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Calcium Effects on Yeast Hexokinase

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by

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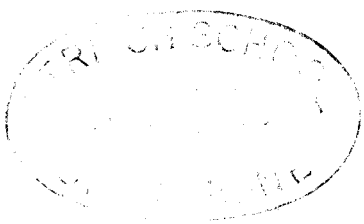

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LIFE

Mary Ann Hurley was born in Cleveland, Ohio, February 4, 1934.

She was graduated from Woodrow Wilson High School, Washington, D. C., June, 1951 and from Trinity College, Washington, D. C., June, 1955, with the degree of Bachelor of Arts.

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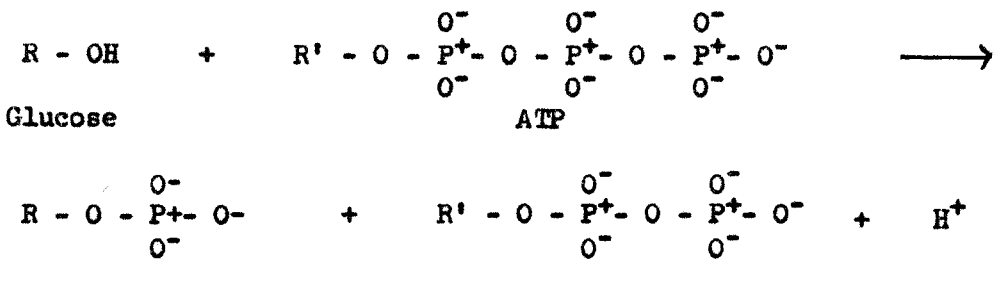
CHAPTER I

INTRODUCTION

An absolute requirement in living matter is some mechanism whereby the free energy liberated in the oxidation of organic metabolites is trapped so that it can be transferred and used for the various vital processes which require energy. The work of Meyerhof ('27, '34), Lundsgaard ('30a, '30b, '31), Lohmann ('31, '34), Warburg ('39,'41) and the Coris ('40), served as the foundation for uncovering such a mechanism which depends on the formation of energy-rich phosphate compounds. It became apparent from their work and the work of succeeding investigators that adenosinetriphosphate (ATP) is a uniquely important molecule. It is essential for the biosynthesis of organic molecules, mechanical work and work against osmotic forces in absorption and secretion.

As part of extensive research into the nature of high-energy phosphates, many investigators have studied the reaction catalyzed by hexokinase as defined by Colowick and Kalckar in 1943:



The enzyme which catalyzes this reaction in yeast has been obtained in crystalline form by several workers. Yeast hexokinase is a protein with a molecular weight of 96,600, an isoelectric point at pH 4.5 - 4.8, and contains three atoms of phosphorus per mole. (Kunitz and McDonald, '46). In the presence of ATP and magnesium, the enzyme phosphorylates glucose, fructose and mannose. The affinity for glucose is much greater than that for fructose, (K_m = 1 X 10^{-4} and 7 X 10^{-4} moles per liter, respectively) but V_{max} with fructose is twice that with glucose. The enzyme is stable in the pH range from 5 to 8, with activity rapidly lost at pH's above 9 or below 4. According to Sols et al ('58), the enzyme possesses highest activity at pH 7.5 with about one-half maximal activity at pH 5.4 and 9.4. Darrow ('57), however, states that the activity at pH 8.5 is at least two times the activity at pH 7.5.

The equilibrium constant of the reaction as determined by Robbins and Boyer ('57) is 3.86 X 10^2 at pH 6.0 and increases by a factor of approximately 10 per pH unit in the region of pH 6 to 8.

Darrow has recently developed a more convenient procedure for obtaining a crystalline preparation which is reported to be free from contaminating pyrophosphatase, phosphohexose isomerase and triosephosphate dehydrogenase which contaminated previous preparations. (Robbins and Boyer, '57).

The enzyme requires magnesium for activity (See Figure 5). This requirement has usually been presented as due to the formation of a magnesium-enzyme complex and the appropriate Michaelis constant has been evaluated by experiments in which all other substances (except the enzyme) were present in excess.

The demonstration that ATP forms complexes with many positive ions including magnesium forces a re-evaluation of the assumption that the magnesium functions only as an enzyme activator.

DiStefano and Neumann ('53) studied the distribution of calcium 45 between ion exchange resin and aqueous solution with and without ATP. They found that the addition of ATP to a fixed amount of cation and resin increased the amount of cation which was removed from the resin, indicating a calcium-nucleotide complex. Their data indicated that a mono-metal-mononucleotide complex was the major form of the complex, at least in dilute solutions.

Spicer ('52) while studying the effects of pH, ionic strength on the contractile phases of the actomyosin response to ATP found that addition of calcium or magnesium ions to the solutions of actomyosin containing ATP resulted in a decrease in pH. He also noted that in the presence of either or both of these ions, there is an increase in the rate of hydrolysis of ATP at 100°.

Burton and Krebs ('53) estimated the formation constant of $MgATP^{-2}$ complex by the titration technique. N. C. Melchior ('54) by titrating tetraalkylammonium ATP with and without sodium or potassium ions found that in certain pH ranges, less hydrochloric acid was required to reach a given pH in the presence of these ions than in their absence, indicating complex formation.

Martell and Schwarzenbach ('56) also by the titration method showed that ATP as well as other polyphosphates formed complexes with calcium and magnesium ions.

Smith and Alberty ('56) demonstrated complex formation of ATP with lithium, sodium and potassium by the titration technique, and Walaas ('58) by the ion-exchange method found that calcium and magnesium form mono-metal-mono-nucleotide complexes with ATP and other nucleotides.

Several authors have attempted a qualitative interpretation of the effects of the ATP-magnesium complex on the velocity of the hexokinase reaction. Hers ('52) found that the optimum Mg:ATP ratio depended upon the concentrations of alkaline cations and reached unity in the presence of molar potassium or sodium chloride or acetate. Liebecq ('53) concluded that since optimal activity occurred when the ratio of magnesium to ATP was 1, $MgATP^{-2}$ was the true substrate for the enzyme.

Rauflaub and Leupin ('56) were of the opinion that the active enzyme is a magnesium-protein complex which dissociates into magnesium ion and inactive protein when the magnesium ion concentration is decreased.

Sols et al ('58) found that at high, constant magnesium, the rate of the reaction increased with increasing amounts of ATP^{-4}. Melchior and Melchior ('58) studied the effects of varying the concentration of magnesium and ATP on the rate of the yeast hexokinase reaction. They found that at constant magnesium total, the initial velocity of the yeast hexokinase catalyzed reaction increased with ATP total until the ATP concentration approximated magnesium total. A further increase in ATP resulted in a decrease in velocity. From the observation that excess ATP^{-4} inhibits the reaction, they developed the following rate law:

$$V_o/V_m = (MgATP^{-2}) / \left[(MgATP^{-2}) + 0.13 + 0.27(ATP^{-4})\right]$$

which is valid for a pH range of 8.2 to 8.6 and an ionic strength of 0.3.

The rate law does not prove a definite mechanism; however, it excludes any mechanisms which give different rate laws. Several mechanisms were found which yielded kinetic equations of the same form as that derived from experiment. Each of these mechanisms included the formation of a complex involving ATP^{-4} and the enzyme. None of these mechanisms permits appreciable reaction of magnesium with the active site of the enzyme without ATP. In view of this equation and the fact that calcium forms a complex with ATP, a statement by Bailey and Webb ('48) concerning the yeast hexokinase reaction caused some perplexity. They reported: "The enzyme was inactive in the absence of magnesium ions, and these could not be replaced by calcium. The activity in the presence of 5×10^{-3}M magnesium was not affected by the addition of an equal concentration of calcium; the Ca:Mg antagonism which is so pronounced in the case of Mg-activated yeast pyrophosphatase is thus absent in the case of yeast hexokinase."

This statement appears to be based upon a single experiment and should be examined very carefully before it is accepted as fact. As has been mentioned, calcium forms a complex of considerable stability with ATP, and it can be shown that the addition of calcium chloride to a solution containing comparable amounts of magnesium and ATP will cause marked changes in the concentrations of magnesium ion, magnesium ATP and free ATP. (See Figure 1.) This list, incidentally, includes all the substances which various authors have indicated as the species responsible for the change in the rate of the hexokinase catalyzed reaction with change in magnesium and or ATP concentrations.

Figure 1 -- Test Conditions: 18 micromoles of glucose, 1.06 micrograms of enzyme, volume, 3.0 ml. The concentrations of the molecular species present in an aqueous solution, pH 8 to 9, which contain $MgCl_2$, $CaCl_2$ and tetraalkylammonium ATP were determined by solving the following simultaneous equations by approximations.

$$(ATP)_t = (HATP^{-3}) + (ATP^{-4}) + (MgATP^{-2}) + (CaATP^{-2})$$

$$(Mg)_t = (Mg^{++}) + (MgATP^{-2})$$

$$(Ca)_t = (Ca^{++}) + (CaATP^{-2})$$

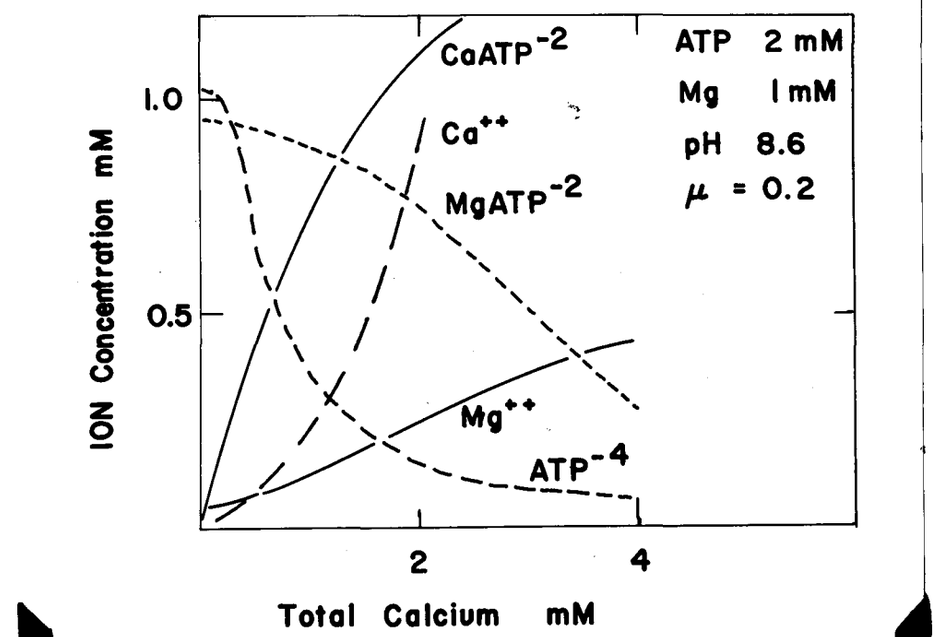
$$Ka_{HATP^{-3}} = \frac{(H^+) \times (ATP^{-4})}{(HATP^{-3})} = 1.26 \times 10^{-4} \text{ mM} \quad \text{(N. C. Melchior, '54)}$$

$$Kf_{MgATP^{-2}} = \frac{(MgATP^{-2})}{(Mg^{++}) \times (ATP^{-4})} = 20 \text{ mM}^{-1} \quad \text{(N. C. Melchior, unpublished results)}$$

$$Kf_{CaATP^{-2}} = \frac{(CaATP^{-2})}{(Ca^{++}) \times (ATP^{-4})} = 8 \text{ mM}^{-1} \quad \text{(N. C. Melchior, unpublished results)}$$

→

FIGURE 1



It therefore seemed worthwhile to re-examine the effect of calcium salts on the rate of the hexokinase catalyzed reaction, in order to ascertain whether or not any effect could be observed; since if the observation of Bailey and Webb were confirmed, the formulation of the mechanism of the magnesium-activated yeast hexokinase reaction would have to be modified to include the new information. However, if the addition of calcium salts did change the observed reaction rate, quantitative measurements of the various rates might lead to a better understanding of the mechanism of the reaction.

