the gestation period ranged from 3.64 - 4.61, 6.99 – 8.68, and from 8.13 – 11.61 g D-ribose/kg/bw/day for the low-, mid- and high-dose group, respectively [Wolterbeek, *et al.*, 2000 Vol 7 Tab 69].

External observations of the fetuses and placentas on Caesarian section did not reveal any remarkable findings that could be related to treatment. Furthermore, no significant differences in the mean fetal body weight and placenta weights were observed between the control group and the groups fed D-Ribose.

Absolute and relative weights of the full and empty caecum were statistically significantly, dose dependently increased in all groups fed D-Ribose. There was no treatment related effect on the weight of the liver.

Upon fetal examination there were no treatment related changes in fetal soft tissues. Skeletal examination revealed various variations in skeletal ossification in the D-Ribose-treated rats, which were considered to be not related to D-Ribose treatment. The statistically significant increases in the incidences of fetuses showing wavy ribs

and incompletely ossified inter parietals in the mid- and high-dose groups were considered to be related to D-Ribose treatment [Wolterbeek, *et al.*, 2000 Vol 7 Tab 69].

In order to clarify the incidence of fetuses with wavy ribs, and retarded interparietal ossification the Expert Panel requested the Opinion of Dr. Frank Sullivan, of the United Kingdom, a noted Expert in the area of toxicological studies. Dr. Sullivan presented the following Expert Opinion [Sullivan, 2001 Expert Opinion, Appendix I]:

1. "I am surprised that such high doses were used since we normally do not recommend higher than 5% in the diet. This could cause malnutrition though of course there are many other causes of reduced bodyweight. An important question is whether the fetal bodyweight was also reduced. If so, then it would be normal to expect reduced ossification. The skull bones are late to ossify so retarded interparietal ossification is not regarded as very important and would almost certainly disappear (or rather would fully ossify) within the next few days. Wavy ribs is a not uncommon variant, seen for example with beta-blockers and uterotonics, and can also disappear after birth, by weaning. Not regarded as very important. Increase in variants is a common finding in studies and is often dose related. Unless very marked and widespread, it is not usually regarded as very important (mainly because it is so common that lots of things would be affected if we did) however, some people do regard them as a sign of disturbed gene control and so might cause effects in people with a specific genetic tendency. Reduced food intake can cause a variety of defects, especially in mice and rabbits, but rats are more resistant.

2. Poor nutrition can cause many effects including malformations since so many vitamins and minerals are necessary for good reproduction.

3. Variations are not considered adverse since they are so common, often 90% of control fetuses show one or more variations (so what does 'normal' mean?). e.g. rats can have 12, 13, 14 or 15 pairs of ribs and the percentages of each number vary with strains.

4. When do variations become terata? When does a short finger become a malformation? How long is a piece of string? Most people define major malformations as 'sufficient to interfere with the life of the baby', and minor malformations are less than this."

For developmental toxicity, the No Observed Adverse Effect Level (NOAEL) of dietary D-Ribose is 5% (w/w). At this dose level, maternal effects on body weights, food consumption and caecum weights were observed. Most probably, these effects were manifestations of an impaired nutritional status of the dams due to the feeding of relative high doses of the low-digestible test substance [Wolterbeek, *et al.*, 2000 Vol 7 Tab 69].

Wagner found that certain 5-carbon sugars are avoided by rodent species [Wagner, 1971, Vol 8 Tab 100]. The relatively large amount of D-ribose fed here, up to 10% - 20% of the diet, may have compromised the nutritional status of the animals in this study.

O. Humans and D-ribose

In order to establish the safety of long-term consumption in healthy adults, Bioenergy, Inc. sponsored four studies, which were designed to assess the biochemical, metabolic and toxicological effects of D-ribose. Two of the studies investigated the effects of 20 grams per day of D-ribose in non-fasted subjects, who consumed the D-ribose in two 10-gram portions with meals. The second set of studies investigated the effect of 0-10 grams of D-ribose dissolved in 250 ml of water on resting subjects, who had been fasting for 12 hours. All

the studies were carried out at the Human Performance Laboratory, St. Cloud State University, St. Cloud, Minnesota. The studies examined:

1. The hematological and hepatological effects of 14 days of D-ribose consumption
2. The effects of gender on the toxicology of 14 days of D-ribose consumption
3. The effects of D-ribose ingestion on blood and urine components, following a 12-hour fast, while at rest
4. The effects of D-ribose ingestion on carbohydrate and purine metabolism following a 12-hour fast.

The paragraphs below describe the results of the studies.

1. The Toxicological Effects of 14 Days of D-Ribose Consumption

Nineteen healthy subjects completed a study in which 20 grams of D-ribose was fed per day for 14 consecutive days [Seifert, *et al.,* 2001 Vol 5 Tab 54]. The subjects (8 female and 11 male) were given 10 grams of D-ribose and 10 grams at dinner. The subjects were allowed to range in age from 20 to 65 years, and to consume a normal diet. Before each test day, subjects were instructed to fast after 10:00 P.M.

Blood samples were collected during seated rest on days 0, 7, and 14. The blood was analyzed for triglycerides, gamma glutamyltransferase (γGT), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin, creatinine, uric acid, red blood cells, white blood cells, platelets, hematocrit, and hemoglobin.

The results of the study are presented in Table 17. The investigators concluded that ingestion of 20 grams of D-ribose /day over a 14 day period did not change the hematological or hepatological variables tested [Seifert, *et al.,* 2001 Vol 5 Tab 54].

However, it was noted that uric acid increased by day 7, but returned to baseline by day 14.

Table 17
Effect of 20 grams per day of D-ribose consumption on Human Volunteers

Variable	Day 0 (Mean)	Day 14 (Mean)
Hemoglobin (g/L)	14.3±0.9	14.3±0.1
Hematocrit (%)	42.2±0.3	42.4±0.3
Red Blood Cells (Mil)	4.69±0.03	4.68±0.03
White Blood Cells (K/μL)	6.12±0.2	6.00±0.2
Platelet Count (K/μL)	235.4±3.2	238.8±3.2
ALP (U/L)	66.9±0.9	69.2±1.0
γGT (U/L)	21.2±1.4	20.3±1.4
ALT (U/L)	24.5±1.5	24.3±1.5
AST (U/L)	16.9±1.5	20.6±1.5
Uric Acid (mM/L)	340.8±8.3 (Day 7)	324.9±8.3

2. The Effects of Gender on the Toxicology 14 Days of D-Ribose Consumption

Frehlich, *et al.*, 2001, compared the biochemical response of the male and female subjects in the study described above [Frelich, *et al.*, 2001 Vol 5 Tab 51]. Again, the subjects consumed 20 grams of D-Ribose per day divided into two 10 gram portions. The investigators concluded that the females in the study exhibited lower values than the males for most of the variables tested, however, there was no significant interaction of gender with the length of time that D-Ribose was consumed [Frehlich, *et al.*, 2001 Vol 5 Tab 51]. The results of this study are presented in Table 18.

