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An Effect of Cyclotetraphosphate, Cyclotriphosphate and Hexametaphosphate on Glycogenesis in Rat Liver

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To the Graduate Council:

I am submitting herewith a dissertation written by Virginia Ann Laiming entitled "An Effect of Cyclotetraphosphate, Cyclotriphosphate and Hexametaphosphate on Glycogenesis in Rat Liver." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Human Ecology.

John T. Smith, Major Professor

We have read this dissertation and recommend its acceptance:

Ada Marie Campbell, Gail W. Disney, Robert H. Feinberg

Accepted for the Council:

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AN EFFECT OF CYCLOTETRAPHOSPHATE, CYCLOTRIPHOSPHATE AND HEXAMETAPHOSPHATE ON GLYCOGENESIS IN RAT LIVER

A Dissertation

presented for the

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ABSTRACT

In order to investigate the effect of dietary phosphate additives on glycogenesis, rats were fed standard diets supplemented with cyclotetraphosphate, cyclotriphosphate or hexametaphosphate at a 0.1% level of phosphorus. A control group was fed orthophosphate at a similar level. After two weeks half of the animals received a test dose of ^{14}C-glucose administered by stomach tube. After 2-1/2 hours their livers were removed to determine the incorporation of ^{14}C-glucose into glycogen and total glycogen recovered. The remaining animals were sacrificed and their livers were removed to determine glycogen synthetase activity. Animals fed cyclotetraphosphate exhibited lower levels of glycogen synthetase activity, ^{14}C-glucose incorporation into glycogen and total glycogen synthesized than did those rats fed orthophosphate. In contrast, animals fed cyclotriphosphate and hexametaphosphate showed a stimulatory effect on the same three parameters when compared to orthophosphate controls.

To determine the minimum length of feeding time necessary to achieve these metabolic effects experimental diets were fed to animals for increasing lengths of time (24 hours, 48 hours, 96 hours, 144 hours and 192 hours) at a 0.1% level of phosphorus. An inhibition of the independent (I) and total glycogen synthetase activity relative to that observed with feeding of orthophosphate was evident in female rats fed cyclotetraphosphate for 24, 48, 144 and 192 hours but not at 96 hours and in males at all time intervals except 24 hours. Males and females fed cyclotriphosphate and hexametaphosphate had glycogen synthetase

activity (I and total) comparable at all time intervals to that of animals fed orthophosphate suggesting that longer dietary periods may be necessary. ^{14}C-glucose incorporated into liver glycogen and total liver glycogen in male rats was lower in animals fed cyclotetraphosphate than in rats fed orthophosphate for all time intervals except 24 hours. Female rats fed cyclotetraphosphate had essentially the same values as females which were fed orthophosphate.

To determine if there was a threshold level for the development of metabolic effects diets containing increasing percentages (0.01%, 0.02%, 0.04%, 0.06%) of phosphate supplementation were fed to animals for two weeks. In general, at all levels of supplementation male and female rats fed cyclotetraphosphate exhibited lower glycogen synthetase activity than animals fed orthophosphate. A stimulatory effect on enzyme activity in males fed cyclotriphosphate was evident at 0.04% and 0.06% and in females at 0.02% and 0.04% phosphate supplementation. The incorporation of ^{14}C-glucose into liver glycogen and total glycogen recovered from male rats fed cyclotriphosphate and hexametaphosphate was increased and in rats fed cyclotetraphosphate decreased at levels as low as 0.01% phosphate supplementation. Similar effects on these parameters were seen in females at 0.04% and 0.06% phosphate supplementation.

The activity of the dependent form of glycogen synthetase may be estimated by subtracting the independent activity from the total enzyme activity. The percentage of total activity made up of the dependent form of the enzyme was higher in rats fed cyclotetraphosphate than in rats fed orthophosphate. However, rats fed cyclotriphosphate and hexametaphosphate had a lower percentage of dependent activity than

animals fed orthophosphate. Similar results were evident in male and female rats.

These data suggest an inhibition by cyclotetraphosphate and a stimulation by cyclotriphosphate and hexametaphosphate on the phosphorylation system controlling glycogen synthetase in the liver.

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CHAPTER I

INTRODUCTION

The metabolism of condensed phosphates has been investigated in view of their extensive use as food additives. Dietary studies using various condensed phosphates have shown only the tripolyphosphates had a similar tissue distribution to orthophosphate when supplemented in experimental animal diets (1), suggesting that longer chain polyphosphates and cyclic phosphates may not be readily hydrolyzed at the intestinal level. The appearance of the intact polyphosphate molecules as urinary constituents and the tissue distribution patterns of the ^{32}P activity of ^{32}P-cyclotetraphosphate suggest a role of cyclotetraphosphate in influencing the phosphorylation of tissue components (2).

Rats fed diets supplemented with cyclotetraphosphate at a 0.1% level of total phosphorus incorporated less of a test dose of ^{14}C-glucose into liver glycogen than rats fed diets supplemented with orthophosphate (3). These results suggest a metabolic effect of cyclotetraphosphate on the regulation of glycogenesis, possibly related to the nature of its ring structure. Furthermore, since cyclotriphosphate also contains a ring structure and is a hydrolytic product of the food additive hexametaphosphate, a series of studies was undertaken to examine the effects of cyclotetraphosphate, cyclotriphosphate and hexametaphosphate on mammalian carbohydrate metabolism. In an attempt to answer these questions, standard rat diets were supplemented with cyclotetraphosphate, cyclotriphosphate and hexametaphosphate and the incorporation of a test dose of ^{14}C-glucose into liver glycogen, total liver glycogen formation, and

glycogen synthetase activity were measured. In addition, the minimum time necessary to achieve a metabolic effect and the threshold level for the development of the metabolic effect were investigated.

CHAPTER II

REVIEW OF LITERATURE

It is the purpose of this review to consider some properties of the enzymes involved in glycogen metabolism followed by the controls placed on these enzyme systems. Although the primary goal of this review is to describe the control of glycogen metabolism in the liver, properties of the muscle enzymes are frequently described because the literature is more abundant than for liver enzymes, which have been less extensively studied due to the difficulty of isolation and subsequent examination.

A detailed review of the chemistry, biochemistry and toxicity of the condensed phosphates has been prepared by Allen[1] and will therefore not be reiterated in this review.

A brief review of the nomenclature is necessary to prevent further confusion. Two distinct species of glycogen synthetase exist, one form referred to as the D (dependent) form which has a very low activity in the absence of glucose-6-phosphate and the I (independent) form which is active even in the absence of glucose-6-phosphate. In other nomenclature used the "I form" is also referred to as "synthetase a" (active) and the "D form" as "synthetase b" (inactive). These two

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[1]Allen, A. M. (1978) The metabolism of condensed phosphate by the rat. Unpublished Doctoral Dissertation, The University of Tennessee, Knoxville.

forms are interconvertible through phosphorylation and dephosphorylation by kinases and phosphatases, respectively, and in turn controlled by systems which will be further explained later.

I. CYCLIC AMP-DEPENDENT PROTEIN KINASES

Protein kinases catalyze the transfer of the terminal phosphoryl group of nucleoside triphosphate (ATP) to amino acid residues in protein substrates. The enzyme catalyzed phosphorylation-dephosphorylation involves the following (4):

$$\text{Protein} + n\text{ATP} \xrightleftharpoons{\text{Protein Kinase}} \text{Protein} - P_n + n\text{ADP} \qquad 1.$$

$$\text{Protein} - \text{Pn} + n\text{H}_2\text{O} \xrightleftharpoons{\text{Phosphoprotein Phosphatase}} \text{Protein} + n\text{Pi} \qquad 2.$$

Reaction 1 requires a divalent metal ion, usually Mg^{+2}. The phosphoprotein phosphatase catalyzes the dephosphorylation of phosphoproteins. It can be seen through these reactions that phosphorylation-dephosphorylation reactions may in turn be regulated through control of protein kinase, protein phosphatase or by regulating both steps.

Distribution

Adenosine 3',5'-monophosphate dependent protein kinase (cyclic AMP-dependent protein kinase) has been found in most mammalian tissues including skeletal muscle, heart, brain, adipose tissue and liver (5). In rat liver, Chen and Walsh (6) found 89% of the enzyme in the cytosol, 6% in the nuclei, 47% in microsomes and less than 1% in the mitochondria. Rabbit muscle cyclic AMP-dependent protein kinase is a soluble enzyme located primarily in the cytosol.

Purification of Multiple Forms

In early work, Bishop and Larner (7) identified in liver a cyclic-AMP stimulated kinase to convert the I form of glycogen synthetase to the D form. The complete or partial purification of cyclic AMP-dependent protein kinase has been demonstrated in various other tissues. Many of the tissues which have been examined contain multiple forms of the enzyme including bovine heart muscle (8), rabbit skeletal muscle (9,10) and liver (11,12). Rubin et al. (8) estimated cyclic AMP-dependent protein kinase from bovine heart muscle to have a molecular weight of 280,000 by gel filtration chromatography, and was composed of the cyclic AMP-dependent protein kinase and catalytic subunits with molecular weights of 42,000 and regulatory subunits of 55,000. When the purified protein kinase was subjected to ultracentrifugation or polyacrylamide gel electrophoresis, two smaller forms of cyclic AMP-dependent protein kinase were evident with estimated molecular weights of 140,000 and 90,000, which may correspond to those of the enzyme and the regulatory subunit—cAMP complex, respectively. This is in agreement with Bechtel et al's. (13) estimation of the molecular weight (6) of the catalytic subunit of rabbit skeletal muscle to be 41,000. Kumon et al. (14) partially purified two (B_1 and B_2) fractions of cyclic AMP-dependent protein kinase from rat liver cytosol by ammonium sulfate fractionation followed by DEAE-Sephadex and hydroxyapatite column chromatography. A third fraction (B_0) was also revealed and showed a single peak with a molecular weight of approximately 3×10^4 when analyzed with gel filtration on Sephadex column. This agrees with the estimations of most authors of the molecular weight of the catalytic subunit in rat liver

enzyme. Protein kinases B_1 and B_2 had estimated molecular weights of 120,000-180,000 which is in agreement with those values obtained by Yamamura et al. (15) for rabbit skeletal muscle cyclic AMP-dependent protein kinase. The muscle active kinase showed essentially identical catalytic and kinetic proerties with rat liver active protein kinase. Chen and Walsh (6) found the sedimentation coefficient of two different liver cyclic AMP-dependent protein kinases was 6.8S. Isoelectric focusing of one of these components in the presence of cyclic AMP separates two catalytic subunits each with a sedimentation constant of 4.05.

Kinetics

In general, kinetic studies of most cyclic AMP-dependent protein kinases have revealed properties similar to those of rabbit skeletal muscle which has been more extensively studied. These values have varied depending upon experimental conditions used.

The apparent Km for cyclic AMP with either casein or glycogen synthase as substrate from rabbit skeletal muscle was about 0.2 μM and with histone used as substrate about 0.06 μM (16). The skeletal muscle protein kinase is stimulated between 5 and 20 fold by cyclic AMP. Rabbit skeletal muscle cyclic AMP-dependent protein kinase catalyzes a phosphorylation of casein and protamine. The Km values of cyclic AMP for these reactions are 1×10^{-7} and 6×10^{-8}, respectively (17). Sanborn and Koreaman (18) purified cyclic AMP-dependent protein kinase of bovine endometrium, along with its binding and catalytic subunits. The cyclic AMP-dependent protein kinase and the subunits show a pH optimum between 6 and 6.5.

Mechanism of Action of Cyclic AMP

Cyclic AMP is known to mediate many physiological processes through regulation of cyclic AMP-dependent protein kinases leading to an altered state of phosphorylation of specific protein substrates. Subsequent work has shown that cyclic AMP-dependent protein kinase(s) is a tetramer containing two regulatory (R) and two catalytic (C) subunits and undergoes dissociation in the presence of 2 moles cyclic AMP as follows:

$$R_2C_2 + 2cAMP \rightleftharpoons R_2(cAMP)_2 + 2C$$

R_2C_2 represents the inactive protein kinase or holoenzyme, which when bound to cAMP in an allosteric manner releases the active free catalytic (C) subunit (19,20).

A number of potential models for the interaction of cyclic AMP with protein kinase have been investigated. Builder et al. (21) concluded cyclic AMP must activate protein kinase by first binding to the holoenzyme forming a R · cAMP complex and free catalytic subunit. Ogez and Segal (22) propose an alternative viewpoint in which dissociation of an independent RC unit prior to cyclic AMP binding to the regulatory subunit is seen as a more feasible model.

In a further attempt to understand the process by which cyclic AMP activates cyclic AMP-dependent protein kinase (23) the cyclic AMP binding sites of the regulatory subunits of protein kinase were studied. These data suggested that one of the cyclic AMP binding sites contains a tryptophan residue and that the cyclic AMP binding sites are not identical on the regulatory subunits of cyclic AMP-dependent protein kinase.