A large number of experiments were performed at varying levels of ATP, magnesium and calcium. The measured reaction velocity in systems containing calcium was always lower than that measured in a system lacking calcium, but otherwise identical.

CHAPTER II

MATERIALS AND METHODS

PREPARATION OF SOLUTIONS

Water, redistilled in a glass apparatus was used for preparing all solutions. Carbon dioxide was removed from the water by purging with nitrogen gas for at least 12 hours, and the storage bottle was protected with a tube filled with "Ascarite".

The following materials were used in the preparation of the reagents.

Acetic Acid -- Glacial Acetic Acid, Mallinckrodt Analytical Reagent 2504, lot BRR.

Albumin -- Armour, lot J-4902 Crystalline Albumin.

Ammonium Sulfate -- Mallinckrodt Analytical Reagent 3512.

Calcium Chloride -- Mallinckrodt Analytical Reagent 4160, lot EKS. Standardized against standard silver nitrate, with potassium chromate as indicator.

Cresol Red -- Harleco, 879-11.

Glucose -- Dextrose, Mallinckrodt, C. P., lot YBZ.

Hydrochloric Acid -- Concentrated Hydrochloric Acid, Mallinckrodt Analytical Reagent 2612, lot GPY. Standardized against standard sodium hydroxide, with phenolphthalein as indicator.

Magnesium Chloride -- Mallinckrodt Analytical Reagent 5958, lot CSP, Standardized against standard silver nitrate, with potassium chromate as indicator.

Potassium Phosphate, Monobasic -- Potassium Dihydrogen Phosphate, Mallinckrodt Analytical Reagent 7100, lot ZTB.

Potassium Phosphate Monobasic, Potassium Phosphate Dibasic, 0.05 M, pH 7.0 was prepared by dissolving 2.66 g of potassium dihydrogen phosphate (Mallinckrodt Analytical Reagent 7100, lot ZTB) and 5.31 g of potassium monohydrogen phosphate (Mallinckrodt Analytical Reagent 7092, lot AEL) in 1 liter of redistilled water.

Silver Nitrate -- Mallinckrodt 2169, lot BSY. Standardized against standard hydrochloric acid neutralized with sodium hydroxide, using potassium chromate as indicator.

Sodium Hydroxide -- Fifty-one Percent Sodium Hydroxide, Baker Analytical Reagent, lot 6062. Standardization of dilute solutions was with potassium acid phthalate (Mallinckrodt Primary Standard, lot APMA), with phenolphthalein as indicator.

Sodium Phosphate Dibasic -- Sodium Monohydrogen Phosphate, Mallinckrodt Analytical Reagent 7917, lot BDX.

Sodium Phosphate Tribasic -- Mallinckrodt Analytical Reagent, lot XHM.

Tetraethylammonium Bromide -- Eastman Kodak 1514, lot 21, recrystallized twice from 2-propanol. Standardized against standard silver nitrate with potassium chromate as indicator.

Triethanolamine -- Redistilled 2, 2', 2" nitrilotriethanol, Eastman Kodak, 1599. Standardized against standard hydrochloric acid, with brom cresol green as indicator.

Tris (Trishydroxymethylaminomethane) -- Tris, Sigma 121, lot 46-160.

Versene (Disodium Ethylenediamine Tetraacetate) -- Versene, Fisher Reagent, lot 771313.

TABLE I

COMPOSITION OF BUFFER-DYE SOLUTIONS FOR KINETIC STUDIES

A. Ionic Strength: 0.11

Solution	0.1 W	0.1 I	0.1 S
Redistilled Triethanolamine	14.93 mM	29.8 mM	59.6 mM
Hydrochloric Acid	1.04 mM	2.09 mM	4.18 mM
Cresol Red	0.03 mg/ml	0.03 mg/ml	0.03 mg/ml
Et_4NBr	0.75 M	0.75 M	0.75 M

B. Ionic Strength: 0.3

Solution	0.3 W	0.3 I	0.3 S
Redistilled Triethanolamine	15.24 mM	30.48 mM	60.96 mM
Hydrochloric Acid	1.04 mM	2.09 mM	4.18 mM
Cresol Red	0.03 mg/ml	0.03 mg/ml	0.03 mg/ml
Et_4NBr	2.14 M	2.14 M	2.14 M

Appropriate aliquots of solutions were pipetted into volumetric flasks. The mixtures were made to volume at room temperature (24 - 26°C) and stored at 4 - 6°C.

ISOLATION AND PURIFICATION OF YEAST HEXOKINASE (From the Thesis by R. A. Darrow ('57). A preprint of this method was obtained through the courtesy of Dr. S. P. Colowick.)

Four pounds (1816 g) of fresh Fleischmann's baker's yeast were crumbled by hand into small fragments and spread out in a layer from 1 to 1.5 cm deep on Whatman No. 1 filter paper which was elevated by a plastic-coated wire screen. The fragments were allowed to dry at room temperature for ten days with thorough mixing each day to insure even drying. Five hundred and seventy grams of dried yeast were obtained.

Into 1 liter of 0.2 M Na_2HPO_4 were suspended 333 g of dried yeast. The mixture was placed in a water bath at 37° and allowed to autolyze for 3 hours with occasional stirring. To the autolysate were added 130 g of celite (Johns-Manville Celite Analytical Filter Aid 1-655-A). This formed a viscous mixture, which was filtered by suction in a Buchner funnel with Whatman filter paper No. 42 covered by a layer of celite about 1/8" deep. A clear, yellowish filtrate, total volume 420 ml was obtained. The filtrate was stored overnight in the refrigerator. In order to change the pH from 5.83 to 4.41, 3 N acetic acid was added, at room temperature, dropwise with vigorous stirring by means of a glass mechanical stirrer. The acidified extract was chilled to ice-bath temperature and the precipitate which formed during acidification was removed by centrifugation in the International Centrifuge, PR 1, at 4400 rpm in the 840 head, at 0°C for 10 minutes. All subsequent steps were carried out at icebath temperature. To the supernatant was added gradually with constant stirring, 97.2 g of solid ammonium sulfate, bringing the calculated ammonium sulfate saturation to 0.4.

The material was stirred for 55 minutes after the addition of the salt. The precipitate was removed by spinning at 4400 rpm (3500 g's) for 20 minutes in the PR 1, using head 840. To the supernatant (430 ml) were added slowly and with constant stirring 42 g of solid ammonium sulfate. The mixture was stirred for 10 minutes after the completion of the addition of the salt. The copious precipitate was collected by centrifugation for 30 minutes at 4400 rpm in the PR 1, head 840, and dissolved in 40 ml of ice cold redistilled water. The resulting solution was placed in a dialysis bag and dialyzed against 1 liter of 0.05 M potassium phosphate buffer, pH 7.0 for approximately 4 hours. The dialysis was continued overnight against 2 liters of fresh buffer solution. The precipitate formed during dialysis was removed by centrifugation in the PR 1 at 4400 rpm, head 840, for 20 minutes. The volume of the supernatant (91 ml) was adjusted with the same buffer so that the optical density at 2800 A was 0.500.

To the supernatant were added 3.6 g of solid bentonite (Fisher USP, No. B-235, lot 773466). Thorough mixing was obtained by the use of a glass homogenizer. The material was spun in the Spinco Ultracentrifuge, Model L, rotor 21, at 14,000 rpm for 25 minutes at 29°F. The supernatant was decanted, and the residue was washed by suspension in 85 ml of 0.05 M potassium phosphate buffer, pH 7.0 and centrifugation as before. The supernatant solution was discarded, and the adsorbed hexokinase was eluted by the addition of 85 ml of 0.05 M Tris, 0.001 M disodium versenate buffer, pH 9.75. The mixture was spun for 60 minutes at 14,000 rpm in the Spinco, model L, rotor 21. The supernatant was decanted and neutralized with 3.9 ml of 1 M KH_2PO_4.

To the neutralized tris eluate, were added gradually and with constant stirring, 42.3 g of solid ammonium sulfate. Stirring was continued for 20 minutes after the addition of the salt. The precipitate which formed was collected by centrifugation for 12 minutes at 14,000 rpm in the 21 rotor of the Spinco model L at 29°F. The residue was dissolved in 4.1 ml of 0.1 M $K\text{-}PO_4$, 0.002 M versene, pH 7.0. The insoluble material was removed by spinning the mixture at 20,000 rpm for 30 minutes in the Spinco Model L, 40 rotor. The clear supernatant was placed in a 20 ml beaker containing a Kel F covered magnetic stirrer arranged for stirring in an ice bath. To induce crystallization, saturated ammonium sulfate, (adjusted with ammonium hydroxide so that after four-fold dilution with distilled water, the pH was 7.0 $\pm$ 0.5) was added until the solution became slightly turbid. Stirring was continued for 30 minutes after the onset of turbidity. The beaker was covered with parafilm and placed in the refrigerator at 4°C for 11 days.

Microscopic examination of the material in the beaker after this time showed an abundance of long needle-like crystals (I). To separate the crystals, the mixture was spun in the PR 1, head 823, at 2000 rpm at 0°C. The supernatant was decanted, and the crystals were dissolved in 0.4 ml of 1 M potassium phosphate, 0.002 M versene buffer, pH 7.0. The specific activity of the crystals was determined and found to be low, therefore the mother liquor of those crystals was seeded with crystals obtained from Dr. Colowick. A second crop of crystals (II) formed which were of high specific activity. They were dissolved in the phosphate-versene buffer, and neutral saturated ammonium sulfate was added dropwise until turbidity was observed. The formation of the crystals (III) appeared to be complete after three days.

When the specific activities of the crystals (III) and the mother liquor was assayed, it was found that most activity had remained in solution. The mother liquor (III) was dialyzed against 0.05 M triethanolamine, 0.033 M hydrochloric acid buffer. The dialysate was divided into aliquots of suitable quantity for our experiments and stored frozen. The specific activity remained fairly stable for about 6 months.