Table 18
Effect of 20 grams per day of D-ribose consumption on Human Volunteers; Comparison of Female and Male Subjects

Variable	♀ Day 14 (Mean)	♂ Day 14 (Mean)
Hemoglobin (g/L)	13.4±0.8	15.2±0.06
Hematocrit (%)	39.8±0.2	45.0±0.2
Red Blood Cells (Mil)	4.5±0.03	4.9±0.02
Platelet Count (K/μL)	233.0±3.0	241.0±2.3
ALP (U/L)	65.2±0.9	70.4±0.7
γGT (U/L)	15.0±1.2	25.2±1.0
ALT (U/L)	20.1±1.4	28.5±1.1
AST (U/L)	15.9±1.3	23.6±1.0
Uric Acid (mM/L)	290.0±7.6	357.0±5.8

3. The Effects of D-Ribose Ingestion on Blood and Urine Components, While at Rest

Seifert, *et al.,* 1999 reported on the results of a double-blind counterbalanced study designed to determine the effects of D-ribose ingestion on oxidation and clearance rates in healthy subjects during seated rest, following a 12-hour fast [Seifert, *et al.,* 1999 Vol 5 Tab 53]. Subjects consumed either a placebo, or 2, 5, or 10 grams of D-ribose in 250 ml of distilled water. Ten non-diabetic subjects participated in this study. Six of the subjects were aged 20-30 years of age, and four subjects ranged in age from 40 to 50 years. No alcoholic or caffeine containing beverages were allowed 24 hours prior to treatment.

During the performance of the study, all subjects were instructed to empty their bladder, prior to the insertion of a venous catheter into the forearm. Blood samples (10 ml each) were drawn at 0, 15, 30, 45, 60, 120, 180, and 240 minutes after ingestion. A sodium chloride solution was used to maintain catheter volume

[Seifert, *et al.,* 1999 Vol 5 Tab 53]. Treatments were repeated at one week intervals.

Blood samples were analyzed for uric acid, lactic acid, and glucose. A 24-hour urine collection was used to analyze for oxalate, and carbohydrate oxidation was estimated from oxygen uptake, and respiratory exchange ratio values using a Medgraphics analyzer [Seifert, *et al.,* 1999 Vol 5 Tab 53].

Results for uric acid showed that there was no statistical difference associated with treatment and time, or treatment and age. However, mean uric acid levels for the 2 gram treatment was statistically less than for the 5 or 10 gram levels. Table 19 shows the mean effect for the 10 subjects, as compared to the non-caloric placebo.

Table 19
Effect of Various Quantities of Ingested D-ribose on Uric Acid Concentration in 10 Healthy Subjects (mg/dL)

	Time (Min.)				
D-Ribose Intake (g)	0	30	60	120	180
0	5.4	5.78	5.08	5.36	4.7
2	5.13	5.18	5.03	5.18	4.46
5	5.65	5.55	5.56	6.12	4.69
10	5.39	5.54	5.62	5.42	4.73

The investigators found no statistical differences in insulin response to D-ribose consumption, and no differences were observed between treatment means (p= 0.49) [Seifert, *et al.,* 1999 Vol 5 Tab 53]. The mean insulin values for the controls, 2, 5, and 10 gram D-ribose groups were 2.57±.1, 2.68±.1, 2.77±.11, and 2.77±.11 μIU/ml, respectively. Interestingly, there were also no statistical differences observed between age groups. The mean insulin concentration for the young group was 2.51±.12, while the

mean insulin concentration for the older group was 2.88±.14 μIU/ml.

An identical pattern was observed for oxalate values. No differences were observed between treatment means (p= 0.85), and there were no treatment x time interactions (p=0.2) [Seifert, *et al.,* 1999 Vol 5 Tab 53]. The mean oxalate concentrations for the controls, 2, 5, and 10 gram D-ribose groups were 0.76±.13, 0.71±.1, 0.87±.13, and 0.76± .13 mM/24 hours, respectively. Again, there were also no statistical differences observed between age groups. The mean oxalate concentration for the young group was 0.89±.08, while the mean insulin concentration for the older group was 0.66±.09 mM/24 hours.

However, there was a significant interaction with time for blood glucose response for all subjects (p=0.000) [Seifert, *et al.,* 1999 Vol 5 Tab 53]. Mean glucose levels were also different by age. The older group maintained a higher mean glucose level compared to the younger group (p=0.001). Mean glucose levels for the younger and older groups were 3.05±.06 and 3.57±.07 mM/L respectively. Table 20 presents the effects of D-ribose intake on glucose response for the 10 subjects.

No significant differences were observed between treatment means (p= 0.09), and there were no treatment x time interactions (p=0.98) for lactic acid [Seifert, *et al.,* 1999 Vol 5 Tab 53]. The mean lactic acid concentrations for the controls, 2, 5, and 10 gram D-ribose groups were 0.93±.06, 1.01±.06, 1.04±.06, and 1.17±.06 mM/L, respectively. With lactic acid, the younger group exhibited a higher mean lactic acid concentration (1.13±.04 mM/L) than the older group (0.95±.05 mM/L).

Table 20
Effect of Various Quantities of Ingested D-ribose on Blood Glucose Concentration in 10 Healthy Subjects (mM/L)

D-Ribose Intake (g)	Time (Min.)					
	0	15	30	45	60	120
0	3.61	3.29	3.3	3.35	3.36	3.47
2	3.6	3.62	3.41	3.63	3.58	3.61
5	3.54	3.34	2.99	3.0	3.13	3.49
10	3.57	3.36	2.83	2.38	2.48	3.47

Likewise, no significant differences were observed between treatment means (p= 0.56), and there were no treatment x time interactions (p=0.48) for estimated carbohydrate oxidation [Seifert, *et al.,* 1999 Vol 5 Tab 53]. The mean oxidation values for the controls, 2, 5, and 10 gram D-ribose groups were 0.89±.08, 0.87±.09, 0.91±.08, and 1.02±.08 kcal/min, respectively. No differences were found between age groups for carbohydrate oxidation (p=0.25). The younger group exhibited a mean value of 1.05±.12 kcal/min than the older group 0.80±.16 kcal/min.

The purpose of the metabolic studies by Seifert, *et al.,* 1999 and Fenstad, *et al.,* 1999 was to determine the effects of one-time ribose ingestion on carbohydrate and purine metabolism [Seifert, *et al.,* 1999 Vol 5 Tab 53 and Fenstad, *et al.,* 1999 Vol 2 Tab 12]. The studies also investigated the influence of age on different biochemical responses and carbohydrate oxidation.

The authors explained that during strenuous exercise adenosine triphosphate (ATP) degrades to adenosine diphosphate (ADP), and adenosine monophosphate (AMP) [Seifert, *et al.,* 1999 Vol 5 Tab 53 and Fenstad, *et al.,* 1999 Vol 2 Tab 12]. The AMP moiety is further catabolized. These metabolites may be either pooled for ATP resynthesis, or they may be further catabolized, eventually

forming uric acid, which is the end product of purine metabolism. De novo synthesis can initiate ATP resynthesis. However, according to the authors, this process is slow and energy consuming, costing up to 8 ATP molecules for each adenine that is produced. The current hypothesis is that D-ribose may increase the rate of ATP resynthesis by increasing the rate of de novo synthesis using the pentose phosphate pathway [Seifert, *et al.,* 1999 Vol 5 Tab 53 and Fenstad, *et al.,* 1999 Vol 2 Tab 12].

