THESIS

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STUDIES ON MAMMALIAN METAPHASE CHROMOSOMES
AND
CHARACTERIZATION OF PHOSPHOFRUCTOKINASE MEMBRANE BINDING
AND ACTIVITY INHIBITION BY HEXACYANOFERRATE(11)

presented by

David Phillip Lapenson

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William C. Dahl Jr
Major professor

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STUDIES ON MAMMALIAN METAPHASE CHROMOSOMES AND CHARACTERIZATION OF PHOSPHOFRUCTOKINASE MEMBRANE BINDING AND ACTIVITY INHIBITION BY HEXACYANOFERRATE(II)

By

David Phillip Lapenson

A THESIS

Submitted to
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ABSTRACT

STUDIES ON MAMMALIAN METAPHASE CHROMOSOMES
AND
CHARACTERIZATION OF PHOSPHOFRUCTOKINASE MEMBRANE BINDING
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1. Two methods, moving-boundary electrophoresis and a rapid isoelectric focusing procedure, were investigated as possible techniques for obtaining quantities of pure individual types of mammalian metaphase chromosomes. In both cases, the experiments were hampered by precipitation of chromosomes in the electric field. Conditions were found under which precipitation could be minimized, but not eliminated.

2. At pH 6.8, pig kidney phosphofructokinase, yeast glucose-6-phosphate dehydrogenase, and yeast phosphoglucose isomerase were competitively inhibited 90, 30, and 30%, respectively, by 1 mM hexacyanoferrate(II), in the presence of 0.2 mM hexose-phosphate substrate. Unlike all previously reported inhibitions of glycolytic enzymes by hexacyanoferrate, this inhibition does not involve oxidation of enzyme, substrate, or enzyme-substrate complex. Rather, it appears to be due to a reversible binding of hexacyanoferrate at, or near, the hexose-phosphate binding site of the enzyme.

3. In differential centrifugation studies at pH 7.4, we found that 50 to 75% of the phosphofructokinase activity in pig liver homogenates is particulate, and associated with a specific membrane fraction. In sucrose density gradient equilibrium centrifugation experiments with step-gradients, particulate phosphofructokinase floats, under conditions where nuclei and aggregated proteins sediment to the bottom. These studies suggest that pig liver phosphofructokinase binds to the plasma membranes.

4. The particulate phosphofructokinase is solubilized immediately by addition of millimolar concentrations of fructose-6-P, fructose-1,6-P_2, glucose-6-P, or AMP, to the homogenate. When the added hexose-phosphates are depleted, the solubilized phosphofructokinase again becomes particulate. There is a direct correlation between the disappearance of solubilizer metabolites and formation of particulate phosphofructokinase. Triton X-100 (1%) solubilizes, but relatively slowly, requiring nearly an hour for maximum solubilization.

5. In contrast, rat liver phosphofructokinase appears to be entirely soluble. Rabbit liver phosphofructokinase sediments with the microsomal fraction, but is not bound to microsomes (or glycogen), and appears to be highly self-associated.

6. In experiments with pig hepatocytes, most of the phosphofructokinase activity appeared to be lost during the hepatocyte isolation, and the low remaining activity was soluble. Total phosphofructokinase activity (measured at high fructose-6-P concentration) increased or

decreased predictably when hepatocytes were incubated at 37° in high concentrations of glucose or lactate, respectively.

VITA

David P. Lapenson was born February 24, 1954, in Philadelphia, PA. He attended junior high school and high school in Glastonbury Connecticut. In addition to academic studies, Mr. Lapenson had a strong interest in music, and played french horn in the band, piano in the dance band, and also played folk guitar and jazz piano. He graduated from high school in June 1972.

For his undergraduate work, Mr. Lapenson pursued a curriculum in pre-veterinary medicine at the University of Connecticut, with a strong emphasis on chemistry and biology. He also did an independent research project on mycoplasmas and mycoplasma viruses, under the direction of Dr. Allan Liss. He graduated with a Bachelor of Science degree in May 1976. Mr. Lapenson then attended graduate school at Michigan State University to study biochemistry, and worked under the guidance of Dr. William C. Deal, Jr. Mr. Lapenson's major research project involved studies on the regulation and properties of mammalian phosphofructokinase. He also obtained much experience in cell culture and electron microscopy.

For postdoctoral training, Mr. Lapenson has accepted a position in the Department of Microbiology at the Boston University School of Medicine, working under the direction of Dr. Se-Kyung Oh. He will be studying immune regulation.

ORGANIZATION OF THE THESIS

The major areas of research are covered individually in the five chapters of this thesis. For convenience to the reader, each chapter is presented as an independent entity in the format of a scientific paper, with its own Abstract, Introduction, Materials and Methods, Results, and Discussion sections. The only deviation from this format is that the references for all the chapters of the thesis are combined at the end of the thesis.

Chapter 2 has been published under the title "Novel Pronounced Reversible Inhibition of Phosphofructokinase, Glucose-6-Phosphate Dehydrogenase, and Phosphoglucose Isomerase by Hexacyanoferrate(II)," by David P. Lapenson and Willian C. Deal, Jr., (1979), Archives of Biochemistry and Biophysics 193:521-528. A preliminary report of the results of Chapter 2 was presented at the 1979 meeting of the Federation of American Societies for Experimental Biology, in Dallas Texas (David P. Lapenson (1979), Federation Proceedings 38:654, Abstract No. 2237).

Chapter 3 is being prepared for publication, and it is hoped that some of the unpublished results in Chapters 1, 4, and 5 will provide a stimulus for further study, and hopefully publication.

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ABBREVIATIONS

Those abbreviations not defined below are listed in the Journal of Biological Chemistry Instructions to Authors, Volume 258 (1983).

AS	ammonium sulfate
BSA	bovine serum albumin
CHO	chinese hamster ovary
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
Fructose-1,6-P_2	D-fructose 1,6-bisphosphate
Fructose-2,6-P_2	D-fructose 2,6-bisphosphate
Fructose-6-P	D-fructose 6-phosphate
FCS	fetal calf serum
Glucose-6-P	D-glucose 6-phosphate
G6PD	glucose 6-phosphate dehydrogenase
αGPDH	α-glycerolphosphate dehydrogenase
HCM solution	1 m$\underline{M}$ HCl, 0.7 m$\underline{M}$ $CaCl_2$, 0.3 m$\underline{M}$ $MgCl_2$, pH 3
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HK	hexokinase
HPC buffer	1 $\underline{M}$ hexylene glycol, 0.1 m$\underline{M}$ PIPES, pH 6.8, 0.5 m$\underline{M}$ $CaCl_2$
HSCM solution	3.3 m$\underline{M}$ HCl, 0.1 $\underline{M}$ sucrose, 0.7 m$\underline{M}$ $CaCl_2$, 0.3 m$\underline{M}$ $MgCl_2$

LDH	lactate dehydrogenase
MK	myokinase
PEP	phosphoenol pyruvate
PFK	phosphofructokinase
PGI	phosphoglucose isomerase
PIPES	piperazine-N,N'-bis(2-ethanesulfonic acid)
RPM	revolutions per minute
SCM solution	0.1 M sucrose, 0.7 mM $CaCl_2$, 0.3 mM $MgCl_2$
T-PBS buffer	(TRIS-phosphate buffered saline) 25 mM TRIS, 7 mM NaH_2PO_4, 137 mM NaCl, 5 mM KCl, pH 7.4
TPI	triosephosphate isomerase
U	standard (micromolar) unit of enzyme activity
v	catalytic reaction velocity
V_{max}	maximum reaction velocity

STUDIES ON MAMMALIAN METAPHASE CHROMOSOMES

CHAPTER 1

ELECTRON MICROSCOPY AND ELECTROPHORESIS OF CHINESE HAMSTER OVARY METAPHASE CHROMOSOMES.

A. ABSTRACT

Our long-term goal in this research was to isolate one (or more) individual type of mammalian metaphase chromosome, and characterize the proteins and the DNA comprising it. Two methods, moving-boundary electrophoresis and a rapid method for isoelectric focusing, were investigated as separation techniques for metaphase chromosomes, isolated from partially synchronized CHO cells.

Moving boundary electrophoresis appeared to yield a rough size separation of chromosomes. However the chromosomes gradually precipitated in the electric field, limiting the resolution obtainable. The precipitation was minimized by running the electrophoresis at neutral pH, in low ionic strength, and in the presence of high (1 or 2 M) concentrations of hexylene glycol.

Using a rapid, multi-sample isoelectric focusing technique, CHO metaphase chromosomes were focused in 4 hr, which is much faster than the 24 to 48 hr required by conventional methods under similar conditions. Chromosomes isolated in hexylene glycol at pH 7 focused as a band in the pH range of 3.0 to 3.9, and remained focused in this region over a 12 hr period.

An electron microscopic study showed that chromosomes isolated at neutral pH in hexylene glycol are much more intact than those isolated at pH 3. Chromosomes isolated in hexylene glycol were also found to be highly resistant to the uncoiling effects of high NaCl concentration.

B. INTRODUCTION

Our long-term goal in this research was to isolate one (or more) individual type of mammalian metaphase chromosome, and characterize the proteins and the DNA comprising it. CHO cells were selected as a source of metaphase chromosomes because CHO cells have only 11 different types of chromosomes; this would provide one of the best systems for separating chromosomes, since there are so few to separate from each other. We investigated two techniques, moving-boundary electrophoresis and a rapid isoelectric focusing procedure, as possible techniques for obtaining quantities of pure individual types of metaphase chromosomes. Previous workers in this laboratory have developed valuable, new, improved methods for both of these techniques.

The most frequently used approach for fractionating mammalian metaphase chromosomes has involved velocity sedimentation in density gradients (1-12). These techniques are useful for obtaining a rough fractionation of chromosomes into size classes (9). The greatest resolution has been obtained using zonal rotors, with large gradient volumes (10-12). However, to separate different types of chromosomes having the same size, other techniques are needed in addition to velocity sedimentation.

Isopycnic banding has also been investigated as an approach to separating metaphase chromosomes (9, 13-16); however, native chromosomes were all found to band at approximately the same density (at 1.36

g/ml). However, preincubating the chromosomes with trypsin or 0.25 N HCl (prior to centrifugation) resulted in a broadening of the band, and yielded a rough size-fractionation (9). (This was attributed to a larger proportion of protein being extracted from the smaller chromosomes, resulting in an increased density.) However this technique did not have an advantage over velocity sedimentation. Also, the chromosomes tended to aggregate, since they were highly concentrated in a small region of the gradient.

Conventional isoelectric focusing has been used by previous workers (4, 17) as a method for fractionating chromosomes. Electrofocusing of the small chromosomes fraction from HeLa cells, obtained from density gradients, yielded sub-populations of chromosomes having an enrichment of ribosomal RNA genes. In using conventional methods however, focusing times of 24 to 48 hr were often required, and the long exposure of the chromosomes to the electric field caused a gradual change in the isoelectric point; also some precipitation occured.

Using a variety of conditions, we investigated a rapid isoelectric focusing procedure, recently developed in our laboratory, and also moving boundary electrophoresis, for their ability to separate CHO metaphase chromosomes. Our goal was to determine whether these methods, in addition to standard sedimentation techniques, would allow us to obtain pure individual types of chromosomes. Electron microscopy was used to determine the purity of the degree of intactness of the isolated chromosomes.

C. MATERIALS AND METHODS

The procedures described below were adapted from the work of Boezi et al. (23), and Thompson and Baker (24).

1. Materials

MEM-alpha medium (plus ribonucleotides, deoxyribonucleotides, and L-glutamine) and fetal calf serum (FCS) were obtained from Grand Island Biological Company (Gibco). MEM-alpha is an enriched cell culture medium, containing essential and non-essential amino acids, and 5.6 mM glucose (122). Penicillin-G (K salt), streptomycin sulfate, and colcemid were purchased from Sigma. Dimethyl sulfoxide (DMSO) and hexylene glycol (99%+) were from Aldrich. Sucrose was obtained from Mallinckrodt. Ampholytes were purchased from Bio-Rad (pH 3-10) and Pharmacia (pH 2.5-5.0). All chemicals were reagent grade or better.

Stock culture media (serum-free) was prepared by dissolving 10.16 g of powdered media, 50,000 units (31 mg) penicillin-G (K salt), 50 mg streptomycin sulfate, and 2.2g $NaHCO_3$, in deionized-distilled H_2O, bringing the final volume to 900 ml. Media was sterilized by filtration, using plastic 0.2 μ millipore filter units (Nalge no. 120-0020) and stored at 4°. Before use, media was supplemented with fetal calf serum, to a final concentration of 10%. FCS was heat-inactivated by incubating at 56° for 30 min.

Most solutions used for cell culture were sterilized by membrane filtration (0.2 μ millipore filters). For smaller volumes, a 25 mm membrane filter holder (Gelman) was used, with a syringe. The filter and holder were autoclaved on the wet cycle (120° for 30 min). DMSO was autoclaved before use.

2. Cell Culture

Chinese hamster ovary (CHO) cells (23, 25) were maintained in suspension culture at 37°, in MEM-alpha medium plus ribonucleotides and deoxyribonucleotides, 10% FCS, and antibiotics, as described previously. The cell line is auxotrophic for proline (26). Stock cultures were maintained in 10 ml plastic culture tubes (Corning or Falcon), on a roller drum (Bellco). The doubling time was about 11 to 12 hr, and cells grew to densities of about 1-2x 10^6/ml. (Cultures could be stored for 1 week at 4° without loss in viability.) For obtaining larger amounts of cells, spinner flasks (50 to 1000 ml capacity; Bellco, no. 1969) were used. Flasks were incubated either with caps tightened, in a 37° warm room, or with caps loosened, in a 5% CO_2-100% humidity incubator.

3. Cell Counting

Cells were counted using a Coulter Counter (model ZBI, Coulter Electronics), with the following instrument settings:

Matching switch	20K
Gain trem	6
Amplification	2
Lower gate	10 (may vary)
Upper gate	110
Aperture current	2
Sample volume	500 µl

Aliquots (0.2 ml) of culture were diluted into 20 ml of isotonic buffered saline (Scientific Products; S/P) in 20 ml counting vials (S/P), and inverted several times immediately before counting. Five counts were taken and averaged together. To convert the counts (in particles per 1/2 ml) in the diluted aliquots, to cell/ml in the undiluted suspension, the count is multiplied by the following dilution factor:

$$\text{Dilution factor} = \frac{1}{0.5\ \text{ml}} \times \frac{20.2}{0.2} = 202$$

4. Frozen Storage of CHO Cells.

Stock CHO cell lines can be stored frozen, and new stocks thawed periodically, to reduce the risk of mycoplasma contamination. Freezing is done as follows: A CHO cell suspension is centrifuged (in a clinical centrifuge) and the cell pellet is resuspended in complete medium (medium plus 10% FCS) plus 10% DMSO, to a concentration of 10^6

cells/ml. One ml aliquots are transferred to plastic freezer vials (Bellco), and placed in liquid N_2. The vials are then stored in a -80 freezer.

5. Thawing of Frozen CHO Cell Lines.

Vials are removed from the freezer and placed immediately in a 37° water bath. After thawing, the contents are diluted in 9 ml of complete medium at 37°, and incubated on a roller drum as described previously.

6. Metaphase Chromosome Isolation.

a. Accumulation of CHO Cells in Metaphase.

For accumulating CHO cells in metaphase, we generally used a low temperature incubation to induce partial synchrony (121), followed by treatment with colcemid. The procedure is as follows:

Suspension cultures are started at initial densities of 5 to 10 x 10^4 cells/ml. When the cell density reaches about 2 x 10^5/ml, flasks are transfered to a 4°C cold room, and incubated, while stirring, for at least 2 hr, or overnight. Flasks are then incubated again at 37°, for about 12 to 14 hr. When the cell density reaches about 4 x 10^5/ml, colcemid (100 µg/ml, in absolute ethanol) is added, to a final concentration of 40 ng/ml. Cultures are incubated with colcemid 8 hr, and harvested as described in the following section. (Incubations with colcemid longer than 8 hr cause micronuclei to form, and should be avoided.) The yield of metaphase cells was about 35 to 40%.

b. Chromosome Isolation at pH 6.8, in 1 M Hexylene Glycol.

This chromosome isolation procedure is based on the sucrose gradient procedures described by Huberman and Attardi (2), and the buffer described by Wray and Stubblefield (27, 28). Procedures were done at 0 to 4° unless otherwise noted. The chromosome isolation buffer (28) contained 1 M hexylene glycol, 0.1 mM PIPES, pH 6.8, and 0.5 mM $CaCl_2$ (HPC buffer). Colcemid-blocked CHO cells, obtained as described in the previous section, were transfered to 250 ml polycarbonate bottles and centrifuged in the Sorvall GSA rotor at 2500 RPM, in the Sorvall RC2-B centrifuge. The cell pellets were transfered to 29 x 102 mm plastic centrifuge tubes (Sorvall; nominal capacity-50 ml), with a stainless steel scoopula, and resuspended in about 35 ml of HPC buffer, using a pipet. The tubes were centrifuged in the Sorvall SS-34 rotor at 2500 RPM for 10 min. Supernatants were decanted and pellets were combined, and resuspended in 25 ml of HPC buffer. The suspension was transfered to a 40 ml Dounce homogenizer (Wheaton) and incubated at 37° for 10 min. Homogenization was done at 37°, with 50 to 80 strokes of a close-clearance pestle. Cell disruption was monitored microscopically, by phase contrast, or brightfield, staining with 0.2% crystal violet. The homogenate was then transfered to 50 ml centrifuge tubes, on ice, and centrifuged in the SS-34 rotor at 5000 RPM for 7 min.

The pellets were resuspended in a total of 5 to 8 ml of HPC buffer, using a Dounce homogenizer with a loose-clearance pestle. Four equal aliquots of the suspension were layered on 4 linear 0.1 to 0.8 M sucrose gradients (37 ml each, in HPC buffer), in 1 x 3.5 inch cellulose nitrate tubes (Beckman). The gradients were centrifuged in the Beckman

SW-27 rotor at 2000 RPM for 9 min, in a Beckman model L3-50 ultracentrifuge, using medium acceleration and no brake.

Gradient fractions were taken from the top, by withdrawing liquid from the meniscus, with a pipet and propipet. The top 2 ml, which usually contained debris, was discarded. A large middle fraction of about 30 ml was next collected. This fraction contained most of the chromosomes and no nuclei. The remaining part of the gradient (about 5 ml) was collected as separate 1 ml fractions. The pellet was resuspended in about 1 ml of HPC buffer. Corresponding fractions from the 4 gradients were combined and examined under the microscope. Those fractions containing chromosomes and no nuclei were pooled. If lower fractions, which contained nuclei, also contained a significant amount of large or aggregated chromosomes, the fractions were combined, redispersed in a Dounce homogenizer, and applied to another sucrose gradient. This gradient was centrifuged for a shorter time period, usually about 5 min, and fractionated as before. The pooled chromosome fractions were diluted 2- to 3-fold with HPC buffer and centrifuged in the SS-34 rotor at 15,000 RPM for 20 min. Pellets were combined and resuspended in 8 ml of HPC buffer.

The dispersed chromosome pellet was then layered on top of 30 ml of 2.2 M sucrose (in HPC buffer), in a 1 x 3.5" cellulose nitrate tube. The tube was stirred with a glass rod, to form a rough gradient, taking care not to distrub the lower 5 ml. The tube was then centrifuged in the SW-27 rotor at 25,000 RPM for 1 hr. The chromosome pellet was resuspended in 8 ml of HPC buffer and stored at 0 to 4°. Chromosomes were stable for at least one month (19). The yield of chromosomes was approximately 20 mg (wet weight) per 10^9 cells.

c. Chromosome Isolation at pH 3.