TABLE II

RELATIVE ACTIVITIES OBTAINED DURING PREPARATION OF HIGH SPECIFIC ACTIVITY HEXOKINASE

Test Conditions: 18 micromoles glucose, 15 micromoles ATP, 30 micromoles $MgCl_2$, 12 micromoles triethanolamine, 0.162 micromoles HCl, 0.127 mg/ml cresol red, volume, 3.0 ml.

Material	Micromoles of Acid Produced per Minute per Milligram of Protein	
	Batch I	Batch II
Mother Liquor I	88	61
Crystals I	19	22
Crystals II	73	134
Crystals III	--	57
Mother Liquor III	---	190

Evaluation of the Enzyme Preparation Used

In order to make sure that the material isolated from yeast after a number of steps actually had the properties of hexokinase, a number of experiments were necessary. Most of these experiments depended upon the measurement of the rate of acid production in a buffered medium as affected by changes in reaction conditions. It was therefore necessary to make sure that the method used for measuring acid production was valid.

Spectrophotometric Measurement of Acid Production -- This method was introduced by Wajzer in 1949 and consists of the spectrophotometric measurement of the change in concentration of the basic form of a suitable indicator as a function of time. The method was further developed by Darrow ('57) and modified by Melchior and Melchior ('58). The buffering substance is triethanolamine: $N(CH_2CH_2OH)_3 + H^+ \rightleftharpoons H^+N(CH_2CH_2OH)_3$. Cresol red is the indicator, which acts as a weak acid which dissociates into the colored dinegative ion and hydrogen ion depending on the pH of the solution.

$$(C_6H_4SO_2OC(C_6H_3\text{-}3\text{-}CH_3\text{-}4\text{-}OH)C_6H_3\text{-}3\text{-}CH_3\text{-}4O)^- \rightleftharpoons H^+ + C_6H_4SO_2OC(C_6H_3\text{-}3\text{-}CH_3\text{-}4O)^{=}_2$$

At 5720 A, the absorption of the dinegative ion of cresol red is so much greater than that of the mononegative ion, that the observed optical density is proportional to the concentration of the dinegative ion of the dye and the difference between the optical density of cresol red in a sufficiently basic solution to obtain complete dissociation and the observed optical density is proportional to the acid concentration of the dye.

In order to show that the measured change in optical density is proportional to the change in the basic form of the buffer, aliquots of buffer-dye (described on page 11) were mixed with known quantities of standard HCl and made to a known volume. The pH and the absorption around the wavelength 5720 A of these solutions were measured at 25°C. See Table III.

TABLE III

SPECTROPHOTOMETRIC TITRATION OF BUFFER-DYE SOLUTIONS

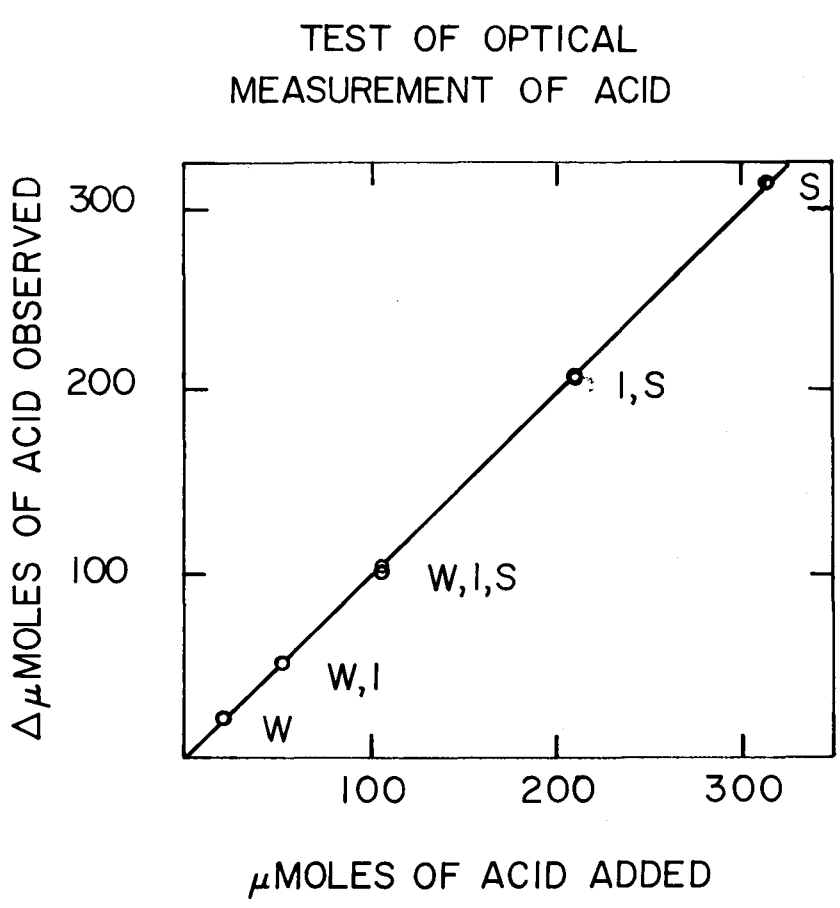
Test Conditions: 15.0 ml aliquots of the buffer-dye solutions described on page 11 were diluted to 100 ml after the addition of acid.

Buffer	umoles of HCl	Optical Density	pH
0.3 W	15.68	0.584	--
0.3 W	36.60	0.519	--
0.3 W	67.98	0.435	--
0.3 W	120.28	0.288	--
0.3 W	basic	0.643	12
0.3 I	31.35	0.590	8.89
0.3 I	83.65	0.513	8.49
0.3 I	135.90	0.440	8.22
0.3 I	240.55	0.292	7.81
0.3 I	basic	0.643	12
0.3 S	62.70	0.598	--
0.3 S	167.30	0.519	--
0.3 S	271.90	0.444	--
0.3 S	376.50	0.368	--
0.3 S	basic	0.643	12

From the data presented in Table III for the solutions prepared from the Intermediate Buffer-Dye solution, the pKa for cresol red was calculated to be 7.84, 7.89, 7.88, 7.89. It was therefore concluded that the pKa for the second dissociation of cresol red was 7.89. The first measurement was not given great weight since it was made at too high a salt to acid ratio of the dye. From the amounts of amine and HCl which had been added the apparent pKa for the ammonium component of the buffer was calculated to be 7.76, 7.84, 7.85, 7.86. Again excluding the first solution, the agreement is acceptable, but it is possible that the observed slight trend was real. This kind of trend would occur if a small amount of carbon dioxide had been absorbed by the solutions during the storage period after distillation of the amine. This possibility was explored by calculating the apparent increase in acid over that present in the first solution as calculated from the optical measurement, and comparing it with the amount actually added. The best fit to the data was with pKa of the amine 0.03 units less than that of cresol red, 7.86.

On the basis of this value for the difference between the pKa of the indicator and the buffer, the amount of acid added to each of the three buffers was calculated from the data in Table III and plotted against the amounts actually added. The agreement is satisfactory. See Figure 2.

FIGURE 2



Test Conditions: Wavelength, 5720 A, slit width, 0.03, 25°C. W = weak, I – intermediate, S – strong buffer-dye solutions. Ionic strength, 0.3.

Method of Calculation -- Since the relative pK's of cresol red and triethanolamine were measured at the ionic strengths used, and the concentration of the amine is known, the amount of acid present can be calculated from the spectrophotometric measurement of the concentration of cresol red ion.

In a given mixture, the following relationships exist:

$$pK_{amine} + \log \frac{salt}{acid}_{amine} = pH = pK_{cresol\ red} + \log \frac{salt}{acid}_{cresol\ red}$$

Buffer Acid + Buffer "Salt" (amine) = total amine.

$$\text{Buffer Acid} = \frac{\text{total amine}}{\left[\frac{K_{amine}}{K_{cresol\ red}} \times \frac{\text{Cresol Red (salt)}}{\text{Cresol Red (acid)}}\right] + 1}$$

It should be noted that the calculation depends upon the difference in the two pK's and not upon the absolute value of either.

Tables based on the measurements of the pK's at the ionic strengths used in our experiments were set up from which could be read the micromoles of buffer acid for each optical density. In experiments in which the amount of ATP^{-4} exceeded the amounts of magnesium and/or calcium, the buffering effect of the uncomplexed ATP^{-4} was corrected for by calculating the amount of $HATP^{-3}$ present in the system at each optical density observed using the pK of $HATP^{-3} = 6.90$. (Melchior,'54).

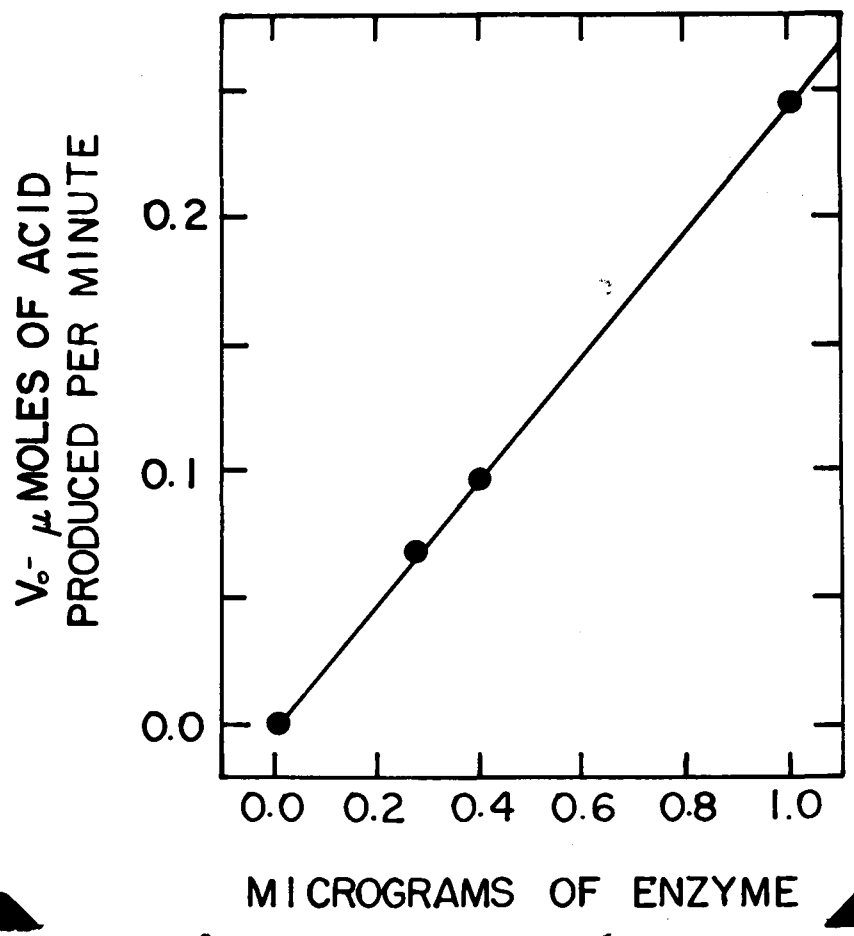
Effect of Enzyme Concentration -- It can be seen from Figure 3 that the rate of product formation is directly proportional to the amount of enzyme present.