Thus, the physiological changes associated with D-ribose administration seem to differ from those of glucose, because of the effect of the five carbon sugar on the purine nucleotide cycle, the pentose phosphate pathway, and de novo synthesis Seifert, *et al.,* 1999 Vol 5 Tab 53 and Fenstad, *et al.,* 1999 Vol 2 Tab 12]. In contrast, the administration of glucose to healthy populations plays a role in supplementing glycogen stores.

While most of the variables tested showed no change with the consumption of D-ribose, significant effects on blood glucose levels were noted with high single doses following fasting. Other authors have also noted drops in blood glucose following high doses of D-ribose. This phenomenon had not been previously explained. Therefore, Seifert, *et al.,* 1999 examined the rate of carbohydrate oxidation as a means of explaining the effect of D-ribose on blood glucose. In this study D-ribose administration (10g) resulted in a significant glycemic decline (35% from baseline). However, subjects receiving either 2g or 5g of D-ribose appeared to maintain blood glucose throughout the trial, leading the investigators to speculate that the optimal dose might lie between 2g and 5g in order to avoid a glycemic decline [Seifert, *et al.,* 1999 Vol 5 Tab 53 and Fenstad, *et al.,* 1999 Vol 2 Tab 12].

The authors explained that D-ribose administration did not elicit a significant insulinemic rise over time. This study may not have reached the threshold for an insulin response, as found in other studies where the administration of higher doses of D-ribose have

shown insulinemic effects. This study also showed no difference in the estimated rate of carbohydrate oxidation. Thus, according to the authors, the hypoglycemic effect of 10g of D-ribose did not result from an increase in the rate at which glucose was oxidized. Red blood cells, kidney cells, and brain cells possess glucose transporters (predominantly GLUT-1 and GLUT-5) that do not require insulin for glucose uptake. Thus, the investigators speculated that it was possible that cellular uptake within these tissues increases with ribose ingestion and may account for the noted glycemic drop with the 10g dose [Seifert, *et al.,* 1999 Vol 5 Tab 53 and Fenstad, *et al.,* 1999 Vol 2 Tab 12].

Other possible explanations would require further study, including: an increase in the rate of fatty acid synthesis, an increase in amino acid synthesis, or conversion to glycogen. The inhibition of glycogenolytic enzymes observed may explain why the liver did not compensate for the glycemic decline [Seifert, *et al.,* 1999 Vol 5 Tab 53 and Fenstad, *et al.,* 1999 Vol 2 Tab 12].

Although no significant treatment related differences were found with carbohydrate oxidation (Coxr), it appeared that a dose threshold might exist. In the 5g and 10g treatments, carbohydrate oxidation rates peaked at 45-min. In the placebo and the 2g treatment, rates decreased to 45-min and then steadily approached or exceeded the baseline measurements. The authors speculated that no difference was seen with Coxr due to the high variability between subjects and the small sampling size (n=10). Therefore, future studies should focus on higher sampling groups when assessing Coxr effects.

Other researchers have examined peripheral glucose utilization with ribose administration to determine the fate of blood glucose. As a product of glycolysis, lactic acid (LA) has often been measured to assess glycolytic activity. If the decline in blood glucose resulted from the increased glycolysis and Kreb's Cycle

activity, one would expect an increase in lactic acid [Seifert, *et al.*, 1999 Vol 5 Tab 53 and Fenstad, *et al.*, 1999 Vol 2 Tab 12].

In the current study, ribose supplementation did not result in any significant lactic acid change for the different treatments. Similar studies have reported non-significant changes in LA, pyruvic acid, and inorganic phosphate. However, these investigations have not looked at age differences with ribose ingestion and LA production. Between age groups, it should be noted that LA in the young subjects increased while LA in the older subjects decreased. The minute individual variations observed were accounted for by the investigators, using instrumentation error, since the precision of the instrument was one tenth of a millimole, which made the smallest changes difficult to interpret. Therefore, increased glycolytic activity in this study cannot explain the glycemic decline.

Contrary to what other researchers have reported with ribose ingestion, uric acid increased in this study. Although the study did not measure adenine levels, the authors suspected declines in adenine concentration with ribose ingestion. They explained that when ribose binds to adenine, adenosine is formed. Excess ribose results in higher adenosine concentrations. An increase in reagent (adenosine) will result in an increase in the end product (uric acid).

The authors concluded that their studies demonstrated that D-ribose ingestion did not influence LA production and Coxr, and that the optimal single dose may lie between 2g and 5g, since the 10g dose resulted in a hypoglycemic drop and increased uric acid concentration. However, further studies using a larger subject population might answer whether carbohydrate supplementation along with ribose would maintain blood glucose and, determine if ribose is indeed an adenine nucleotide scavenger during and after intense exercise, and establish the optimal doses for adenine nucleotide salvage.

P. Summary

The No Observed Adverse Effect Level (NOAEL) of dietary D-Ribose (5% (w/w)) found in the animal studies discussed above provides a margin of safety for human consumption. In humans 5% of the diet would be equivalent to approximately 60 grams per day, well below the recommended maximum daily consumption of ribose suggested herein, and equal to that given to humans in the 1992 study by Pliml, *et al.* [Enns, *et al.*, 1997 Vol 7 Tab 78 and Pliml, *et al.*, 1992 Vol 5 Tab 45].

Other authors cited in this Notification, also reported that humans have a high tolerance for D-ribose. Gross and Zollner, 1991, administered from 83.3 to 222.2 mg/kg of D-ribose per hour to 9 healthy subjects [Gross *et al.*, 1991 Vol 2 Tab 20]. A dose of 166.7 mg/kg of D-ribose per hour was administered orally to 8 of the subjects for a minimum of 4 hours. Assuming a 60 kg person, this dosage is equivalent to 10.0 grams per hour for 5 hours (50 grams). No adverse effects were reported, and the authors stated that doses equal to or less than 200 mg/kg/hr were not associated with side effects [Gross *et al.*, 1991 Vol 2 Tab 20].

Pliml, *et al.*, 1992, studied the effects of D-ribose administration to patients with coronary artery disease. These subjects received 60 grams of D-ribose daily for 3 days. The doses were divided into four 15 gram portions during the day [Pliml, *et al.*, 1992 Vol 5 Tab 45]. A second study by the same group concluded that ribose plasma levels associated with a high dose of (3.5 mM) oral administration did not inhibit human lymphocyte proliferation *in vitro* [Pliml, *et al.*, 1993 Vol 5 Tab 46].

Goodman *et al.*, 1970, writing in *Metabolism* administered 0.5g of D-ribose/kg body weight as a 7.5% solution orally over a 35-minute period (equal to 51.5 grams per hour for a 60 kg person) to 10 healthy subjects [Goodman *et al.*, 1970 Vol 2 Tab 18]. No adverse effects were noted.