Regulation

The equation equilibrium may be influenced by the amounts of RC and cAMP in the tissues or by any other substance within a cell that may bind one of the components. One such endogenous protein kinase inhibitor has been purified from skeletal muscle (24). It inhibits the activity of cyclic AMP-dependent protein kinases from skeletal muscle, heart, liver, adipose tissue and brain. The total specific activity of cyclic AMP-dependent protein kinase was inversely related to cellular levels of the inhibitor (25). Interaction of the protein inhibitor with the catalytic subunit of protein kinase has been described along with kinetic analysis of casein phosphorylation which reveals noncompetitive interaction between the inhibitor and the catalytic subunit (26). The inhibitor protein is heat stable, has acidic characteristics and an estimated molecular weight of 11,300 (27). More recently, two molecular forms of the protein inhibitor referred to as Type I and Type II inhibitor, with molecular weights of 24,000 and 15,000, respectively, have been resolved by gel chromatography (28).

(Mg^{++}) ATP in rabbit skeletal muscle has been found to facilitate the recombination of R and C and frees cyclic AMP (29,30) resulting in an impairment of cyclic AMP binding and activation of the cyclic AMP-dependent protein kinase, by decreasing the affinity of the R unit for cyclic AMP.

Phosphorylation of protein kinase is reversible upon addition of ADP and Mg^{++}, resulting in a transfer of phosphate from the protein to ADP forming ATP (31). This reverse reaction is optimal at a pH of 5.5 whereas the forward reaction has a more broad optimal alkaline pH.

Ueda et al. (32) investigated the regulation of protein phosphorylation from rat brain by cyclic AMP. The concentration of cyclic AMP required for maximum stimulation of the phosphorylation was approximately 5×10^{-6} M. Guanosine 3',5'-monophosphate (cyclic-GMP) had no significant effect.

Miyamoto et al. (33) examined cyclic AMP-dependent protein kinase from bovine brain. Low concentrations of histone or cyclic AMP caused a dissociation of the enzyme into subunits. This suggests an alternative mechanism of activation of the protein kinase without the participation of cyclic AMP.

Specificity

Cyclic AMP-dependent protein kinases in most mammalian tissues appear to be similar enzymes which show the same broad substrate specificities (34). The enzymes catalyze the incorporation of the terminal phosphate of ATP into the same specific residues of substrate proteins such as glycogen synthetase and lipase.

When pig liver pyruvate kinase was incubated with (^{32}P) ATP and cyclic AMP-dependent protein kinase, one major (^{32}P) phosphopeptide was isolated with the amino acid sequence Leu-Arg-Arg-Ala-(^{32}P)SerP-Leu (35). Kemp et al. (36) studied the protein substrate specificity of the catalytic subunit of rabbit skeletal muscle cyclic AMP-dependent protein kinase using genetic variants of β casein. The variant most readily phosphorylated was β-casein-B. The phosphorylation occurred at a single serine 124 site near the arginine substitution position of 122. Results obtained using synthetic peptides derived from rabbit skeletal muscle (37,38) support the concept that arginine is an important residue in

determining the substrate specificity of protein kinase and influencing the Vmax and apparent Km of the phosphorylation reaction. The results of other studies (39,40) further substantiate the importance of the arginine and serine residues.

During incubation with cyclic-AMP dependent protein kinase, cyclic-AMP and Mg-ATP the phosphorylation of glycogen synthetase incorporates between 1.0 (41-45) and 4.0 (46) molecules of phosphate per subunit. Although the phosphorylation and a to b conversion of glycogen synthetase have been shown to be catalyzed by both cyclic-AMP dependent and cyclic AMP-independent protein kinase in skeletal muscle, there is little definitive knowledge with regard to the cyclic-AMP independent form functioning in the liver system. Jett and Soderling (47) investigated the stoichiometry of the phosphorylation using both cyclic AMP-dependent and cyclic AMP-independent protein kinases from rat liver. The cyclic AMP-dependent protein kinase conversion of the b form of synthetase to the a form was associated with the incorporation of 3 mol of phosphate/mol of synthetase subunit. With cyclic AMP-independent protein kinase only 2 mol phosphate/mol of subunit were required to achieve the same degree of phosphorylation and inactivation.

II. CONTROL OF GLYCOGEN METABOLISM

A diagram illustrating the control of glycogen metabolism in the liver is shown in Figure 1 (48). Glycogen synthesis and degradation in the liver are catalyzed by glycogen synthetase and glycogen phosphorylase, respectively. The two enzymes are found in both active (a) and inactive (b) forms which are converted by phosphorylation by phosphorylases and

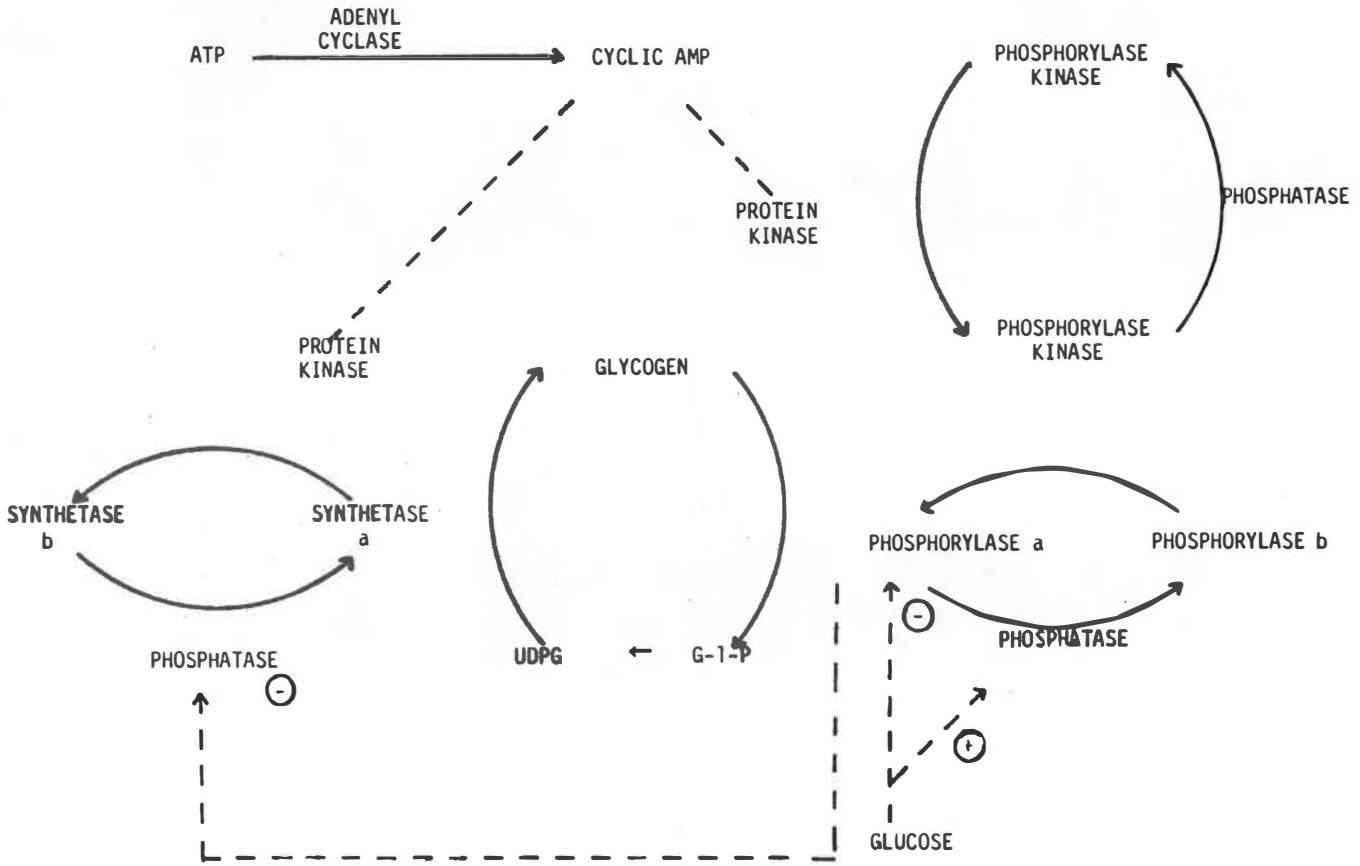

Figure 1. The control of liver glycogen metabolism adapted from Hers et al. (48).

dephosphorylation by phosphatases. It is important to remember that the cyclic AMP-dependent protein kinase which catalyzes the phosphorylation and inactivation of glycogen synthetase and that which catalyzes the phosphorylation and activation of phosphorylase kinase are the same enzyme (49). This suggests a mechanism which through protein kinase can simultaneously inhibit glycogenesis and activate glycogenolysis.

Glycogen Synthetase Phosphatase

In mammalian systems the conversion of glycogen synthetase in its phosphorylated form ("inactive," D or b) to the dephosphorylated ("active," I or a) form is catalyzed by glycogen synthetase phosphatase (50). Inhibition of the phosphatase reaction in skeletal muscle was found in the presence of Na_2SO_3, F^- and Pi or PPi (51). On the other hand, phosphatase activity was increased by more than 2-fold in the presence of divalent metal cations: $Mn^{++} > Ca^{++} > Mg^{++}$ and glucose-6-phosphate.

There have been some discrepant reports on the molecular weight and subcellular distribution of the phosphatase enzyme in rat liver. Synthetase phosphatase was found to sediment in the microsomal fraction (52); however in the cytosolic fraction when the liver was depleted of glycogen by fasting (53). These observations suggested an association of synthetase phosphatase with the glycogen particles of liver. Doperé et al. (54) further investigated synthetase phosphatase in rat liver identifying two components: the G-component of the phosphatase cosediments with particulate glycogen; the S-component remains in the supernatant. Although the S-component behaves as a phosphatase with synthetase b as substrate, the G-component does not, leaving its role open to speculation.

Doperé et al. also isolated three fractions of glycogen synthetase (b_1, b_2, b_3) from the glycogen fraction of the dog liver which showed progressively less affinity for glucose-6-phosphate. These characteristics are compatible with the hypothesis of various states of phosphorylation from b_1 towards b_3 fractions of glycogen synthetase. As previously shown in Figure 1, glucose stimulates the deposition of glycogen in the liver by stimulating phosphorylase phosphatase and removing the inhibition of phosphorylase upon synthetase phosphatase (55,56). The inhibition of liver synthetase phosphatase by phosphorylase-a was kinetically noncompetitive with substrate.

Glycogen Phosphorylase

Rabbit muscle phosphorylase, the controlling enzyme in the breakdown of glycogen, is controlled by phosphorylation by phosphorylase kinase and dephosphorylation by phosphorylase phosphatase. Phosphorylase b contains two identical polypeptide chains with individual estimated molecular weights of 100,000 (57). This evidence seems to be consistent with Seery et al. (58), who found the subunit of rabbit muscle glycogen phosphorylase has a molecular weight of 92.5×10^3 g/mol as determined by high-speed sedimentation equilibrium experiments. The enzyme can exist in two states: phosphorylase b, a dimer with a molecular weight of 185×10^3 g, and phosphorylase a, a tetramer with a molecular weight of 370×10^3 g.

The "b" form of the enzyme can be activated allosterically (59, 60) by the binding of AMP and be inhibited by the binding of glucose, glucose-6-phosphate and ATP. Phosphorylase b is converted to active phosphorylase a when a seryl residue (Ser-14) is phosphorylated.

Dreyfus et al. (61) proposed that two arginine residues of phosphorylase are involved in the binding of nucleotides at the activator site on one residue, and in the binding of phosphorylated substrates at the active site on the other residue. The latter residue was modified by arginine-specific 2,3-butanedione in the active conformation of the molecule. Presumably, modification of the arginine residue results in conformational changes involved in the binding of phosphorylated substrates at the active sites. The amino acid sequence of the site phosphorylated in the phosphorylase reaction has been investigated (62,63).

In hepatocytes isolated from fed rats, insulin and glucose play major regulatory roles in the modulation of glycogen phosphorylase and in turn glycogen synthetase. Glucose administration led to a decrease in phosphorylase a and insulin plus glucose led to a further decrease in phosphorylase a. A reciprocal relationship was noted between phosphorylase a inactivation and synthetase activation in response to glucose and insulin plus glucose (64). The inactivation of phosphorylase by glucose can be attributed partially to the activation of phosphorylase phosphatase.