This procedure was adapted from that of Huberman and Attardi (2). Solutions used for chromosome isolation, and abbreviations are as follows:

HCM 1 mM HCl, 0.7 mM $CaCl_2$, 0.3 mM $MgCl_2$ (pH 3)

HSCM 3.3 mM HCl, 0.1 M sucrose, 0.7 mM $CaCl_2$, 0.3 mM $MgCl_2$ (pH 3)

SCM 0.1 M sucrose, 0.7 mM $CaCl_2$, 0.3 mM $MgCl_2$

T-PBS (TRIS-phosphate buffered saline) 25 mM TRIS, 7 mM NaH_2PO_4, 137 mM NaCl, 5 mM KCl, pH 7.4

All procedures were done at 0 to 4°. Colcemid-blocked CHO cells were prepared from a 250 to 1000 ml suspension culture, as described previously, and centrifuged in the GSA rotor at 2500 RPM for 10 min. Pellets were combined, resuspended in 30 ml of T-PBS buffer using a Pasteur pipet, and centrifuged in the SS-34 rotor, at 2500 for 10 min. Pellets were washed two more times in T-PBS buffer. The washed cell pellet was transferd to a 15 ml Dounce homogenizer and resuspended gently in 6 ml of SCM solution. The suspension was transfered to a 150 ml beaker surrounded by ice, and incubated 5 min. Twenty ml of HSCM solution was then added dropwise, over a 10 min period. The cells were transfered to a 40 ml Dounce homogenizer, and homogenized with about 50 strokes of a close-clearance pestle. During homogenization the pH was maintained at 2.5 to 3.5 by adding additional drops of 1 N HCl, as required. The homogenate was centrifuged in the SS-34 rotor at 4500 RPM for 15 min, and the pellet was resuspended in 8 ml of HCM.

At this point, chromosomes are separated from nuclei and debris, following the same procedure as described in the previous section,

starting with the 0.1 to 0.8 M sucrose gradient step. The HCM system is substituted in place of the HPC buffer, and additional 1 N HCl is added to sucrose solutions if needed, to maintain the pH at 3.

7. Sucrose Gradient Velocity Sedimentation.

This method was adapted from the procedures described by Stubblefield et al. (12), and Maio and Schildkraut (1). Linear 10 to 40% sucrose gradients (38 ml, in HPC buffer) were prepared in 1 x 3.5" cellulose nitrate tubes, using an automatic gradient former (Isco model 570). Suspensions of isolated chromosomes were dispersed in a Dounce homogenizer, with a few strokes of a loose-clearance pestle, and 1 ml aliquots were layered on top of the gradients. Gradients were then centrifuged in the SW-27 rotor, in the Beckman model L3-50 ultracentrifuge, at 2000 RPM for 12 min, using medium acceleration and no brake. Fractions (1 to 2 ml) were collected using an automataic gradient fractionator (Isco model 183), by injecting 55% sucrose at the bottom of the tube, and collecting fractions from the top.

8. Light Microscopic Visualizataion of Chromosomes from Gradient Fractions.

Aliquots of chromosome fractions were placed in 12 ml glass centrifuge tubes (Sorvall), and diluted with H_2O to a volume of 2 to 4 ml. Round (12 mm diameter) coverslips (Propper Manufacturing Co.) were placed in the tubes, and tubes were centrifuged in a clinical centrifuge, with a swinging bucket rotor, at top speed for 5 min. Coverslips were then removed with forceps and excess liquid was blotted by touching the edges to a paper towel. Coverslips were then placed on a drop

of stain (0.1% crystal violet in glycerol), on a microscope slide, and observed under a brightfield microscope.

9. Isoelectric Focusing of Metaphase Chromosomes.

Chromosomes were electrofocused using the rapid, multisample technique described by Behnke et al. (18, 37), and including recently developed modifications for improving the pH gradient linearity and decreasing the focusing time (19).

Polyacrylamide gel plugs (7.5%) were prepared as described previously (18) and included 35% sucrose (19). Gel plugs were soaked overnight in the lower reservoir (acidic) solution.

Linear 5 to 25% sucrose gradients were prepared in the tubes, to a volume of 6.2 ml; both heavy and light gradient solutions contained 1.5 to 2 mg/ml of chromosomal protein (measured by the Lowry procedure (29), using BSA as a standard), and either a 1/33 dilution of stock pH 3-10 ampholytes, or a 1/40 dilution of the pH 2.5-5.0 ampholytes.

The upper and lower reservoir solutions were either 3% ethanolamine and 0.15% H_2SO_4, for the pH 3-10 system, or 50 mM NaOH and 330 mM phosphoric acid, for the pH 2.5-5.0 system. The power supply was attached with the cathode on the upper reservoir, and the voltage was set to 200V.

Fractions (100 µl) were collected from the top, by injecting 50% sucrose through the gel plus, as described previosuly (19). Chromosomes were prepared for brightfield microscopy as described in the previous section.

10. Moving Boundary Electrophoresis of Metaphase Chromosomes.

This procedure was carried out in the same apparatus as used for isoelectric focusing. Gel plugs containing 7.5% acrylamide and 35% sucrose were prepared as described previously, and soaked overnight in the electrophoresis buffer.

Chromosome suspensions were mixed with concentrated (75 to 80%) sucrose, to a final sucrose concentration of 30%. About 200 µl of this suspension was layered on top of the gel plus. A 5 to 25% sucrose gradient (5.8 ml, in the appropriate electrophoresis buffer) was then formed on top of the chromosome band. The upper and lower reservoirs contained the same buffer as the gradients and gel plugs, except hexylene glycol was not included.

For electrophoresis at neutral or alkaline pH, the (+) terminal of the power supply was connected to the upper reservoir. For electrophoresis at acid pH, the (-) terminal was on top. The current of the power supply was adjusted to give 2 mAmp per tube. Fractions (50 to 200 µl) were collected the same way as described for isoelectric focusing.

11. Transmission Electron Microscopy.

a. Preparation of Metaphase Chromosomes for Transmission Electron Microscopy.

Parlodion-coated grids (300 mesh) were prepared as described by Paulson and Laemmli (21). Aliquots (50 µl) of chromosome suspensions were mixed with 20 µl of 1% uranyl nitrate (pH 7), on a square of parafilm. A parlodion-coated grid was touched to the drop, and excess

liquid was blotted with filter paper. Grids were allowed to air-dry, and shadowed with Pt, from an angle of 8°.

b. Preparation of Histone-Depleted Metaphase Chromosomes.

The procedure of Paulson and Laemmli (21) was adapted as follows: Aliquots (5 to 40 µl) of chromosomes isolated in hexylene glycol were added to 1/2 x 2" cellulose nitrate tubes (Beckman) containing 5 M NaCl, to give a total volume of 100 µl. The tubes were mixed gently by rolling, and incubated overnight, at 0°. Fifty µl of cytochrome-c (4 mg/ml in 6 M ammonium acetate, pH 6.5) was then added, mixed by gentle rolling, and incubated for 30 min.

The cytochrome-c spreading technique (21, 30, 31) was carried out as follows: A drop (200 µl) of 125 mM ammonium acetate (pH 6.5) was placed on a square of parafilm, on an ice-filled petri dish. Aliquots (10 to 30 µl) of the chromosome suspension were taken in a micropipet, with a wide-bore tip, and allowed to flow down a glass rod, placed in the drop at a 45° angle. Parlodion-coated grids were touched to the surface of the drop, and immediately immersed in 90% ethanol for 10 seconds. Grids were then immersed in the stain solution (50 mM uranyl nitrate in 50 mM Hcl, diluted 1/50 in 90% ethanol before use), for 1 min, rinsed in hexane for 10 sec, and air-dried. Contrast was enhanced by shadowing with Pt from an angle of 8°.

c. Preparation of Nuclei for Transmission Electron Microscopy.

CHO cell nuclei, isolated in HPC buffer (27), were suspended in 0.1 M phosphate buffer, pH 7, and centrifuged in the SS-34 rotor at 5000 RPM, for 5 min. The pellet was resuspended in about 2 to 4 ml of

liquefied 2% agar in the phosphate buffer, in a 45° water bath. The suspension was spread on microscope slides and allowd to form a gel, about 2 mm thick. The gel was cut into small (about 2 mm square) pieces, and fixed in 5% glutaraldehyde in buffer, and then 0.1% OsO_4 in buffer, according to standard methods (32, 33). Nuclei were dehydrated in ethanol, and embedded in epon/araldite/spurrs resin, with acetone as the transition solvent (32). Sections were taken and stained with uranyl nitrate and lead citrate, according to standard procedures (32).

D. RESULTS

1. Electron Microscopy of Metaphase Chromosomes Isolated at pH 6.8 in Hexylene Glycol, and those Isolated at pH 3.0.

Electron micrographs of chromosomes isolated at neutral pH, in 1 M hexylene glycol, and those isolated at pH 3, are shown in Figures 1 and 2, respectively. The chromosomes isolated in hexylene glycol appear much more intact, and bundled chromatin fibers are visible in the micrographs. The chromosomes isolated at pH 3 are basically intact; however they have a somewhat frayed appearance, due to stretched DNA around the periphery. Apparently the acid-isolated chromosomes are not as resistant to mechanical damage during the isolation procedure as chromosomes isolated in hexylene glycol.

2. Effect of Incubating Chromosomes Isolated in Hexylene Glycol in High Salt Concentrations.

Previous workers (21, 22) have found that when metaphase chromosomes not isolated in hexylene glycol are incubated in 2 M NaCl for 1 hr, all of the histones are extracted and the chromosomes become completely uncoiled. Incubation in hexylene glycol has been found to increase the compactness of chromosomes (20), and previous workers have suggested that it may make them irreversibly compacted (20). We would therefore expect that chromosomes isolated in hexylene glycol would be

FIGURE 1. ELECTRON MICROGRAPHS OF CHINESE HAMSTER OVARY (CHO) METAPHASE CHROMOSOMES, ISOLATED AT pH 6.8 IN 1 M HEXYLENE GLYCOL.

Metaphase chromosomes were isolated by a modification of the methods of Huberman and Attardi (2), and Wray and Stubblefield (27), as described in Materials and Methods. Chromosomes were stained in uranyl nitrate, picked up on parlodion-coated grids, and shadowed with Pt, also as described in Methods. Grids were viewed in a Philips 201 TEM, at magnifications of 2000X to 45,000X, with an accelerating voltage of 60 kV. Images were photographed on Kodak electron microscope film (no. 4489), and developed in D-19, according to standard procedures (32). The reference bars represent a distance of 0.5 μm.

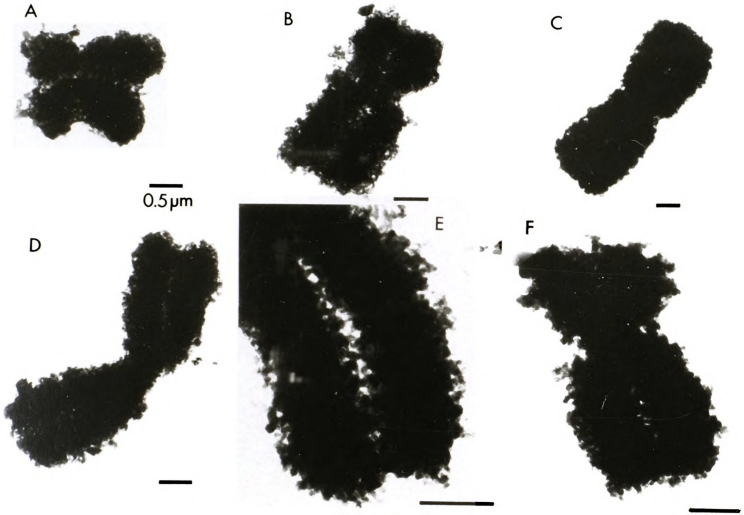
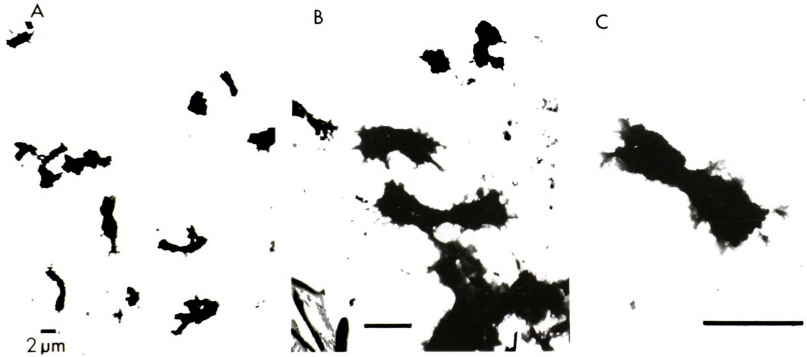


Figure 1

FIGURE 2. ELECTRON MICROGRAPHS OF CHO METAPHASE CHROMOSOMES, ISOLATED AT pH 3.

Chromosomes were isolated by a modification of the method of Huberman and Attardi (2), as described in Materials and Methods. Chromosomes were prepared for electron microscopy and photographed the same way as described in Figure 1. The reference bars represent a distance of 2 μm.



Figure

FIGURE 3. ELECTRON MICROGRAPHS OF CHO INTERPHASE NUCLEI, ISOLATED AT pH 6.8, IN 1 M HEXYLENE GLYCOL.

Nuclei were isolated according to the method of Wray and Stubblefield (27), and prepared for TEM as described in Materials and Methods, and in reference 32. Electron microscopy was carried out as described in Figure 1.

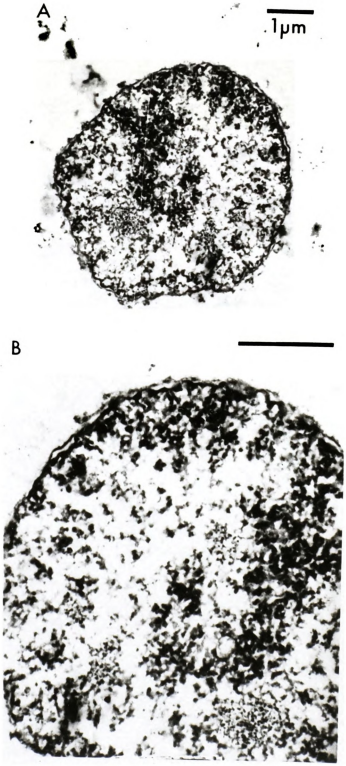


Figure 3

FIGURE 4. ELECTRON MICROGRAPHS OF PARTIALLY HISTONE-DEPLETED CHO METAPHASE CHROMOSOMES.

Metaphase chromosomes were isolated at pH 6.8, in 1 M hexylene glycol, and incubated in 4.5 M NaCl, as described in Materials and methods. Chromosomes were then spread with cytochrome c, according to the method of Paulson and Laemmli (21), and observed in the TEM, as described in Figure 1.

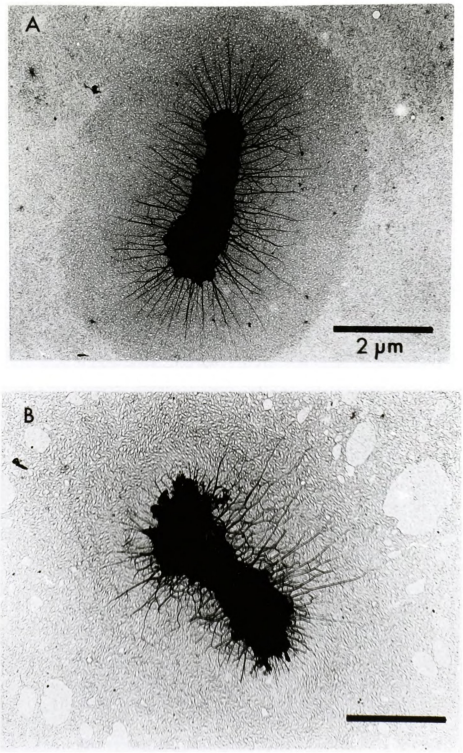
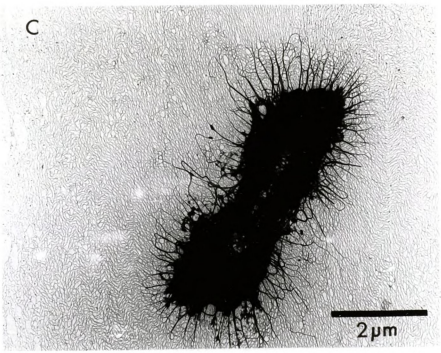


Figure 4



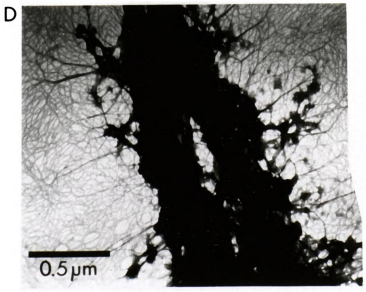


Figure 4 (continued)

much more resistant to mechanical damage, and other denaturing conditions.

We next carried out a similar set of experiments, where chromosomes isolated in hexylene glycol were incubated in NaCl solutions, and prepared for electron microscopy, as described in Materials and Methods. In order to give stronger denaturing conditions, we increaed the NaCl concentration from 2 M to 4.5 M (saturated = 6.1 M, at 4°), and increased the incubation time (at 0-4°) from 1 hr to 10 hr. Several of the electron micrographs we obtained are shown in Figure 4. These micrographs appear somewhat similar to the histone-depleted chromosomes obtained by Paulson ad Laemmli (21), with chromosomes not isoalted in hexylene glycol; they contain a surrounding carpet of DNA, and a darker staining central structure. However the DNA carpet is much smaller, and the central structure is also much smaller, and has the appearance of an intact chromosome, rather than a loose, fibrous appearance. Apparently only the periphery of the chromosome was depleted of histones and uncoiled, while the interior portions remained coiled and compact. We thus refered to these chromosomes as being partially histone-depleted (Figure 4).

3. Sucrose Gradient Fractionation of Chromosomes.

Chromosomes isolated at neutral pH in hexylene glycol, were initially separated on a 10 to 40% sucrose gradient, as described in Materials and Methods. Figure 5 shows the distribution of chromosomes from a typical gradient experiment, starting with a previously unfractionated chromosome suspension. A rough size fractionation was generally obtained: the top fractions contained the small (group D)

FIGURE 5. PHOTOMICROGRAPHS OF CHROMOSOME FRACTIONS OBTAINED BY SUCROSE GRADIENT VELOCITY SEDIMENTATION.

Metaphase chromosomes isolated at neutral pH in 1 M hexylene glycol were fractionated on a 10 to 40% sucrose gradient, as described in Materials and Methods. Twentyfive 1.5 ml fractions were collected and prepared for brightfield microscopy, also as described in Methods. (Top of gradient = fraction-1) Images were photographed with Kodak Tri-X pan film (ASA-400), and developed in D-76. Total magnification = 900 X. Those fractions shown represent the typical size fractionation obtainable by this technique, starting with an unfractionated chromosome suspension.

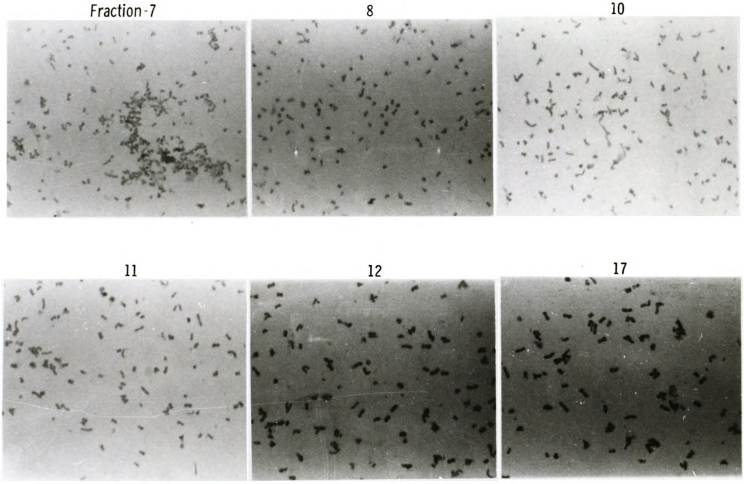


Figure 5

chromosomes. The middle fractions contained mostly the intermediate-sized chromosomes, and also some aggregates of small chromosomes. The bottom fractions contained the large (group A) chromosomes, and some aggregates of smaller chromosomes. The size distribution could generally be improved by re-dispersing the fractions in a Dounce homogenizer, and running a second gradient, for a longer or shorter time period, where appropriate.