Seifert, *et al.,* 1999 studied nineteen healthy subjects who received 20 grams of D-ribose per day for 14 consecutive days [Seifert, *et al.,* 1999 Vol 5 Tab 52 and Seifert, *et al.,* 2001 Vol 5 Tab 54]. The subjects (8 female and 11 male) were given 10 grams of D-ribose at breakfast and 10 grams at dinner. The investigators concluded that ingestion of 20 grams of D-ribose /day over a 14 day period did not change the hematological or hepatological variables tested [Seifert, *et al.,* 1999 Vol 5 Tab 52 and Seifert, *et al.,* 2001 Vol 5 Tab 54]. It was noted, however, that uric acid increased by day 7, but returned to baseline by day 14. In another study, involving the same research group, fasted subjects tolerated up to 5 grams of D-ribose as a single bolus without a change in blood chemistries, including blood glucose concentration, while 10 grams produced a drop in blood glucose. The authors speculated, based on these results that D-ribose may be utilized to increase the supply of nucleotides in the body, rather than to increase or supplement the supply of glycogen, as is the case with glucose [Seifert, *et al.,* 1999 Vol 5 Tab 53 and Fenstad, *et al.,* 1999 Vol 2 Tab 12].

These results suggest that humans may be able to tolerate D-ribose at much higher levels than other species such as rodents [Wagner, 1971 Vol 8 Tab 100]. Bioenergy, Inc. has recommended that ribose, when used as a dietary supplement, be administered in doses of 2-3 grams every 4 hours before exercise, during each 4 hours of exercise, and 4 hours following exercise to a maximum of 20 grams/day. This level is well within the range of the results discussed above.

Further, the D-ribose products produced by Bioenergy, Inc. are derived from fermentation with a non-pathogenic, non-sporulating *Bacillus* species. The recovery of D-ribose from the fermentation medium and its subsequent purification and packaging is conducted under current Good Manufacturing Practice conditions. A crystallization step during manufacturing further assures that no incidental contaminants are present. Documentation of lots, storage

of retainer samples, and a written recall program ensure that product can be tracked and recalled if necessary.

Based on its own sponsored research, review of the literature, and its product specifications Bioenergy, Inc. submits that D-ribose can be considered generally recognized as safe, when manufactured and used according to current Good Manufacturing Practice.

Expert Opinion

000196

Bioenergy, Inc. D-Ribose Expert Panel Opinion

Introduction

The undersigned, an independent panel of recognized experts (Expert Panel), qualified by their scientific medical training and relevant national and international experience to evaluate the safety of food and food ingredients, was convened by Bioenergy, Inc. to determine the Generally Recognized As Safe (GRAS) status of Bioenergy D-ribose for use as a food ingredient. The resumes of each Panelist are on file at the company's headquarters in Ham Lake, Minnesota. The qualifications of the Panel satisfy the requirements set forth in the Federal Food, Drug, and Cosmetic Act's definition of generally recognized as safe (GRAS) substances (§ 201(s)) and 21 CFR 170.30(a) "Eligibility for classification as generally recognized as safe (GRAS)".

A comprehensive search of the scientific literature for information on the safety/toxicity of D-ribose and Bioenergy D-ribose through September 2001 was undertaken by Lee Dexter and Associates and was made available to the Expert Panel. The members of the Expert Panel independently evaluated information provided by Bioenergy and other materials deemed appropriate or necessary. Following this independent and critical evaluation, the Expert Panel conferred by telephone with representatives of Bioenergy, Inc., who presented the pertinent safety and functionality information associated with Bioenergy D-ribose. The information critically evaluated by the Panel was compiled into a GRAS Report, which supports the eligibility of Bioenergy D-ribose as a GRAS ingredient in accordance with proposed regulation 21 CFR 170.36. The information provided for the Panel's review was presented in the form stipulated in 21 CFR 170.35. The Panel unanimously agreed to the GRAS status of Bioenergy D-ribose as a food ingredient when produced and used in accordance with current Good Manufacturing Practices and meeting the specifications described herein.

Notice of GRAS exemption claim:

Bioenergy D-ribose is exempt from premarket approval requirements of the Federal Food, Drug and Cosmetic Act because it has been determined to be generally recognized as safe (GRAS) under conditions of intended use by experts qualified by scientific training and experience.

A Summary of the Basis of the GRAS Determination

Chemical Identity and Manufacturing

- D-ribose (α-D-ribofuranose) is a naturally occurring 5-carbon sugar, that is associated with most living organisms.
- D-ribose is classified as an aldopentose, since it contains an aldehyde group.
- Ribose (in the form of ribose-5-phosphate) is formed in the body from the conversion of glucose via a metabolic system known as the pentose phosphate pathway (PPP) [Lehninger, 1982 Vol 8 Tab 94].
- Ribose-5-phosphate (R-5-P) is an intermediate that is utilized in a number of physiological reactions to:

    1. Form PRPP
    2. Generate ATP
    3. Synthesize nucleotides and nucleosides
    4. Regenerate glucose via the PPP
       [Hiatt, 1957 Vol 3 Tab 28, Segal, *et al.,* 1958 Vol 5 Tab 50, Bloom, *et al.,* 1954 Vol 2 Tab 5 and Coffey, *et al.,* 1965 Vol 2 Tab 8].

- Ribose-5-phosphate is the building block for purine and pyrimidine synthesis.
- The Bioenergy D-ribose crystalline product is a reducing monosaccharide sugar produced by fermentation of a starch-derived carbohydrate.
- The D-ribose product is composed of ≥ 97% D-ribose, ≤ 2.0% moisture, and ≤ 0.2% ash.
- The Bioenergy production process results in D-ribose, which contains low levels of chloride, sulfate, heavy metals, and arsenic. The average lead level measured over four (4) lots was less than 0.1 ppm.
- Bioenergy D-ribose may be produced as undried syrup. The syrup is a clear syrupy liquid with a pale yellow – pale yellowish-brown appearance. The solids content of the syrup is 62 – 74% on a weight/weight basis. The minimum ribose content, as measured by HPLC is 92.0%, on a dry basis.

- Final product specifications for Bioenergy D-ribose are shown below:

FINAL PRODUCT SPECIFICATIONS OF BIOENERGY D-RIBOSE

Food Grade Chemical Specifications	
D-ribose	97.0 – 103.0%
Loss on Drying (Moisture)	≤ 2.0 %
Ash	≤ 0.2%
Lead	≤ 0.1 ppm
Microbiological Specifications	
Aerobic Plate Count	≤ 100 CFU/g
Salmonella sp.	Negative/125g
Coliforms	≤ 10 CFU/g
Yeast & Mold	≤ 100 CFU/g
Product Form	
Consistency	Powder or Granular
Color	White to Slightly Yellow
Flavor	Slightly Sweet

FINAL PRODUCT SPECIFICATIONS OF BIOENERGY D-RIBOSE SYRUP

Food Grade Chemical Specifications	
Solids	62 – 74% (w/w)
D-ribose	≥ 92.0%
pH	3.5 – 6.0
Specific Gravity	1.28 – 1.35 [d] [20]
Lead	< 0.1 ppm
Arsenic	< 1.0 ppm
Product Form	
Consistency	Syrup
Color	Pale Yellow – Yellowish Brown
Microbiological Specifications	
Aerobic Plate Count	≤ 100 CFU/g
Salmonella sp.	Negative/125g
Coliforms	≤ 10 CFU/g
Yeast & Mold	≤ 100 CFU/g

- In the mid-1970s Takeda Chemical Industries of Osaka, Japan filed two patents describing the production of D-ribose via fermentation from a D-ribose producing strain of the genus *Bacillus* [US Patent 3,919,046, 1975 Vol 8 Tab 105 and US Patent 3,970,522, 1976 Vol 8 Tab 106]. The same basic method is used with modifications to produce Bioenergy, Inc. D-ribose.