Phosphorylase Phosphatase

The dephosphorylation of phosphorylase a to phosphorylase b is catalyzed by phosphorylase phosphatase. Phosphorylase phosphatase in rabbit muscle (65,66) and mouse liver (67) extract is bound to particular glycogen. It is stimulated by glucocorticoids and glucose and inhibited by glycogen and AMP. The interconversion of the two forms of phosphorylase exerts an indirect influence on the synthesis of

concentrations of calmodulin. Embi et al. (76) found homogeneous preparations of rabbit skeletal muscle phosphorylase kinase can catalyze the phosphorylation of glycogen synthetase a from the same tissue. These results suggest that one of the protein kinases present in purified preparations of glycogen synthetase and phosphorylase kinase are the same enzyme.

In brain (77) the calmodulin-Ca^{++} complex has been reported to mediate both the inhibition and stimulation of adenylate cyclase. In addition, the Ca^{++} dependent form of phosphodiesterase has been reported to be stimulated by calmodulin in most tissues (78). The implication of these findings in the control of glycogen synthetase in muscle tissue may be significant.

III. GLYCOGEN SYNTHETASE

Glycogen synthetase catalyzes a reaction which results in the storage of glycogen polymers in mammalian liver as shown below:

$$\text{UDP - glucose} + (\text{glucose})_n \longrightarrow \text{UDP} + (\text{glucose})_{n+1}$$

In the above reaction UDP-D-glucose is transferred to the terminal glucose residue at the nonreducing end of an amylose chain forming an $\alpha(1 \rightarrow 4)$ glycosidic linkage between the number one carbon atom of the added glycosyl residue and the 4-hydroxyl of the terminal glucose residue in the chain (79). An $\alpha(1 \rightarrow 4)$ polyglucose chain, containing at least four glucose residues, serves as a primer for the glycogen synthetase reaction. The branch points in the glycogen structure are formed by 1,6-α-transglucosylase branching enzyme which catalyzes the transfer of

several glucosyl residues from the end of a glycogen chain to a 6-hydroxyl group in a chain forming a branch point joined by an $\alpha(1 \rightarrow 6)$ linkage.

Forms

The existence of two forms of glycogen synthetase has been well documented. The interconversion of these two forms required ATP and Mg^{++} and was shown to involve the transfer of the terminal phosphate from ^{32}P-labeled ATP to the enzyme (34,80,81).

The two forms of rat liver glycogen synthetase differ in their affinity for uridine diphosphoglucose (UDPG) and glucose-6-phosphate (G-6-P), with the b form being highly dependent on the presence of G-6-P as well as Pi concentrations; however, in the presence of both G-6-P and Pi, the enzyme is insensitive to either. It was, therefore, suggested the b form is physiologically inactive whereas the a form functions at one-half full capacity under conditions likely to occur in vivo. For both enzyme forms, the apparent Km for UDPG decreased with increasing G-6-P to a minimum value of 0.2 mM and 0.9 mM for synthetase a and synthetase b, respectively (82). In the presence of G-6-P, the Km of synthetase b for UDPG was 9×10^{-4} and 6.7×10^{-4} M for synthetase a at pH 8.9.

The two forms of glycogen synthetase are also distinguishable by a pH activity curve. Synthetase b had a sharp maximum activity at pH 8.9 in the presence of G-6-P as did synthetase a; however, synthetase a had a broader pH-activity curve. Furthermore, the b form of the enzyme was inhibited 50% by 6.7×10^{-3} M ATP, ADP and AMP whereas the a form was inhibited only 0-5% by the same nucleotides (83). These data suggest the a form of the enzyme exhibits more stability than the b form.

Physical Properties and Kinetics

Rat liver glycogen synthetase b has been purified by Jett and Soderling (47) and Lin and Segal (83). A subunit molecular weight of 85,000 was determined by electrophoresis in sodium dodecyl sulfate. These results are in general agreement with those of McVerry and Kim (84), but are smaller than the estimated molecular weight of 90,000 for the rabbit skeletal muscle enzyme (85,86). Determinations of the molecular weight of the native enzyme have varied greatly from 260,000 to 470,000.

In rabbit skeletal muscle glycogen synthetase b, it has been reported that 7 moles of phosphate and 6 sulfhydryl groups are required for each subunit of 90,000 molecular weight to be phosphorylated. Determinations of alkali-labile phosphate from the liver enzyme estimated a value of 12.4/subunit of 85,000 molecular weight. Values for reactive sulfhydryl groups were similar for the liver enzyme and the muscle enzyme. The data (84) suggest a sequential random BiBi mechanism for the rat liver enzyme. Since both the kinetics and stoichiometry of glycogen synthetase phosphorylation depend upon the reaction conditions it may be premature to base conclusions on the presently available literature.

Control Mechanisms

Glycogen synthetase, the rate-limiting enzyme for glycogen deposition, is a classic example of the complexity and sensitivity of regulatory mechanisms. The enzyme is found in two forms: the D or b form which requires glucose-6-phosphate for activity and the I or a form which is active in the absence of glucose-6-phosphate. The two forms are subject to regulation by cellular metabolites (87) which in turn may be

influenced by various hormonal (88) and metabolic states (89,90). Watts and Malthus (91) have suggested glycogen is a major factor in controlling glycogen synthesis in rats.

Insulin

The effect of insulin to promote the D to I conversion and concomitant activation of glycogen synthetase has been well documented. Miller and Larner (92) investigated the effects of insulin on the activation of glycogen synthetase in perfused livers from normal rats and when injected into fed alloxan diabetic rats. Insulin alone activated glycogen synthetase by converting the D form to the I form. The effect was maximal within 6 to 15 minutes and was associated with a decrease in synthetase kinase activity. Insulin also activated the enzyme previously inactivated by glucagon. These changes were also associated with a decrease in protein kinase and cyclic-AMP tissue levels. Bishop and Larner (93) found insulin infusion intravenously with glucose resulted in a rapid increase (7-13 minutes) in liver glycogen synthetase activity associated with a decrease in the activity of phosphorylase. An increased activity of the synthetase enzyme has been found in extracts of diaphragms incubated with insulin when compared to controls (94,95). Insulin treatment of the tadpole (96) results in the transformation of the hepatic enzyme into a form with a lower Km for UDP-glucose than the control enzyme. Thus, the insulin-activated enzyme exhibits less dependency on glucose-6-phosphate indicating a probable increased conversion from the D to I form.

With respect to the mechanism of insulin action, the present findings indicate insulin promotes the conversion of glycogen synthetase b to a and decreases cyclic-AMP levels in the liver tissue (79).

Controls Mediated Via Cyclic-AMP

The conversion in vivo of mice liver glycogen synthetase b to a with a simultaneous decrease in the activity of phosphorylase induced by the administration of glucose or glucocorticoids has been demonstrated (97). The stimulatory effects on liver glycogen synthesis are additive when both treatments are combined. A further administration of glucagon, epinephrine or cyclic-AMP resulted in a reconversion or inactivation of synthetase.

The effects of glucagon and epinephrine (98) may be explained by a cascade effect in which adenylate cyclase is increased followed by an increase in cyclic-AMP and protein kinase. This cascade of events not only increases the activity of phosphorylase but inactivates glycogen synthetase.

The activation of liver glycogen synthetase b into a is greatly enhanced by caffein and other methyl xanthines, which inhibit phosphodiesterase resulting in a decrease in cyclic-AMP (99).

CHAPTER III

EXPERIMENTAL PROCEDURE

I. GENERAL PLAN

Previous work has shown cyclotetraphosphate may be influencing the phosphorylation of tissue components. Therefore, the effect of cyclotetraphosphate, cyclotriphosphate and hexametaphosphate on glycogen formation and glycogen synthetase activity was investigated and compared to that of orthophosphate. Experimental diets supplemented with the condensed phosphates were fed to animals for various lengths of time (24, 48, 96, 144, 192 and 336 hours) to determine if there was a minimum time for the development of a metabolic effect. Based upon the periods of time indicated, diets containing increasing percentages (0.01%, 0.02%, 0.04%, 0.06%, 0.1%) of total phosphorus supplementation were fed to animals to determine if there was a threshold level for the development of metabolic effects.

Experiment I

Sprague-Dawley rats (100-125 g) were purchased from Harlan Industries[2] and maintained in steel galvanized group cages for approximately two weeks to mature. At the initiation of the study, four groups of 12 adult rats were randomly selected, weighed and placed in group cages with no more than three animals per cage. Both male (350-400 g) and female (240-300 g) rats were used but only one sex of animal was used

[2]Harlan Industries, Indianapolis, Indiana.

in each experiment. The animals were weighed again before sacrificing. There were no significant differences in the mean weights among the groups of animals by Duncan's Multiple Range Test (100) (Table 1).

Each group of animals was fed a standard diet modified from Caputto et al. (101) and supplemented with one of the following sodium phosphates at a 0.1% level of total phosphorus:cyclotetraphosphate ($Na_4P_4O_{12} \cdot 4H_2O$), cyclotriphosphate ($Na_3P_3O_9$), or hexametaphosphate ($Na_6P_6O_{18}$). All cyclic sodium phosphates were supplied by Monsanto Company.[3] Purity of the sodium phosphate salts was determined by paper chromatography according to the method of Karl-Kroupa (102) as described by Reiman and Beukenkamp (103). A control group was fed sodium orthophosphate at a 0.1% level of phosphorus (Table 2). After the 2-week dietary period, six animals from each group were stunned by a blow to the head, decapitated and their livers removed to Tris (hydroxymethyl aminomethane) buffer, pH 7.2, to determine glycogen synthetase activity according to the method of Villar-Palasi et al. (104). The other six animals were fasted for 24 hours and then administered a 1.5 ml test dose of 25% ^{14}C-glucose (average specific activity 1.86×10^4 cpm/mg glucose) by stomach tube. Two and one-half hours later, the animals were stunned by a blow to the head, decapitated and their livers removed to hot (boiling water bath) 30% KOH to isolate glycogen according to the method of Lifson et al. (105). Anthrone reagent (106) was used to determine the glycogen purity. All radioactive samples were transferred to liquid scintillation vials, containing 6 ml of 2-ethoxyethanol and 10 ml

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[3]Donated by Monsanto Company, St. Louis, Missouri.

TABLE 1

Mean weights of animals (experiment 1)

0.1% Dietary phosphate	Male		Female	
	Initial	Final	Initial	Final
	g	g	g	g
Ortho	386 ± 7	396 ± 10[1]	251 ± 6	255 ± 8
Cyclotetra	385 ± 6	401 ± 8	253 ± 6	259 ± 7
Cyclotri	380 ± 6	386 ± 6	247 ± 5	256 ± 8
Hexameta	381 ± 7	406 ± 7	284 ± 6	244 ± 7

[1]All values represent means ± SEM of twelve animals.

TABLE 2

Composition of diets

Component	Dietary phosphate			
	Ortho	Cyclotetra	Cyclotri	Hexameta
	g/100 g	g/100 g	g/100 g	g/100 g
Casein	18.000	18.000	18.000	18.000
Sucrose	30.000	30.000	30.000	30.000
Cornstarch	30.000	30.000	30.000	30.000
Vegetable oil[1]	2.000	2.000	2.000	2.000
Vegetable shortening[2]	8.000	8.000	8.000	8.000
Vitamin mixture[3]	2.000	2.000	2.000	2.000
Salt mixture[4]	3.000	3.000	3.000	3.000
NaH_2PO_4	0.458	---	---	---
$Na_4P_4O_{12}\cdot 4H_2O$	---	0.387	---	---
$Na_3P_3O_9$	---	---	0.329	---
$Na_6P_6O_{18}$	---	---	---	0.329
Nonnutritive bulk[5]	6.542	6.613	6.671	6.671

TABLE 2 (continued)

[1]Wesson oil, Hunt-Wesson Foods, Inc., Fullerton, California 92634.

[2]Crisco, Procter and Gamble, Cincinnati, Ohio 45202.

[3]Nutritional Biochemicals Corp., Cleveland, Ohio 44128. Vitamin Diet Fortification Mixture formulated to supply the following amounts of vitamins (g/kg vitamin premix): thiamin hydrochloride 1.0, riboflavin 1.0, niacin 4.5, p-aminobenzoic acid 5.0, calcium pantothenate 3.0, pyridoxin hydrochloride 1.0, ascorbic acid 45.0, inositol 5.0, choline chloride 75.0, menadione 2.25, biotin 0.020, folic acid 0.090, vitamin B-12 0.00135, α-tocopherol 5.0, vitamin A 9 X 10^5 units, vitamin D 1 X 10^5 units and sufficient glucose to make 1000 g.

[4]Hubbell, R. B., Mendel, L. B. & Wakeman, A. J. (1937) A new salt mixture for use in experimental diets. J. Nutr. 14, 273-285.

[5]Alphacel, Nutritional Biochemicals Corp., Cleveland, Ohio 44128.

2,5-diphenyloxazole (PPO) in toluene. Analyses of radioactivity were done in a Beckman LS 100C liquid scintillation counter.