4. Moving Boundary Electrophoresis of Metaphase Chromosomes.

Electrophoresis studies were done initially in HPC buffer at pH 6.8, as described in Materials and Methods. The chromosomes were anionic under these conditions, and thus migrated toward the positive electrode. Typically, the chromosomes migrated into the gradient at an initial rate of about 1 to 1.5 cm/hr, and the band began to broaden. Examination of the upper and lower parts of the band under the microscope showed a rough size distribution, with the small chromosomes toward the top. However after bout 1 to 2 hr, the initially finely turbid band began to have a coarse appearance, and larger precipitates eventually formed and sank towards the gel plug. This precipitation prevented further broadening of the band from occuring, thus limiting the resolution.

In attempting to decrease the precipitation of chromosomes, and increase the resolution, studies were done to determine which variables affect the stability and mobility of chromosomes in the electric field. Several of the variables tested were: hexylene glycol concentration, divalent cation concentration, pH, and several other organic solvents.

At concentrations of 1 to 2 M, hexylene glycol markedly improved the stability and migration rate. In the absence of hexylene glycol, the migration was relatively slow, and precipitation was rapid. However, concentrations greater than 2 M decreased mobility. High concentrations of diethylene glycol also decreased mobility. These effects may be due to the large decrease in electric current which occured at high glycol concentrations.

$CaCl_2$ and $MgCl_2$ gave a slight decrese in precipitation at a total cation concentration of 0.5 mM (in the presence of 1 M hexylene glycol). However, concentrations above 0.5 mM decreased the chromosome migration rate. The addition of 10% formamide to the system also slowed the chromosome migration.

As an alternative approach, electrophoresis was carried out at pH 3, with chromosomes isoalted in HCM solution, as described in Materials and Methods. In the presence of HCM solution however, there was a lot of precipitation and very little migration in the gradient. A significant increae in migration (toward the negative electrode) was obtained when the 1 mM HCl was substituted with 10 to 20% acetic acid (v:v). A further improvement in the chromosomes stability during electrophoresis was obtained by including 1 to 2 M hexylene glycol in the system.

The fastest chromosome migration rate in the acidic system (about 0.4 cm/hr), was obtained when phosphoric acid was added to the system, at an optimum concentration of 0.1 M. This may have been the result of lowering the pH further below the isoelectric point of the chromosomes, where they would be more cationic.

The least amount of precipitation and fastest chromosome migration rate (1.5 cm/hr) occured in the neutral pH system, in the presence of

1 to 2 M hexylene glycol. One reason for the greater mobility could be that the chromosomes are further from their isoelectric point at neutral pH. Chromosomes are also more stable at neutral pH than at pH 3, where some extraction of histones may occur (27). The presence of hexylene glycol must also have a stabilizing effect (previous electron microscopy results and reference 20). Thus, the neutral pH/hexylene glycol system appears to be the method of choice so far for moving boundary electrophoresis. However the problem of precipitation was still not completely overcome, and we did not obtain the resolution hoped for. As an alternate method for separating chromosomes, we were next interested in using the rapid isoelectric technique of Behnke et al. (18), which had not previously been used for separating chromosomes.

5. Isoelectric Focusing of Metaphase Chromosomes.

Chromosomes were isolated at neutral pH in hexylene glycol, and electrofocused in the pH 3-10, and pH 2.5-5.0 systems, as described in Materials and Methods. Initial experiments with the wide-range (pH 3-10) ampholytes resulted in a single narrow band, at about pH 4. In order to obtain a broader band, and increase the resolution, the narrow-range (pH 2.5-5.0) ampholytes were used in most of the experiments.

The focusing time with the pH 2.5-5.0 system was approximately 4 hr; markedly faster than the 24 to 48 hr required by conventional methods, under similar conditions (17). Figure 6 shows the results of two parellel experiments, allowed to run for 4 hr and 12 hr, respectively. In the 4 hr run, the chromosomes focused as a broad band in

FIGURE 6. ISOELECTRIC FOCUSING OF METAPHASE CHROMOSOMES FROM CHO CELLS.

Chromosomes were isolated in the presence of 1 M hexylene glycol, and electrofocused using the rapid, multi-sample technique, as described in Materials and Methods (1.C.9). The absorbance profiles shown are from two parallel experiments, allowed to electrofocus for 4 hr or 12 hr, as indicated. The pH values of the peaks and boundaries of the chromosome bands are also indicated.

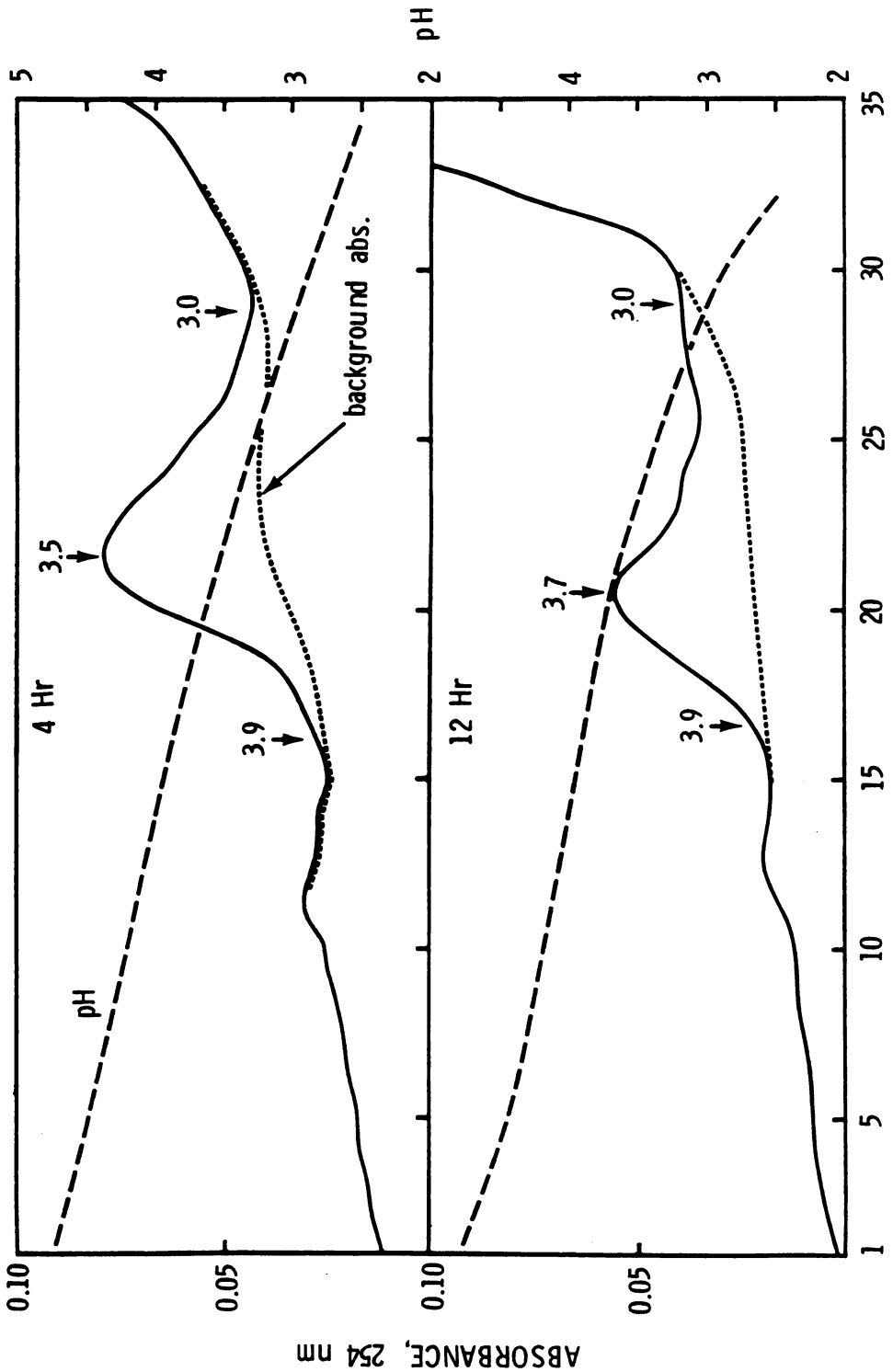

ABSORBANCE, 254 nm
0.10
0.05
0.10
0.05
pH
5
4
3
2
4 Hr
12 Hr
3.5
3.0
3.9
3.7
3.0
3.9
background abs.
1
5
10
15
20
25
30
35
FRACTION

Figure 6

the pH range of 3.0 to 3.9. A single peak in the UV absorbance scan occured at pH 3.5. On examining the fractions under the microscope, the chromosome distribution appeared homogeneous, without obvious differences in size or morphology.

The results of the 12 hr run were very similar to those of the 4 hr run (Figure 6). The absorbance scan is slightly different in shape, which is partially due to the decrease in the background absorbance of the ampholytes. The chromosomes remained focused in the same general area of the gradient although the peak shifted slightly, to pH 3.7.

E. DISCUSSION

1. Aggregation of Metaphase Chromosomes During Electrophoresis and Isoelectric Focusing.

Previous workers ahve found that during prolonged isoelectric focusing of metaphase chromosomes with conventional techniques, the isoelectric point of the chromosomes gradually changes, and some aggregation and precipitation occurs (4, 17). This was found to be due to proteins being stripped from the chromosomes, during prolonged exposure to the electric field (4).

We suspect that the precipitation which occured during our moving boundary electrophoresis experiments with chromosomes was also caused by protein stripping. We found that the amount of precipitation was decreased in the presence of hexylene glycol and low concentrations of divalent cations. These compounds have been previously found to increase the compactness of isolated chromosomes (20), and apparently this increases the stability of chromosomes in the electric field.

In addition, we found that chromosomes isolated in the presence of hexylene glycol aare much more resistant to the uncoiling effect of high NaCl concentrations. Previous workers (21, 22) have found that metaphase chromosomes not isolated in hexylene glycol become completely uncoiled when incubated in the presence of 2 M NaCl. However in our experiments with chromosomes isolated in hexylene glycol, we observed only a partial uncoiling of the chromosomes around the periphery

(Figure 4), when the chromosomes were incubated in near saturated (4.5 M) NaCl solution.

We also saw very little precipitation or change in isoelectric point during prolonged isoelectric focusing runs (Figure 6), with chromosomes isolated in hexylene glycol (Figure 6). High concentrations (1 or 2 M) of hexylene glycol reduced the amount of precipitation during electrophoresis. However, precipitation was not prevented completely. This limited the resolution, and we could not obtain individual types of chromosomes by electrophoreis.

2. Isoelectric Focusing of Metaphase Chromosomes, using Conventional and Rapid Techniques.

Previous workers, using conventional isoelectric focusing techniques, found that Hela metaphase chromosomes focused in the pH range of 3.5 to 4.1 (4, 17). Chromosomes isolated at neutral pH were found to focus as a single braod band. However when the electrofocusing experiments were done with chromosomes isolated at pH 3, or during prolonged electrofocusing, multiple peaks were observed in the focusing patterns. This was attributed to the gradual extraction of histones from the chromosomes when they were incubated at pH 3 (27), or subjected to prolonged exposure to the electric field. Apparently the extraction of chromosomal proteins occurs at different rates in different types of chromosomes, and results in a gradual change in isoelectric point.

In our rapid isoelectric focusing experiments, with chromosomes isolated in hexylene glycol, we also obtained a single broad band, at pH 3.5 ± 0.5. The focusing time was also much faster than with

conventional methods (4 hr versus 24 hr). We found no differences in the morphology of the chromosomes from different areas of the band. Perhaps with other techniques such as RNA hybridization (17) it might be possible to detect differences in the chromosomes from different areas of the gradient.

CHARACTERIZATION OF PHOSPHOFRUCTOKINASE MEMBRANE BINDING AND ACTIVITY INHIBITION BY HEXACYANOFERRATE(II)

CHAPTER 2

NOVEL PRONOUNCED REVERSIBLE INHIBITION OF PIG KIDNEY PHOSPHOFRUCTOKINASE BY HEXACYANOFERRATE(II).

A. ABSTRACT

At pH 6.8, pig kidney phosphofructokinase (PFK) is inhibited 90% by 1 mM hexacyanoferrate(II), in a reaction mixture containing 0.2 mM fructose-6-P and 1 mM ATP. Glucose 6-phosphate dehydrogenase and phosphoglucose isomerase are inhibited 70% by 5 mM hexacyanoferrate(II), at a 0.2 mM concentration of their respective substrates. Unlike all previously reported inhibitions of glycolytic enzymes by hexacyanoferrate, this inhibition seems not to involve an oxidation of enzyme, substrate, or enzyme-substrate complex. It appears to be due to reversible binding of the hexacyanoferrate at, or near, the hexose phosphate binding site of each enzyme. These inhibition studies were carried out in 50 mM 2-mercaptoethanol, and spectral studies showed that these conditions ensured that all the hexacyanoferrate was in the reduced (II) state. The inhibition of PFK was competitive with respect to the substrate fructose-6-P. Some reaction between hexacyanoferrate(II) and the substrate could not be definitely ruled out, but such reactions cannot be the major basis for the inhibitions observed. Increasing the magnesium concentration did not overcome the PFK inhibition. For all three enzymes, addition of a high concentration of hexose phosphate substrate to an assay mixture containing highly inhibited enzyme resulted in removal of the inhibition. The inhibiiton was instantaneous and there was no increase in inhibition with time of incubation with hexacyanoferrate(II).

These results may provide an approach to active site labeling of these three enzymes at their hexose phosphate binding sites. These results should also be of interest to other workers, especially those involved in oxidative phosphorylation studies, who use ferro-and ferricyanide as research tools. The effects from such experiments may, in some cases, be due to binding of these compounds at, or near, hexose phosphate binding sites in the system.

B. INTRODUCTION

Phosphofructokinse (PFK; ATP:D-fructose 6-phosphate 1-phosphotransferase EC 2.7.1.11) has unique kinetic properties and a key role in the regulation of glycolysis (36). A method of isolation and several unique properties of pig kidney and liver PFK have been described by Massey and Deal (37, 38). A preliminary investigation in this laboratory showed that the enzymatic activity of pig kidney PFK was markedly inhibited when potassium hexacanoferrate was added to the assay mixture in concentrations in the range of 0.1 to 1 m<u>M</u>. Since the inhibition was so great it seemed desirable to determine whether the hexacyanoferrate was interacting with the enzyme, one of the substrates (ATP, fructose-6-P, Mg^{2+}), or the enzyme-substrate complex.

Virtually all previous reports of effects of hexacyanoferrate on enzymes involve oxidation. Bloxham and Lardy (39) reviewed the report (40) of Engelhardt and Sakov in 1943 of the inhibition of PFK by hexacanoferrate(III) and several other oxidizing agents. Engelhardt and Sakov attributed this inhibition to oxidation of sulfhydryl groups of the enzyme. Later, Mason, Anson, and Mirsky used hexacyanoferrate(III) as an oxidizing agent for the determination of sulfhydryl groups (for review, see 41).

More recent investigations have shown that an entire group of enzymes undergo <u>irreversible</u> inhibition in the presence of substrate and hexacyanoferrate(III) (42-44). This class of inhibition has been

ascribed to paracatalytic enzyme modification (42). This type of reaction is of considerable interest as an approach to specific active site labeling.

One characteristic of paracatalytic reactions is that they occur in an environment which allows oxidation of an enzyme-substrate enamine complex (42); however, the inhibition we observed was in the presence of a high concentration of 2-mercaptoethanol, so that the hexacyanoferrate would be in a reduced form and not able to oxidize the enzyme. Hence, the inhibition we have observed with PFK is totally different from that described in all the previous studies.

Therefore a detailed investigation of the mechanism of inhibition was undertaken. In the course of this investigation the study was broadened to include phosphoglucose isomerase (PGI) and glucose 6-phosphate dehydrogenase (G6PD), to determine whether the hexacyanoferrate-(II) reaction was specific for the enzyme or substrate (fructose-6-P, glucose-6-P).

C. MATERIALS AND METHODS

1. Enzymes

Pig kidney phosphofructokinase (PFK) was purified according to the method of Massey and Deal (37). Phosphoglucose isomerase (PGI), glucose 6-phosphate dehydrogenase (G6PD), aldolase, and a trisephosphate isomerase-α-glycerolphosphate dehydrogenase mixture were obtained from Sigma Chemical Company.

2. Chemicals

NADP, NADH, and ATP were obtained from P-L Biochemicals, Inc. Fructose 6-phosphate, fructose 1,6-diphosphate, glucose 6-phosphate, 2-mercaptoethanol and imidazole were obtained from Sigma Chemical Company. Analytical reagent grade K_3 $Fe(CN)_6$ was obtained from Mallincrodt. Imidazole was recrystallized two times in 2:1 (V:V) chloroform:ether. All other chemicals were analytical reagent grade.

3. Enzyme Assays

All assays were carried out at 25° in the standard assay buffer containing 50 mM imidazole-HCl, pH 6.8, and 50 mM 2-mercaptoethanol. In the inhibition experiments, a solution of potassium hexacyanoferrate in the (III) oxidation state was added to the reaction mixture, where it was immediately reduced to the (II) oxidation state upon mixing. Unless otherwise indicated, this was done prior to adding the enzyme or

substrate which was to be tested. In all assays the change in absorbance at 340 nm was measured. (a) Phosphofructokinase Assay - The coupled spectrophotometric assay at ph 6.8 described by Massey and Deal (38) was used. In addition to the standard assay components mentioned above, the standard PFK assay buffer contained 50 mM KCl, 5 mM $MgCl_2$, 0.2 mM F-6-P, 1 mM ATP, 0.2 mM NADH and the coupling enzymes described in (38). The reaction was started by the addition of fructose-6-P. (b) Glucose 6-Phosphate Dehydrogenase Assay - In addition to the components of the standard assay buffer, the assay mixture contained 30 mM $MgCl_2$, 0.5 mM NADP, and 0.2 mM glucose 6-phosphate (45). The reaction was started by adding G6PD to a final concentration of 0.006 U/ml in a 0.5 ml assay mixture. The same procedure was used for the end-point determination of glucose-6-P concentration, except the mixture contained 10 mM NADP and 5 U/ml G6PD and the glucose-6-P was added separately, not included in the assay mix. (c) Phosphoglucose Isomerase Assay - The procedure was the same as that used to assay G6PD, except the final mixture contained 0.2 mM fructose-6-P and 1.2 U/ml G6PD (46) and had no glucose-6-P.

D. RESULTS

1. Inhibition of PFK by Hexacyanoferrate(II).

In the standard PFK assay containing 0.2 mM fructose-6-P, pig kidney PFK was inhibited 90% by 1 mM hexacyanoferrate; however there was very little inhibition in the presence of 6 mM fructose-6-P (Figure 7). Furthermore, adding more fructose-6-P directly to an assay containing highly inhibited enzyme completely overcame the inhibition (not shown), suggesting that the inhibition was completely reversible. Adding more ATP did not relieve the inhibition. One characteristic of the inhibition was that at equal concentrations of hexacyanoferrate and substrate, about 50% inhibition was observed (Figure 7). This suggested that hexacyanoferrate might compete with fructose-6-P for its binding site, with a binding constant approximately equal to that of fructose-6-P. An alternative possibility was that hexacyanoferrate bound fructose-6-P directly, in a stoichiometry of two hexacyanoferrate molecules per fructose-6-P molecule. Further analysis of this question follows several control experiments.

2. Proof That the Observed Inhibition of PFK Was Not an Artifact.

With the PFK assay system, assays of the coupling enzymes, using fructose-1, 6-P_2 as substrate in place of fructose-6-P, were not significantly affected by hexacyanoferrate; the coupling enzyme

FIGURE 7. EFFECT OF VARYING CONCENTRATION OF FRUCTOSE-6-P (F6P) ON THE INHIBITION OF PIG KIDNEY PHOSPHOFRUCTOKINASE (PFK) BY HEXACYANOFERRATE(II).

PFK activity was assayed in the presence of various concentrations of hexacyanoferrate(II) and fructose-6-P, as described in Materials and Methods. The standard PFK reaction mixture was used. In the experiments containing 0.1, 0.2, and 0.3 m$\underline{M}$ concentrations of fructose-6-P, the PFK concentration was 4 µg/ml. In the experiment containing 6 m$\underline{M}$ fructose-6-P, the PFK concentration was 0.4 µg/ml. At each concentration, the activity in the absence of hexacyanoferrate(II) was normalized to 100%.