- The Bioenergy, Inc. process involves the cultivation of a D-ribose producing microorganism of the genus *Bacillus*, whose transketolase activity is very low. These organisms are non-pathogenic and are non-sporulating.

- When transketolase is limiting, certain strains accumulate D-ribose, which can then be recovered from the culture broth [US Patent 3,919,046, 1975 Vol 8 Tab 105].

- The cultural conditions for D-ribose production include a controlled temperature of 25 - 40°C, a preferred pH level of 5.5 - 8, and a cultivation time of between 36 - 120 hours [US Patent 3,919,046, 1975 Vol 8 Tab 105]. After incubation, the fermentation broth can be harvested by a conventional method for the recovery of D-ribose.

- All medium ingredients are food grade, or are used per food cGMP guidelines.

- The cells of the producing strain are removed through filtering or centrifugation. The filtrate or supernatant is then purified, decolorized and desalted by treatment with successive steps using activated carbon, ion exchange or similar purification techniques. These purification steps are followed by concentration through evaporation, or other appropriate means. An organic solvent, such as ethanol, is then added to facilitate crystallization. The crystals are recovered by centrifugation, and evaporation, and are finally, dried, milled, and packaged [US Patent 4,904,587, 1990 Vol 8 Tab 107].

- The manufacturers of D-ribose for Bioenergy, Inc. have developed a system of critical process controls for the manufacture of D-ribose. These controls assure that the product will consistently meet the company's specifications.

- Bioenergy has provided standardized analytical methods for the testing of its D-ribose product. These procedures assure that the product is tested consistently against specification parameters.

Use and Functionality

- The use of D-ribose as a supplement to human nutrition has been investigated for several decades [Quraishi, *et al.*, 1999 Vol 5 Tab 47].

- Bioenergy, Inc. and its associates have developed food formulations in which D-ribose may be utilized across a wide range of food categories.

- Bioenergy, Inc. D-ribose is a multiple-use direct food additive.

- The technical effects of D-ribose are among those codified at 21 CFR § 170.3 (o) (1 - 32), including:

  (11) "Flavor enhancers": Substances added to supplement, enhance, or modify the original taste and/or aroma of a food, without imparting a characteristic taste or aroma of its own.
  (16) "Humectants": Hygroscopic substances incorporated in food to promote retention of moisture, including moisture-retention agents and antidusting agents.
  (20) "Nutritional supplement": Substances added to food to supplement the diet, or increase the nutritional value of a food.
  (21) "Nutritive sweeteners": Substances having greater than 2 percent of the caloric value of sucrose per equivalent unit of sweetening capacity.
  (28)"Stabilizers and thickeners": Substances used to produce viscous solutions or dispersions, to impart body, improve consistency, or stabilize emulsions, including suspending and bodying agents, setting agents, jellying agents, and bulking agents, etc.
  (31) "Synergists": Substances used to act or react with another food ingredient to produce a total effect different or greater than the sum of the effects produced by the individual ingredients.
  (32) "Texturizers": Substances, which affect the appearance or feel of the food.

- The estimated mean and 90th percentile exposure levels for Bioenergy D-ribose, given conservative assumptions, are 24.98 grams and 49.96 grams, respectively.

- The use of Bioenergy D-ribose as a dietary supplement with a recommended dosage of 20 grams per day has also been noted separately.

- Data was presented, which showed that if D-ribose was substituted for 25% of all sugars added to the diet, consumption would equal 20 grams/day, with a 90th percentile of 40 grams/day. The company stated that these levels would represent the maximum consumption that could be expected from even the most brand loyal consumer.

- Nutritional analysis of 7 lots of Bioenergy's D-ribose showed a minimum carbohydrate content of 99% [Bioenergy Raw Data, 1999-2000 Vol 7 Tab 80].

- Total calories derived from D-ribose range from 4.5 - 5.0 kcal/g.

- D-ribose is not available in the free form in foods.

- The average level of D-ribose in human blood is 1.6 mg/100ml [Burke, 1981 Vol 8 Tab 97]. Blood levels for D-ribose range from zero to 3.6 mg/100ml.

- Research has shown that approximately 3 to 5 grams of D-ribose taken daily will provide a moderate level in the blood stream from which compounds in the pentose phosphate pathway can be produced.

Safety Studies

Biochemical Studies

- D-ribose occurs in nearly every living organism. While it is formed in the body from glucose, humans are most likely to encounter D-ribose in red meats or organ meats. Therefore, small quantities of D-ribose have been a continuous part of the human diet.

- Detailed biochemical studies showing the fate of D-ribose in the digestive tract after oral administration and following intravenous injection were published nearly 60 years ago [Naitô, 1942 Vol 5 Tab 38].

- In 1954 Bloom, *et al.,* established that ribose was involved in energy cycling in the liver [Bloom, *et al.*, 1954 Vol 2 Tab 5].

- In 1957 Hiatt described glycogen formation via the pentose phosphate pathway in mice. The author concluded that D-ribose appeared to be as efficient a precursor of mouse liver glycogen as glucose under the conditions of his study [Hiatt, 1957 Vol 3 Tab 28].

Metabolism in Man

- The classic research on the metabolism of D-ribose in man was carried out by Stanton Segal and Joseph Foley from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md. in 1958 [Segal, *et al.*, 1958 Vol 5 Tab 50]. The authors stated that the pentose, D-ribose, in the

form of the ester, ribose-5-phosphate, had been shown to be an important intermediate in the pentose phosphate pathway of glucose metabolism.

- Intravenously administered D-ribose was found to clear the bloodstream in an entirely linear fashion when 3 gm amounts were injected. Doses larger than 3 gm produced a lag phase before the onset of a linear disappearance [Segal, *et al.*, 1958 Vol 5 Tab 50].

- In normal subjects the rate constant for blood clearance ranged from 3.8 to 5.8 per cent per minute after a 20 gm dose. As the dose decreased, the rate constant increased to as high as 13.8%/min for 3 gm [Segal, *et al.*, 1958 Vol 5 Tab 50].

- An average of 21 per cent of a D-ribose dose was excreted in urine as an orcinol reactive material by a normal subject after a 15 minute ribose infusion. Nearly all the D-ribose disappeared following a 90-minute postinfusion period [Segal, *et al.*, 1958 Vol 5 Tab 50].

- Data on a subject who received various ribose doses suggested that urinary excretion decreased as the dose was diminished. When 20 gm of ribose was given at a constant slow rate, only 5 to 12 per cent was found in the urine.

- A total of 10 per cent of C^{14} ribose administered to subjects in tracer dosages was found in urine, whereas 40 per cent of a 5 μc dose given as a load (on 20 gm) was excreted. During the first hour after injection, about four times as much label tracer appeared in urine following the larger dose. The authors stated that this difference probably explained the greater rate constant for C^{14} disappearance from blood after the 20 gm dose [Segal, *et al.*, 1958 Vol 5 Tab 50].