Effect of Glucose -- Figure 4 shows that the initial rate of reaction was reasonably constant (130 - 140 micromoles of acid produced per minute per milligram of enzyme) over a period until most of the substrate was used up, after which the reaction rate decreased in proportion to the amount of glucose consumed in the reaction. In the first 15 minutes of reaction, at least 1.7 micromoles of hydrogen ion were produced. The rate after 15 minutes was 0.005 micromoles of hydrogen ion produced per minute. (See Table IV.) There was no significant acid production after the glucose was used up, therefore it was concluded that there were no significant amounts of acid-producing enzymes, such as ATP-ase, or phosphatases.

Effect of Magnesium -- In the absence of magnesium ion, no acid was produced. With increasing amounts of magnesium, there was an increase in the rate of hydrogen ion formation. See Figure 5.

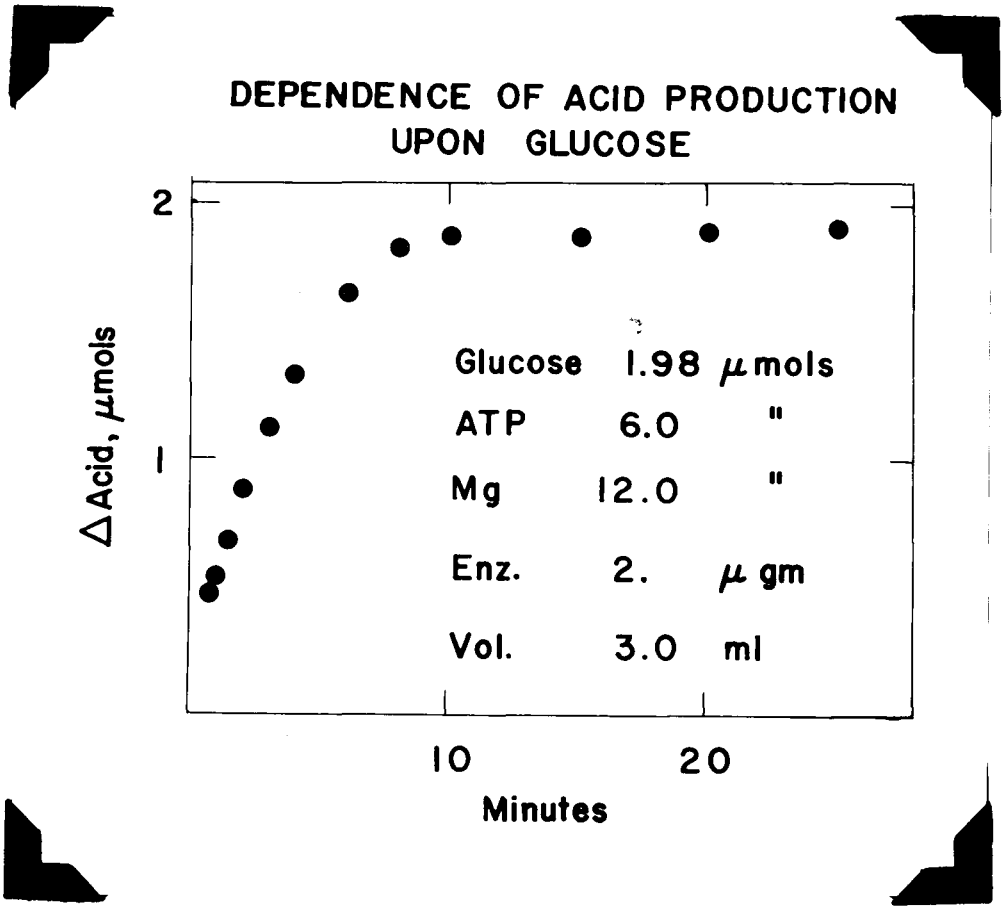
FIGURE 3

EFFECT OF ENZYME CONCENTRATION ON ACID PRODUCTION



Test Conditions: 18 micromoles of glucose, 6 micromoles of ATP, 12 micromoles of magnesium chloride, 300 micrograms of albumin, 0.1 I buffer, volume, 3.0 ml.

FIGURE 4



Test Conditions: 0.1 I buffer, 10 micrograms albumin.

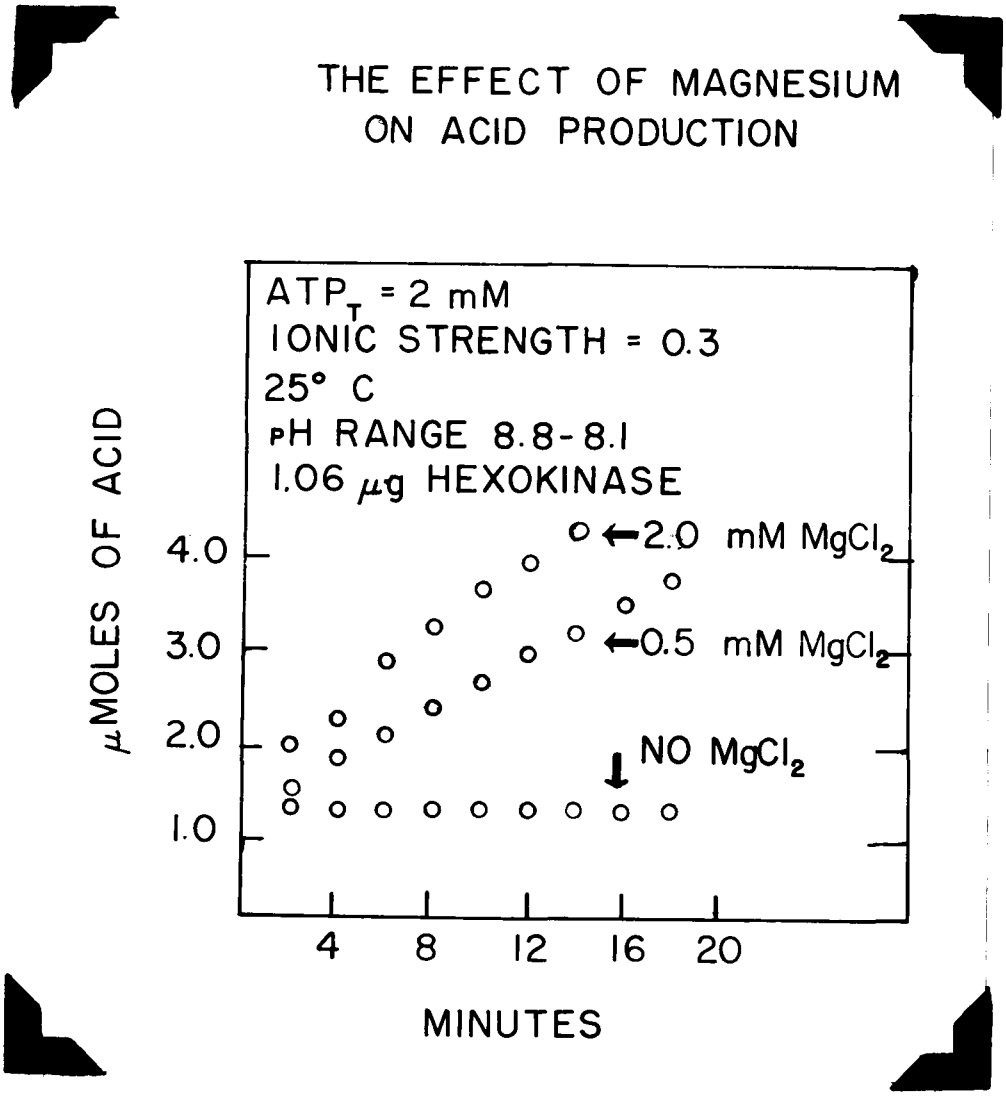
TABLE IV

DEPENDENCE OF ACID PRODUCTION UPON GLUCOSE

Test Conditions: 1.98 micromoles of glucose, 6 micromoles of ATP, 12 micromoles of $MgCl_2$, 2 micrograms of enzyme, 0.1 I buffer, 10 micrograms of albumin, volume, 3.0 ml.

Time in Minutes	Micromoles of Acid Present
Zero	2.663
0.75	2.968
1.0	3.046
1.5	3.182
2.0	3.378
3.0	3.635
4.0	3.835
6.0	4.156
8.0	4.339
10.0	4.380
15.0	4.380
20.0	4.400
25.0	4.421
33.0	4.441
45.0	4.461

FIGURE 5



Test Conditions: 18 micromoles of glucose, 300 micrograms of albumin, 0.3 I buffer, volume, 3.0 ml.

DETERMINATION OF PROTEIN CONCENTRATION (Method of Warburg and Christian, '41, as tabulated by E. Layne, '57.)

The optical density of the enzyme dissolved in 18 mM glucose was read against 18 mM glucose at 260 and 280 millimicrons. The hydrogen lamp was used with a slit width of 0.1. The protein concentration in milligrams per milliliter is equal to 1.55 X O. D. at 280 minus 0.76 X O. D. at 260.

SPECTROPHOTOMETRIC MEASUREMENTS

A Beckman Model DU spectrophotometer with 1 cm fused silica cells was used for all determinations. The temperature of the cells was maintained constant by the circulation of water from a constant temperature bath through hollow metal plates on both sides of the cells. Tap water was circulated through the lamp housing to prevent overheating of the lamp. Factors to correct for variation in the path length and light transmittance of the cells were determined to aid in obtaining accurate results. In all kinetic studies, the following conditions were used: wavelength, 5720 A; temperature 25°C; tungsten lamp; slit width, 0.03.

CELL CORRECTIONS

The absorbance of the cells filled with redistilled water was observed against a reference cell at the wave lengths used in these studies. The maximum correction was 0.004 density units or 1% of the usual reading.

PATH LENGTHS

The readings of a solution of the intermediate buffer-dye in its most basic form were obtained against a redistilled water blank.

The ratio of the optical density of the solution in the reference cell to the optical densities of the solution in the other cells was calculated using 1.000 cm as the path length of the reference cell, thus giving the relative path lengths of each cell. All cells were within 0.7% of the same length.

PREPARATION OF ADENOSINETRIPHOSPHATE

A) For assay of enzyme -- To 0.234 g of crystalline disodium ATP (Pabst lot 132) were added 300 micromoles of triethanolamine, 40.4 micromoles of hydrochloric acid, and sodium hydroxide to obtain a pH between 8.5 and 9.0. This was made to 25 ml with redistilled water. The concentration of ATP was determined from the absorbance of an accurate 1:500 dilution at 2590 A, slit width, 0.1.