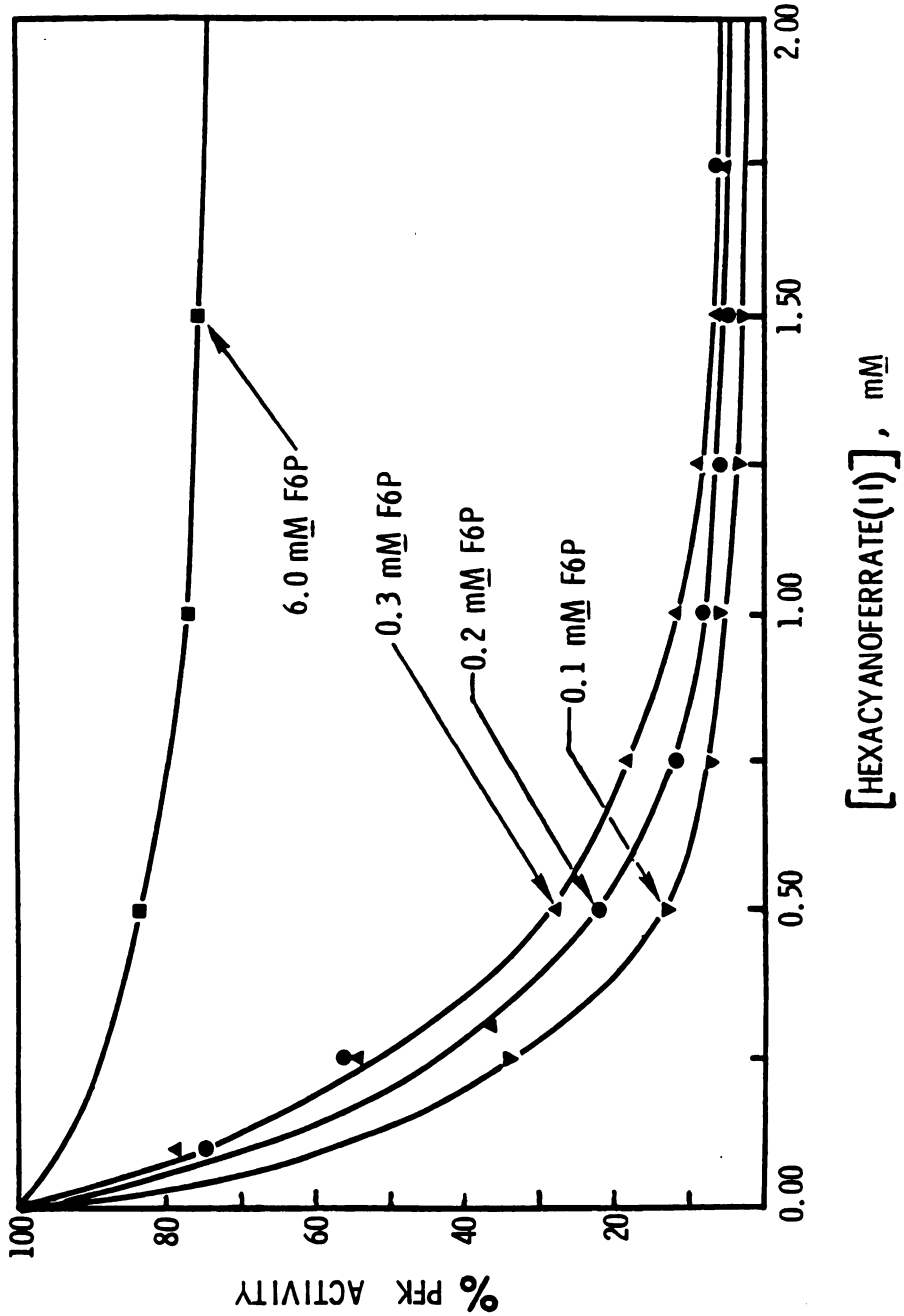

% PFK ACTIVITY
100
80
60
40
20
0.00
0.50
1.00
1.50
2.00
[HEXACYANOFERRATE(II)], mM
6.0 mM F6P
0.3 mM F6P
0.2 mM F6P
0.1 mM F6P

Figure 7

activity was still 10- to 100- fold in excess, even in the presence of 2 mM hexacyanoferrate.

Furthermore, the extinction coefficient of NADH at 340 nm was not affected by hexacyanoferrate. This control was important, since it would have appeared that the enzyme was inhibited if the extinction coefficient of the NADH at 340 nm had been decreased in the presence of hexacyanoferrate.

3. Oxidation State of Hexacyanoferrate In the Assay

Since the inhibition experiments were conducted by adding hexacyanoferrate in the oxidized (III) form to assay mixtures which contained a high concentration of reducing agent (50 mM 2-mercaptoethanol), it was necessary to determine the state of oxidation of hexacyanoferrate in such assays. In the absence of 2-mercaptoethanol, the absorbance spectrum of hexacyanoferrate in the (III) state shows three major peaks, at wavelengths of approximately 270, 320, and 420 nm respectively; shoulders are apparent on both sides of the 320 nm peak (Figure 8). A 390 nm shoulder is apparent on the 420 nm peak. The addition of an equimolar quantity of 2-mercaptoethanol to a solution contianing 0.5 mM hexacyanoferrate(III) largely abolished the two absorbance peaks at the higher wavelengths. These two peaks are completely abolished in the presence of 1 mM 2-mercaptoethanol, so reduction is complete with only two 2-mercaptoethanol molecules per hexacyanoferrate molecule. Since the PFK assay contains a 50-fold excess of 2-mercaptoethanol, the hexacyanoferrate must be totally in the reduced (II) form.

FIGURE 8. SPECTRAL ANALYSIS OF THE REDUCTION OF HEXACYANOFERRATE(III) BY 2-MERCAPTOETHANOL.

Varying concentrations of 2-mercaptoethanol were added to solutions originally containing 0.5 m$\underline{M}$ potassium hexacyanoferrate(III), $K_3Fe(CN)_6$. The sample cuvettes also contained standard assay buffer with 0 to 50 m$\underline{M}$ 2-mercaptoethanol (shown by arrows). The reference cuvettes were identical except they had no hexacyanoferrate. Spectra were taken on a Cary 15 recording spectrophotometer using 50 nm per chart division. The tungsten lamp was used to scan from 500 to 350 nm; the hydrogen lamp was used to scan below 350 nm.

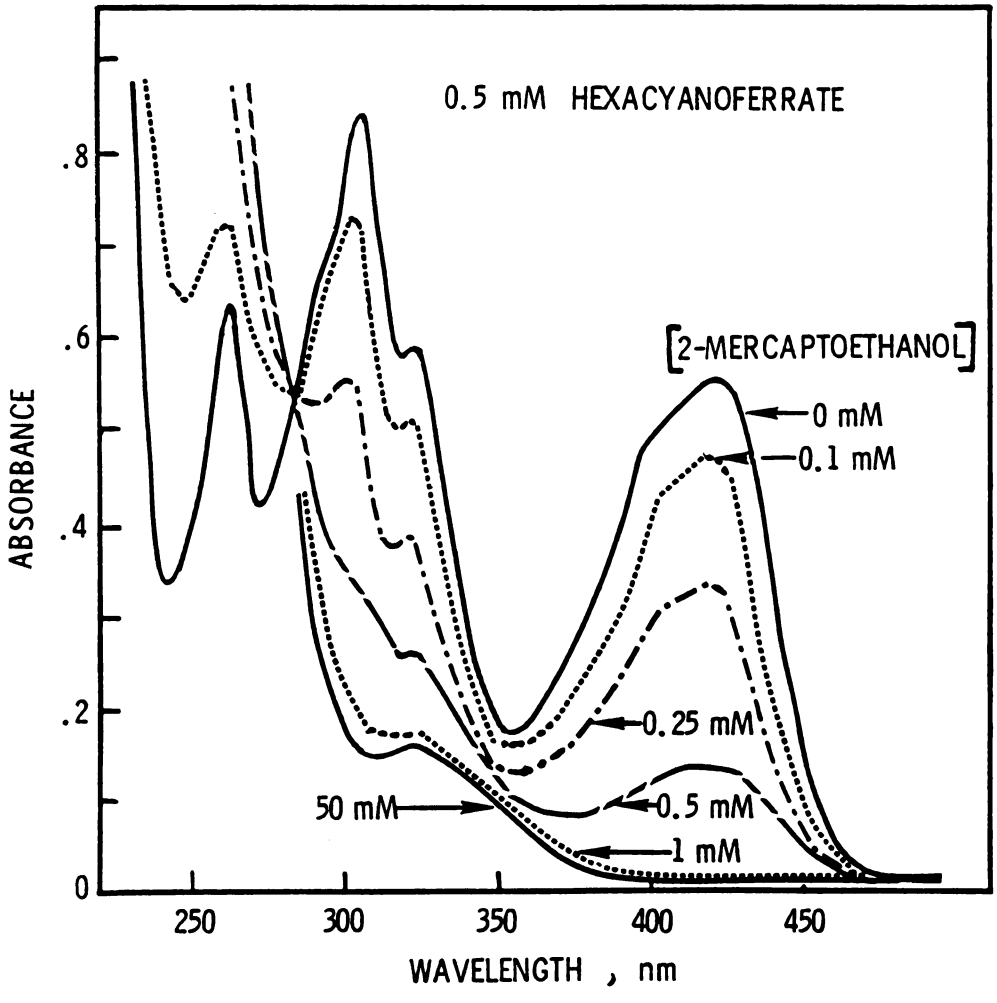

0.5 mM HEXACYANOFERRATE
[2-MERCAPTOETHANOL]
0 mM
0.1 mM
0.25 mM
0.5 mM
1 mM
50 mM
ABSORBANCE
0
.2
.4
.6
.8
250
300
350
400
450
WAVELENGTH , nm

Figure 8

4. Proof That the Inhibition Is Not Due to Competition of Hexacyanoferrate(II) With Mg^{2+}.

Increasing the concentration of Mg^{2+} in the assay did not relieve the inhibition (Figure 9), and therefore hexacyanoferrate(II) must not compete for Mg^{2+}.

5. Test for Competitive Inhibition With Respect to Fructose-6-P.

The previous results (Figure 7) suggested that the inhibition of PFK by hexacyanoferrate(II) might be competitive with respect to fructose-6-P. The results from a detailed kinetic study are consistent with this possibility (Figure 10A). The lines on the Linewever-Burk plot approach approximately the same V_{max} value. However, the lines intersect slightly to the right of the ordinate, which might indicate an increase in V_{max} in the presence of hexacyanoferrate and high concentrations of fructose-6-P substrate. Further studies showed that hexacyanoferrate(II) did not increase the PFK V_{max}, even at concentrations of fructose-6-P above 5 mM, so this apparent intercept to the right of the ordinate was assumed to be due to experimental error. A K_i value of 0.15 ± 0.03 mM was calculated for both concentrations of hexacyanoferrate. These overall results are consistent with hexacyanoferrate(II) reversibly bindng at, or near, the catalytic fructose-6-P binding site to produce the inhibition.

The v vs. s graph in Figure 10B shows the hexacyanoferrate(II) effects on PFK at low substrate concentrations, where allosteric effects are observed. The sigmoidicity of the curves become more pronounced as the hexacyanoferrate(II) concentration is increased; also, the maximal velocity appears to be lower. Both of these results would be produced

FIGURE 9. EFFECT OF $MgCl_2$ ON THE INHIBITION OF PIG KIDNEY PHOSPHOFRUCTOKINASE BY HEXACYANOFERRATE(II).

PFK was assayed in the presence and absence of 0.5 mM hexacyanoferrate(II) as described in the legend to Figure 7, with the modification that additional amounts of $MgCl_2$ were added to the assays to give the final concentrations shown on the graph. The concentration of PFK was 4 µg/ml.

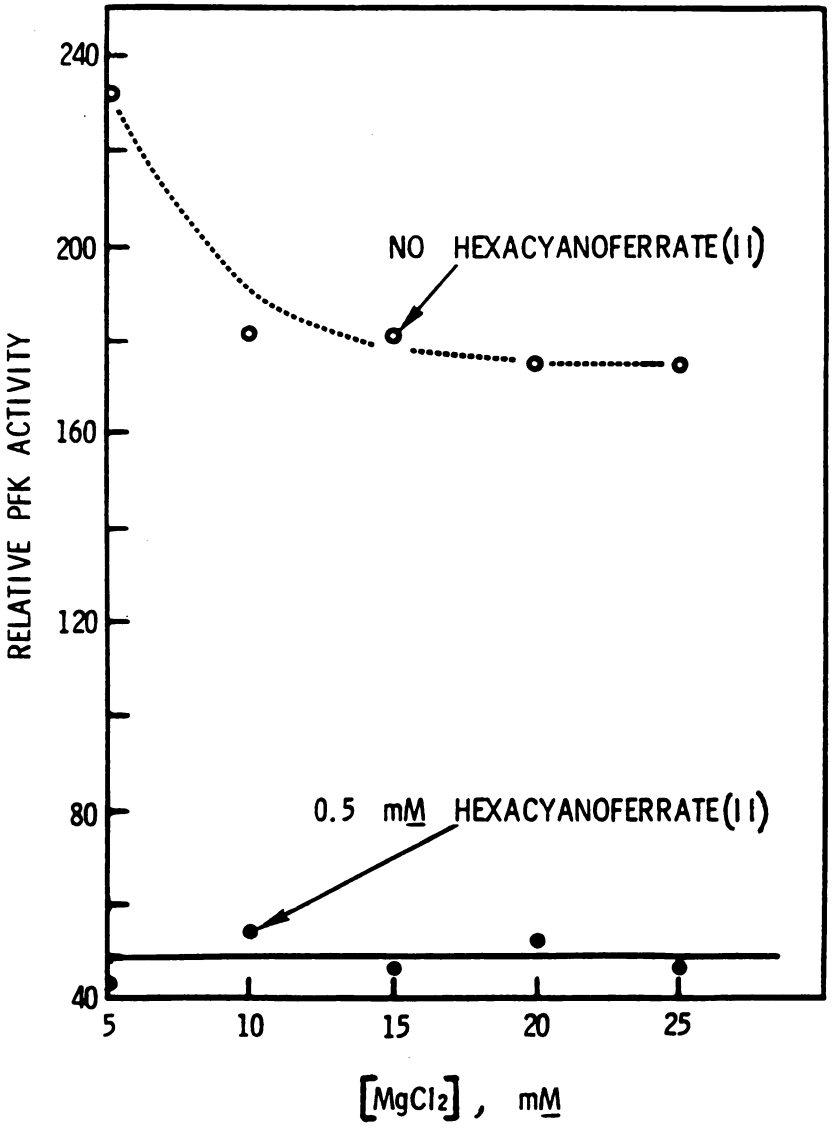

240
200
160
120
80
40
RELATIVE PFK ACTIVITY
NO HEXACYANOFERRATE(II)
0.5 mM HEXACYANOFERRATE(II)
5
10
15
20
25
[MgCl2], mM

Figure 9

FIGURE 10. MECHANISM OF INHIBITION OF PIG KIDNEY PHOSPHOFRUCTOKINASE BY HEXACYANOFERRATE(II).

PFK at a concentration of 4 μg/ml was assayed as described in the legend to Figure 7. The slopes and intercepts for Figure 10A were determined by a computer program which used the method of Wilkinson (48).

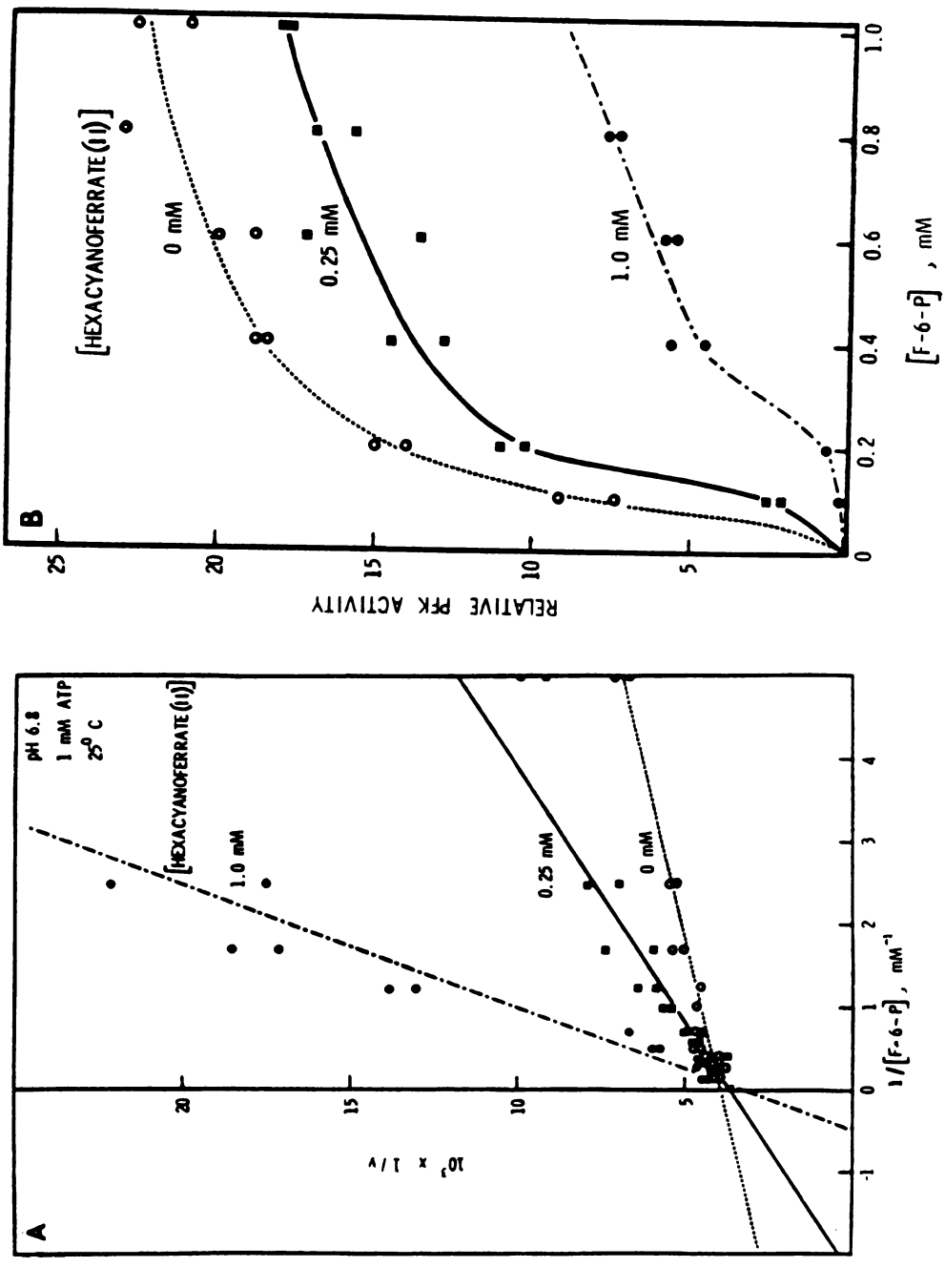

A
pH 6.8
1 mM ATP
25° C
[HEXACYANOFERRATE(II)]
1.0 mM
0.25 mM
0 mM
10³ x 1/v
1/[F-6-P], mM⁻¹
B
[HEXACYANOFERRATE(II)]
0 mM
0.25 mM
1.0 mM
RELATIVE PFK ACTIVITY
[F-6-P], mM

Figure 10

if the effective fructose-6-P concentrations were proportionately less than the nominal concentrations shown. If hexacyanoferrate(II) and the fructose-6-P are competing for the same sites, or overlapping sites, the effect would be to lower the fraction of fructose-6-P bound and thus lower its effective concentration. However, with this data alone, it is not possible to exclude the alternative possibility that hexacyanoferrate(II) is binding at some other site and causing a conformational change which produces the effects seen. It seems unlikely, though, because similar effects are seen with three different enzymes.