Liver proteins were determined by the Folin-Ciocalteu method as described by Lowry et al. (107).

Experiment II

To determine if there was a minimum time period necessary to achieve a metabolic effect, 4 groups of 10 adult Sprague-Dawley rats were fed the standard diets shown in Table 2 for 24, 48, 96, 144, or 192 hours. The animals were weighed before the beginning of the dietary period and at the end of the dietary period. There were no significant differences found in the mean weights of the groups of animals by Duncan's Multiple Range Test (100) (Tables 3-7). Both male (275-330 g) and female (175-225 g) rats were used in the experiment, but only one sex of animal was used at any one time.

At the end of the dietary period 5 rats from each group were fasted for 24 hours and then received a test dose of ^{14}C-glucose as previously mentioned. Two and one-half hours later, they were stunned by a blow to the head and decapitated. Livers were removed from the rats and the incorporation of ^{14}C-glucose into liver glycogen and glycogen purity were determined according to Lifson et al. (105) and Carroll et al. (106), respectively. The other five animals from each group were killed by decapitation after the dietary period and their livers removed to determine glycogen synthetase activity as described by Villar-Palasi et al. (104).

TABLE 3

Mean weights of experimental animals fed diets for 24 hours

0.1% Dietary phosphate	Males		Females	
	Initial	Final	Initial	Final
	g	g	g	g
Ortho	318 ± 19	306 ± 16[1]	182 ± 3	165 ± 4
Cyclotetra	309 ± 16	297 ± 14	189 ± 5	177 ± 5
Cyclotri	316 ± 10	304 ± 8	181 ± 5	166 ± 2
Hexameta	315 ± 12	301 ± 11	187 ± 5	172 ± 4

[1]All values represent means ± SEM for ten animals.

TABLE 4

Mean weights of experimental animals fed diets for 48 hours

0.1% Dietary phosphate	Males		Females	
	Initial	Final	Initial	Final
	g	g	g	g
Ortho	309 ± 20	307 ± 13[1]	208 ± 4	204 ± 3
Cyclotetra	309 ± 20	303 ± 12	204 ± 4	196 ± 2
Cyclotri	313 ± 21	311 ± 14	210 ± 3	206 ± 2
Hexameta	306 ± 19	301 ± 13	210 ± 3	206 ± 5

[1]Values represent means ± SEM for ten animals.

TABLE 5

Mean weights of experimental animals fed diets for 96 hours

0.1% Dietary phosphate	Males		Females	
	Initial	Final	Initial	Final
	g	g	g	g
Ortho	321 ± 8	321 ± 5[1]	184 ± 2	193 ± 4
Cyclotetra	327 ± 9	324 ± 6	188 ± 2	194 ± 4
Cyclotri	323 ± 9	324 ± 8	187 ± 3	192 ± 4
Hexameta	321 ± 8	321 ± 5	189 ± 4	195 ± 5

[1]Values represent means ± SEM for ten animals.

TABLE 6

Mean weights of experimental animals fed diets for 144 hours

0.1% Dietary phosphate	Males		Females	
	Initial	Final	Initial	Final
	g	g	g	g
Ortho	278 ± 19	288 ± 11[1]	186 ± 6	194 ± 8
Cyclotetra	279 ± 17	280 ± 9[2]	183 ± 4	190 ± 7
Cyclotri	274 ± 17	275 ± 9	182 ± 5	188 ± 8
Hexameta	284 ± 17	287 ± 8	183 ± 4	195 ± 6

[1]Values represent means ± SEM for nine animals.

[2]All other values represent means ± SEM for ten animals.

TABLE 7

Mean weights of experimental animals fed diets for 192 hours

0.1% Dietary phosphate	Males		Females	
	Initial	Final	Initial	Final
	g	g	g	g
Ortho	303 ± 9[1]	315 ± 6	186 ± 5	199 ± 6
Cyclotetra	308 ± 8	316 ± 6	177 ± 5	192 ± 7
Cyclotri	303 ± 8	318 ± 6	183 ± 5	200 ± 8
Hexameta	311 ± 7	322 ± 8	183 ± 3	184 ± 11

[1]Values represent means ± SEM for ten animals.

Experiment III

This experiment was designed to determine if there was a threshold level for the development of a metabolic effect. Four groups of 10 male rats (240-300 g) and an equal number of female rats (160-200 g) were fed purified diets (Table 2, p. 24) containing increasing percentages (0.01, 0.02, 0.04, and 0.06) of the sodium phosphates (Table 8) mentioned previously for 2 weeks.

Animals were randomly selected and placed into galvanized group cages according to the phosphate diet. Each individual experiment used only one sex of animal at any given time. The rats were weighed before the initiation of the dietary period and again before sacrificing. There were no significant differences in the means among the 4 groups by Duncan's Multiple Range Test (100) as seen in Tables 9-12.

At the end of the dietary period, 5 rats from each group were stunned by a blow to the head, decapitated and their livers removed to determine glycogen synthetase activity according to the method of Villar-Palasi et al. (104). The other five rats in each group were decapitated and their livers removed to determine ^{14}C-glucose incorporation into liver glycogen and total liver glycogen as described earlier. Glycogen purity was determined by the method of Carroll et al. (106) and liver protein by using folin reagent.

II. METHODS

Two Dimensional Paper Chromatography for Phosphate Detection and Quantitation

Two dimensional paper chromatography was used to check the purity of the sodium phosphates. The method according to Karl-Kroupa (102) as described by Reiman and Beukenkamp (103) was used.

TABLE 8

Composition of diets (Experiment III)

Dietary phosphates and nonnutritive bulk	Percent dietary phosphate			
	0.01	0.02	0.04	0.06
	g/100 g	g/100 g	g/100 g	g/100 g
Ortho	0.046	0.092	0.184	0.276
nonnutritive bulk[1]	8.450	16.900	33.800	50.700
Cyclotetra	0.039	0.078	0.156	0.234
nonnutritive bulk	8.460	16.920	33.840	50.760
Cyclotri	0.033	0.066	0.132	0.198
nonnutritive bulk	8.466	16.932	33.864	50.796
Hexameta	0.033	0.066	0.132	0.198
nonnutritive bulk	8.466	16.932	33.864	50.796

[1]Alphacel, Nutritional Biochemicals Corp., Cleveland, Ohio 44128.

TABLE 9

Mean weights of experimental animals (0.01% dietary phosphate)

0.01% Dietary phosphate	Males		Females	
	Initial	Final	Initial	Final
	g	g	g	g
Ortho	274 ± 33	326 ± 22[1]	168 ± 3	195 ± 4
Cyclotetra	275 ± 33	305 ± 28	170 ± 3	196 ± 2
Cyclotri	283 ± 36	316 ± 26	167 ± 4	193 ± 6
Hexameta	271 ± 31	320 ± 21	164 ± 3	195 ± 4

[1]Values represent means ± SEM for ten animals.

TABLE 10

Mean weights of experimental animals (0.02% dietary phosphate)

0.02% Dietary phosphate	Males		Females	
	Initial	Final	Initial	Final
	g	g	g	g
Ortho	243 ± 15	304 ± 17[1]	179 ± 5	208 ± 4
Cyclotetra	252 ± 18	309 ± 22	184 ± 6	212 ± 6
Cyclotri	254 ± 17	310 ± 18	183 ± 6	204 ± 7
Hexameta	351 ± 19	290 ± 19	183 ± 7	207 ± 6

[1]Values represent means ± SEM for ten animals.

TABLE 11

Mean weights of experimental animals (0.04% dietary phosphate)

0.04% Dietary phosphate	Males		Females	
	Initial	Final	Initial	Final
	g	g	g	g
Ortho	265 ± 21	324 ± 16[1]	170 ± 6	195 ± 11
Cyclotetra	252 ± 18	300 ± 15	179 ± 8	205 ± 6
Cyclotri	246 ± 18	309 ± 19	174 ± 7	197 ± 7
Hexameta	252 ± 21	301 ± 18	172 ± 7	194 ± 5

[1]Values represent means ± SEM for ten animals.

TABLE 12

Mean weights of experimental animals (0.06% dietary phosphate)

0.06% Dietary phosphate	Males		Females	
	Initial	Final	Initial	Final
	g	g	g	g
Ortho	292 ± 25	334 ± 26[1]	163 ± 2	190 ± 3
Cyclotetra	283 ± 19	328 ± 15	165 ± 3	193 ± 3
Cyclotri	286 ± 22	334 ± 22	165 ± 5	188 ± 5
Hexameta	267 ± 20	326 ± 23[2]	163 ± 3	188 ± 3

[1]Values represent means ± SEM for ten animals.

[2]Value represents mean ± SEM for nine animals.

Whatman No. 1 paper was cut into 9" by 9" squares. A line was penciled one inch from the bottom horizontal and one inch from the left vertical edge of the paper. At the point where the lines met, 25 µl of phosphate sample was applied in a spot not to exceed 3/8 inch in diameter. The paper was then rotated 90° to the left and rolled into a cylinder, allowing separation between the vertical edges and stapled. Each phosphate was spotted in a similar manner on an individual sheet of paper. Cylinders were then placed in an equilibration jar for 45 minutes with a beaker containing 50 ml of the alkaline solvent containing 400 ml 2-propanol, 200 ml sec-butanol, 390 ml distilled H_2O and 10 ml 15N ammonia. The cylinders were then placed in individual one gallon jars lined with Whatman No. 3 chromatography paper and containing 150 ml of alkaline solvent. The chromatograms were allowed to develop until the solvent front was 1-1/2-2 inches from the upper edge of the paper (6-7 hrs.). The papers were allowed to dry overnight after which they were rotated 90°, stapled and placed in an equilibration jar with acid solvent as previously described. The acid solvent contained 750 ml 2-propanol, 250 ml of distilled H_2O containing 50 g trichloracetic acid (TCA) and 2.5 ml 15N ammonia. The cylinders were allowed to develop for 6-7 hrs. and dried. The chromatograms were then sprayed with ammonium molybate-perchloric acid solution (dissolve 1 g ammonium molybate in mixture of 5 ml 9-12N perchloric acid, 1 ml 12N hydrochloric acid (HCl) and enough distilled H_2O to give a volume of 100 ml) and placed in a convection oven (55°C) for 5-7 minutes to dry. The dried chromatograms were placed under a long wave UV light to view the blue spots formed.

In preparation for elution, spots were cut from the paper chromatograms. A similar size spot was cut from a clear area of the

paper to be used as a correction factor. All paper spots were individually cut into 1/8 inch strips and dropped into tapered stems of separate funnels, and the funnels placed in the necks of 25 ml volumetric flasks.

Elutions were started by adding 4 drops of 8N ammonia with a medicine dropper into the funnels. This was followed by 4 drops of distilled water. The procedure was repeated three times and followed by several drops of distilled water to rinse. The procedure was then repeated using 8N sulfuric acid instead of 8N ammonia. Two ml of 8N sulfuric were then added to each flask.

Hydrolysis of the condensed phosphates involved placing the flasks into beakers containing boiling water for 20 minutes. The flasks were then allowed to come to room temperature.

For the colorimetric determination of the phosphates, 10 ml of benzene-butanol mixture (1:1) plus 10% ammonium molybate were added to each flask and the flasks were diluted to the mark with water, stoppered and shaken for 20 seconds. Five ml of the top organic layer was transferred to 25 ml volumetric flasks. Then 10 ml sulfuric acid-methanol solution (2% by volume in absolute methanol) and 1 ml reducing agent (add .5 ml 25% stannous chloride in 12N HCl to 100 ml 1N sulfuric acid) which must be prepared daily, were added. After 10 mins. the absorbance of each solution was determined at 650 nm in a Beckman B spectrophotometer. To obtain the percent phosphorus present in each sample, the following equation was used:

$$\frac{\text{total absorbance of sample - correction factor}}{\text{total absorbance of sample}} \times 100 = \%\ \text{purity}$$

Determination of Glycogen Synthetase Activity

Animals were stunned by a blow to the head, decapitated and their livers removed to a weighed 25 ml beaker packed in ice and containing approximately 10 ml of Tris (hydroxymethyl aminomethane) buffer, pH 7.2. After recording the weight of each liver a 15% homogenate was prepared by diluting with Tris buffer pH 7.2. The tissue homogenates were prepared using a motor driven Thomas homogenizer with a Teflon pestle. The samples were then placed in a refrigerated centrifuge and centrifuged at 8,000 X g for 10 minutes. Approximately 5 ml of each liver homogenate was frozen for protein determinations.