B) For kinetic studies -- Dibarium ATP (Pabst lot 129) was added to Dowex 50 WX 8 cation exchange resin in the tetraethylammonium form. (1 g of ATP per 20 ml of resin.) This mixture was shaken vigorously for 30 minutes on a mechanical shaker. A few drops of supernatant, were tested for traces of barium by the addition of 1 M H_2SO_4. If no precipitate was evident, the supernatant was filtered through Whatman No. 50 filter paper. The pH of the solution was adjusted to a range between 8.5 and 9.0 by the addition of 0.03 M tetramethylammonium hydroxide. The concentration of ATP was determined from the the absorption of an accurate 1:1000 dilution at 2590 A, slit width, 0.1.

ENZYME ASSAY

Solutions

A) 15 mM tetrasodium ATP. For preparation, see page 29.

B) 30 mM $MgCl_2$ containing 0.0127 mg/ml cresol red.

C) Enzyme sample in 18 mM glucose. (In this assay, a change of 5 optical density units per minute is convenient. The amount of the enzyme sample used was adjusted by trial to give approximately this activity.)

Procedure

Exactly one milliliter of solution A was added to 1.00 ml of solution B in a Beckman cell which was placed in the cell compartment of the spectrophotometer to allow for temperature equilibration. At zero time, 1.00 ml of the enzyme-glucose solution was added to the cell. The contents were well mixed, and the optical density of the solution was read against a water blank every 2 minutes for a period of 20 minutes. The rate of the reaction was determined from the slope of the line obtained by plotting the micromoles of acid (converted from the optical density) against time.

PROCEDURE FOR KINETIC STUDIES

Solutions

A) Enzyme in 18 mM glucose containing 300 micrograms per milliliter albumin.

B) An accurate 2:5 dilution with redistilled water of either weak, intermediate, or strong buffer-dye solutions (see page 11) with tetraethylammonium adenosinetriphosphate of the desired concentration.

C) $MgCl_2$ and $CaCl_2$ of varying concentrations.

Procedure

Into a Beckman cell were placed 1.00 ml of buffer-dye-ATP solution (pipetted with a Kimax A pipette which has a tolerance of ± 0.6%) and 1.00 ml of enzyme-glucose-albumin solution (pipetted with a Kimax B pipette which has a tolerance of ± 1.2%.) These solutions were usually pipetted about 10 to 20 minutes before placing the cell in the Beckman cell compartment for temperature equilibration. After allowing about 5 minutes for temperature equilibration, 1.00 ml (Kimax B pipette) of the appropriate magnesium and/or calcium solution was added at zero time to start the reaction. The pipette was drained for 20 seconds after which a parafilm cap was placed on the cell and the contents were mixed by inversion. Three cells were prepared at the same time and the reactions were started at zero, 30 and 180 second intervals. The optical density of each solution was read every 2 minutes for a period of 20 minutes. The rate of the color-change was regulated by using the buffer-dye of appropriate strength, so that the change in optical density was about 5 density units per minute. The optical density observed was converted into micromoles of acid and the rate of the reaction was determined from the slope of the line obtained when micromoles of acid were plotted against time. Whenever a curved line resulted, the rate was determined from a tangent drawn to the curve at the point where the pH was equal to 8.6.

pH MEASUREMENTS

pH measurements were obtained by use of the Beckman pH meter, Model G, using Beckman extension calomel and glass electrodes. For measurements in which temperature control was critical, the solutions were placed in glass vessels with outer jackets, through which water, controlled at 25°C by means of a constant temperature bath, was circulated. Through a rubber top were inserted the electrodes and a capillary tube by means of which nitrogen gas was bubbled into the solutions. An additional opening in the rubber top provided an exit for the gas. The solutions were stirred by a grounded magnetic stirrer. Readings were taken after allowing sufficient time for temperature and electrode equilibration.

PREPARATION OF TETRAETHYLAMMONIUM RESIN

Dowex 50 WX 8 resin in the barium form was poured into a glass chromatography column 60 cm high by 5 cm in diameter to a height of 45 cm. The resin was backwashed with 4 liters of 3 N HCl at the rate of 3 ml per minute. It was then washed with redistilled water until no precipitate was obtained by testing the effluent with 0.1 N $AgNO_3$. The resin was transferred to an Erlenmeyer flask. The supernatant solution was decanted and 10% aqueous tetraethylammonium hydroxide (Eastman Kodak 2078) was added until the pH of the supernatant solution was the same as that of the original tetraethylammonium hydroxide - approximately 11 - as tested with pHydrion paper. (188 g of the base were required.)

The resin stood in the base for 24 hours, after which the supernatant was decanted, and the resin was transferred to the column with 0.1 M tetraethylammonium bromide, with which it was backwashed until the pH of the effluent was about 6.5. The resin was then washed with redistilled water until the test with 0.1 N $AgNO_3$ for halide was negative.

COMPARISON OF Et_4NBr WITH Me_4NCl

In previous work with hexokinase, (Melchior and Melchior, '58), a high ionic strength was maintained by using tetramethylammonium chloride in the reaction mixture. Tetraethylammonium bromide, a salt of similar structure, is more easily purified and therefore its use was preferred. The following data show that the rate of the yeast hexokinase reaction was not altered by the use of tetraethylammonium bromide.

Test Conditions:

3 micromoles of ATP, 6 micromoles of glucose, 15 micrograms per milliliter of Sigma Enzyme, Type III, 3 micromoles of $MgCl_2$, 6 micromoles of triethanolamine, volume, 3.0 ml.

Relative Rates:

With 0.3 M Me_4NCl -- 1.00 micromole of acid per minute per microgram of enzyme.

With 0.3 M Et_4NBr -- 1.04 micromoles of acid per minute per microgram of enzyme.

CHAPTER III

EXPLORATORY ENZYME STUDIES

In the first kinetic studies with purified yeast hexokinase, it was found that replicate experiments run approximately four hours apart were not in quantitative agreement. Investigation of the reasons for this lack of reproducibility showed at least two effects were present. First, there is a measurable production of acid when mixtures of ATP and magnesium are allowed to stand for several hours. See Table V. This amount of acid, however, is insignificant when compared to the rapid production of acid which occurs in the same system in the presence of minute amounts of enzyme.

The second effect was more difficult to identify, but proved to be due to the very low concentration of some of the hexokinase solutions used in this study. This may be related to the phenomenon reported by Berger et al ('46) but does not seem to be identical with it. These authors report an immediate loss of half of the enzyme activity upon dilution beyond a certain point (4.2 micrograms/ml). Darrow ('57) found that this effect was reproduced with his preparation when the same assay system (manometric) was used, but not when his spectrophotometric method was used. Berger et al ('46) found that numerous proteins would prevent loss of activity upon dilution and Darrow confirmed this with bovine serum albumin (which had no effect on the assay by the spectrophotometric method).

To evaluate our assay system with respect to the variables time, dilution and bovine serum albumin stabilization, several dilutions of yeast hexokinase were prepared with and without albumin. These solutions were assayed immediately, then after 4 and 24 hour storage at room temperature (23 - 26°C).

From Table VI, it is clear that the solutions containing albumin retained enzymic activity more effectively. It may be of interest to note that while V_o was zero in the most dilute solutions after 4 and 24 hours, a finite rate of acid production, (181 and 49 micromoles per minute per milligram of enzyme, respectively) was observed after 8 to 10 minutes incubation with magnesium, ATP, and glucose.

It can be seen from TABLES VII and VIII that albumin does not change the absorption of the dinegative ion of cresol red nor the absorption maximum of cresol red. It appears from the slight change in pH which occurred in the more weakly buffered regions, that albumin has a slight buffering action in the reaction system.

TABLE V

ACID PRODUCTION IN ABSENCE OF ENZYME

Test Conditions: 18 micromoles of glucose, 15 micromoles of ATP, 100 micrograms per milliliter albumin, 0.3 I and 0.3 S buffers, volume, 3.0 ml.

Time in Minutes	Increase in Acid - Micromoles	
	0.5 mM Mg	8.0 mM Mg
2	0.0	0.0
6	0.0	0.039
10	0.0	0.039
18	0.059	0.140
19 Hours	1.124	1.394

TABLE VI

THE EFFECT OF ALBUMIN ON THE REACTION RATE OF HEXOKINASE

Test Conditions: 18 micromoles of glucose, 12 micromoles of magnesium, 6 micromoles of ATP, 0.1 I buffer, *100 micrograms per milliliter of albumin, volume, 3.0 ml. V_o = initial velocity.

Concentration of Enzyme During		V_o - Micromoles of Acid Produced per Minute per Milligram of Enzyme		
Storage	Assay	0 Hours	4 Hours	24 Hours
0.99 ug/ml	0.33 ug/ml	208	213	115
		211*	247*	222*
0.39 ug/ml	0.13 ug/ml	204	131	152
		228*	242*	219*
0.27 ug/ml	0.09 ug/ml	144	0	0
		220*	258*	216*

TABLE VII

THE EFFECT OF ALBUMIN ON THE pK OF CRESOL RED

Test Conditions: 15.0 ml aliquots of 0.3 I buffer were made to 100 ml after the addition of hydrochloric acid and *100 micrograms of albumin. Temperature, 25°C, Tungsten lamp, 5720 A, wavelength, slit width, 0.03.

Micromoles of Hydrochloric Acid	Optical Density	pH	pK Cresol Red
83.65	0.513	8.49	7.89
	0.504*	8.46*	7.90*
135.95	0.440	8.22	7.88
	0.429*	8.21*	7.91*
240.55	0.292	7.81	7.89
	0.279*	7.80*	7.92*
basic	0.643		
	0.643*		

TABLE VIII

THE EFFECT OF ALBUMIN ON THE ABSORPTION PEAK OF CRESOL RED

Test Conditions: 18 micromoles of glucose, 6 micromoles of ATP, 12 micromoles of magnesium chloride, 0.1 I buffer, volume, 3.0 ml. Temperature, 25°C, Tungsten lamp, 5720 A, wavelength, slit width, 0.03.

Wave Length	Optical Density Without Albumin	Optical Density with 100 micrograms per ml albumin
600	0.099	0.098
590	0.235	0.229
580	0.409	0.392
576	0.447	0.430
574	0.452	0.434
572	0.454	0.435
570	0.450	0.431
568	0.439	0.421
564	0.411	0.393
560	0.372	0.357

CHAPTER IV

RESULTS

The following tables give the results of experiments run at two levels of ATP and several concentrations of magnesium and calcium. Typical results obtained in these experiments are presented in Figure 6.

TABLE IX

THE EFFECT OF MAGNESIUM ON THE VELOCITY AND IONIC SPECIES WITH 2 mM ATP

Test Conditions: 18 micromoles of glucose, 6 micromoles of ATP, 1.06 micrograms of enzyme, 300 micrograms of albumin, 0.3 I and 0.3 S buffers, volume, 3.0 ml. Velocity measured at pH 8.6, concentrations in millimoles per liter.