Another experiment was carried out to directly test for reversibility of the inhibition as well as to see if the degree of inhibition changed with time. A relatively high concentration of PFK was preincubated in the standard assay buffer, in the presence of 0.5 mM hexacyanoferrate(II) plus 50 mM KCl and 5 mM $MgCl_2$. At timed intervals after adding hexacyanoferrate, 5 µl aliquots of this concentrated solution were diluted 100-fold with PFK assay mix (minus fructose-6-P) and assayed for PFK activity. This effectively diluted the hexacyanoferrate far below inhibitory concentrations. A control experiment was run with no hexacyanoferrate(II), and the enzyme was assayed in the presence of the amount of hexacyanoferrate(II) carried into the assay in the inhibition test samples. The expeirment was also carried out with 0.5 mM substrate, fructose-6-P, in the incubation and control mixtures. Both experiments showed no inhibition of the diluted samples and no decrease in activity over a period of one hour. This suggested that hexacyanoferrate(II) did not bind irreversibly to the enzyme. It should be noted that the inhibition appears to be instantaneous.

6. Effect of Hexacyanoferrate(II) on Other Enzymes Which Use Fructose-6-P or Glucose-6-P as Substrates.

The previous results had suggested that hexacyanoferrate(II) was bound at the catalytic site rather than involved in a direct reaction with the substrate alone. Therefore it was of interest to see whether other enzymes using fructose-6-P or glucose-6-P as substrates would be affected by hexacyanoferrate(II). If hexacyanoferrate(II) were reacting directly with the substrate alone, than we would expect other enzymes using fructose-6-P or glucose-6-P substrates to be inhibited. Surprisingly, tests for inhibition of glucose 6-phosphate dehydrogenase (G6PD) and phosphoglucose isomerase (PGI) by hexacyanoferrate(II) were also positive (Figures 11 and 12). This left open the possibility that hexacyanoferrate(II) could be binding to either enzyme or substrate. Rabbit muscle and pig liver PFK also showed a similar inhibition by hexacyanoferrate.

The inhibition of G6PD and PGI by hexacyanoferrate(II) appeared to be simlar to that observed for PFK: adding high concentrations of substrate overcame the inhibition of both enzymes, which suggested that the inhibition was reversible and competitive. In a detailed kinetic study with PGI (Figure 13) the inhibition by hexacyanoferrate(II) was indeed found to be competitive with respect to fructose-6-P. The K_i for hexacyanoferrate(II) was found to be about 1.68 ± 0.05 m$\underline{M}$.

Although the pattern of inhibition for all thre enzymes appeared similar, PGI and G6PD were inhibited much less than was PFK, at a given concentration of hexacyanoferrate(II) and substrate. For all three enzymes, the affinity of the enzyme for hexacyanoferate(II) correlates directly with the affinity of the enzyme for its hexose-phosphate

FIGURE 11. INHIBITION OF YEAST GLUCOSE 6-PHOSPHATE DEHYDROGENASE BY HEXACYANOFERRATE(II).

G6PD at a concentration of 17 μg/ml was assayed as described in Materials and Methods. The reaction was started by adding G6PD.

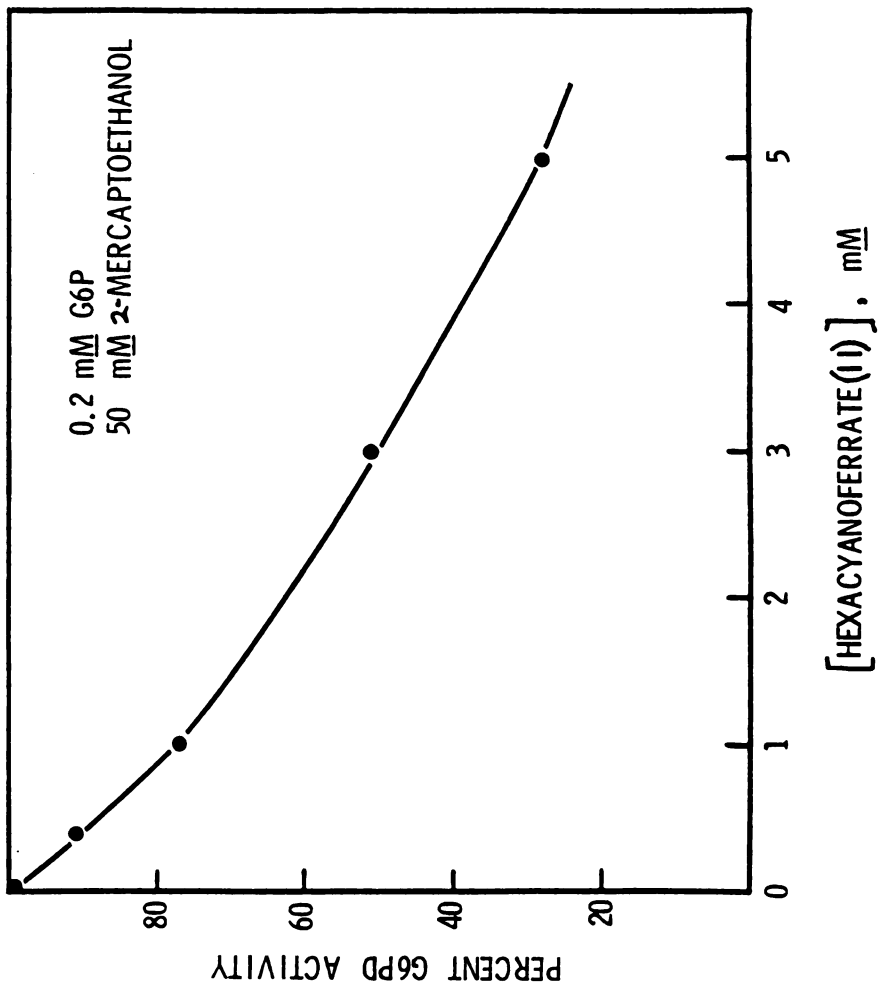

0.2 mM G6P
50 mM 2-MERCAPTOETHANOL
PERCENT G6PD ACTIVITY
20
40
60
80
0
1
2
3
4
5
[HEXACYANOFERRATE(II)], mM

Figure 11

FIGURE 12. INHIBITION OF YEAST PHOSPHOGLUCOSE ISOMERASE BY HEXACYANOFERRATE IN THE PRESENCE AND ABSENCE OF 2- MERCAPTOETHANOL.

PGI at a concentration of 10 µg/ml was assayed as described in Materials and Methods with the modification that 2-mercaptoethanol was added to the cuvettes separately. The assay was started by adding fructose-6-P to a concentration of 0.2 m<u>M</u>.

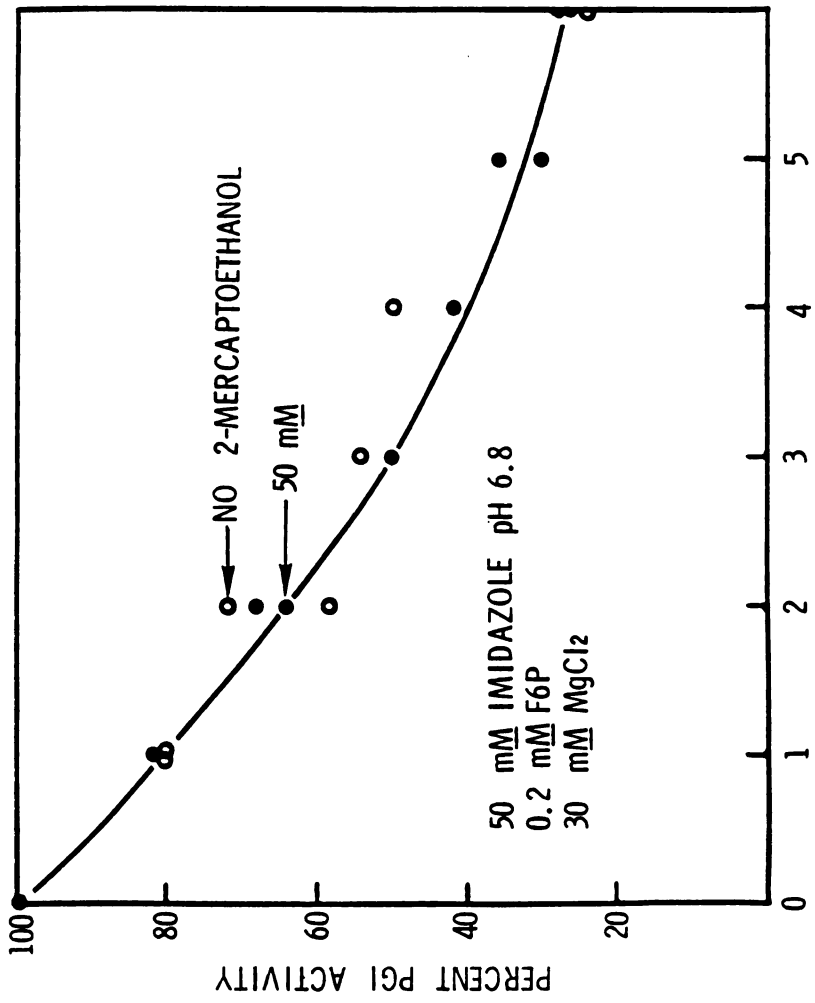

PERCENT PGI ACTIVITY
100
80
60
40
20
0
1
2
3
4
5
[HEXACYANOFERRATE] , mM
NO 2-MERCAPTOETHANOL
50 mM
50 mM IMIDAZOLE pH 6.8
0.2 mM F6P
30 mM MgCl2

Figure 12

FIGURE 13. MECHANISM OF INHIBITION OF YEAST PHOSPHOGLUCOSE ISOMERASE BY HEXACYANOFERRATE(II).

Phosphoglucose isomerase was assayed at a concentration of 22.8 ng/ml, in the presence of varying concentrations of fructose-6-P and hexacyanoferrate(II), as described in the Materials and Methods section of this chapter.

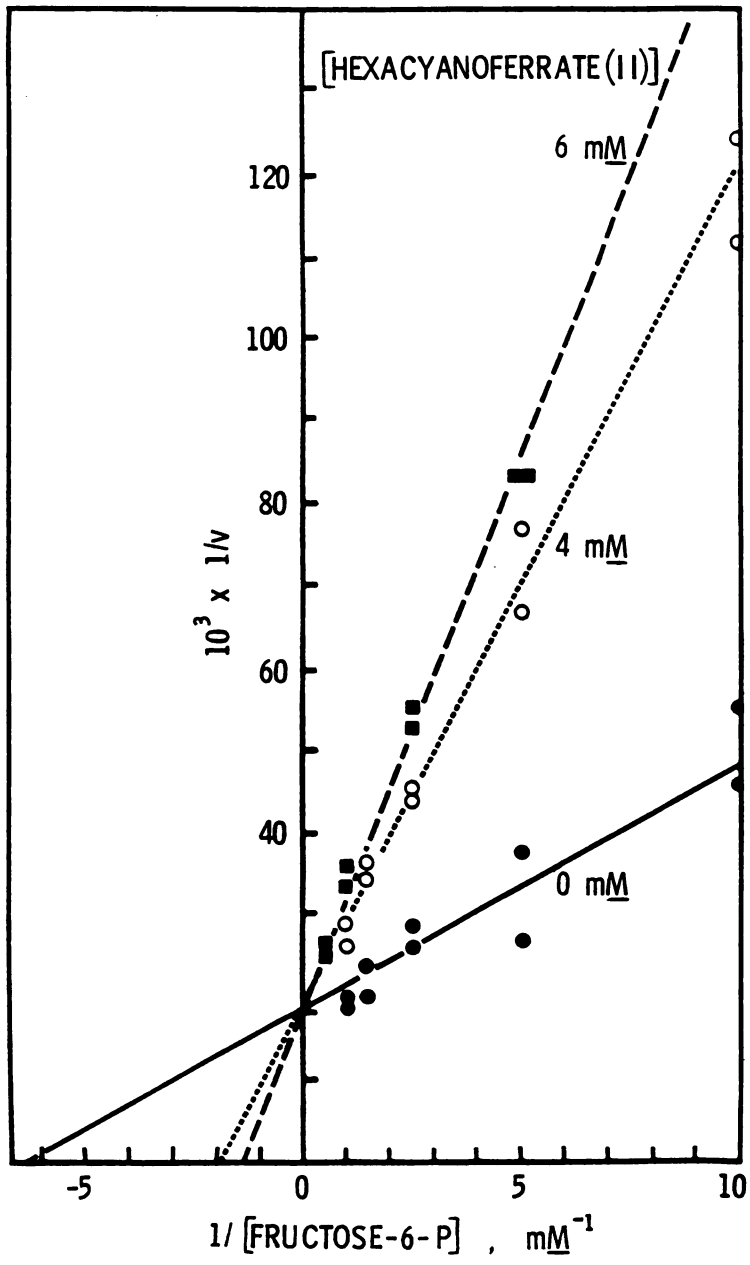

[HEXACYANOFERRATE(II)]
6 mM
4 mM
0 mM
120
100
80
60
40
10^3 x 1/v
-5
0
5
10
1/ [FRUCTOSE-6-P] , mM^{-1}

Figure 13

substrate. The K_m values are listed in Table 1, as well as the K_i and K_m/K_i ratios which we determined for PFK and PGI. The K_m value of PGI for fructose-6-P (0.16 m$\underline{M}$) is 6.9 times greater than the K_m of PFK for fructose-6-P (0.023 m$\underline{M}$); similarly, the K_i of PGI for hexacyanoferrate (1.68 m$\underline{M}$) is 11.2 times greter than the K_i of PFK (0.15 m$\underline{M}$). (The K_m/K_i ratios for both enzymes were in the range of about 0.1.)

TABLE 1. K_m AND K_i VALUES FOR THE ENZYMES STUDIED.[a]

ENZYME	K_m FOR SUBSTRATE		K_i FOR	K_m/K_i
	FRUCTOSE-6-P	GLUCOSE-6-P	HCF(II)	
PIG-KIDNEY PHOSPHOFRUCTOKINASE	0.023[b]	---	0.15[c]	0.15
YEAST GLUCOSE-6-P DEHYDROGENASE	---	0.058-10.0[d]		
YEAST PHOSPHOGLUCOSE ISOMERASE	0.16[e]	0.8[e]	1.68[f]	0.095

[a] K_m and K_i are in millimolar units.

[b] Massay, T.H., and Deal, W.C., Jr. (1975) Methods Enzymol. 42:99.

[c] This work (2.D.5)

[d] Noltman, E.A., and Kuby, S.A. (1963) in The Enzymes (Boyer, P.D., Lardy, H., and Myrback, K., eds.) 2nd Ed., vol. 7, p. 232, Academic Press, N.Y.

[e] Noltman, E.A. (1972) in The Enzymes (Boyer, P.D., ed.) 3rd Ed., vol. 6, p. 271, Academic Press, N.Y.

[f] This work (2.D.6)

Unlike PFK, PGI is not sensitive to sulfhydryl group oxidation so it can be assayed in a non-reducing environment. This allowed us to test the effect of hexacyanoferrate in the (III) oxidation state. As shown in Figure 12 the inhibition of PGI by hexacyanoferrate was identical in both reducing and non-reducing environments; this is further evidence that the inhibition does not involve oxidation.

7. Other Tests to Determine Whether Hexacyanoferrate(II) Binds to the Enzyme or Substrate.

a. Analysis for Loss of Glucose-6-P Due to a Reaction With Hexacyanoferrate(II).

Glucose-6-P was preincubated in standard assay buffer at a concentration of 0.5 mM, in the presence of 10 mM hexacyanoferrate(II). At timed intervals after adding hexacyanoferrate, 100 and 200 µl aliquots were assayed for glucose-6-P as described in the Materials and Methods section of Chapter 3. The control experiment had no hexacyanoferrate-(II). The background absorbance at 340 nm of hexacyanoferrate(II) was subtracted from the total absorbance change, and the decrease in absorbance due to dilution of the assay by the glucose-6-P mixture was added. In these experiments there was a rapid initial 10% decrease in glucose-6-P and then no further change with time. This decrease was small, and may be due to technical problems created by the relatively high absorbance of the high concentration of hexacyanoferrate. Alternatively, it could represent a weak reverisble association of hexacyanoferrate with glucose-6-P at these relatively high concentrations of hexacyanoferrate.

b. Test for Ability of Fructose-6-P to Spare Glucose-6-P From Potential Binding to Hexacyanoferrate(II).

The effect of hexacyanoferrate(II) on the activity of G6PD (using glucose-6-P as a substrate) was tested in the presence and absence of 4 mM fructose-6-P. The fructose-6-P is neither a substrate for G6PD nor does it affect G6PD activity. If hexacyanoferrate(II) were reversibly or irreversibly binding exclusively to the hexose phosphate substrate, then the excess fructose-6-P (4 mM) should largely react with the hexacyanoferrate(II) and leave a much larger fraction of the glucose-6-P substrate unreacted. However, the added fructose-6-P was found to have no effect on the inhibition of G6PD. This suggests that the mechanism of the inhibition of G6PD, and presumably the other enzymes, by hexacyanoferrate does not involve either reversible or irreversible bindng of the substrate by hexacyanoferrate.

8. Preliminary Study Using Photo-Oxidized PFK

PFK which had been photo-oxidized according to the method of Ahlfors and mansour (47) was shown to be still inhibited by hexacyanoferrate(II). Since photo-oxidation inactivates the ATP regulatory site, this is evidence that the regulatory site does not have an essential role in the inhibition.

E. DISCUSSION

1. Mechanism of the Inhibition.

The inhibition of PFK, PGI, and G6PD by hexacyanoferrate(II) appears to be unique since it does not involve an oxidation of either enzyme, substrate, or enzyme-substrate complex. Previous reports concerning the effect of hexacyanoferrates on other enzymes involved oxidation (39-44). The mechanism for the inhibition we observed seems to involve a competition with substrate by reversible binding of hexacyanoferrate(II) at, or near, the fructose-6-P (or glucose-6-P) binding site.

2. Molecular Basis for the Inhibition.

This raises the question of how hexacyanoferrate(II) could compete with fructose-6-P and in particular, what structural similarity hexacyanoferrate(II) might have to fructose-6-P. The strucutral properties of hexacyanoferrate have been described in detail (49-50) and summarized (51). The overall net charge of hexacyanoferrate is similar to that of the phosphate moiety of the hexose phosphate, but the molecular size of the hexacyanoferrate is clearly much greater than that of the phosphate moiety. This suggests that a substructure of the hexacyanoferrate might be involved. The bond electron orbitals of hexacyanoferrate are hybrids of the type, $3d^2 4s 4p^3$. The coordination positions of this complex with iron have octahedral symmetry, with all positions

equivalent (49). In contrast, the P-O bonds of phosphate have tetrahedral symmetry because sp^3 hybrid orbitals are involved (49). The bonding of cyanide to iron is neither covalent nor ionic, but rather belongs to a bonding group called complex cyanides (49). The hexacyanoferrate complexes are remarkably stable (49). The evidence is strongly in favor of bonding through a metal-carbon bond rather than a metal-nitrogen bond (52), although the latter is not absolutely ruled out by present data (49). The C-N bond distance in cyanide ion is only 1.05 A (49), which is considerably shorter than the P-O bonds in phosphate.

In summary, the propeties of hexacyanoferrate do not show any pronounced similarities to those of the phosphate ion. Their anionic character appears to be almost the only obvious common denominator structurally; but it does not provide an explanation for the structural specificity of hexacyanoferrate for these three enzymes.

3. Potential Active Site Directed Reagents.

So far there have been no successful attempts to label the hexose phosphate catalytic sites of PFK, PGI and G6PD. Hexacyanoferrate(II) has a specific affinity for the hexose phosphate active sites of these three enzymes. It may be possible to use this property to label the active site of these enzymes by using the oxidized form of hexacyanoferrate.

4. Contrast with other Inhibitory Compounds, Especially Those Involving Oxidation.

Hexacyanoferrate(III) has been shown (42, 44) to chemically modify the active sites of fructose-1,6-P_2 aldolase, transaldolase, transketolase, and ribulose bisphosphate carboxylase oxygenase. This process, which has been termed paracatalytic modification (42), involves a non-specific oxidation since the reaction is also carried out by a number of other oxidizing agents, such as tetranitromethane, H_2O_2, porphyrindin, and 2,6-dichlorophenolindophenol. Similarly, ferrate ion, FeO_4^{2-}, appears to be structurally similar to phosphate. It binds to the adenine nucleotide binding site of phosphorylase b, producing a specific modification, through oxidation, of the active site (54). Ferrate ion has also been found to be a powerful specific inhibitor ofphosphatases (55), presumably also as a reuslt of binding to the phosphate binding site.