- Segal, *et al.*, 1958 concluded that the kinetics of the disappearance of D-ribose from blood differ from those of D-glucose or D-xylose by being dose dependent. Their kinetic data indicated that the system for clearing ribose from blood is saturated after the infusion of small amounts of the sugar [Segal, *et al.*, 1958 Vol 5 Tab 50]. Furthermore, a C^{14} ribose experiment in a diabetic showed that the

ribose not excreted in urine was primarily converted to glucose by the sequence of reactions of the pentose phosphate pathway [Segal, *et al.*, 1958 Vol 5 Tab 50].

Exercise in Man

- The activity of the pentose phosphate pathway in normal skeletal muscle has been found to be low. Consequently, the formation of ribose-5-phosphate by this pathway is thought to set an upper limit on the de novo synthesis of nucleosides and bases. According to Tullson, *et al.,* 1991a, this limitation can be overcome via ribose supplementation, which leads to increased provision of 5-phosphoribosyl-1-pyrophosphate (PRPP) and stimulation of de novo synthesis *in vitro* and *in vivo* skeletal muscle [Tullson, *et al.,* 1991a Vol 6 Tab 62].

- Gross, *et al.,* studied D-ribose administration during-exercise and its effects on end products of energy metabolism in healthy subjects and a patient with myoadenylate deaminase deficiency [Gross, *et al.,* 1991b Vol 2 Tab 19]. Previous studies had demonstrated an almost complete absorption of orally administered ribose. Ribose given to healthy subjects at rest was shown to lower serum glucose levels as much as 25%.

- Gross, *et al.,* stated that ribose administration was not associated with side effects in doses under 200 mg/kg per hour [Gross, *et al.*, 1991b Vol 2 Tab 19].

- Nine healthy men and a patient with myoadenylate deaminase deficiency were exercised on a bicycle ergometer (30 minutes, 125 Watts) with and without oral ribose administration at a dose of 2 g every 5 minutes of exercise. Plasma or serum levels of glucose, free fatty acids, lactate, ammonia and hypoxanthine and urinary hypoxanthine excretion were determined. After 30 minutes of exercise without ribose, the healthy subjects showed significant increases in plasma lactate ($p<0.05$), ammonia ($p<0.01$) and hypoxanthine ($p<0.05$) concentrations and a decrease in serum glucose concentration ($p<0.05$). When ribose was administered, the plasma lactate concentration increased significantly ($p < 0.05$) and the increase in plasma hypoxanthine concentration was no longer significant. A patient with myoadenylate deaminase deficiency

showed the same pattern of changes in serum or plasma concentrations with exercise with the exception of hypoxanthine in plasma, which increased when ribose was administered [Gross, *et al.*, 1991b Vol 2 Tab 19].

- When ribose was given, the increase in plasma hypoxanthine concentration was no longer significant, and the increase in plasma lactate concentrations was significantly higher.

- D.R. Wagner, *et al.* investigated the effects of oral ribose on muscle metabolism during bicycle ergometer in three AMPD-deficient patients [Wagner, *et al.,* 1991a Vol 7 Tab 65]. The subjects performed exercise on a bicycle ergometer with increasing workload with and without administration of D-ribose (3 g orally every 10 min, beginning 1 hour before exercise until the end). The patients performed exercise until their heart rate was 200 minus age. Maximum capacity was not increased by administration of ribose, but post-exertional muscle stiffness and cramps disappeared almost completely in 2 of 3 AMPD-deficient patients. Plasma concentrations of lactate and inosine were increased in AMPD-deficient patients after oral administration of ribose. The researchers concluded that ribose may both serve as an energy source and enhance the de novo synthesis of purine nucleotides [Wagner, *et al.,* 1991b Vol 7 Tab 65].

- Zollner *et al.,* first demonstrated that the oral administration of high doses of ribose might be a successful therapy in AMPD deficiency. Gross *et al.* found that orally administered ribose was 88-100% absorbed intestinally in both healthy subjects and AMPD-deficient patients. Since the studies of Segal and Foley (1958) it was known that after absorption ribose is converted either to glucose via the pentose phosphate pathway or to nucleotides via ribose-5-P and PRPP. As the response of ribose to insulin is small, it may be metabolized more rapidly than glucose [Wagner, *et al.,* 1991a Vol 7 Tab 65].

Blood Glucose Levels

- Fenstad, *et al.,* designed a blinded counterbalanced study to investigate the effects of ribose ingestion on blood glucose (BG), blood lactate (LA), and estimated glucose oxidation rates. Six non-diabetic subjects (24-30 years old)

ingested either 0 (PL), 2, 5, or 10 g of D-ribose in 300 ml of water following a 12 h fast. Data were collected over a 2-hr period. Results showed that no significant difference was seen for LA between treatments. A significant difference (p=0.00) was observed between treatments for BG. Mean BG concentrations- for PL, 2, 5, and 10 g were 3.98 (.17), 3.38 (.07), 3.08 (.23), and 2.77 (.5) mmol/L, respectively. Two sample t-tests revealed significant differences between 0 and 2 g and 2 and 10 g treatments. No significant differences were seen for carbohydrate oxidation rates between treatments. The researchers concluded that, ingestion of 2 and 5 g of ribose maintained blood glucose over 120 minutes, however, there was a strong trend (p= 0.07) for oxidation rates to be greater with 5 g and 10 g than with 0 g and 2 g [Fenstad, *et al.,* 2000 Vol 2 Tab 12].

- Goodman and Goetz, 1970, studied the effects of administration routes of oral and intravenous D-ribose (0.5 gm/kg body weight as a 7.5% solution) and plasma insulin in healthy humans. Following oral administration ribose concentration for the 10 subjects was not measurable until a 15-minute sample was taken. Results showed a steady rise to a peak level of about 30-mg/per 100 ml of blood at 60 minutes. Despite a prolonged rise in blood ribose, the mean insulin level was first observed to rise slightly at 15 minutes, and then peak at 20 minutes at levels approximately double base line values. Blood insulin concentrations, then fell rapidly. The authors showed that insulin levels following orally administered D-ribose took longer to reach a peak, than when D-ribose was administered intravenously.

- In a study by Steinberg, *et al.*, nine normal subjects received D-ribose intravenously at a rate of 750 mg/min for 20 minutes and 3 normal subjects received a similar infusion at the same rate for a 5-minute period. D-ribose, 7 ½ per cent in water, was infused for 20 minutes in 9 subjects and for 5 minutes in 3 subjects. Blood specimens were obtained at frequent intervals for 105 minutes following the beginning of the infusion. Infusions resulted in a total dose of 15 and 3.75 gm of D-ribose, respectively. Although similar transient elevations in concentration of serum immuno-reactive insulin occurred with all infusions, hypoglycemia appeared only with the 15 gm infusion. When the serum insulin concentration was subsequently elevated to similar levels as those attained

during the D-ribose infusions in 2 subjects by the intravenous injection of crystalline insulin, no hypoglycemia occurred [Steinberg, *et al.*, 1967 Vol 6 Tab 58].