$(Mg)_t$	Micromoles of Acid Produced per Minute per Milligram of Enzyme			(Mg^{++})	$(MgATP^{-2})$	(ATP^{-4})
	Experiment Series I	II	III			
0.0	0	0	0	0	0	1.96
0.5	117	128	116	0.0163	0.484	1.486
	121	126	117			
	119	129	111			
1.0	176	190	159	0.0463	0.954	1.030
	184	179	166			
	171	177	163			
2.0	200	202	180	0.294	1.706	0.290
	193	208	161			
	161	181	161			
			161			
4.0	188	161	165	2.048	1.952	0.0471
	187	171	176			
	171	179	170			
8.0	183	162	187	6.017	1.983	0.0166
	179	167	168			
		160	160			

TABLE X

THE EFFECT OF MAGNESIUM ON THE VELOCITY AND IONIC SPECIES WITH 5 mM ATP

Test Conditions: 18 micromoles of glucose, 15 micromoles of ATP, 1.06 micrograms of enzyme, 300 micrograms of albumin, 0.3 I and 0.3 S buffers, volume 3.0 ml. Velocity measured at pH 8.6, concentrations in millimoles per liter.

$(Mg)_t$	Micromoles of Acid Produced per Minute per Milligram of Enzyme			(Mg^{++})	$(MgATP^{-2})$	(ATP^{-4})
	Experiment Series I	II	III			
0.0	0	0	0	0	0	4.90
0.5		81 80 76	63 61	0.0056	0.4944	4.42
1.0	121 119 122	121 125 124	96 91 95	0.0125	0.9875	3.93
2.0	200 193 198	173 176 184	135 153 144	0.033	1.967	2.97
4.0	203 203	195 247 223	182 160 167	0.167	3.833	1.143
5.0	191 198 197	202 193 197	167 173 165	0.48	4.52	0.47
8.0	179 182 191	194 190 186	155 149 146	3.081	4.919	0.079

TABLE XI

THE EFFECT OF CALCIUM ON THE VELOCITY AND IONIC SPECIES WITH 2 mM ATP

Test Conditions: 18 micromoles of glucose, 6 micromoles of ATP, 1.06 micrograms of enzyme, 300 micrograms of albumin, 0.3 I and 0.3 S buffers, volume, 3.0 ml. V = velocity measured at pH 8.6 = micromoles of acid produced per minute per milligram of enzyme. Concentrations are tabulated in millimoles per liter.

Salts	V	(Mg^{++})	$(MgATP^{-2})$	(Ca^{++})	$(CaATP^{-2})$	(ATP^{-4})
1.0 mM Mg 0.5 mM Ca	120 104 111	0.072	0.928	0.081	0.419	0.642
1.0 mM Mg 1.0 mM Ca	51 51 51	0.120	0.880	0.254	0.746	0.367
1.0 mM Mg 2.0 mM Ca	34 35 34	0.249	0.751	0.906	1.094	0.151
2.0 mM Mg 1.0 mM Ca	57 63 66 76 54	0.601	1.399	0.517	0.483	0.1167
2.0 mM Mg 2.0 mM Ca	40 57 56	0.812	1.188	1.262	0.738	0.073
2.0 mM Mg 4.0 mM Ca	38 32 31 39 47	1.072	0.928	2.972	1.028	0.0433

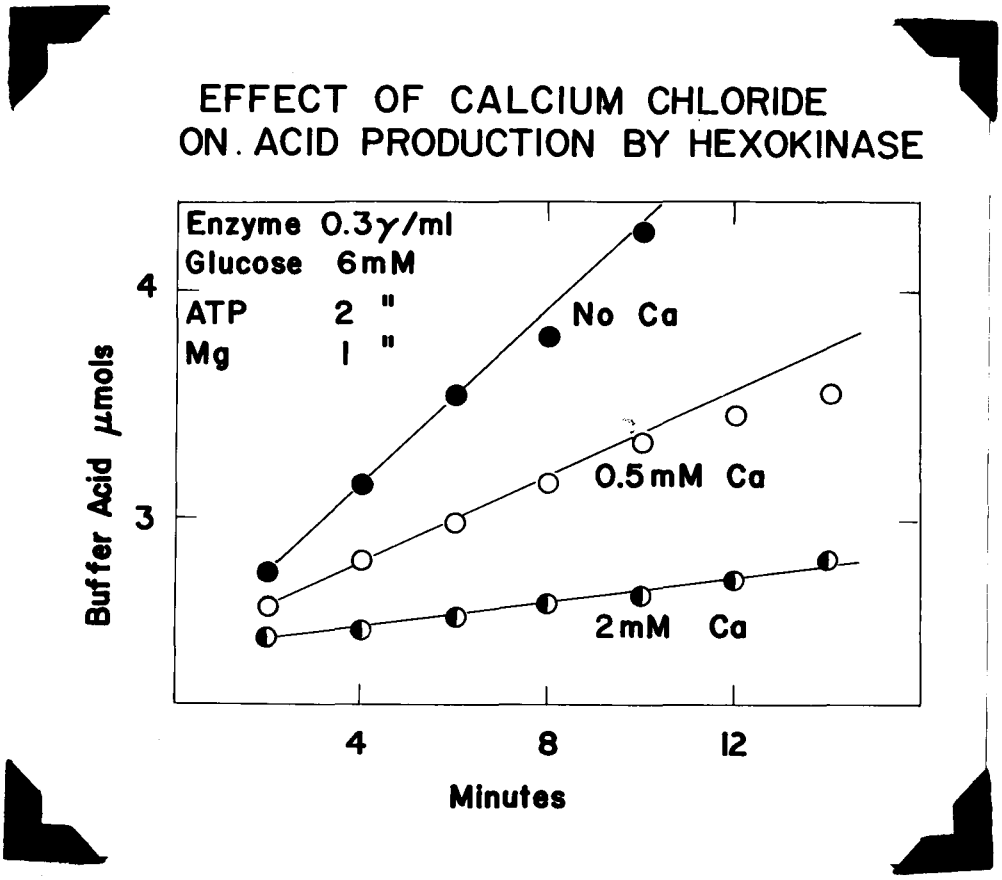
TABLE XII

THE EFFECT OF CALCIUM ON THE VELOCITY AND IONIC SPECIES WITH 5 mM ATP

Test Conditions: 18 micromoles of glucose, 15 micromoles of ATP, 1.06 micrograms of enzyme, 300 micrograms of albumin, 0.3 I and 0.3 S buffers, volume, 3.0 ml. V = velocity measured at pH 8.6 = micromoles of acid produced per minute per milligram of enzyme. Concentrations are tabulated in millimoles per liter.

Salts	V	(Mg^{++})	$(MgATP^{-2})$	(Ca^{++})	$(CaATP^{-2})$	(ATP^{-4})
2.0 mM Mg 1.0 mM Ca	103 109 104	0.046	1.954	0.056	0.944	2.102
2.0 mM Mg 2.0 mM Ca	77 70 71	0.077	1.923	0.181	1.819	1.258
2.0 mM Mg 4.0 mM Ca	31 33 37	0.258	1.742	1.079	2.921	0.337
8.0 mM Mg 4.0 mM Ca	44 47	4.136	3.864	2.911	1.089	0.047
8.0 mM Mg 8.0 mM Ca	37 39	4.754	3.246	6.28	1.72	0.034

FIGURE 6



Test Conditions: 300 micrograms of albumin, 0.1 I or 0.1 S buffer, volume, 3.0 ml.

CHAPTER V

DISCUSSION

The analysis of an enzyme catalyzed reaction can be considered complete only when the entire course of the reaction can be quantitatively explained. Understanding of the variables which influence the rate of the yeast hexokinase reaction has not progressed far enough to make this quantitative explanation a practical goal for short term research. In the work reported here the plan was to study the kinetic effects of the addition of calcium salts to reaction mixtures containing yeast hexokinase, glucose, ATP, magnesium salts, buffer components, and (presumably inert) salts to maintain a reasonably constant ionic strength. The major part of the research has been concerned with attempting to establish a system in which such comparisons would be meaningful.

The first experimental difficulty encountered was the lack of stability of the very dilute solutions of enzyme which were necessary in these experiments. As has been discussed earlier, the addition of a relatively large quantity of bovine serum albumin appears to remove this difficulty, but its use does raise questions concerning its effect upon the other components of the kinetic system. At the moment it is not possible to determine whether it is more meaningful to use a fraction of a microgram of high specific activity enzyme plus 100 micrograms of a known protein, or to use larger quantities (five to twenty-five micrograms) of low specific activity enzyme with its attendant uncertainties of composition.

These observations of lack of stability are not in agreement with those of Darrow using a similar enzyme preparation and assay system. However, the reduction in stability observed here takes place relatively slowly and would not have been detected by Darrow. The most interesting observation in this connection is that a considerable part of the enzyme activity is restored if magnesium and ATP are added to the glucose-enzyme mixture. That is, when the components of the reaction mixture are combined, there is at first no measurable release of acid. However, after a lapse of time which is related to the age of the diluted enzyme solution, acid production begins and continues during the period of measurement. See Appendix I, Tables XIV and XV. This apparent increase in in vitro enzyme activity following exposure to substrate is of intrinsic chemical interest, and may prove to be interesting from a biochemical standpoint.

A second interesting characteristic of this reaction is the effect of the ratio of magnesium to ATP upon the kinetic order of the reaction as calculated in the usual fashion. In a reaction mixture containing 2 mM ATP and 4 mM magnesium, the rate of acid production (utilization of ATP) is first order in total ATP over the entire reaction period, during which more than 50% of the ATP was used. In contrast to this, in a reaction mixture identical to that above except that total magnesium was reduced to 0.5 mM the production of acid was substantially constant over the entire reaction period during which approximately 50% of the ATP was consumed. In other words, the reaction was zero order in total ATP, although the velocity was significantly lower.

These apparently discordant observations are reconciled if one considers the molecular species present in each of these systems. Using the constant for the formation of magnesium-ATP complex given by Melchior and Melchior, it is possible to show that in the first system (excess magnesium) the concentration of the complex is practically equal to the total ATP present at any time and that the reaction is really first order with respect to the concentration of the magnesium-ATP complex. In the second system, ATP is in excess over the entire range of measurement, the concentration of the complex changes only slightly, and the observed constant rate is proportional to the (unchanging) concentration of the complex. It is interesting to note that toward the close of the observation period, the rate observed had diminished by 7% at a point at which the calculated $MgATP^{-2}$ concentration had diminished by 5%. It should be noted here that the observed relationship to the concentration of the magnesium-ATP complex does not prove that this complex is the reacting substrate. As Melchior and Melchior have indicated, the same kinetic relationship would be given by a sequential reaction of ATP^{-4} with the enzyme followed by interaction of the enzyme-ATP complex with the magnesium ion, provided that the dissociation of the magnesium-ATP complex is sufficiently rapid. At the present time, no information concerning the rate of this reaction is available.

A second difficulty encountered was that upon addition of calcium to the system, the initial rates of reaction of replicate experiments were not always in agreement. In some instances, only after 6 to 8 minutes did the rates measured on identical reaction mixtures converge to give comparable velocities.