All of these processes involve failry strong oxidizing agents, and they therefore differ from the process described in the present paper. Since the experiments described in this paper were carried out in the presence of a strong reducing agent, the effects observed cannot be attributed to oxidation. Rather, they appear to be due to a specific, reversible binding of hexacyanoferrate(II) at, or near, the hexose-phosphate binding sites.

CHAPTER 3

MEMBRANE-BOUND LIVER PHOSPHOFRUCTOKINASE AND METABOLITE-DEPENDENT REVERSIBLE SOLUBILIZATION

A. ABSTRACT

Approximately 50 to 75% of the phosphofructokinase is fresh pig liver homogenates at pH 7.4 is particulate and associated with specific membrane fractions. The particulate phosphofructokinase is solubilized essentially immediately in fresh homogenates by addition of relatively high levels of fructose-6-P, fructose-1,6-P_2, or glucose-6-P. Moreover, the solubilization is reversible; the soluble enzyme becomes particulate again as the added metabolites are converted to other products. Direct kinetic measurements of metabolite levels and particulate and soluble enzyme levels show a clear correlation between the disappearance of solubilizing metabolites and formation of particulate phosphofructokinase. The hexose monophosphates mentioned above are rapidly interconvertible and quickly formed from fructose-1,6-P_2 in the homogenates, so it was not possible to identify which of the individual hexose phosphates actually solubilize the particulate enzyme. AMP also rapidly solubilizes the particulate enzyme. The detegent Triton X-100 solubilizes the particulate enzyme, but that process takes almost an hour, in contrast to the very rapid solubilization by the metabolites. In sedimentation equilibrium experiments with the particulate phosphofructokinase in sucrose solutions, the phosphofructokinase floats with a membrane fraction under conditions where nuclei and large protein aggregates sediment. The evidence thus far suggests that the enzyme binds to the plasma membranes.

Isoelectric focusing and DEAE-cellulose chromatography of the particulate and soluble pig liver phosphofructokinase suggest that both forms are identical in charge, and therefore probably do not differ in degree of phosphorylation.

B. INTRODUCTION

For a long time it was assumed that, with the exception of hexokinase in some tissues (58), virtually all the glycolytic enzymes existed solely in the soluble fraction of the cell. There is now quite a bit of evidence that certain glycolytic enzymes in contractile tissues may bind to contractile proteins; the list includes aldolase (59-61), glyceraldehyde-3-P dehydrogenase (60), and phosphofructokinase (61). This apparently explains observations of a number of years ago (62, 64) that phosphofructokinases in a number of different muscle (62, 64) and other contractile tissues (63) seemed to be particulate; they sedimented at very low centrifugal fields.

In some cases (63) the proportion of enzyme sedimenting at low centrifugal fields increased if the tissue was allowed to age for several hours before the homogenization. Freezing also seems to cause or enhance particulate phosphofructokinase in several contractile tissues (62, 63), but not in rabbit liver (62). Kemp found particulate phosphofructokinase in rabbit muscle but not in fresh muscle (62); in contrast, he found that freezing did not produce particulate phosphofructokinase in rabbit liver (62).

Two points just mentioned are espcially pertinent to the present studies. First, the phosphofructokinase in liver, a gluconeogenic tissue, is quite different from that in muscle, a highly glycolytic contractile tissue. Second, freezing and aging are treatments which

can sometimes give rise to the artifactual appearance of, or increases in, the amount of particulate phosphofructokinase (62).

It appears that the particulate phosphofructokinase, aldolase, and glyceraldehyde-3-P dehydrogenase complexes in contractile tissues involve association of the enzymes with myofibrils, based on the known *in vitro* binding of these enzymes to actin (60, 65-67). This idea is supported by the fact that the amount of enzyme bound to a particulate fraction is increased by electrical stimuli (59-61).

In addition to particulate phosphofructokinase formed by protein-protein associations, recent *in vitro* studies showed that phosphofructokinase and several other glycolytic enzymes bind to erythrocyte ghost membranes under certain conditions, namely low ionic strength and slightly acidic pH (68-77). The physiological significance of these results at first seemed questionable, because tight binding occured only at pH values of about 6.8 and below, and only at low ionic strength; also, at higher ionic strength (150 mM KCl), all of the enzymes were dissociated from the membranes. However recent binding experiments with glyceraldehyde-3-P dehydrogenase suggest that it actually is membrane-bound *in vivo*, despite the *in vitro* results suggesting that it would be soluble (78).

A particulate phosphofructokinase from yeast has recently been discovered (79), and has been found to bind to the plasma membranes. This particulate phosphofructokinase remains firmly attached to the membrane at high ionic strength and at high concentrations of substrates, and appears to be distinct from the soluble form of the enzyme.

In some preliminary studies of the effects of hormones on pig liver phosphofructokinase, we found that a large portion of the phosphofructokinase activity in the homogenates sedimented at very low (900 x g) centrifugal fields. We then carried out an extensive study to characterize this phenomenon, using precautions to avoid possible artifacts. The results presented in this thesis show that a large portion of the phosphofructokinase in fresh pig liver homogenates is particulate, and appears to be membrane-bound. The results suggest that particulate phosphofructokinase may exist *in vivo* in pig liver, and may be important in the regulation of the enzyme.

C. MATERIALS AND METHODS

1. Reagents

Most biochemicals were purchased from Sigma Chemical Co., except NADH, NADP, ATP, and other nucleotides, which were purchased from P-L Biochemicals, Inc. All chemicals were reagent grade or better. Imidazole was recrystallized twice in 3:10:5 (w:v:v) imidazole: chloroform:ether. DEAE-cellulose, Triton X-100, ampholytes, and sucrose were purchased from Whatman, Rohm and Haas, LKB, and Mallinckrodt, respectively.

2. Tissues

Pig liver was obtained from the Michigan State University Meats Laboratory. Most livers were obtained during the fall, winter, and early sring, from pigs which had been fasted 15 hr. Livers were removed from the animals within 20 min after slaughter, and immediately placed on ice.

3. Preparation of Liver Homogenates

All procedures were done at 0 to 4°C. The homogenization procedure was begun promptly after obtaining the liver, usually within 15 min. Liver was cut into 2 to 4 mm pieces using a single-edge razor blade, and the pieces were washed in several changes of 0.25 M sucrose, to remove erythrocytes. The washed liver pieces were then transferred to

a 40 ml teflon-glass homogenizer (Potter-Elvehjem type) and 2 or 4 ml of homogenization buffer per gram tissue was added. For differential centrifugation experiments, 4 ml per gram was used; in other experiments, 2 ml per gram was used. The homogenization buffer consisted of: 50 mM imidazole-HCl, pH 7.4, 0.25 M sucrose, and either 20 mM 2-mercaptoethanol or 5 mM dithiothreitol. Liver was homogenized with 5 strokes of a size-C pestle, rotating at about 900 RPM. The homogenate was then filtered by pouring through 400-mesh nylon cloth, or several layers of cotton gauze.

4. Differential Centrifugation

This procedure was adapted from the method described by Fleisher and Kervina (80) for isolating subcellular organelles from rat liver. Figure 14 outlines the general procedure, rotors, and centrifugation conditions, and volumes of buffer used to resuspend pellet fractions. The homogenates were prepared from 12 to 48 grams of liver, using 4 ml of buffer per gram, as described previously. The standard buffer used for resuspending pellet fractions contained the same components as the homogenization buffer, plus the addition of 1 M $MgCl_2$. This buffer was used in all procedures after homogenization, unless otherwise noted. Centrifuge tubes for the Sorvall SS-34 rotor were the 29 x 102 mm polycarbonate types; tubes for the Beckman 42.1 rotor were the 1 x 3.5" cellulose or polyallomer types. For resuspending the 1000 x g pellet (and other fractions enriched in plasma membranes), a Dounce homogenizer was used, with a loose-clearance pestle (80). For most other pellet fractions, teflon- glass homogenizers were used.

FIGURE 14. PROCEDURE FOR DIFFERENTIAL CENTRIFUGATION OF LIVER HOMOGENATES.

The procedure was carried out at 0 to 4°. Other experimental details are described in this Materials and methods section (3.C.4).

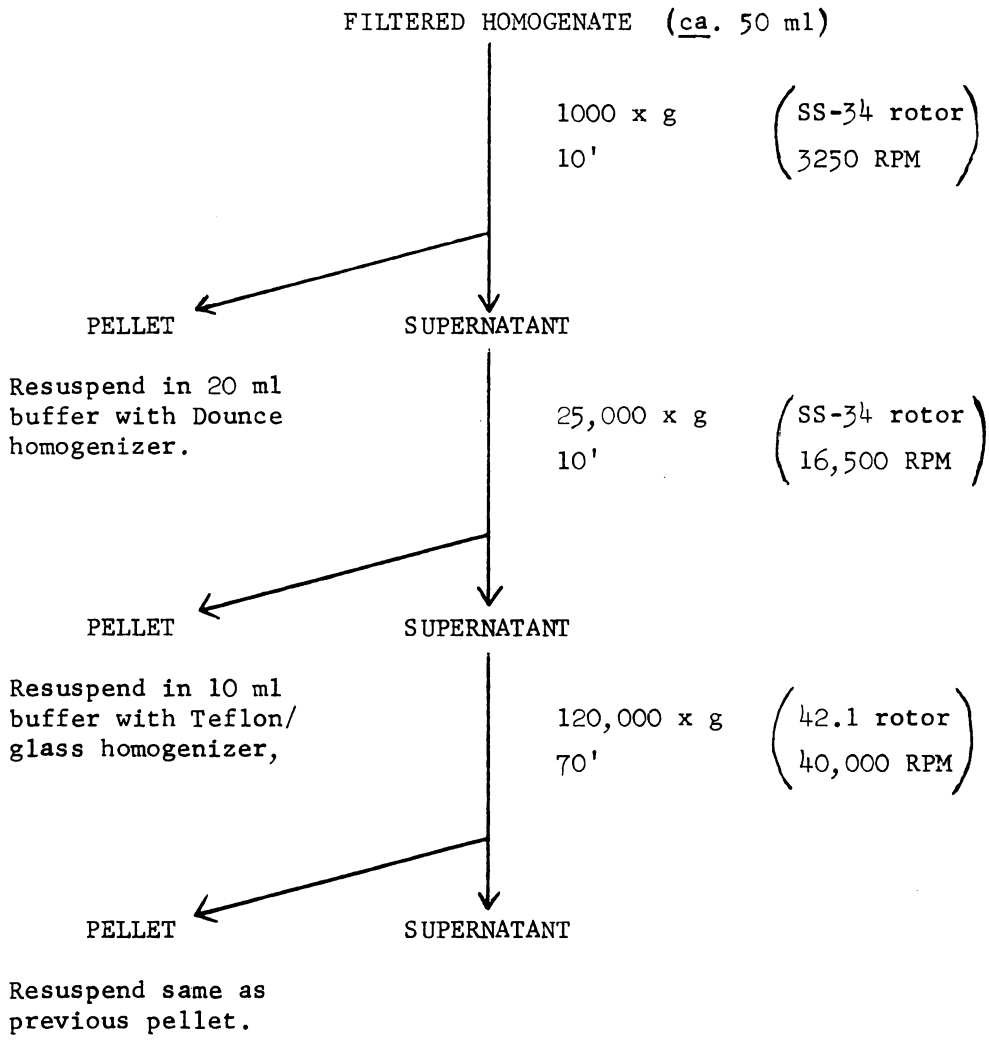

FILTERED HOMOGENATE (ca. 50 ml)
1000 x g
10'
SS-34 rotor
3250 RPM
PELLET
SUPERNATANT
Resuspend in 20 ml buffer with Dounce homogenizer.
25,000 x g
10'
SS-34 rotor
16,500 RPM
PELLET
SUPERNATANT
Resuspend in 10 ml buffer with Teflon/ glass homogenizer,
120,000 x g
70'
42.1 rotor
40,000 RPM
PELLET
SUPERNATANT
Resuspend same as previous pellet.

Figure 14

5. Sucrose Step-Gradient Centrifugation of Particulate Pig Liver PFK.

Suspensions of 100 x g pellet fractions from liver homogenates were prepared as described previously, and mixed with concentrated (2.4 M) sucrose (in the standard buffer), to bring the final sucrose concentration to 1.46 or 1.6 M (54.8 or 50% [w/v], respectively). Aliquots (about 30 ml) of this suspension were then transferred to 1 x 3.5" cellulose nitrate centrifuge tubes. The tubes were then filled carefully with buffer and centrifuged in the Beckman SW-27 rotor at 25,000 RPM for 1 hr (at 4°). Figure 24 shows the appearance of a tube before and after centrifugation.

Gradients were then fractionated as follows: The clear buffer on top was removed with a pipet and discarded. The floating band, at the heavy sucrose interface, was collected with a stainless steel scoopula. These bands were pooled and resuspended in 2 ml of the standard buffer per initial gram liver, in a Dounce homogenizer. The liquid below the floating band was poured off, and the pellets were combined and resuspended in a teflon-glass homogenizer, using about 1 ml buffer per initial gram of liver. The buffer used to resuspend this pellet fraction was modified to contain 3 mM $MgCl_2$, in order to stabilize the nuclei (80, 81), which were in this fraction.

6. Enzyme Assays.

Phosphofructokinase activity was determined according to the standard procedure of Massay and Deal (82), with the modifications that imidazole-HCl, pH 7.4, was substituted for TRIS buffer, and the volume of the reaction mixtures was adjusted with H_2O to allow 10 to 50 µl aliquots of enzyme solution to be added to the cuvettes. The

spectrophotometer slit-width was set a maximum (2 mm) to minimize background interference when assaying turbid fractions. The relatively high concentrations of fructose-6-P and ammonium sulfate in the cuvettes (4 and 40 mM, respectively) ensured that the added phosphofructokinase was solubilized and maximally activated.

Lactate dehydrogenase activity was determined according to the method of Kornberg (83). This enzyme, which is higly active and predominantly soluble in liver, was assayed as a control for unbroken cells and soluble supernatant protein carried over in pellet fractions.

Fructose-1,6-diphosphatase activity was determined spectrophotometrically according to the method of Racker (84).

7. Determination of Relative Particulate and Soluble Enzyme Activity Distributions in Pig Liver.

Aliquots (1 to 1.5 ml) of homogenates, prepared as described previously, were incubated under various conditions, transferred to 12 ml centrifuge tubes, and centrifuged in the SS-34 rotor at 8000 RPM (12,800 x g at r_{max}) for 5 minutes, at 0 to 4° or 23°. Supernatants (soluble fractions) were carefully transferred to calibrated tubes. Pellets (particulate fractions) were resuspended in 1 to 1.5 ml of standard buffer (3.C.3), with a Teflon pestle. Aliquots (10 to 20 µl) of the fractions were then assayed for PFK and LDH activity as described previously.

8. Metabolite Determinations

a. Reagents

perchloric acid ($HClO_4$), 6 N, (stock 70% [w/w] = 116.9 N)

K_2CO_3, 2.5 M

triethanolamine · HCl, pH 7.6/$MgCl_2$, 400/10 mM

triethanolamine · HCl, pH 7.6/EDTA, 400/40 mM

NADH, 20 mM, in 1 mM NaOH

NADP, 50 mM

ATP, ADP, AMP, 100 mM, pH 7

glucose, 100 mM

phosphoenol pyruvate (PEP), 40 mM

sodium pyruvate, 80 mM

F-6-P, F-1,6-P_2, 100 mM

Enzymes used as reagents are listed in Table 2. Commercial stock enzymes were diluted with 3.2 M ammonium sulfate and H_2O, as shown in Table 2, and stored in screw-capped vials, at 4°. The triethanolamine buffers (with $MgCl_2$ or EDTA) were described by previous workers (85, 86), and were also stored in amber bottles at 4°.

b. Preparation of Perchloric Acid Extracts of Liver Homogenates.

Perchloric acid has been commonly used by previous workers (85,86) in preparing tissue extracts for metabolite determinations. The procedure was done at 0°. Aliquots (1 to 1.5 ml) of homogenate, previously incubated with various metabolites, were diluted into 2 volumes of cold (0°) 6 N $HClO_4$, in 12 ml glass centrifuge tubes, and immediately mixed, using a vortex mixer. (The final $HClO_4$ concentration was 4 N.) Tubes were then centrifuged in the SS-34 rotor at 8000 RPM for 10

TABLE 2. STOCK CONCENTRATIONS AND DILUTIONS OF ENZYMES USED IN METABOLITE DETERMINATIONS.

ENZYME	SOURCE	CONCENTRATIONS OF STOCK SUSPENSIONS[1] enzyme conc. (mg/ml)	AS[2] conc. (M)	specific activity (U/mg)	FINAL CONCENTRATION mg/ml	U/ml	DILUTIONS OF STOCK SUSPENSIONS µl liquid in mixture stock enzyme	3.2 M AS	H_2O
Glucose-6-P Dehydrogenase	yeast	1.66	3.2	300	0.5	150	150	350	-
Hexokinase	yeast	9.5	3.2	430	0.38	163	40	960	-
Phosphoglucose Isomerase	yeast	3.64	2.6	550	0.36	200	100	740	160
Fructose-1,6-bisphosphate Aldolase	rabbit muscle	10.0	3.2	12	1.0	12	100	900	-
Trisosephosphate Isomerase/ α-Glycerol-P Dehydrogenase	rabbit muscle	10.8	3.2	2322/ 243	2.7	860/ 90	250	750	-
Pyruvate Kinase	rabbit muscle	10.0	2.2	465	0.5	233	50	650	300
Lactate Dehydrogeanse	rabbit muscle	10.0	2.1	870	0.5	435	50	625	325
Myokinase	pig muscle	1.6	3.2	1190	0.16	190	100	900	-

[1]The concentrations and specific activities of commercial stock enzymes are listed. To obtain the desired final concentrations for metabolite determiantions, the commerical stocks are diluted with 3.2 M ammonium sulfate and H_2O, as indicated on the right side of the table.

[2]AS, ammonium sulfate.

min. Supernatants were poured into calibrated tubes, and neutralized with potassium carbonate. About 7 drops of 2.5 M K_2CO_3 per 0.5 ml of perchloric acid added was required. The volume of the extract was then brought to 3 initial homogenate volumes with H_2O. The precipitate which formed from the neutralization was allowed to settle before assaying.

c. Spectrophotometer Instrument Settings.

A Gilford 240 spectrophotometer with a tungsten lamp was used; the wavelength was set to 340 nm. A chart recorder was useful for determining when absorbances of reaction mixtures were stabilized. The slit width was set to either 0.5 or 1.0 mm, for assays using NADH or NADP, respectively. These slit widths were chosen for the following reasons: For reactions coupled to oxidation of NADH, the initial A_{340} should be in the range of 1.5, to ensure that the final A_{340} will be above zero when the reaction is completed. For those assays coupled to reduction of NADP, the initial absorbance reading should be close to zero so that the final absorbance reading will not be too high or off scale. The slit width should be kept the same for a series of assays because the extinction coefficient of the reduced pyridine nucleotides may vary slightly with changes in slit width.

d. Metabolite Assay Procedure.

Metabolite determinations were done in 0.5 ml quartz cuvettes. Three groups of metabolites, shown in Table 3, were assayed together in the same cuvette. Each cuvette contained 250 µl of the appropriate triethanolamine buffer, and neutralized perchlorate extract plus H_2O,

TABLE 3. ORDER OF ADDITION OF REAGENTS FOR METABOLITE ASSAYS.

METABOLITES TO BE ASSAYED[a]	BUFFER SYSTEM (250 µl stock added)		REAGENTS TO ADD TO CUVETTE (5 µl of stock solutions)
	with $MgCl_2$	with EDTA	
glucose-6-P, fructose-6-P, glucose, or ATP	*		NADP, G6PD, PGI, HK + ATP or glucose
fructose-1,6-P_2 triosephosphates		*	NADH, TPI/α-GPDH, aldolase
pyruvate, PEP[b] or ADP, AMP or ATP	*		NADH, LDH, PK + ADP or PEP, MK + ATP or AMP

[a]For assaying the metabolites shown on the left, the reagents on the right are added to the cuvettes (in 5 µl quantities), in the sequence shown. After each addition, the reaction is allowed to go to completion. The A_{340} is then read, before addition of the next reagent(s). The "+" sign indicates two reagents are added together.