Test solutions were prepared according to the method of Villar-Palasi et al. (104). Test solution a, for the determination of the I activity of glycogen synthetase, contained 8 ml of 0.01M ^{14}C-UDP-glucose (average specific activity 5.23 X 10^7 cpm/μm), 1.5 ml glycogen 80 mg/ml, 1.2 ml of 0.5M Tris-HCl-0.05M EDTA, pH 7.8, and 1.3 ml of distilled H_2O. Test solution b for the determination of the total (I+D) activity of glycogen synthetase contained 8 ml of 0.01M ^{14}C-UDP-glucose, 1.5 ml glycogen 80 mg/ml, 1.2 ml 0.5M Tris-HCl-0.05M EDTA, pH 7.8, and 1.3 ml 0.1M glucose-6-phosphate, pH 8.0. Both test solutions were frozen and stored for three weeks. Storage times exceeding three weeks resulted in the unincubated blank showing trace amounts of radioactivity incorporated into glycogen.

A blank consisting of 1 ml 6% TCA containing 1 mg/ml glycogen, 0.1 ml test solution and 0.05 ml enzyme diluted 2-fold with 0.05M Tris-HCl-0.05M mercaptoethanol-0.005M EDTA-0.3% glycogen, pH 7.8 was prepared. Two test tubes, one containing 0.05 ml enzyme (2-fold dilution) and 0.1 ml test solution a and the other containing 0.05 ml enzyme

(4-fold dilution) and test solution a were prepared. Two other test tubes both containing 0.1 ml of test solution b and either 0.05 ml of 2-fold diluted enzyme or 0.05 ml of 4-fold diluted enzyme were also put on ice.

The test tubes were placed in a 30°C waterbath and shaken for 15 minutes. The reaction was stopped with the addition of 1 ml cold 6% TCA containing glycogen 1 mg/ml. After centrifuging for 5 minutes at 671 X g, the supernatant was poured into 15 ml centrifuge tubes, precipitated with 2 ml cold 95% ethanol (EtOH), mixed thoroughly and allowed to stand at room temperature for 20 minutes. After centrifuging for 10 minutes at 671 X g, the precipitates were washed twice with 3 ml 66% EtOH, shaken and recentrifuged for 10 minutes at 671 X g and inverted to drain. The precipitates were dissolved in 0.4 ml of H_2O and allowed to stand overnight to completely dissolve the glycogen. Samples were then transferred to liquid scintillation counting vials containing 6 ml 2-ethoxyethanol and 10 ml PPO in toluene. Analyses of the radioactivity were done in a Beckman LS 100C liquid scintillation counter.

Determination of ^{14}C-Glucose Incorporation into Rat Liver Glycogen

Two and one-half hours after the administration of a 25% ^{14}C-glucose solution by stomach tube the animals were sacrificed and livers removed to 50 ml centrifuge tubes containing 10-15 ml of hot (boiling waterbath) 30% KOH for digestion. The livers were stirred, with a glass stirring rod, intermittently to insure complete tissue digestion. After approximately 30 minutes, the tubes were removed and

cooled to 40-50°. The glycogen was precipitated with the addition of 18 ml 95% EtOH to the tubes which were then placed in an 80° water bath for 5 minutes. After cooling the tubes to room temperature, they were centrifuged at 671 X g for ten minutes. The supernatant was poured off and the protein impurities removed by the addition of 15 ml 10% TCA by first adding enough TCA to form a paste. The remaining TCA was added in three parts with thorough mixing between additions. The samples were then centrifuged at 671 X g and the supernatant transferred to clean centrifuge tubes. The purified glycogen was precipitated with the addition of 25 ml 95% EtOH and centrifugation at 671 X g for 10 minutes. After inverting the tubes to drain, the precipitate was dried with the addition of a small volume of acetone, drained and rinsed with ether. The samples were drained and left to dry overnight. The glycogen was transferred to 10 ml volumetric flasks and diluted to the mark with distilled water. Duplicate 0.5 ml samples were pipetted into liquid scintillation vials containing 6 ml 2-ethoxyethanol and 10 ml PPO (12 g in 1 liter toluene). All samples were analyzed in a Beckman LS 100c liquid scintillation counter.

Determination of Glycogen Purity

As previously mentioned, the purified glycogen was transferred to 10 ml volumetric flasks and diluted to the mark with distilled water. These samples were further diluted by pipetting 1 ml into 100 ml volumetric flasks and diluting with distilled water.

Anthrone reagent was prepared daily as needed according to the method of Carroll et al. (106). It consisted of 0.05% anthrone, 1.0% thiourea and 72.0% by volume H_2SO_4. For each 250 ml of reagent, 180 ml

concentrated H_2SO_4 was carefully added to a flask containing 70 ml distilled H_2O followed by the addition of 125 mg anthrone and 2.5 g thiourea. Duplicate 9.0 mg percent (0.5 μ moles/ml) glucose standards were prepared by quantitatively weighing out two 9.0 mg samples of glucose which were transferred to 100 ml volumetric flasks and diluted with distilled water. Four ml of anthrone reagent were added to duplicate 1.0 ml samples of glycogen, glucose standards and two distilled water blanks. After mixing thoroughly the test tubes were chilled to 0° in an ice bath. A marble was placed in the mouth of each test tube to prevent evaporation and the samples were heated for 10 minutes in a boiling water bath. The reaction was stopped by immersing the tubes in cold water. After bringing the samples to room temperature the absorbances were read at 620 nm.

Liver Protein Determination

The amount of protein in each liver homogenate was determined according to the method of Folin-Ciocalteu as described by Lowry et al. (107). Reagents were prepared as follows:

Reagent A was prepared by adding 1 ml of sodium-potassium tartrate ($NaKC_4H_4O_6 \cdot 4H_2O$) followed by 1 ml 1% cupric sulfate ($CuSO_4 \cdot 5H_2O$) to 100 ml of 2.0% sodium carbonate (Na_2CO_3). This reagent must be prepared for each experiment and may not be stored.

Reagent B was prepared by diluting commercial Folin-Ciocalteu reagent (2N) 2-fold with distilled water to make a 1N reagent.

A standard containing 0.2 mg protein/ml was prepared by diluting 20 mg of bovine serum albumin to 100 ml with 0.5N NaOH.

Liver homogenates were diluted 250-fold by pipetting 0.4 ml of liver homogenate into a 50 ml volumetric flask and bringing it up to volume with 0.5N NaOH. Two ml of this solution was added to 2 ml of 1N NaOH (1:1) and mixed thoroughly. One ml of this final dilution was used in the determination.

Procedure:

Five ml reagent A was added to duplicate 1.0 ml protein samples and each protein standard mixing well. The samples were allowed to stand at room temperature for 10 minutes. A blank containing 1.0 ml of 0.5N NaOH was treated in a similar manner. Then 0.5 ml of reagent B was added to each test tube and mixed. After allowing the tubes to sit for 30 minutes the absorbances were read at 750nm in a Beckman B spectrophotometer. The following equation was used to calculate the mg protein in each sample:

$$\frac{\text{absorbance of unknown}}{\text{absorbance of standard}} \times \left(\text{dilution factor}\right) \times \frac{0.2}{\text{concentration of standard}} \times 100 = \text{mg protein}$$

III. STATISTICS

Duncan's Multiple Range Test for equal sample sizes and unequal sample sizes was used to measure differences between mean animal weights according to the procedure described by Steel and Torrie (100). The method of paired comparisons for equal sample sizes was utilized to analyze the results because one animal from each dietary group was sacrificed on the same day. When necessary, due to experimental animal deaths, the method of paired comparisons for unequal sample sizes was also used. All computations were done using the Olivetti-Underwood 101 programmer.

CHAPTER IV

RESULTS

I. THE EFFECT OF 0.1% PHOSPHATE SUPPLEMENTATION ON GLYCOGEN SYNTHETASE ACTIVITY AND GLYCOGEN FORMATION

Some of the results that are reported in this chapter were not statistically significant but appeared to reflect strong trends. Those effects that were statistically significant are identified.

Liver glycogen synthetase activity was 49% (I) and 24% (T) lower in male rats fed diets supplemented with 0.1% cyclotetraphosphate than in livers from rats fed equal levels of orthophosphate (Table 13). Similar results were found in female rats; however, the magnitude of the decrease in both activities of the enzyme (9%) was less. In contrast, male rats fed diets supplemented with hexametaphosphate demonstrated a 20% increase in glycogen synthetase activity (I or total) when compared to those rats fed orthophosphate at the same level of supplementation. Furthermore, there were apparent sex differences between the animals. The I enzyme activity represented a larger percent of the total activity in female rats than in male rats regardless of the phosphate supplementation.

The uptake of ^{14}C-glucose, expressed as total counts per minute (Tcpm), was approximately 37% lower in livers from rats fed cyclotetraphosphate than in those from rats fed orthophosphate (Table 14). Comparable results were found when values were expressed in terms of body weight. These results were evident in both male and female

TABLE 13

Liver glycogen synthetase activity

Dietary phosphate	Sex of experimental animals	
	Males (units)[1]	Females (units)
Ortho		
independent	42.2 ± 13.8[2]	15.4 ± 2.3[2]
total	117.15 ± 35.7	32.7 ± 3.8
Cyclotetra		
independent	21.1 ± 5.1	13.5 ± 2.0
total	89.6 ± 19.8	30.5 ± 3.1
Cyclotri		
independent	37.1 ± 6.0	14.5 ± 4.5
total	128.3 ± 18.5	26.3 ± 6.6
Hexameta		
independent	51.2 ± 14.6	13.0 ± 2.4
total	145.6 ± 20.7	28.3 ± 2.6

[1] 1 unit = 10 cpm/mg protein.

[2] All values represent means ± SEM for 6 animals.

TABLE 14

^{14}C-Glucose recovered in liver glycogen

Dietary phosphate	Sex of experimental animals	
	Males	Females
Ortho		
Tcpm x 10^{-4}	26.1 ± 11.0[1]	10.3 ± 2.3[1]
$\frac{\text{cpm x } 10^{-4}}{\text{100 g body weight}}$	7.0 ± 3.0	4.4 ± 0.8
Cyclotetra		
Tcpm x 10^{-4}	19.5 ± 6.9[1]	7.1 ± 0.7[2]
$\frac{\text{cpm x } 10^{-4}}{\text{100 g body weight}}$	4.8 ± 1.9	2.9 ± 0.2
Cyclotri		
Tcpm x 10^{-4}	31.2 ± 9.5[2]	18.5 ± 1.3[1]
$\frac{\text{cpm x } 10^{-4}}{\text{100 g body weight}}$	15.0 ± 8.1	7.9 ± 0.4
Hexameta		
Tcpm x 10^{-4}	27.6 ± 4.7[1]	13.4 ± 2.6
$\frac{\text{cpm x } 10^{-4}}{\text{100 g body weight}}$	6.8 ± 1.1	5.7 ± 1.1

[1]Values represent means ± SEM for 6 animals.

[2]Values represent means ± SEM for 5 animals.

animals. Furthermore, there was a 17% and 44% increase in the incorporation of ^{14}C-glucose into liver glycogen in male and female rats respectively, fed cyclotriphosphate when compared to those rats fed orthophosphate. The magnitude of increase in ^{14}C-glucose incorporation into liver glycogen by female animals that were fed hexametaphosphate was not as great as the magnitude of increase in those animals fed cyclotriphosphate. In general, male animals fed each of the four phosphate diets incorporated more of the test dose of ^{14}C-glucose into liver glycogen than the female animals. These results were demonstrated whether expressed as total cpm or cpm relative to animal body weight.

The results of total liver glycogen recovered, expressed as mg glucose and mg glucose/100 g body weight, are shown in Table 15. Rats fed cyclotetraphosphate had approximately 15% and 53% less total liver glycogen in male and female rats respectively than did rats fed orthophosphate. The decreases in mg glucose or mg glucose expressed relative to body weight in female rats were significant at $p < 0.05$. In contrast, males fed cyclotriphosphate and hexametaphosphate had approximately a 15% increase in total glycogen recovered when compared to those rats which were fed orthophosphate. This was true when expressed as mg glucose or relative to animal body weight.

In female rats there was a significant ($p < 0.01$) increase in the total liver glycogen recovered in those rats fed hexametaphosphate when compared to controls. Although the differences were not significant, female rats fed cyclotriphosphate also had more liver glycogen expressed as mg glucose or relative to body weight than those female rats fed orthophosphate. Furthermore, more liver glycogen was recovered from

TABLE 15

Total liver glycogen recovered

Dietary phosphate	Sex of animals	
	Males	Females
Ortho		
mg glucose	130.8 ± 19.1[1]	74.9 ± 12.0[1]
mg glucose / 100 g body weight	34.9 ± 5.3	32.2 ± 4.9
Cyclotetra		
mg glucose	112.1 ± 12.5[1]	35.4 ± 6.1[2,3]
mg glucose / 100 g body weight	27.7 ± 3.2	15.7 ± 3.3[3]
Cyclotri		
mg glucose	163.5 ± 33.6[2]	82.5 ± 26.4[1]
mg glucose / 100 g body weight	43.1 ± 9.3	36.0 ± 11.6
Hexameta		
mg glucose	162.0 ± 19.0[1]	134.6 ± 18.2[1,4]
mg glucose / 100 g body weight	38.5 ± 4.1	57.6 ± 8.3[4]

[1]Values represent means ± SEM for 6 animals.