The velocity at pH 8.6 was chosen as the reference velocity since at this pH, the steady-state velocity seemed to have been achieved, and under the conditions used, the reaction was still in its initial state; the amount of substrate present was very nearly the same as the initial substrate. Comparison of the results at the same pH eliminates the possibility of variation in the reaction due to the acceleration in the velocity of the reaction as the pH optimum of the enzyme is approached. The data obtained in this research did not present evidence of the existence of such a phenomenon, but Sols et al ('58) reported that the rate of the reaction increases as the pH changes from 9 toward 7.4.

Approximately 90 comparisons were made of yeast hexokinase reaction mixtures which were identical except for the addition of calcium chloride to one and not to the other. In every case the observed rate was lower in the presence of calcium. See Figure 6. Figures 7 and 8 show typical results plotted as percent reduction of the rate observed in the same system in the absence of calcium.

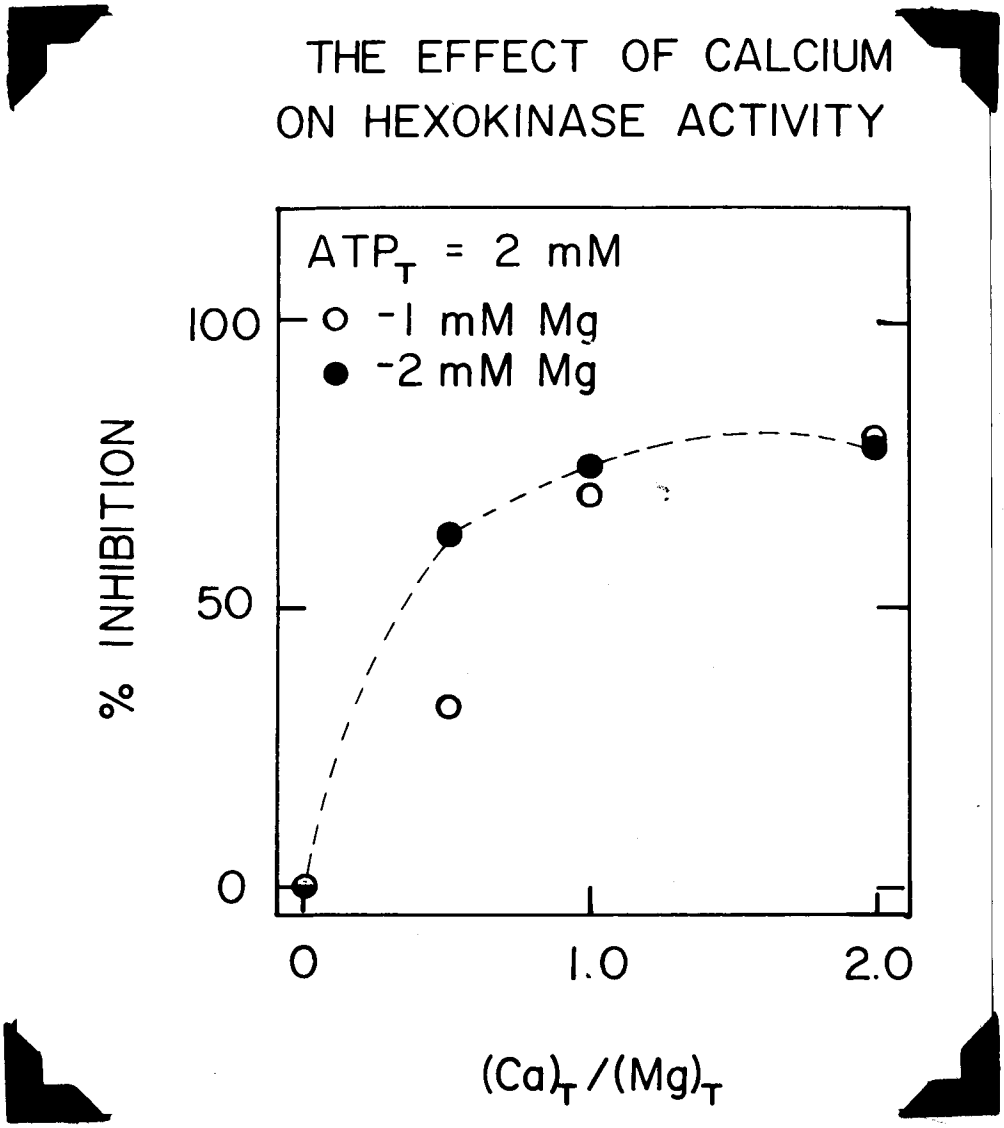
It is difficult to determine exactly the cause of the reduction of velocity since when calcium is introduced into the reaction system, ($MgATP^{-2}$), and (ATP^{-4}) decrease, whereas (Mg^{++}), (Ca^{++}) and ($CaATP^{-2}$) increase. See Figure 1. The inhibition is largest in the systems in which there is a substantial fraction of calcium in excess of the ATP available to react with calcium. (Total ATP minus total magnesium). See Tables XI and XII.

This would indicate that free calcium ion is a potent inhibitor of yeast hexokinase ---a conclusion which is in harmony with that of Melchior and Melchior concerning the small inhibition in the presence of relatively high concentrations of sodium chloride, but which contradicts the results obtained by Bailey and Webb ('48).

It is very difficult to determine the reason for this disagreement since their assay method seems to be without defect. The incubation mixture contained 10 mM $MgCl_2$, 0.12 M glucose, 10 mM veronal-acetate buffer - pH 7.9 and enzyme in a quantity sufficiently small to give a linear breakdown for 7 to 8 minutes. The reaction was started by the addition of 0.5 ml of Na_2H_2ATP, the final concentration of which was 5.14 mM. The incubation mixture (total volume, 5.0 ml) was placed in the water bath at 38°. Samples of 1 ml were removed at 1, 3, 5, and 7 minutes and pipetted directly into 0.12 ml of 10 N HCl and the orthophosphate was determined by the Fiske-Subarrow method.

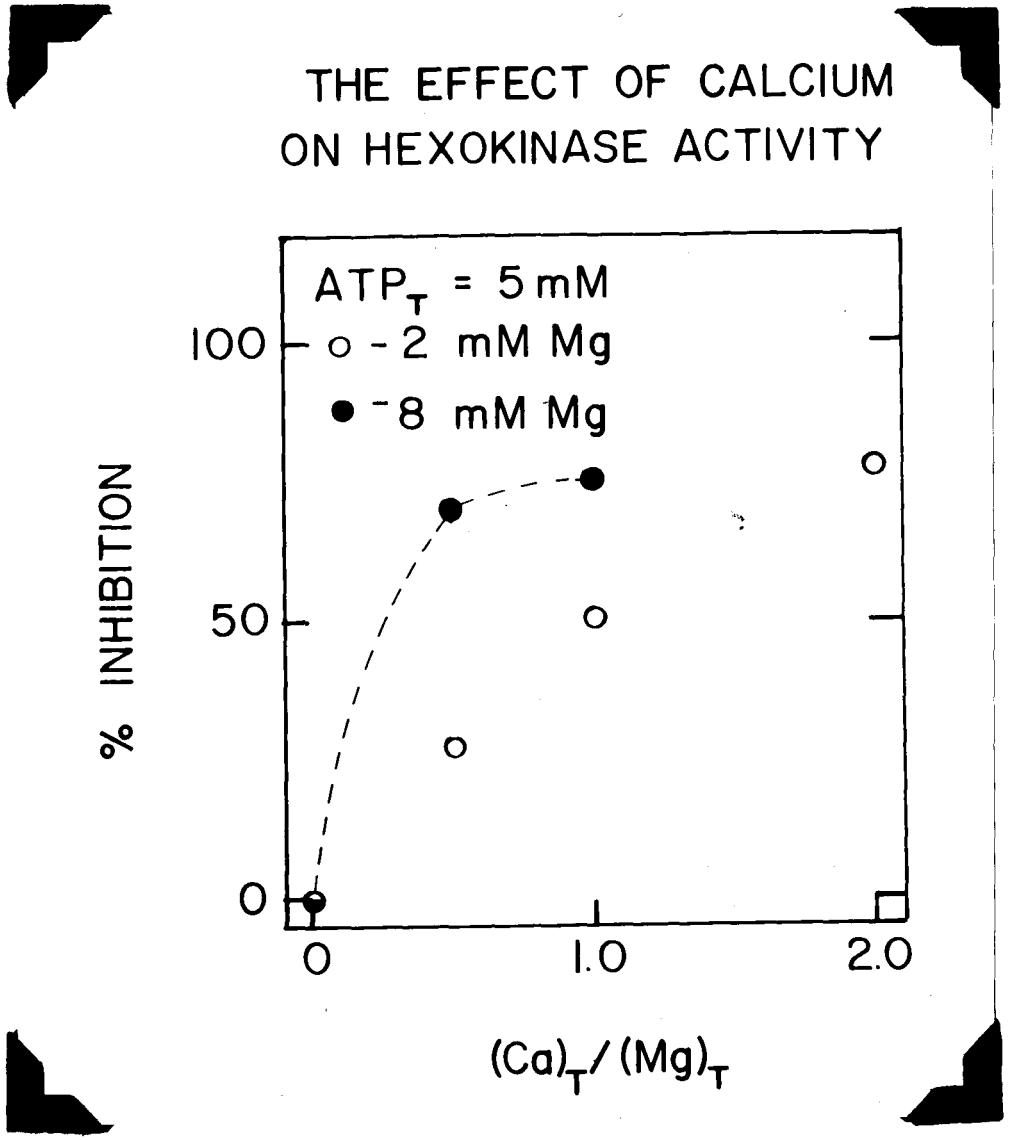
No specific data were given for the calcium experiment. The only detail presented was that calcium and magnesium ions were both present in the incubation mixture in equal concentrations -- 5×10^{-3} M which differs from their standard assay conditions.

Figure 7



Test Conditions: 18 micromoles of glucose, 1.06 micrograms of enzyme, 300 micrograms of albumin, 0.3 I or 0.3 S buffers, volume, 3.0 ml.

Figure 8



Test Conditions: 18 micromoles of glucose, 1.06 micrograms of hexokinase, 300 micrograms of albumin, 0.3 I and 0.3 S buffers, volume, 3.0 ml.

CHAPTER VI

SUMMARY

A kinetic study was made of the reaction between ATP and glucose in the presence of high specific activity yeast hexokinase. The concentrations of ATP, $MgCl_2$, $CaCl_2$ and albumin were varied. It was found that yeast hexokinase loses activity when stored for several hours at low concentrations and that this loss of activity is prevented by addition of albumin.

The addition of calcium chloride decreased the measured velocity under all conditions tried.

BIBLIOGRAPHY

Bailey, K. and Webb, E. C. 1948 Purification of yeast hexokinase and its reaction with BB'-dichlorodiethyl sulphide. Biochem. J., 42:60-68.

Berger, L., Slein, M. W., Colowick, S. P. and Cori, C. F. 1946 Isolation of hexokinase from baker's yeast. J. Gen. Physiol., 29: 379-391.