[b]If assaying PEP and subsequent metabolites, add an additional 5 µl of stock PEP after the pyruvate kinase (PK) reaction and wait for absorbance to stabilize before adding myokinase (MK).

in a total volume of 500 µl. Details of the procedure are given in Table 3. To assay the metabolites listed, the appropriate enzymes and co-substrates are added, in 5 µl quantities, and the final A_{340} is measured after the reactions are completed. For example, to assay the first set of metabolites listed in Table 3, first 5 µl of NADH is added, and the A_{340} is recorded. G6PD is added next, and the A_{340} is read after the absorbance stabilizes. The change in absorbance caused by adding G6PD, $\Delta A_{(G6PD)}$, is used to calculate the glucose-6-P concentration. Next PGI is added and $\Delta A_{(PGI)}$ is obtained. Then HK, together with either glucose or ATP is added, and $\Delta A_{(HK+glucose)}$ or $\Delta A_{(HK+ATP)}$ is obtained, to calcualte ATP or glucose concentration, respectively.

In order to ensure that limiting reagents are not depleted in an assay, an excess amount of metabolite being assayed is added, and the maximum ΔA_{340} is obtained. If a given assay causes the maximum absorbance to be reached, it is repeated with a smaller volume of the extract.

In order to correct for the absorbances of the added reagents, an assay is run with H_2O and no extract. The ΔA's obtained are subtracted from the appropriate measured ΔA's to give the corrected absorbance change, corr. ΔA_{340}.

e. Calculation of Metabolite Concentration.

The number of moles of metabolite reacted in the cuvette equals the number of moles of pyridine nucleotide oxidized or reduced in the coupled reaction, multiplied by a stoichiometric factor. The following equation is based on an extinction coefficient of 6.22 liter/(mole · cm)

for NADH and NADPH (87), a light path of 1 cm, and a reaction volume of about 500 µl:

$$\text{Metabolite concentration in extract (m}\underline{\text{M}}\text{)} = \frac{(\text{corr. } \Delta A_{340}) \times 80.39 \times (\text{stoichiometric factor})}{(\mu\text{l of extract in cuvette})}$$

The stoichiometric factor equals 1/2 for F-1,6-P_2 and AMP, and 1.0 for the other metabolites listed in Table 2. (The factor equals 1/2 for ATP if the myokinase reaction is used, and 1.0 if hexokinase is used.) The corr. ΔA_{340} was explained in the preceding paragraph. The metabolite concentration in the homogenate is obtained by multiplying the concentration in the extract by 3, since 3 volumes of extract are obtained per volume of homogenate.

9. DEAE-Cellulose Chromatography of Pig Liver PFK.

a. Extraction and Solubilization of Phosphofructokianse from Pig Liver.

In order to maintain the initially particulate phosphofructokinase in a soluble form, EDTA (5 m$\underline{\text{M}}$) was included in the chromatography buffer. This was based on a previous observation that EDTA gradually solubilizes the particulate enzyme. The chromatography buffer contained the same components as the standard buffer, except $MgCl_2$ was omitted, and 5 m$\underline{\text{M}}$ EDTA was included. The procedure for extracting particulate and soluble phosphofructokinases is shown in Figure 15.

b. DEAE-Cellulose Batch Chromatography.

Supernatant fractions containing soluble, or soluble plus particulate phosphofructokinase (Figure 15) were pre-chromatographed on a

FIGURE 15. PROCEDURE FOR EXTRACTING SOLUBLE AND PARTICULATE PFK FOR DEAE-CELLULOSE CHROMATOGRAPHY.

The procedure was done at 0 to 4°. Pig liver homogenates were prepared as described previously (3.C.3). In an alternate procedure to extract soluble and particulate phosphofructokinases together, 5 mM EDTA was included in the homogenization buffer, and the procedure for obtaining the soluble enzyme was that shown on the right side of the above diagram.

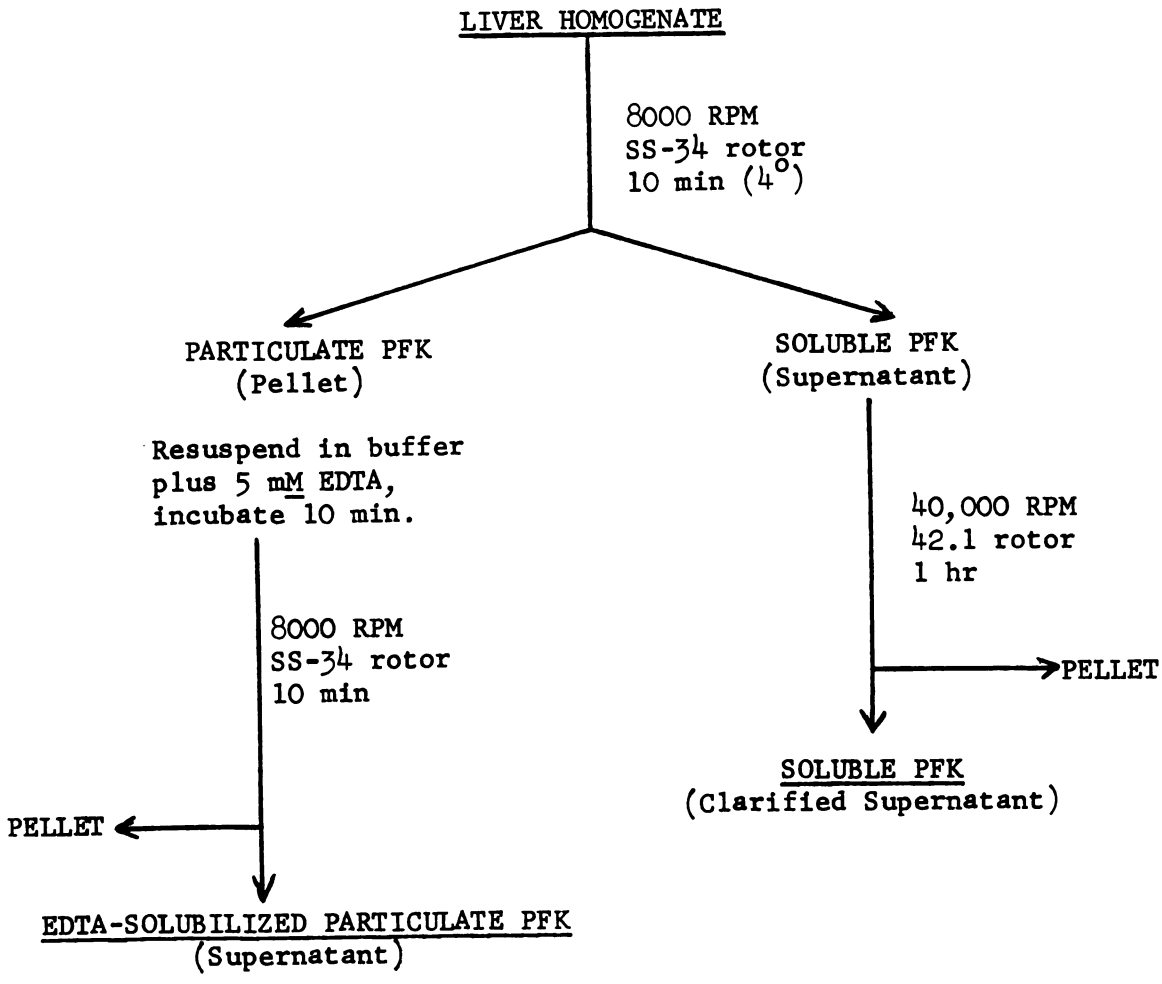

LIVER HOMOGENATE
8000 RPM
SS-34 rotor
10 min (4°)
PARTICULATE PFK
(Pellet)
SOLUBLE PFK
(Supernatant)
Resuspend in buffer
plus 5 mM EDTA,
incubate 10 min.
40,000 RPM
42.1 rotor
1 hr
8000 RPM
SS-34 rotor
10 min
PELLET
PELLET
SOLUBLE PFK
(Clarified Supernatant)
EDTA-SOLUBILIZED PARTICULATE PFK
(Supernatant)

Figure 15

DEAE-cellulose pad, in order to remove other proteins which bind tightly to the resin and decrease the resolution. Those fractions which contained only the EDTA-solubilized enzyme did not need to be pre-chromatographed.

DEAE-Cellulose (Whatman, standard grade) was acid-base washed according to the procedure described in the Whatman manual. The washed resin was stored in 3 M NaCl, at 4°.

A pad was formed in a 30 ml Pyrex funnel with a fritted glass filter, as follows: a nylon cloth screen was placed on the filter, and the DEAE-cellulose suspension was added, to form a bed volume of 20 to 30 ml. A nylon screen was placed on top of the pad, and the pad was equilibrated with the chromatography buffer. The chromatography buffer contained the same components as the standard buffer, except $MgCl_2$ was omitted, 5 mM EDTA was included, and ammonium sulfate was added in varying concentrations. Generally the reducing agent (DTT) was not included in buffer used to equilibrate the resin. The resin was washed with buffer including DTT (5 mM) just prior to adding the enzyme extract.

The chromatography was done at room temperature (23°). The DEAE-cellulose pad was washed with 10 ml buffer (containing 5 mM DTT). The enzyme extract (10 to 20 ml) was diluted 2-fold with the same buffer and applied to the pad. The pad was washed with 20 ml of buffer, which was allowed to filter through the resin by gravity. All of the red-colored hemoglobin in the extract eluted in the wash fraction. The pad was then eluted with 30 ml of buffer containing 150 mM ammonium sulfate. The yellow-colored band which eluted was collected in one fraction, of about 10 ml. This fraction was found to contain all of

the phosphofructokinase activity of the extract. The fraction was then dialized against 500 ml of buffer to remove ammonium sulfate. The PFK in the dialysate was stable for several days at 0 to 4°.

c. DEAE-Cellulose Column Chromatography.

Column chromatography was done at 23°, and 2.5 mM DTT was included in the buffers. A 0.9 x 10 cm DEAE-cellulose column was formed in glass tubing, equilibrated with buffer (minus reducing agent), and the flow rate adjusted to 1.2 ml/min. The dialyzed extract (at 23°) was diluted with 0.25 M sucrose plus 2.5 mM DTT to bring the conductivity to 0.85 ± 0.05 mmho. (A Radiometer type CDM2e conductivity meter was used.) The diluted dialysate was applied to the column, followed by 10 ml of buffer. The column was eluted with a 64 ml linear 0 to 150 mM ammonium sulfate gradient, in buffer. Sixty 22-drop fractions were collected, using an automatic fraction collector (ISCO model 328). Aliquots (50 µl) were assayed for phosphofructokinase activity (3.C.6).

The ammonium sulfate concentrations in the fractions were estimated from the conductivity, by using a standard curve of conductivity versus ammonium sulfate concentration in the buffer.

10. Isoelectric Focusing of Pig Liver Phosphofructokinase.

Liver homogenates were prepared in the presence of 5 mM EDTA, and clarified supernatant fractions were obtained as described in the previous section and in Figure 15. The supernatants (about 10 ml), were dialized against 500 ml of buffer (containing EDTA). Isoelectric focusing was done as described in Chapter 1. The heavy and light

gradient solutions both contained 1.2% ampholyte, and 75 to 225 µl of the dialized supernatant. Experiments were done with wide-range (pH 3-10) and narrow-range (pH 4-6) ampholytes. Focusing times were about 5 to 7 hr. After focusing, gradients were fractionated as described in Chapter 1, and phosphofructokinase activity in the fractions was determined by the standard procedure.

11. Phosphofructokinase-Membrane Complex Reconstitution Experiments.

Pig liver phosphofructokinase was purified according to the procedure of massay and Deal (82, 88), or partially purified to the first washed Mg/alcohol pellet. Subcellular fractions were isolated from pig liver using the procedure of Fleisher and Kervina (80). Membrane protein was determined by the Lowry procedure (29).

In one series of experiments, purified or partially purified pig liver phosphofructokinase was incubated with various subcellular fractions, in the standard buffer, at 0 or 23°. After incubating 45 minutes, the mixtures were centrifuged at 27,000 x g, and phosphofructokinase activity was determined in the supernatant and pellet fractions.

In another series of experiments, pig liver homogenates were treated with either 1 mM fructose-6-P or 150 mM KCl, to solubilize the phosphofructokinase, and centrifuged at 10,000 x g. Supernatants were incubated 1 hr (to deplete fructose-6-P), or dialized (to remove KCl), and then mixed with the pellet fractions. The mixtures were incubated 20 min to 1 hr, centrifuged at 10,000 x g, and phosphofructokinase activity was determined in supernatant and pellet fractions, as described in Materials and Methods (3.C.7).

12. Synthesis of Fructose-2,6-P_2.

Fructose-2,6-P_2 was synthesized according to the basic method of Van Schaftingen and Hers (89). Specific details on the procedure, as well as some additional modifications and comments, are given below.

(1) Formation of the Pyridinium Salt of Fructose-1,6-P_2.

The trisodium salt of fructose-1,6-P_2 (487.3 mg; FW = 406.1) was dissolved in distilled, deionized H_2O, to give a final volume of 2.4 ml, and a concentration of 0.5 M. The solution was applied to a 12 x 8 mm Dowex-50 column (X8, 50-100 mesh, H^+ form) and eluted with H_2O. Fractions (0.5 to 2 ml) were collected, and those with a pH less than 2 (which contained the free acid of fructose-1,6-P_2) were pooled, and neutralized with 2.1 ml of distilled pyridine. The volume of the mixture was brought to 6 ml with H_2O.

(2) Formation of Cyclic-1,2-phosphate.

Triethanolamine (0.5 ml) and 20 ml of pyridine were added to the above mixture. Dicyclohexylcarbodiimide (DCC; 2.4 g) was dissolved in 10 ml of pyridine, added to the above mixture, and incubated at room temperature (23°) for 24 hr. (Note: the DCC does not completely dissolve in the 10 ml of pyridine, but eventually dissolves in the reaction mixture.)

(3) Ether Extraction.

The reaction was then stopped by adding 40 ml of H_2O, and extracting the mixture with 200 ml of ether. The aquesous phase was then filtered through Watman no. 1 filter paper, to remove the large amount of crystals which formed during the reaction. The filtrate was extracted with 200 l of ether 4 more times. (Note: during the ether extraction, care must be taken to allow the emulsion to separate

completely, to avoid loss of some of the aqueous phase.) The mixture was then flushed with N_2 to remove traces of ether.

(4) Ring-opening of the Cyclic-1,2-phosphate.

Ring-opening of the cyclic-1,2-phosphate formed in step 2 was accomplished by alkaline hydrolysis, as follows: 0.2 volumes of 2.5 N NaOH was added to the mixture and the mixture, was incubated at 37° for 30 min. Solid glycine was then added to give a final concentration of 20 mM, and the mixture was cooled on ice. The pH was adjusted to 9.4 by dropwise addition of 2 N HCl.

(5) Hydrolysis of Fructose-1,6-P_2 by Fructose-1,6-bisphosphatase.

Eighteen units of rabbit muscle fructose-1,6-bisphosphatase (FDPase; Sigma; crystalline suspension in 3.2 M $(NH_4)_2SO_4$) were centrifuged in the Sorvall SS-34 rotor at 15,000 RPM for 10 min. The supernatant was discarded and the pellet was dissolved in 4 ml of 5 mM glycine, pH 9.4, and dialized in 500 ml of the same buffer, to remove ammonium sulfate. The dialized FDPase was added to the reaction mixture from step 4, and $MnCl_2$ (stock concentration = 1 M) was added to give a final concentration of 0.5 mM. The mixture (90 ml) was then incubated at 30° for 2-3 hr. The mixture was then diluted with 4 volumes of H_2O and stored at 0 to 4° until beginning the next step.

(6) First Dowex-1 Chromatography. Fructose-2,6-P_2 was purified from the mixture by chromatography on Dowex-1, at 23°. The diluted mixture from step 5 (450 ml) was applied to a 30 x 0.9 cm Dowex-1 column (hydroxide form), at a flow rate of about 1.4 ml/min. (The column was fitted with a safety loop, and allowed to run overnight.) The column was then eluted with a 250 ml linear 0.1-0.4 M NaCl gradient, at a flow rate of about 0.72 ml/min. Fractions (70-drops; 3.65 ml) were

collected using an automatic fraction collector (Isco, model 328). Fractions were first assayed for the presence of carbohydrate, using the phenol- H_2SO_4 method (described in the following section). For an accurate determination of fructose-2,6-P_2 concentration, we measured acid- revealed fructose-6-P (89), as follows: test samples were incubated in the presence of 10 m$\underline{M}$ HCl (or the amount of HCl required to reach pH 2), for 10 min at 23°. The fructose-6-P produced during the acid treatment was assayed as described earlier (3.C.8). The elution profile is shown in Figure 16. All of the fructose-2,6-P_2 was found in the second major carbohydrate peak (fractions 32-39), corresponding to an NaCl concentration of about 0.27 $\underline{M}$. These fractions were pooled, cooled to 0°, and the pH (initially 7.5) was adjusted to about 9.5 with 10 m$\underline{M}$ NaOH.

(7) Second Dowex-1 Chromatography.

The pooled fructose-2,6-P_2 fractions were rechromatographed on another Dowex-1 column, the same way as done before, except that smaller fractions (55-drops) were collected. The elution profile (Figure 17) shows one major carbohydrate peak, containing the fructose-2,6-P_2. A trace of fructose-1,6-P_2 was found on the leading edge of the peak (fraction 41). We also observed a 340 nm absorbing contaminant (not reported by the original authors) which eluted on the trailing edge of the fructose-2,6-P_2 peak (Figure 17). (A spectrum of the contaminant fraction revealed a single absorbance peak at 315 nm.) The fractions (nos. 41-49) containing fructose-2,6-P_2 were adjusted to pH 9.5, with 10 m$\underline{M}$ NaOH, and stored frozen, at -20°. The yield of fructose-2,6-P_2 was abaout 4%, similar to that reported by the original authors (89).

FIGURE 16. FIRST DOWEX-1 CHROMATOGRAPHY IN FRUCTOSE-2,6-P_2 PREPARATION.

After treating the fructose-2,6-P_2 reaction mixture with FDPase, the mixture was chromatographed on Dowex-1, as described in Materials and Methods (3.C.12.6). The column was eluted with a 250 ml linear 0.1 to 0.4 M NaCl gradient, at a flow rate of 0.72 ml/min. Fractions (70-drops) were collected using an automatic fraction collector (Isco, model 328). Relative carbohydrate concentrations were determined using the phenol/H_2SO_4 method described in section 3.C.13. Fructose-2,6-P_2 was determined by measuring acid-revealed fructose-6-P (3.C.12.6).