[2]Values represent means ± SEM for 5 animals.

[3]Means are significantly different ($p < 0.05$) from ortho means by the method of paired comparison for unequal sample sizes (100).

[4]Means are significantly different ($p < 0.01$) from ortho means by the method of paired comparisons (100).

male rats than female rats regardless of the dietary phosphate supplementation.

II. EFFECT OF LENGTH OF FEEDING TRIAL ON THE DEVELOPMENT OF METABOLIC EFFECTS

The I activity of glycogen synthetase in male rats fed cyclotetraphosphate for 24, 96, 144 and 192 hours was less than the enzyme activity in male rats fed orthophosphate (Table 16) for equal time periods. The decrease in enzyme activity in rats fed cyclotetraphosphate for a time interval of 192 hours, when compared to those rats fed orthophosphate for 192 hours, was significant ($p < 0.05$).

However, there was a decrease in total enzyme activity at only 24 and 144 hours for rats fed cyclotetraphosphate when compared to control rats. In contrast, those rats fed cyclotriphosphate had an increase in both the I and total glycogen synthetase activity at all time periods with an exception of 24 hours. Rats fed hexametaphosphate showed increases in glycogen synthetase activity (I and total) at 48, 96 and 192 hours. These differences were not as prominent for females (Table 17). Decreases in enzyme activity (I) were seen in females fed cyclotetraphosphate at 24, 48, 144 and 192 hours when compared to those females which were fed orthophosphate. These results were also apparent when examining the total enzyme activity. Rats fed cyclotetraphosphate for 144 hours had significantly lower ($p < 0.05$) glycogen synthetase activity (I and total) than rats fed orthophosphate for similar period of time.

Glycogen synthetase activity in males and females did not appear to increase with increasing time intervals regardless of the dietary phosphate supplementation.

TABLE 16

Liver glycogen synthetase activity in male rats fed experimental diets for increasing lengths of time

Dietary phosphate	Time interval				
	24 hours	48 hours	96 hours	144 hours	192 hours
	(units)[1]	(units)	(units)	(units)	(units)
Ortho					
independent	14.2 ± 2.2[2]	4.2 ± 0.8[2]	15.0 ± 3.1[2]	14.0 ± 2.7[2]	10.6 ± 1.6[2]
total	43.8 ± 8.3	22.4 ± 3.4	40.7 ± 4.9	51.6 ± 6.4	31.2 ± 5.7
Cyclotetra					
independent	12.5 ± 1.9	7.2 ± 1.0	10.6 ± 1.2	11.1 ± 2.1	6.1 ± 0.6[3]
total	37.4 ± 3.1	26.9 ± 2.6	41.0 ± 4.7	35.2 ± 6.2	33.4 ± 6.7
Cyclotri					
independent	11.2 ± 3.4	7.6 ± 1.2	31.3 ± 3.9	23.5 ± 5.6	14.5 ± 2.4
total	42.5 ± 7.0	31.5 ± 2.9	50.2 ± 7.0	51.2 ± 4.2	47.6 ± 9.1
Hexameta					
independent	13.6 ± 3.4	6.8 ± 1.1	23.2 ± 2.2	18.2 ± 2.2	12.2 ± 2.0
total	37.2 ± 1.1	33.9 ± 5.8	52.9 ± 3.5	37.4 ± 2.7	41.9 ± 7.0

[1] 1 unit = 10 cpm/mg protein.

[2] All values represent means ± SEM for 5 animals.

[3] Means are significantly different ($p < 0.05$) from ortho means by the method of paired comparisons (100).

TABLE 17

Liver glycogen synthetase activity in female rats fed experimental diets for increasing lengths of time

Dietary phosphate	Time interval				
	24 hours	48 hours	96 hours	144 hours	192 hours
	(units)[1]	(units)	(units)	(units)	(units)
Ortho					
independent	14.3 ± 2.5[2]	17.8 ± 2.1[2]	11.0 ± 3.7[2]	25.1 ± 2.5[2]	18.5 ± 3.7[2]
total	23.7 ± 2.4	38.0 ± 2.4	21.2 ± 4.7	30.6 ± 3.5	30.4 ± 7.3
Cyclotetra					
independent	8.6 ± 1.6	11.8 ± 4.3	13.2 ± 3.5	10.7 ± 1.4[3]	12.8 ± 1.8
total	12.6 ± 2.8	27.8 ± 7.9	22.2 ± 5.7	15.3 ± 1.3[3]	22.7 ± 3.9
Cyclotri					
independent	8.5 ± 2.3	13.6 ± 2.6	6.5 ± 2.3	15.7 ± 0.5	11.2 ± 1.3
total	14.6 ± 3.3	35.9 ± 5.7	18.0 ± 6.2	21.0 ± 1.1	16.8 ± 2.8
Hexameta					
independent	10.8 ± 1.5	12.6 ± 5.8	8.5 ± 1.6	15.6 ± 2.3	11.5 ± 2.8
total	15.7 ± 3.8	31.2 ± 7.8	17.7 ± 3.8	20.3 ± 2.4	16.9 ± 4.3

[1] 1 unit = 10 cpm/mg protein.

[2] All values represent means ± SEM for 5 animals.

[3] Means are significantly different ($p < 0.05$) from ortho means by the method of paired comparisons (100).

Uptake of ^{14}C-glucose expressed as total cpm was significantly ($p < 0.01$) lower in male rats fed cyclotetraphosphate for 144 hours than in those fed orthophosphate (Table 18). When expressed in terms of body weight there were also significant differences. ^{14}C-glucose recovered in liver glycogen expressed as total cpm and cpm relative to body weight in livers from rats fed cyclotriphosphate and hexametaphosphate was comparable to that for rats fed orthophosphate at all time intervals investigated. In females no differences were seen in the recovery of ^{14}C-glucose in liver glycogen regardless of the dietary phosphate or whether the results were expressed as total cpm or cpm relative to body weight (Table 19).

Total liver glycogen recovered was significantly lower ($p < 0.01$) in male rats fed cyclotetraphosphate for 144 hours than in rats fed orthophosphate when expressed as mg glucose but not when expressed relative to body weight (Table 20). Although there were no significant differences at 24, 48 and 96 hours rats fed cyclotetraphosphate in general, had less glycogen recovered when compared to rats fed orthophosphate. These results were evident when expressed as mg glucose or relative to animal weights. Rats fed cyclotetraphosphate for 144 hours had significantly ($p < 0.01$) less glycogen recovered, expressed as mg glucose, than in those fed orthophosphate. Slight increases in these parameters were seen in male rats fed cyclotriphosphate and hexametaphosphate for 24 hours when compared to rats fed orthophosphate but not at other time intervals investigated. Similar patterns were seen with female animals (Table 21).

TABLE 18

^{14}C-Glucose recovered in liver glycogen from male rats fed experimental diets for increasing lengths of time

Dietary phosphate	Time interval				
	24 hours	48 hours	96 hours	144 hours	192 hours
Ortho					
Tcpm x 10^{-4}	10.0 ± 1.3[1]	11.6 ± 3.4	9.9 ± 1.9[1]	13.1 ± 2.5[2]	7.9 ± 0.5[1]
cpm x 10^{-4} / 100 g body weight	3.2 ± 0.6	3.4 ± 1.0	3.1 ± 0.6	4.4 ± 1.1	2.5 ± 0.2
Cyclotetra					
Tcpm x 10^{-4}	11.0 ± 0.5	8.3 ± 1.6	8.5 ± 1.7	10.8 ± 1.8[1,3]	9.1 ± 2.3
cpm x 10^{-4} / 100 g body weight	3.6 ± 0.3	2.5 ± 0.6	2.6 ± 0.5	3.5 ± 0.6[3]	2.7 ± 0.6
Cyclotri					
Tcpm x 10^{-4}	11.4 ± 2.4	6.8 ± 1.6	9.5 ± 2.7	9.2 ± 2.1[1]	8.9 ± 1.6
cpm x 10^{-4} / 100 g body weight	3.8 ± 0.9	1.9 ± 0.4	3.0 ± 0.9	3.3 ± 0.8	2.9 ± 0.5
Hexameta					
Tcpm x 10^{-4}	12.7 ± 2.3	6.7 ± 1.3	6.8 ± 0.5	10.8 ± 0.7[1]	5.5 ± 1.3
cpm x 10^{-4} / 100 g body weight	4.2 ± 0.7	2.1 ± 0.5	2.1 ± 0.2	3.6 ± 0.3	1.7 ± 0.4

[1]All values represent means ± SEM for 5 animals.

[2]This value represents mean ± SEM for 4 animals.

[3]Means are significantly different ($p < 0.01$) from ortho means by the method of paired comparisons for unequal sample sizes (100).

TABLE 19

^{14}C-Glucose recovered in liver glycogen from female rats fed experimental diets for increasing lengths of time

Dietary phosphate	Time interval				
	24 hours	48 hours	96 hours	144 hours	192 hours
Ortho					
Tcpm x 10^{-4}	25.5 ± 9.8[1]	29.9 ± 7.3[1]	33.4 ± 7.6	13.5 ± 3.9[1]	8.2 ± 4.0[1]
cpm x 10^{-4} / 100 g body weight	15.7 ± 6.4	14.6 ± 3.5	17.5 ± 3.7	7.7 ± 2.1	4.7 ± 2.0
Cyclotetra					
Tcpm x 10^{-4}	20.5 ± 12.8	29.3 ± 8.7	34.2 ± 11.7	18.1 ± 9.1	12.8 ± 5.3
cpm x 10^{-4} / 100 g body weight	12.1 ± 7.6	14.4 ± 4.2	18.1 ± 6.0	10.9 ± 5.7	7.4 ± 5.3
Cyclotri					
Tcpm x 10^{-4}	22.8 ± 6.7	18.5 ± 3.9	15.2 ± 5.4	18.6 ± 6.3	14.7 ± 4.5
cpm x 10^{-4} / 100 g body weight	13.5 ± 3.9	9.1 ± 2.0	8.1 ± 2.6	11.2 ± 3.8	8.6 ± 2.9
Hexameta					
Tcpm x 10^{-4}	20.5 ± 2.8	24.1 ± 9.2	16.3 ± 4.6	14.5 ± 3.7	13.7 ± 4.3
cpm x 10^{-4} / 100 g body weight	12.0 ± 1.9	12.0 ± 4.6	10.2 ± 1.5	7.9 ± 1.9	7.5 ± 2.3

[1]All values represent means ± SEM for 5 animals.

TABLE 20

Total liver glycogen recovered in male rats fed experimental diets for increasing lengths of time

Dietary phosphate	Time interval				
	24 hours	48 hours	96 hours	144 hours	192 hours
Ortho					
mg glucose	118.6 ± 25.4[1]	174.4 ± 47.4[1]	137.6 ± 26.5	274.4 ± 51.0[2]	107.4 ± 12.3[1]
mg glucose / 100 g body weight	38.0 ± 9.4	50.0 ± 12.2	43.2 ± 7.8	90.3 ± 21.3	33.9 ± 4.9
Cyclotetra					
mg glucose	112.3 ± 13.1	98.0 ± 22.4	122.7 ± 21.2	162.1 ± 43.9[1,3]	155.6 ± 42.0
mg glucose / 100 g body weight	36.2 ± 2.7	29.1 ± 2.7	37.9 ± 7.5	52.5 ± 15.2	46.3 ± 11.1
Cyclotri					
mg glucose	124.7 ± 5.7	107.9 ± 15.0	107.4 ± 40.7	124.6 ± 31.6[1]	166.2 ± 40.7
mg glucose / 100 g body weight	42.4 ± 11.8	30.8 ± 4.1	33.9 ± 13.3	43.6 ± 11.9	52.6 ± 13.5
Hexameta					
mg glucose	150.9 ± 53.2	107.7 ± 25.9	90.6 ± 19.2	174.1 ± 27.2[1]	148.4 ± 37.5
mg glucose / 100 g body weight	49.4 ± 4.0	32.6 ± 8.2	27.5 ± 5.6	56.7 ± 14.8	48.4 ± 14.8

[1]All values represent means ± SEM for 5 animals.

[2]Value represents means ± SEM for 4 animals.

[3]Means are significantly different ($p < 0.01$) from ortho means by the method of paired comparisons for unequal sample sizes (100).