Burton, K. and Krebs, H. A. 1953 The free-energy changes associated with the individual steps of the tricarboxylic acid cycle, glycolysis and alcoholic fermentation and with the hydrolysis of the pyrophosphate groups of adenosinetriphosphate. Biochem. J., 54: 94-107.

Colowick, S. P. and Kalckar, H. M. 1943 Role of myokinase in transphosphorylations. J. Biol. Chem., 148: 117-126.

Colowick, S. P., Welch, M. S. and Cori, C. F. 1940 Phosphorylation of glucose in kidney extract. J. Biol. Chem., 133: 359-373.

Darrow, Robert Arnold 1957 The purification and assay of yeast hexokinase. Thesis, Johns Hopkins University.

DiStefano, Victor and Neuman, W. F. 1953 Calcium complexes of adenosinetriphosphate and adenosinediphosphate and their significance in calcification in vitro. J. Biol. Chem., 200: 759-763.

Hers, H. G. 1952 Role du magnesium et du potassium dans la reaction fructokinasique. Biochim et Biophys Acta, 8: 424-430.

Kunitz, M. and MacDonald, M. R. 1946 Crystalline hexokinase (heterophosphatases) method of isolation and properties. J. Gen. Physiol., 29: 393-411.

Layne, Ennis 1957 The spectrophotometric and turbidimetric methods for measuring proteins. Methods in Enzymology, Volume III by Colowick and Kaplan. Academic Press Inc., New York, 453.

Liebecq, C. 1953 The hexokinase activity of rat muscle extract and the lability of the Mg-ATP complex. Biochem. J., 54: xxii-xxiii.

Lohmann, K. 1931 Darstellung der Adenylpyrophosphosaure aus Muskulatur. Biochem. Z., 233: 460-469.

Lohmann, K. and Meyerhof, O. 1934 Uber die enzymatische Umwandlung von Phosphoglycerinsauer in Brenztraubensaure and Phosphosaure. Biochem. Z., 273: 60-72.

Lundsgaard, Einar 1930 a Untersuchungen uber Muskelkontraktionen ohne Milchsaurebildung. Biochem. Z., 217: 162-177.

Lundsgaard, Einar 1930 b Weitere Untersuchungen uber Muskelkontraktionen ohne Milchsaurebildung. Biochem. Z., 227: 51-83.

Lundsgaard, Einar 1931 Uber die Energetik der anaeroben Muskelkontraktion. Biochem. Z., 233: 322-343.

Martell, Arthur E. and Schwarzenbach, G. 1956 Adenosinephosphate und Triphosphat als Komplexbildner fur Calcium und Magnesium. Helv. Chim. Acta, 39: 653-661.

Melchior, N. C. 1954 Sodium and potassium complexes of adenosinetriphosphate: equilibrium studies. J. Biol. Chem., 208: 615-627.

Melchior, N. C. and Melchior, J. B. 1958 The role of complex metal ions in the yeast hexokinase reaction. J. Biol. Chem., 231: 609-623.

Meyerhof, Otto 1927 Uber die enzymatische Milchsaurebildung in Muskelextrakt. Biochem. Z., 183: 176-215.

Rauflaub, J. and Leupin, I. 1956 Uber die Anwendung von Metallpuffern bei Fermentreaktionen. Die pMg-Aktivitatskurve der Hexokinase aus Hefe. Helv. Chim. Acta, 39: 832-837.

Robbins, E. A. and Boyer, P. D. 1957 Determination of the equilibrium of the hexokinase reaction and the free energy of hydrolysis of adenosinetriphosphate. J. Biol. Chem., 224: 121-135.

Smith, R. M. and Alberty, R. A. 1956 The apparent stability constants of ionic complexes of various adenosine phosphates with monovalent cations. J. Phys. Chem., 60: 180-184.

Sols, Alberto, de la Fuente, Gertrudis, Villar-Palasi, Carlos and Asensio, Carlos 1958 Substrate specificity and some other properties of baker's yeast hexokinase. Biochim. Biophys. Acta, 30: 92-101.

Spicer, Samuel S. 1952 The relation between the magnesium-pyrophosphate complex and the actomyosin reaction to adenosinetriphosphate. J. Biol. Chem., 199: 301-305.

Wajzer, Jacques 1949 Test spectrophotometrique de l'hexokinase. Compt Rend L'Acad des Sciences, 229: 1270-1272.

Walaas, Eva 1958 Stability constants of metal complexes with mononucleotides. Acta Chem. Scand., 12: 528-536.

Warburg, Otto and Christian, Walter 1939 Isolierung und Kristallisation des Proteins des oxydierenden Garungsferments. Biochem. Z., 303: 40-68.

Warburg, Otto and Christian, Walter 1941 Isolierung und Kristallisation des Garungsferments Enolase. Biochem. Z., 310: 384-421.

APPENDIX I

PRELIMINARY EXPERIMENTS

TABLE XIII

THE EFFECT OF MAGNESIUM ON ACID PRODUCTION

Test Conditions: 18 micromoles of glucose, 6 micromoles of ATP, 0.1 I buffer, volume, 3.0 ml. $MgCl_2$ and ATP-buffer-dye solutions were mixed on the day of the experiment. The reaction was started by the addition of the enzyme. pH range was 8.6 to 8.3.

Concentration of $MgCl_2$	V_o - Micromoles of Acid Produced per Minute per Milligram of Enzyme			
	Concentration of Enzyme	0.92 ug/ml	0.57 ug/ml	0.29 ug/ml
0.5 mM		48 47	--	--
1.0 mM		--	71 70 77	--
2.0 mM		--	--	70 67 63 74
4.0 mM		--	--	57 57

TABLE XIV

THE EFFECT OF CALCIUM ON ACID PRODUCTION - I

Test Conditions: 18 micromoles of glucose, 6 micromoles of ATP, 0.1 I buffer, volume, 3.0 ml. The salts and ATP-buffer-dye solution were mixed on the day of the experiment. The reaction was started by the addition of the enzyme. pH range 8.6 - 8.3. max V maximum velocity attained in the particular reaction.

Concentration of Salts	Micromoles of Acid Produced per Minute per Milligram of Enzyme							
Concentration of Enzyme	2.86 ug/ml		0.95 ug/ml		0.57 ug/ml		0.29 ug/ml	
	V_o	max V	V_o	max V	V_o	max V	V_o	max V
2.0 mM Mg	--	--	70	70	58	58	0 12	56 63
2.0 mM Mg 1.66 mM Ca pre-incubated with enzyme	--	--	--	--	0	12	--	--
3.33 mM Ca	0	1.7	--	--	--	--	--	--
2.0 mM Mg 1.66 mM Ca	--	--	5.3 4.5	13 15	7.3	15	0	17

TABLE XV

THE EFFECT OF CALCIUM ON ACID PRODUCTION - II

Test Conditions: 18 micromoles of glucose, 6 micromoles of ATP, 0.1 I buffer, volume, 3.0 ml. The salts and ATP-buffer-dye solution were mixed on the day of the experiment. The reaction was started by the addition of the enzyme. pH range was 8.6 to 8.3.

Concentration of Salts	Micromoles of Acid Produced per Minute per Milligram of Enzyme					
Concentration of Enzyme	0.91 ug/ml		0.46 ug/ml		0.23 ug/ml	
	V_o	max V	V_o	max V	V_o	max V
0.25 mM Mg	27	31	18	27	--	--
	31	31				
0.5 mM Mg	--	--	48	48	36	36
			29	51		
1.0 mM Mg	--	--	51	63	15	46
			36	68		
2.0 mM Mg	63	63	66	66	15	55
	48	55	58	69	15	58
0.25 mM Mg 0.105 mM Ca	13	26	7	22	--	--
	29	29				
0.25 mM Mg 0.21 mM Ca	19	24	21	21	--	--
	25	25				

TABLE XV (Continued)

THE EFFECT OF CALCIUM ON ACID PRODUCTION - II

Concentration of Salts	Micromoles of Acid Produced per Minute per Milligram of Enzyme					
	Concentration of Enzyme 0.91 ug/ml		0.46 ug/ml		0.23 ug/ml	
	V_o	max V	V_o	max V	V_o	max V
0.25 mM Mg 0.42 mM Ca	7.4 20	23 20	0	23	--	--
0.5 mM Mg 0.21 mM Ca	33	33	36 20	44 39	--	--
0.5 mM Mg 0.42 mM Ca	35 36	35 36	34	34	--	--
0.5 mM Mg 0.84 mM Ca	18 20	25 20	7	28	--	--
1.0 mM Mg 0.42 mM Ca	33	33	33 48	41 48	--	--
1.0 mM Mg 0.84 mM Ca	14 21 22	20 23 22	--	--	--	--
1.0 mM Mg 1.67 mM Ca	11 15 13	11 15 13	--	--	--	--

TABLE XV (Continued)

THE EFFECT OF CALCIUM ON ACID PRODUCTION - II

Concentration of Salts	Micromoles of Acid Produced per Minute per Milligram of Enzyme					
	Concentration of Enzyme	0.91 ug/ml		0.46 ug/ml		0.23 ug/ml	
		V_o	max V	V_o	max V	V_o	max V
2.0 mM Mg 0.84 mM Ca		18 18	18 20	21	25	--	--
2.0 mM Mg 1.67 mM Ca		16 15 14	16 15 14	--	--	--	--

TABLE XVI

THE EFFECT OF CALCIUM ON ACID PRODUCTION - III

Test Conditions: 18 micromoles of glucose, 0.85 micrograms of enzyme, 300 micrograms of albumin, 0.1 I and 0.1 S buffers, volume, 3.0 ml. The enzyme solution and ATP-buffer-dye solution were added separately. The reaction was started by the addition of the salts. *Reaction started by the addition of the enzyme.

Concentration of Salts	V_o - Micromoles of Acid Produced per Minute per Milligram of Enzyme	
	2 mM ATP	5 mM ATP
0.5 mM Mg	170 176	--
1.0 mM Mg	213 224	179 175
2.0 mM Mg	235* 237 228	256 250
4.0 mM Mg	231 238	304 284
8.0 mM Mg	221* 250* 247 240	251* 251 245
1.0 mM Mg 0.5 mM Ca	105 106	
1.0 mM Mg 1.0 mM Ca	65 67	
1.0 mM Mg 2.0 mM Ca	37 37	
4.0 mM Mg 2.0 mM Ca	56 60 51	
4.0 mM Mg 4.0 mM Ca	48 50 41	

TABLE XVI (Continued)

THE EFFECT OF CALCIUM ON ACID PRODUCTION - III

Concentration of Salts	V_o - Micromoles of Acid Produced per Minute per Milligram of Enzyme
	2 mM ATP
4.0 mM Mg 8.0 mM Ca	38 38 38

APPROVAL SHEET

The thesis submitted by Mary Ann Hurley has been read and approved by three members of the faculty of the Graduate School.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

Dec 12, 1959
Date

M C McClelland
Signature of Adviser