Cardiac Metabolism

- D-Ribose has been found to be cardioprotective in a number of species including humans [Zimmer *et al.*, 1984 Vol 7 Tab 74]. Supplemental ribose bypasses the low capacity of the oxidative pentose phosphate pathway in the myocardium, elevates the available pool of 5-phosphoribosyl-1-pyrophosphate, and thus stimulates the biosynthesis of adenine nucleotides.

- The limited pool of 5-phosphoribosyl-1-pyrophosphate and the low rate of adenine nucleotide biosynthesis in the heart can be overcome by administration of supplemental ribose, both in the control-state and in various pathophysiological conditions. According to Zimmer *et al.*, ribose enhances cardiac adenine nucleotide biosynthesis in catecholamine-treated rats and in animals recovering from oxygen deficiency and experimental surgery [Zimmer *et al.*, 1984 Vol 7 Tab 74].

Toxicological Studies

- Bioenergy, Inc. has commissioned several classical toxicological studies in order to provide further support for the safety of its specific D-ribose product. These studies are summarized in a table presented below.

Classical Non-Human Toxicity Assessments of D-Ribose

Species	Dosage of D-Ribose	Duration	Effect	Reference
Bacterial Reverse Mutation Test with Salmonella typhimurium strains TA 1535, TA 1537, TA 98, TA 100, and Eschericia coli strain WP2 uvrA	62-5000μg/plate in the first assay and 313-5000μg/plate in the second assay		Slight decrease in the mean number of revertant colonies observed in some strains in one test indicated possible toxicity to these strains by the test substance; however, in both the absence and the presence of a metabolic activator, and in all strains, D-ribose did not cause a reproducible increase in the mean number of revertant colonies appearing in the test plates; Investigators concluded that D-ribose was not mutagenic in this study.	van Ommen, 1999 Vol 7 Tab 64

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Classical Non-Human Toxicity Assessments of D-Ribose (Continued)

Species	Dosage of D-Ribose	Duration	Effect	Reference
Chromosomal Aberration Test - Chinese hamster ovary cells, in both the absence and presence of a metabolic activation system (S9-mix); Two independent chromosome aberration assays were conducted.	Based on the toxicity of the test substance to the cells	Maximum exposure times were 18 hours without, and 32 hours with metabolic activation	D-Ribose did not induce a biologically relevant or statistically significant increase in the number of cells with structural chromosome aberrations at any of the concentrations and time points analyzed; it was concluded that D-Ribose was not clastogenic under the conditions used in the study.	De Vogel, 2000b Vol 6 Tab 9
Gene Mutation Test At The TK-locus Of Mouse Lymphoma L5178Y Cells	10 mM D-ribose (highest concentration tested)		D-Ribose did not induce a reproducible significant increase in mutant frequency and there was no dose related increase in mutant frequency in the absence or presence of metabolic activation; D-ribose was not mutagenic under the study conditions.	Steenwinkel, 2000 Vol 6 Tab 57

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Classical Non-Human Toxicity Assessments of D-Ribose (Continued)

Species	Dosage of D-Ribose	Duration	Effect	Reference
Micronucleus Test In Mice	2000mg/kg-bw	One dose via gavage; erythrocytes examined at 24 and 48 hours	The study indicates that treatment with D-ribose did not result in genotoxicity or cytotoxicity to bone marrow cells. The study concluded that D-ribose did not produce micronuclei in polychromatic erythrocytes in either male or female mice.	De Vogel, 2000a Vol 2 Tab 10
Oral Embryotoxicity/Terato-genicity In Wistar Rats	0,5, and 10% of diet; 1 group was fed 10% from 0-3 days of gestation and 20% from days 3-21 of gestation	Daily in feed	No mortalities were observed; No untoward clinical observations; Body weights were reduced in certain test groups; food consumption was decreased in all groups during the first 2 weeks of gestation, and in all weeks in the high dose group; Maximum intake ranged from 8.13-11.61 g D-ribose/kg/bw/day; No significant differences in mean fetal body weight; Wavy ribs and incompletely ossified interparietals were treatment related for the mid and high dose groups; The NOAEL was set at 5% for developmental toxicity, due to impaired nutritional status of the dams.	Wolterbeek, and Waalkens-Berendsen, 200(Vol 7 Tab 69

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Classical Non-Human Toxicity Assessments of D-Ribose (Continued)

Species	Dosage of D-Ribose	Duration	Effect	Reference
Sub-Chronic (13 Week) Oral Toxicity Study in Wistar Rats	5, 10 and 20% of diet; Highest intake was 15.0 g/kg/bw in males and 15.7 g/kg bw in females	Daily in feed	No treatment related mortalities were observed; Several changes, mainly in the mid- and/or high-dose group, e.g., decreased body weights accompanied by transiently lower food consumption and food efficiency, increased water intake paralleled by increased urinary volume and decreased urinary density, decreased glucose excretion, decreased sodium excretion, decreased urinary pH, changes in certain clinical chemistry variables decreased weight of the thymus and increased relative weights of the kidneys, liver, spleen and caecum; The investigator speculated that it was likely that the high amounts of D-ribose fed in this study were not completely absorbed and that unabsorbed D-ribose was fermented by the gut flora; A number of the above changes were attributed to unspecific effects that are generally observed in rodent feeding studies with incompletely absorbed yet fermentable carbohydrates. The NOAEL was set at less than 5% of the diet (4.0 g/kg/bw average).	Jonker, 2000 Vol 4 Tab 34

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Thirteen (13) Week Sub-Chronic Study

- Bioenergy, Inc. commissioned TNO Nutrition and Food Research (TNO), The Netherlands, to conduct a thirteen-week sub-chronic toxicology study in rats [Jonker, 2000 Vol 4 Tab 34]. The substance was administered in the diet to groups of 20 male and 20 female Wistar rats at dietary levels of 5, 10 or 20%. A control group consisting of 20 rats per sex was kept on a diet containing 20% potato starch, but no D-ribose.

- Results showed that there were no treatment-related mortalities.

- The investigator reported that the feeding of D-ribose resulted in several changes, mainly in the mid- and/or high-dose group, e.g., decreased body weights accompanied by transiently lower food consumption and food efficiency, increased water intake paralleled by increased urinary volume and reduced urinary density, reduced glucose excretion, reduced sodium excretion, reduced urinary pH, changes in clinical chemistry variables (increased alkaline phosphatase and aspartate aminotransferase activity, decreased plasma levels of proteins and lipids), decreased weight of the thymus and increased relative weights of the kidneys, liver, spleen and caecum.

- The investigator hypothesized that it was likely that the high amounts of D-ribose fed in this study were not completely absorbed and that unabsorbed D-ribose was fermented by the gut flora. Hence, a number of the above changes (e.g. growth reduction, decreased urinary pH, increased plasma alkaline phosphatase activity and increased caecum weight) might represent non-specific effects that are generally observed in rodent feeding studies with incompletely absorbed yet fermentable carbohydrates). Also increases in water intake and urinary volume in response to the feeding of high doses of a specific polyol have been reported and discussed previously [Lina, 1996 Vol 8 Tab 99].