TABLE 21

Total liver glycogen recovered in female rats fed experimental diets for increasing lengths of time

Dietary phosphate	Time interval				
	24 hours	48 hours	96 hours	144 hours	192 hours
Ortho					
mg glucose	48.5 ± 14.3	72.3 ± 20.8[1]	81.8 ± 24.1[1]	30.5 ± 7.2[1]	20.9 ± 7.7[1]
mg glucose / 100 g body weight	29.8 ± 9.4	35.6 ± 10.3	43.0 ± 11.9	17.8 ± 4.3	10.9 ± 3.8
Cyclotetra					
mg glucose	45.9 ± 16.7	84.8 ± 25.4	75.3 ± 29.2	28.1 ± 10.6	21.0 ± 8.4
mg glucose / 100 g body weight	27.2 ± 10.2	42.0 ± 12.6	39.8 ± 15.0	16.8 ± 6.7	12.1 ± 4.7
Cyclotri					
mg glucose	59.3 ± 14.7	30.1 ± 9.6	37.6 ± 14.6	22.1 ± 8.1	29.2 ± 4.5
mg glucose / 100 g body weight	35.2 ± 8.2	19.2 ± 4.8	20.0 ± 7.4	13.5 ± 5.3	16.6 ± 3.1
Hexameta					
mg glucose	53.5 ± 7.1	70.4 ± 30.4	37.3 ± 10.2	42.8 ± 6.6	39.3 ± 15.9
mg glucose / 100 g body weight	31.4 ± 4.5	35.0 ± 15.2	19.9 ± 5.4	23.9 ± 3.5	21.8 ± 8.8

[1]All values represent means ± SEM for 5 animals.

III. EFFECT OF PERCENT PHOSPHATE SUPPLEMENTATION ON GLYCOGEN SYNTHETASE AND GLYCOGEN FORMATION

In general, at all levels of supplementation rats fed cyclotetraphosphate had lower glycogen synthetase activity in comparison to those rats fed orthophosphate. This pattern was seen with both the I activity and total activity and for both males and females. Male rats fed cyclotetraphosphate at the 0.02% level of supplementation had significantly ($p < 0.02$) lower I enzyme activity when compared to controls (Table 22). At the 0.04% level of supplementation male rats fed cyclotetraphosphate had significantly lower ($p < 0.05$) I and total glycogen synthetase activity when compared to those rats fed orthophosphate. No significant differences in either form of glycogen synthetase activity were evident when comparing means from male rats fed cyclotriphosphate and hexametaphosphate with those from orthophosphate controls.

Dietary cyclotetraphosphate supplementation at the 0.01% level in females (Table 23) resulted in a significant ($p < 0.05$) decrease in the I glycogen synthetase activity when compared to rats fed orthophosphate using the method of paired comparisons (100). In addition, significant decreases ($p < 0.05$) in both the I and total glycogen synthetase activities in female rats fed 0.06% cyclotetraphosphate were evident when compared to rats fed 0.06% orthophosphate.

Only those male rats fed cyclotetraphosphate at a 0.06% level of supplementation incorporated less ^{14}C-glucose into liver glycogen than did rats fed 0.01% orthophosphate (Table 24).

A significant ($p < 0.01$) increase in the ^{14}C-glucose recovered from rats fed cyclotriphosphate compared to those animals fed

TABLE 22

Liver glycogen synthetase activity in male rats fed diets containing increasing percentages of phosphate supplementation

Dietary phosphate	Level of supplementation (%) 0.01	0.02	0.04	0.06
	(units)[1]	(units)	(units)	(units)
Ortho				
independent	49.4 ± 5.8[2]	51.9 ± 5.3[2]	46.7 ± 4.7[2]	41.5 ± 1.8[2]
total	118.2 ± 6.2	146.9 ± 22.2	159.1 ± 19.4	103.8 ± 13.8
Cyclotetra				
independent	34.4 ± 5.7	31.2 ± 3.7[4]	33.5 ± 2.1[5]	31.8 ± 5.9[2]
total	92.5 ± 16.8	115.3 ± 15.9	107.4 ± 9.7[5]	130.4 ± 16.1
Cyclotri				
independent	41.6 ± 4.2	39.7 ± 6.8	62.6 ± 11.4	45.1 ± 12.6[2]
total	92.1 ± 12.6	119.9 ± 12.3	157.2 ± 11.0	129.3 ± 22.7
Hexameta				
independent	36.6 ± 7.4	34.8 ± 7.1	35.7 ± 2.6	36.9 ± 5.3[3]
total	82.6 ± 7.8	115.1 ± 21.2	161.2 ± 19.5	112.6 ± 13.4

[1] 1 unit = 10 cpm/mg protein. [2] All values represent means ± SEM for 5 animals.

[3] Value represents means ± SEM for 4 animals.

[4] Means are significantly different ($p < 0.02$) from ortho means by the method of paired comparisons (100).

[5] Means are significantly different ($p < 0.05$) from ortho means by the method of paired comparisons (100).

TABLE 23

Liver glycogen synthetase activity in female rats fed diets containing increasing percentages of phosphate supplementation

Dietary phosphate	Level of supplementation (%) 0.01 (units)[1]	0.02 (units)	0.04 (units)	0.06 (units)
Ortho				
independent	26.1 ± 3.3	10.3 ± 2.9	25.5 ± 4.5	23.4 ± 4.4
total	91.4 ± 7.0	61.8 ± 10.0	93.0 ± 12.2	85.4 ± 11.7
Cyclotetra				
independent	14.3 ± 4.2[2]	9.1 ± 1.5	18.6 ± 5.8	8.5 ± 3.6[2]
total	82.3 ± 13.9	57.1 ± 7.2	82.3 ± 5.9	35.9 ± 7.4[2]
Cyclotri				
independent	20.5 ± 3.9	28.3 ± 10.8	28.7 ± 3.9	8.1 ± 1.9[2]
total	77.3 ± 13.5	71.1 ± 13.0	126.5 ± 9.6	53.4 ± 18.9
Hexameta				
independent	17.3 ± 4.5	14.2 ± 5.4	36.0 ± 6.0	11.9 ± 2.2[2]
total	89.9 ± 13.2	87.9 ± 8.5	95.9 ± 9.1	56.9 ± 13.5

[1]All values represent means ± SEM for 5 animals.

[2]Means are significantly different ($p < 0.05$) from ortho means by the method of paired comparisons (100).

TABLE 24

^{14}C-Glucose recovered in liver glycogen from male rats fed diets containing increasing percentages of phosphate supplementation

	Level of supplementation (%)			
Dietary phosphate	0.01	0.02	0.04	0.06
Ortho				
Tcpm x 10^{-4}	53.5 ± 6.7[1]	56.0 ± 3.0[1]	51.2 ± 7.7[1]	44.1 ± 2.5[1]
cpm x 10^{-4} / 100 g body weight	20.6 ± 2.5	21.8 ± 1.4	18.4 ± 2.9	16.6 ± 2.5
Cyclotetra				
Tcpm x 10^{-4}	59.6 ± 8.2	73.5 ± 7.7[1]	67.6 ± 4.2	40.7 ± 3.9
cpm x 10^{-4} / 100 g body weight	27.0 ± 4.1	30.1 ± 5.0	26.1 ± 1.5	14.2 ± 1.2
Cyclotri				
Tcpm x 10^{-4}	74.0 ± 6.1[3]	52.9 ± 6.9[2]	68.4 ± 4.8	66.7 ± 4.8[4]
cpm x 10^{-4} / 100 g body weight	31.0 ± 3.4[3]	20.8 ± 4.1	26.7 ± 2.0	24.4 ± 1.3[4]
Hexameta				
Tcpm x 10^{-4}	66.5 ± 1.9[4]	66.6 ± 6.4[1]	66.6 ± 8.2	64.2 ± 6.3[4]
cpm x 10^{-4} / 100 g body weight	25.1 ± 0.7[4]	28.1 ± 3.2	25.6 ± 3.0	23.5 ± 2.4[4]

[1]All values represent means ± SEM for 5 animals. [2]Value represents means ± SEM for 4 animals.

[3]Means are significantly different ($p < 0.01$) from ortho means by the method of paired comparisons (100).

[4]Means are significantly different ($p < 0.05$) from ortho means by the method of paired comparisons (100).

orthophosphate was evident at the 0.01% level. The level of significance was $p < 0.05$ when comparing the means from animals fed cyclotriphosphate and hexametaphosphate to orthophosphate controls at 0.06% level of phosphate supplementation. Differences between the means were seen whether these data were expressed as total cpm or cpm/100 g body weight.

For females fed cyclotriphosphate, hexametaphosphate and orthophosphate there were small differences between phosphates in the uptake of ^{14}C-glucose at the various percentages of phosphate supplementation (Table 25). However, there was a significant decrease ($p < 0.05$) in the uptake of ^{14}C-glucose in rats fed cyclotetraphosphate when compared to rats fed orthophosphate at the 0.01% level. These data were significant when expressed as total cpm or cpm relative to body weight.

The total liver glycogen recovered in male rats fed cyclotetraphosphate when compared to those fed orthophosphate was approximately 23% less at all levels of supplementation with the exception of 0.02% (Table 26). Rats fed cyclotetraphosphate at a 0.06% level of supplementation had significantly ($p < 0.05$) less total liver glycogen recovered when compared to rats fed orthophosphate at the 0.06% level. Rats fed cyclotriphosphate and hexametaphosphate at the 0.01 and 0.02% levels of supplementation had 20% higher total liver glycogen recovered as mg glucose than rats fed orthophosphate. These results were also evident when expressed relative to body weight; however, not at higher levels of phosphate supplementation.

Similar patterns were seen with females (Table 27). Rats fed cyclotetraphosphate at the 0.01% level were the only animals with

TABLE 25

^{14}C-Glucose recovered in liver glycogen from female rats fed diets containing increasing percentages of phosphate supplementation

	Level of supplementation (%)			
Dietary phosphate	0.01	0.02	0.04	0.06
Ortho				
Tcpm x 10^{-4}	19.5 ± 4.7[1]	12.4 ± 3.1[1]	11.3 ± 1.4[1]	11.2 ± 4.7[1]
$\frac{\text{cpm x } 10^{-4}}{\text{100 g body weight}}$	8.9 ± 1.6	6.4 ± 1.6	5.0 ± 0.8	6.4 ± 2.1
Cyclotetra				
Tcpm x 10^{-4}	11.7 ± 1.9[2]	11.7 ± 2.7	11.1 ± 1.1	12.6 ± 4.1
$\frac{\text{cpm x } 10^{-4}}{\text{100 g body weight}}$	5.2 ± 0.9[2]	5.9 ± 1.4	5.4 ± 0.4	6.3 ± 1.9
Cyclotri				
Tcpm x 10^{-4}	19.1 ± 4.5	6.9 ± 2.3	13.0 ± 3.3	12.8 ± 4.4
$\frac{\text{cpm x } 10^{-4}}{\text{100 g body weight}}$	8.6 ± 2.0	3.7 ± 1.1	6.4 ± 1.9	6.8 ± 2.3
Hexameta				
Tcpm x 10^{-4}	12.5 ± 4.8	5.9 ± 1.8	12.3 ± 3.9	10.0 ± 2.0
$\frac{\text{cpm x } 10^{-4}}{\text{100 g body weight}}$	5.6 ± 0.9	3.0 ± 0.8	6.2 ± 2.0	5.1 ± 0.9

[1]All values represent means ± SEM for 5 animals.

[2]Means are significantly different ($p < 0.05$) from ortho means by the method of paired comparisons (100).

TABLE 26

Total liver glycogen recovered in male rats fed diets containing increasing percentages of phosphate supplementation

Dietary phosphate	Level of supplementation (%)			
	0.01	0.02	0.04	0.06
Ortho				
mg glucose	229.1 ± 30.2[1]	181.9 ± 30.9[1]	279.0 ± 44.0[1]	227.0 ± 27.1[1]
mg glucose / 100 g body weight	87.2 ± 9.7	70.8 ± 9.8	97.4 ± 13.2	86.4 ± 10.9[2]
Cyclotetra				
mg glucose	177.5 ± 9.2	208.3 ± 7.5	207.7 ± 22.6	176.3 ± 24.9[2]
mg glucose / 100 g body weight	78.3 ± 4.3	83.9 ± 6.8	79.7 ± 7.2	61.9 ± 8.7[2]
Cyclotri				
mg glucose	298.9 ± 59.6	225.2 ± 17.1	201.5 ± 25.1	203.7 ± 28.2
mg glucose / 100 g body weight	124.6 ± 24.8	86.1 ± 9.1	77.4 ± 9.0	74.0 ± 5.8
Hexameta				
mg glucose	264.5 ± 24.3	197.3 ± 19.4	224.6 ± 24.2	233.8 ± 12.8
mg glucose / 100 g body weight	100.7 ± 12.1	82.7 ± 7.3	90.0 ± 5.7	77.6 ± 8.7

[1]All values represent means ± SEM for 5 animals.