- No abnormalities attributable to the ingestion of D-ribose were revealed by gross or histopathological examination, and ophthalmoscopic examination revealed no treatment-related ocular changes [Jonker, 2000 Vol 4 Tab

34].

- The investigator stated that although some of the above effects might represent non-specific effects related to the feeding of incompletely absorbed carbohydrates, other effects found in the present study could not be related to this phenomenon. Some of these effects (e.g., a tendency toward reduced glucose excretion, reduced thymus weight and increased kidney, liver and spleen weights) were still observed at the lowest dose level. It was noted that these findings were not accompanied by any histopathological changes and that their toxicological relevance might be in doubt. However, the author stated that they could not be completely disregarded. Therefore, it was concluded that under the conditions of this study the No-Observed-Effect Level (NOEL) of D-ribose was lower than 5% in the diet (equivalent to 3.6 and 4.4 g/kg body weight/day in males and females, respectively) [Jonker, 2000 Vol 4 Tab 34].

- In a study published in *Psychological Reports* the authors noted that rats were immediately adverse to the taste of D-ribose, and that a related sugar, xylose was toxic to some rodents, including rats. Company representatives hypothesized that a simple dislike of D-ribose as a food substance may account for lack of weight gain at the higher levels fed in this study [Wagner, 1971 Vol 8 Tab 100].

Oral Embryotoxicity/Teratogenicity Study With D-Ribose In Rats

- D-Ribose was fed in the diet to mated female Wistar rats (28 animals per dose group) from days 0-21 of gestation at concentrations of 0, 5 and 10%. Furthermore, one group was fed D-Ribose at 10% from gestation days 0-3 and at 20% from gestation days 3-21.

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- Results showed that mean body weights of the pregnant females of the mid- and high-dose groups were statistically significantly lower on gestation days 7 and 14. Weekly body weight gains were lower in the mid- and high-dose groups during the first week, and all treatment groups were lower during the second week of gestation. Body weight increased

during the last week of gestation in the mid- and high dose groups when compared with the control group [Wolterbeek, *et al.*, 2000 Vol 7 Tab 69].

- Food consumption was lower in all treatment groups during the first two weeks of gestation and in the high-dose group during the last week of gestation.

- External observations of the fetuses and placentas on Caesarian section did not reveal any remarkable findings that could be related to treatment. Furthermore, no significant differences in the mean fetal body weight and placenta weights were observed between the control group and the groups fed D-Ribose.

- The statistically significant increases in the incidences of fetuses showing wavy ribs and incompletely ossified inter parietals in the mid- and high-dose groups were considered to be related to D-Ribose treatment by the investigator [Wolterbeek, *et al.*, 2000 Vol 7 Tab 69].

- The investigator concluded that for developmental toxicity, the No Observed Adverse Effect Level (NOAEL) of dietary D-Ribose was 5% (w/w). He commented that at this dose level, maternal effects on body weights, food consumption and caecum weights were observed. Most probably, these effects were manifestations of an impaired nutritional status of the dams due to the feeding of relative high doses of the low-digestible test substance [Wolterbeek, *et al.*, 2000 Vol 7 Tab 69].

- In order to clarify the incidence of fetuses with wavy ribs, and retarded interparietal ossification the Expert Panel requested the Opinion of Dr. Frank Sullivan, of the United Kingdom, a noted Expert in the area of toxicological studies. Dr. Sullivan presented the following Expert Opinion:

  1. I am surprised that such high doses were used since we normally do not recommend higher than 5% in the diet. This could cause malnutrition though of course there are many other causes of reduced bodyweight. An important question is whether

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the fetal bodyweight was also reduced. If so, then it would be normal to expect reduced ossification. The skull bones are late to ossify so retarded interparietal ossification is not regarded as very important and would almost certainly disappear (or rather would fully ossify) within the next few days. Wavy ribs is a not uncommon variant, seen for example with beta-blockers and uterotonics, and can also disappear after birth, by weaning. Not regarded as very important. Increase in variants is a common finding in studies and is often dose related. Unless very marked and widespread, it is not usually regarded as very important (mainly because it is so common that lots of things would be affected if we did) however, some people do regard them as a sign of disturbed gene control and so might cause effects in people with a specific genetic tendency. Reduced food intake can cause a variety of defects, especially in mice and rabbits, but rats are more resistant.

2. Poor nutrition can cause many effects including malformations since so many vitamins and minerals are necessary for good reproduction.

3. Variations are not considered adverse since they are so common, often 90% of control fetuses show one or more variations (so what does 'normal' mean?). e.g. rats can have 12, 13, 14 or 15 pairs of ribs and the percentages of each number vary with strains.

4. When do variations become terata? When does a short finger become a malformation? How long is a piece of string? Most people define major malformations as 'sufficient to interfere with the life of the baby', and minor malformations are less than this."

Human Tolerance

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- The following results suggest that humans may be able to tolerate D-ribose at much higher levels than other species such as rodents.

Bioenergy, Inc. has recommended that ribose, when used as a dietary supplement, be administered in doses of 2-3 grams every 4 hours before exercise, during each 4 hours of exercise, and 4 hours following exercise to a maximum of 20 grams/day. This level is well within the range of the results reviewed in the GRAS Report.

- As cited in the GRAS Report, Gross and Zollner, 1991, administered from 83.3 to 222.2 mg/kg of D-ribose per hour to 9 healthy subjects [Gross *et al.*, 1991 Vol 2 Tab 20]. A dose of 166.7 mg/kg of D-ribose per hour was administered orally to 8 of the subjects for a minimum of 4 hours. Assuming a 60 kg person, this dosage equals 10.0 grams per hour for 5 hours (50 grams). No adverse effects were reported.

- Pliml, *et al.*, 1992, studied the effects of D-ribose administration to patients with coronary artery disease. These subjects received 60 grams of D-ribose daily for 3 days. The doses were divided into four 15 gram portions during the day [Pliml, *et al.*, 1992 Vol 5 Tab 45]. A second study by the same group concluded that ribose plasma levels associated with high dose (3.5 mM) oral administration did not inhibit human lymphocyte proliferation *in vitro* [Pliml, *et al.*, 1993 Vol 5 Tab 46].

- Goodman *et al.*, 1970, administered 0.5g of D-ribose/kg body weight as a 7.5% solution orally over a 35-minute period (equal to 51.5 grams per hour for a 60 kg person) to 10 healthy subjects [Goodman *et al.*, 1970 Vol 2 Tab 18]. No adverse effects were noted.

Conclusion

Based on its independent and collective critical evaluation of the information and data summarized in the GRAS Report, the Panel concluded that Bioenergy D-ribose, meeting the specifications described herein and produced and used in accordance with cGMP, is Generally Recognized As Safe (GRAS) by scientific procedures for use as a food ingredient at the levels cited in the Report in various major food categories.

Name:	Date:
Dr. Joseph F. Borzelleca, Chairman Professor Pharmacology and Toxicology Medical College of Virginia	16 January 2002
Mr. Cleve Denny, Microbiologist, National Food Processor's Assn. (Retired)	January 11, 2002
Dr. Darrell G. Medcalf, Carbohydrate Chemist Vice President (Retired) HealthComm, International, Inc.	Jan. 9, 2002

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Complete List of References

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Complete List of References

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