[2]Means are significantly different ($p < 0.05$) from ortho means by the method of paired comparisons (100).

TABLE 27

Total liver glycogen recovered in female rats fed diets containing increasing percentages of phosphate supplementation

Dietary phosphate	Level of supplementation (%) 0.01	0.02	0.04	0.06
Ortho				
mg glucose	113.8 ± 16.7[1]	66.9 ± 17.1[1]	47.1 ± 1.7[1]	50.8 ± 23.5[1]
mg glucose / 100 g body weight	59.3 ± 9.4	42.7 ± 8.5	24.6 ± 1.4	26.0 ± 11.6
Cyclotetra				
mg glucose	59.2 ± 8.6[2]	69.3 ± 15.3	53.3 ± 5.5	62.3 ± 22.9
mg glucose / 100 g body weight	40.5 ± 7.1	35.0 ± 8.0	25.3 ± 2.0	32.5 ± 10.8
Cyclotri				
mg glucose	107.1 ± 20.7	36.1 ± 8.7	60.5 ± 14.3	61.3 ± 23.0
mg glucose / 100 g body weight	71.5 ± 20.7	28.3 ± 4.6	30.0 ± 7.1	32.4 ± 12.4
Hexameta				
mg glucose	92.5 ± 8.8	31.4 ± 9.3	61.1 ± 24.2	41.1 ± 7.6
mg glucose / 100 g body weight	47.1 ± 4.8	18.2 ± 4.8	31.7 ± 13.0	21.4 ± 3.5

[1] All values represent means ± SEM for 5 animals.

[2] Means are significantly different ($p < 0.05$) from ortho means by the method of paired comparisons (100).

significantly ($p < 0.05$) less total glycogen when compared to orthophosphate controls. Total liver glycogen recovered from females fed cyclotriphosphate and hexametaphosphate was similar to that of orthophosphate controls whether expressed as mg glucose or relative to animal body weight.

IV. EFFECT OF PERCENT PHOSPHATE SUPPLEMENTATION AND LENGTH OF FEEDING TRIAL ON THE DEPENDENT ACTIVITY OF GLYCOGEN SYNTHETASE

The dependent activity of glycogen synthetase may be estimated by subtracting the independent activity from the total enzyme activity.

In rats fed 0.1% cyclotetraphosphate for two weeks, the dependent form of glycogen synthetase represented a higher percent of the total enzyme activity than in rats fed orthophosphate (Table 13, p. 46). In contrast, animals fed cyclotriphosphate and hexametaphosphate had a lower percent of dependent activity when compared to animals fed orthophosphate.

The percent of dependent activity was approximately the same at 24 and 48 hours for all the groups of animals (Table 16, p. 51). Males fed cyclotetraphosphate for 96 and 192 hours had a higher percentage of dependent enzyme activity when compared to animals fed orthophosphate. However, animals fed cyclotriphosphate and hexametaphosphate for 96 and 144 hours had a lower percent of dependent activity than animals fed orthophosphate and cyclotetraphosphate. Similar patterns were evident in females fed cyclotriphosphate and hexametaphosphate for 192 hours (Table 17, p. 52). In males fed cyclotetraphosphate the percent of dependent activity increased with increasing lengths of time with an

exception at 144 hours. In females fed cyclotetraphosphate for 192 hours the percentage of dependent activity was higher than in females fed for 24 hours.

At all levels of phosphate supplementation except 0.04%, males fed cyclotetraphosphate had a higher percent of dependent activity than did males fed orthophosphate (Table 22, p. 59). Similar patterns were evident in female animals at all levels of supplementation (Table 23, p. 60). Also, an increasing percentage of dependent activity was evident with increasing levels of phosphate supplementation in male rats but not in females fed cyclotetraphosphate.

Rats fed cyclotriphosphate and hexametaphosphate had very little difference in the percent of the dependent form of the enzyme when compared to rats which were fed orthophosphate.

CHAPTER V

DISCUSSION

The effect of 0.1% dietary cyclotetraphosphate on glycogen synthetase activity (Table 13, p. 46), ^{14}C-glucose incorporation into liver glycogen (Table 14, p. 47) and total liver glycogen (Table 15, p. 49) revealed a 15-50% decrease in these parameters when compared to rats fed 0.1% orthophosphate. These results were evident in male and female animals and when expressed relative to animal body weight. These data suggest cyclotetraphosphate may be inhibiting glycogen synthetase and thus the other parameters by interfering with the mechanism of cyclic AMP mediated protein phosphorylation. The importance of protein phosphorylation is not limited to the regulation of carbohydrate metabolism (108) and has been shown to modify a number of enzymes to date (109). The precise mechanism by which cyclotetraphosphate affects glycogenesis is open for speculation. It is attractive to consider, due to the cyclic structure of the phosphate, that cyclotetraphosphate is influencing or mimicking cyclic-AMP. Increasing levels of cyclic-AMP would activate protein kinase resulting in the phosphorylation and inactivation of glycogen synthetase and therefore decreasing glycogenesis. Under conditions which should result in low levels of cyclic-AMP, it seems possible cyclotetraphosphate is affecting glycogenesis. In theory, this is not to rule out several other possibilities including altering protein kinase itself or phosphoprotein phosphatase.

Recently it has been found rabbit muscle phosphorylase kinase can catalyze the phosphorylation of glycogen synthetase (76). Furthermore, results have indicated glycogen synthetase kinase-2 may actually be a modified form of phosphorylase kinase which is not regulated by Ca^{++}; however, it is activated by calmodulin in the presence of Ca^{++}. If calcium absorption at the intestinal level was altered by dietary polyphosphate supplementation the possible implication in the control of glycogen synthetase should be considered. The ability of the condensed phosphates to sequester calcium resulting in an increased solubility and excretion varies between linear and cyclic phosphates. A 100-fold difference exists between the binding affinity of the linear phosphates and the less efficient cyclic phosphates which do not readily bind metal cations. The differences are presumably due to the ring conformation which interferes with the formation of a chelate structure (110).

No adverse effects of high dietary levels of orthophosphate (1.3%) and hexametaphosphate (1.2%) on calcium absorption and retention were noted in rats by Dymsza et al. (111). However, rats fed diets supplemented with either 0.46% or 1.2% hexametaphosphate retained 10% more calcium in soft tissues than rats fed orthophosphate at similar levels. In view of the demonstrated effects of dietary polyphosphates on tissue calcium levels it is of interest to consider whether these changes could modulate changes in glycogen metabolism.

Although little, if any, information is available about the regulation of liver glycogen metabolism by Ca^{++}, the calmodulin-Ca^{++} complex has been reported to mediate both the inhibition and stimulation of adenylate cyclase in brain (77). In addition, the Ca^{++} dependent

form of phosphodiesterase has been reported to be stimulated by calmodulin in most tissues (78).

If cyclotetraphosphate were acting as cyclic-AMP one would expect the effects on glycogenesis to become apparent at the initiation of the feeding trial. Both in male and female animals liver glycogen synthetase activity was decreased after a 24-hour dietary period (Table 16, p. 51, and Table 17, p. 52); however, the effects were inconsistent. The inconsistencies could be due to a period of time necessary for the animals to adapt to the powdered diet and resume normal eating patterns. Similar inconsistencies were evident at hourly feeding trials when examining the uptake of ^{14}C-glucose into glycogen (Table 18, p. 54, and Table 19, p. 55) and the total liver glycogen recovered. At all levels of dietary phosphate supplementation rats fed cyclotetraphosphate had decreased levels of glycogen synthetase (Table 22, p. 59, and Table 23, p. 60). The inhibition of glycogen synthetase was evident in both the independent and total activities of the enzyme.

When the effect of cyclotriphosphate and hexametaphosphate on glycogen synthetase, ^{14}C-glucose recovered in liver glycogen and total glycogen formation was investigated, it was found that all three parameters were increased when compared to rats fed orthophosphate.

The stimulatory effect of cyclotriphosphate and hexametaphosphate may be a result of the binding of the phosphate to the regulatory subunit of protein kinase therefore preventing the binding of cyclic-AMP. Cyclic-AMP usually binds to the regulatory subunit of protein kinase releasing the free catalytic unit and activating protein kinase which with ATP phosphorylates and inactivates glycogen synthetase. However,

if the binding of the phosphates to the regulatory subunit resulted in an inhibition of cyclic AMP binding, a corresponding increase in glycogenesis would result in animals fed cyclotriphosphate and hexametaphosphate.

If cyclotetraphosphate were involved in the activation of a protein kinase, the percent of dependent activity of liver glycogen synthetase from rats fed cyclotetraphosphate would be more than from rats which were fed orthophosphate.

The activity of the dependent form of glycogen synthetase may be estimated by subtracting the independent activity from the total enzyme activity. An approximately 16% increase in the activity of the dependent form of glycogen synthetase was found in male rats (Table 13, p. 46) fed 0.1% cyclotetraphosphate when compared to rats which were fed 0.1% orthophosphate. Similar trends were seen in females. When animals were fed cyclotetraphosphate for time intervals of at least 96 hours, the percent of dependent activity was higher than in animals which were fed orthophosphate for similar lengths of time (Table 16, p. 51, and Table 17, p. 52). Furthermore, the differences in the percent of dependent enzyme activity were more prominent at 192 hours when compared with the differences between rats fed cyclotetraphosphate and rats fed orthophosphate for 96 hours. If cyclotetraphosphate supplementation resulted in an increased percent of dependent enzyme activity, under conditions where cyclic AMP levels should be low, it suggests that cyclotetraphosphate is causing an increased phosphorylation of glycogen synthetase.

The percentage of dependent activity in rats fed 0.01% cyclotetraphosphate (Table 22, p. 59, and Table 23, p. 60) also demonstrated increases in the percent of dependent enzyme activity when

compared to rats fed orthophosphate at that level of supplementation. Although the percent of dependent activity remained approximately the same with increasing levels of phosphate supplementation in rats fed orthophosphate, rats fed 0.06% cyclotetraphosphate had a higher percentage of dependent activity than rats fed 0.01% cyclotetraphosphate.

If cyclotriphosphate and hexametaphosphate were binding to the regulatory subunit of protein kinase, resulting in an inhibition of cyclic AMP binding, a lower percentage of the dependent form of glycogen synthetase would be expected in livers from animals fed these phosphates when compared to animals fed cyclotetraphosphate or orthophosphate. These data revealed that animals fed 0.1% cyclotriphosphate or 0.1% (Table 13, p. 46) hexametaphosphate had an approximately 9-16% lower percent of the dependent form of liver glycogen synthetase than animals which were fed orthophosphate, regardless of the animals' sex. Decreases in the percent dependent activity were also seen at all time intervals except at 24 and 48 hours in males and only at 192 hours in females (Table 16, p. 51, and Table 17, p. 52). There was little difference in the percent dependent activity at lower levels of supplementation in rats fed cyclotriphosphate or hexametaphosphate when compared to rats which were fed orthophosphate.

In conclusion, the results of this investigation show an effect of cyclotetraphosphate, cyclotriphosphate and hexametaphosphate on glycogenesis in the liver. Even if hexametaphosphate in its purest form is used as a food additive, it has been documented in vitro (112,113,114) as well as in vivo (115) and in this laboratory[3] that the cyclic

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[3]Allen, A. M., cited on p. 3.

phosphates may be formed during the hydrolysis of hexametaphosphate. In view of these data and the importance of protein phosphorylation in the control of diverse biological processes, of which glycogenesis is but one, the food industry should further evaluate the use of phosphate additives in processed foods.

CHAPTER VI

SUMMARY

The effect of cyclotetraphosphate, cyclotriphosphate and hexametaphosphate has been investigated in view of their extensive use as food additives. It was found cyclotetraphosphate supplementation in rat diets, at phosphate levels commonly used by the food industry in commercial products, resulted in an inhibitory effect on glycogenesis. This was evident by a decreased total and independent activity of liver glycogen synthetase, ^{14}C-glucose incorporation into glycogen and total glycogen recovered from rats fed cyclotetraphosphate when compared to orthophosphate controls. On the other hand, animals fed cyclotriphosphate and hexametaphosphate exhibited an increase in these parameters. When animals were fed the phosphate diets at lower levels of supplementation and for shorter dietary periods similar results were evident. In rats fed cyclotetraphosphate, the dependent form of glycogen synthetase represented a higher percentage of the total enzyme activity than in rats fed orthophosphate. However, animals fed cyclotriphosphate and hexametaphosphate had a lower percentage of dependent activity when compared to animals fed orthophosphate. This suggests, in accordance with the previous data, that cyclotetraphosphate, cyclotriphosphate, and hexametaphosphate may be interfering with the phosphorylation-dephosphorylation control of glycogenesis in the liver.

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