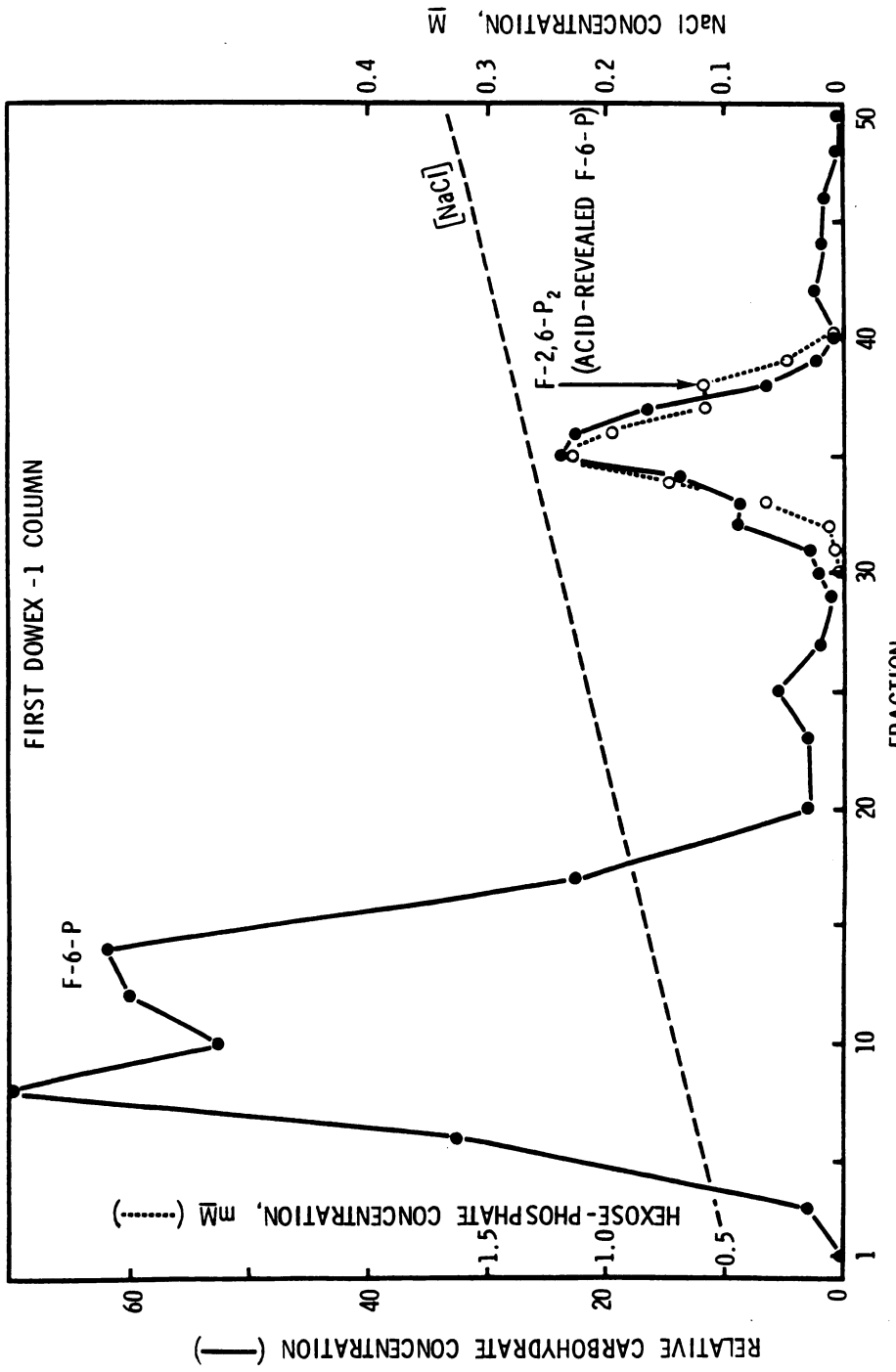

FIRST DOWEX -1 COLUMN
F-6-P
F-2,6-P2
(ACID-REVEALED F-6-P)
[NaCl]
RELATIVE CARBOHYDRATE CONCENTRATION (——)
HEXOSE-PHOSPHATE CONCENTRATION, mM (.....)
NaCl CONCENTRATION, M
FRACTION
0
20
40
60
0.5
1.0
1.5
0.1
0.2
0.3
0.4
1
10
20
30
40
50

Figure 16

FIGURE 17. SECOND DOWEX-1 CHROMATOGRAPHY.

The pooled fractions (nos. 32-39) from the first Dowex-1 column were rechromatographed the same way as described in the legend to Figure 16, except the fraction volume was decreased, to 55 drops. Fructose-2,6-P_2 and fructose-1,6-P_2 were determined as described previously (3.C.8 and 3.C.12.6). For determining absorbance at 340 nm (A_{340}), 100 µl aliquots of the fractions were diluted in 400 µl of H_2O, and A_{340} was measured on a Gilford 240 spectrophotometer, using the tungsten lamp.

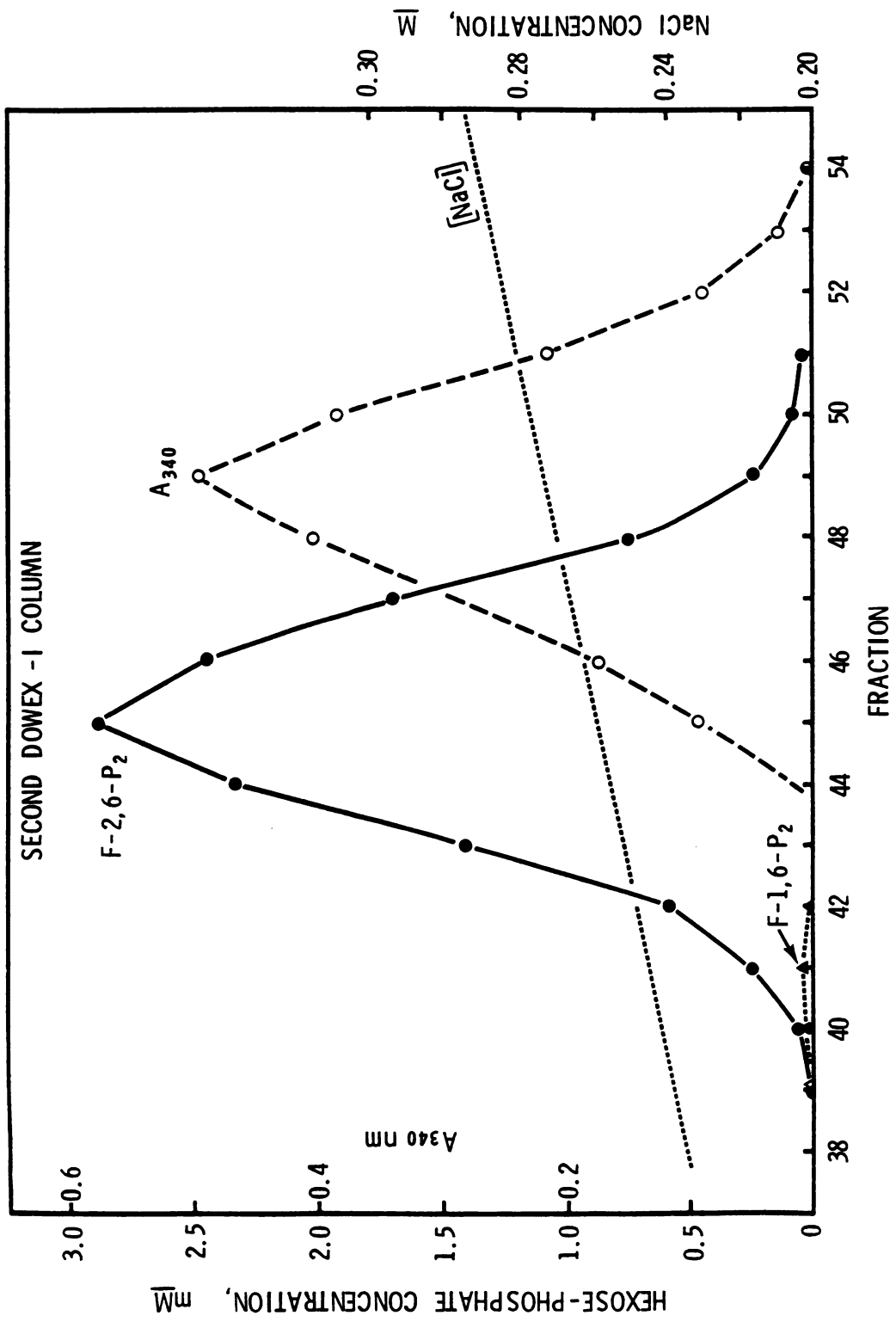

SECOND DOWEX -1 COLUMN
F-2,6-P2
F-1,6-P2
A340
[NaCl]
A340 nm
HEXOSE-PHOSPHATE CONCENTRATION, mM
NaCl CONCENTRATION, M
FRACTION
0
0.5
1.0
1.5
2.0
2.5
3.0
0.2
0.4
0.6
0.20
0.24
0.28
0.30
38
40
42
44
46
48
50
52
54

Figure 17

13. Determination of Carbohydrate by the Phenol-H_2SO_4 Method.

A previously reported method (90) using phenol and H_2SO_4 was adapted as follows: aliquots of column fractions were transferred to 14 x 125 mm Pyrex tubes, and H_2O was added to bring the total volumes to 100 µl. Then 50 µl of 6% phenol was added. Concentrated sulfuric acid (300 µl) was addded to each tube at separate timed intervals and tubes were mixed on a vortex mixer. The tube contents were transferred to a 0.5 ml cuvette, and the absorbance at 486 nm was measured 1 minute after adding the acid. Timing the absorbance readings accurately is important because the absorbance gradually increases with time. A control containing only H_2O was used as the blank.

We found this method gave a fast indication of the relative amount of carbohydrate and was useful for detecting peaks. However it does not give an accurate measure of concentration because the absorbance varies with the type of carbohydrate and the amount of salt in the fraction (90). For accurate determinations of hexose phosphates, we used the enzymatic techniques described previously (3.C.8).

14. Test for Activation of Pig Liver Phosphofructokinase by Fructose-2,6-P_2.

Pig liver (5 g) was homogenized in 2 volumes of buffer as described previously (3.C.3). KCl (2 M) was then added to give a final concentration of 150 mM, to solubilize the particulate phosphofructokinase. The homogenates were centrifuged at 8000 RPM in the SS-34 rotor for 10 min at 4°. The supernatants were used for the activation experiments.

The phosphofructokinase reaction mixture was prepared with the following modifications: the pH of the imidazole buffer was 7.0, (instead

of 7.4; the ATP concentration was 1 m$\underline{M}$, and the coupling enzyme concentration was reduced by half. Before adding the coupling enzymes to the reaction mixture, the ammonium sulfate was removed as follows. Enzyme suspensions were centrifuged in the SS-34 rotor at 10,000 RPM for 10 min. The supernatant was removed and the pellet was disolved in about 1.5 ml of 5 m$\underline{M}$ imidazole-HCl, pH 7, and dialized against 500 ml of the same buffer for 1 to 2 hr. The dialized enzymes were then added to the reaction mixture.

To test for activation of phosphofructokinase by fructose-2,6-P_2, 20 µl aliquots of the KCl-treated extract were assayed in the presence of varying concentrations of fructose-2,6-P_2 added to the cuvettes. The reactions were started by adding fructose-6-P, to a concentration of 50 µ$\underline{M}$. The V_{max} was determined by adding a high concentration (4 m$\underline{M}$) of fructose-6-P. The activation ratio, v/V_{max}, was obtained by dividing the observed rate by the V_{max}.

D. RESULTS

1. Relative Distribution of Phosphofructokinase in Soluble and Particulate Fractions of Pig Liver.

In order to promote stability of membrane fractions, and avoid artifacts, liver homogenates were prepared soon as possible after sacrifice of the animal. The bufers contained iso-osmotic (0.25 M) sucrose, and a Teflon-glass (Potter-Elvehjem type) homogenizer was used.

Homogenates were prepared freshly and incubated 2 minutes or 1 hour at 0 or 23°, and centrifuged at 10,000 x g as described in Materials and Methods. The pellet (particulate) and supernatant (soluble) fractions were assayed for phosphofructokinase activity. The control shown in Table 4, labeled "no additions", had about 55% of the phosphofructokinase activity in the particulate fraction, in samples incubated both 2 min or 1 hr before centrifugation. Hence a large portion of the phosphofructokinase is in the particulate fraction, and there is little or no change upon incubating for 1 hour. (The experiments shown in Table 4 were done at 23°; however the control showed the same results at 0 to 4°.) Assays for lactate dehydrogenase and fructose-1,6-diphosphatase, which are generally believed to be soluble, showed that these enzymes were indeed present only in the soluble fraction. Therefore the presence of phosphofructokinase in the particulate fraction could not have been a result of liquid included in the pellet. A number of

TABLE 4. FRACTION OF PARTICULATE PHOSPHOFRUCTOKINASE (PFK) IN PIG LIVER HOMOGENATES WITH VARIOUS COMPOUNDS AND INCUBATED 2 MINUTES OR 1 HOUR AT 23° PRIOR TO CENTRIFUGATION.

TREATMENT	PERCENT OF TOTAL PFK ACTIVITY IN 10,000 x g PELLET[a]	
	2 minutes	1 hour
NO ADDITION	55	59
2 mM Fructose-6-P	12	44
2 mM AMP	10	11
1% TRITON X-100	53	16
SONICATION[b]	12	12

[a]Liver homogenates were prepared as described in Materials and Methods. Aliquots were brought to 23°, and stock solutions of the indicted compounds were added and mixed. After incubating at 23° for 2 minutes or 1 hour, aliquots were centrifuged at 10,000 x g for 5 minutes, and particulate and soluble PFK activities were determined as described previously.

[b]Aliquots (3-5 ml) of homogenate were placed in 12 ml centrifuge tubes on ice, and sonicated using a Branson sonicator, at 4 amps for 8 seconds, with a stainless stell micro-tip.

livers obtained during the fall and winter showed similar results, with the amount of particulate phosphofructokinase varying from 50 to 75%.

2. Reversible Solubilization of Particulate Phosphofructokinase by Fructose-6-P.

When aliquots of homogenate were brought to 2 mM fructose-6-P and allowed to incubate 2 min or 1 hr at 23° before centrifugation, only 12% of the phosphofructokinase was in the pellet in the 2 min sample (Table 4); this indicated that most of the particulate enzyme had been solubilized. However the aliquot which was incubated 1 hr after adding fructose-6-P, before centrifugation, showed 44% particulate phosphofructokinase. We had expected to observe an increase in soluble phosphofructokinase with time, however this result led us to consider that perhaps the added fructose-6-P might be metabolized by enzymes in the homogenate; later results showed that this had happened.

3. Solubilization by AMP, Triton X-100, and Sonication.

We also found that 2 mM AMP solubilized almost all of the particulate phosphofructokinase in 2 minutes (Table 4). However, in contrast to the results with fructose-6-P, the solubilized enzyme remained soluble when incubated for 1 hr after adding AMP, before centrifugation. Apparently the added AMP was not metabolized away like the fructose-6-P; later direct measurements showed this was the case.

One percent Triton X-100 solubilized almost none of the particulate phosphofructokinase in 2 minutes, but solubilized all of the enzyme in 1 hour (Table 4). Sonication solubilized essentially all of the particulate phosphofructokinase, shown in the 2 minute aliquot, and there

was no change upon standing for 1 hour after the sonication treatment, before centrifugation.

4. Correlation of Reversible Solubilization of Particulate PFK with Fructose-6-P Levels.

In order to understand the previously described re-association of phosphofructokinase with the particulate fraction during incubation in homogenates to which fructose-6-P had been added, we did a series of measurements of metabolite levels at various time intervals after addition of 2 mM fructose-6-P to a homogenate sample. At selected time intervals, aliquots were taken for determination of hexose-phosphates (fructose-6-P and glucose-6-P), and other aliquots were assayed for relative amounts of soluble and particulate phosphofructokinase.

The results of a typical experiment are shown in Figure 18. Before adding fructose-6-P, 60% of the phosphofructokinase activity sedimented with the particulate fraction. One minute after adding 2 mM fructose-6-P, the phosphofructokinase in the supernatant fraction, initially 40%, increased to 71%. Also within the first minute after addition of 2 mM fructose-6-P, 86% of the fructose-6-P was converted to glucose-6-P (Figure 18); within 15 to 20 minutes both hexose phosphates were depleted, the portion of phosphofructokinase activity in the soluble fraction began to decrease, while that in the particulate fraction increased. The re-association of the enzyme with the particulate fraction continued for 40 to 50 minutes, after which time the amount of particulate phosphofructokinase leveled off at a value of 66%, very near the initial value of 60%, before the addition of fructose-6-P. These results indicate that the solubilization process is completely

FIGURE 18. SOLUBILIZATION OF PARTICULATE PHOSPHOFRUCTOKINASE (PFK) BY 2 m$\underline{M}$ FRUCTOSE-6-P, FOLLOWED BY RE-APPEARANCE OF PARTICULATE PFK AFTER FRUCTOSE-6-P DEPLETION.

A homogenate was prepared from pig liver as described in Materials and Methods and allowed to incubate for 5 min at 23° before beginning the experiment by adding fructose-6-P to a concentration of 2 m$\underline{M}$. At the timed intervals, shown on the graph, 0.5 ml aliquots were taken, diluted in 1 ml of cold 6 $\underline{N}$ $HClO_4$, mixed on a vortexer, and used for metabolite determinations (3.C.8). One ml aliquots were also taken, centrifuged at 10,000 x g, and used for determining particulate and soluble PFK activities, as described previously. The controls were run on the same homogenate, before adding fructose-6-P.

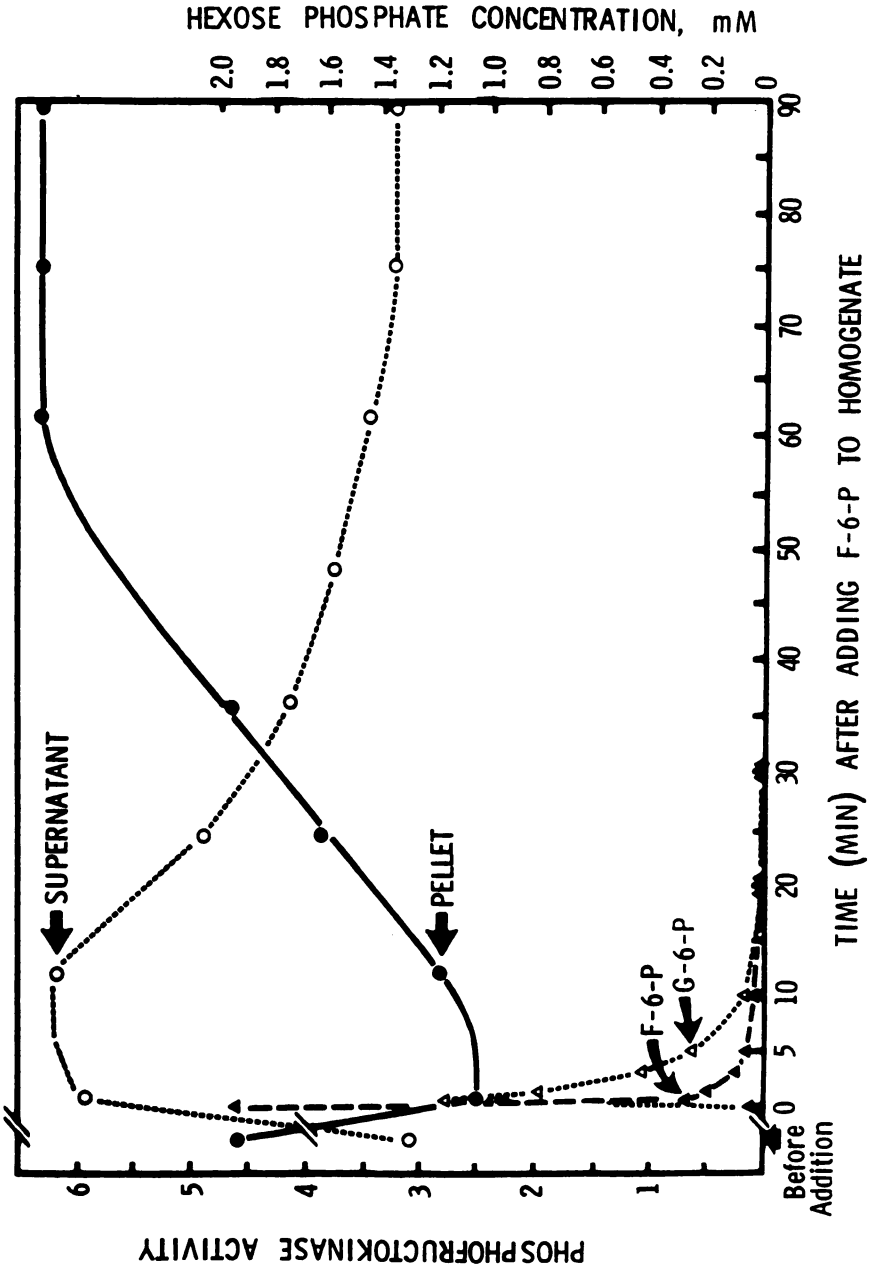

HEXOSE PHOSPHATE CONCENTRATION, mM
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
90
80
70
60
50
40
30
20
10
5
0
Before Addition
TIME (MIN) AFTER ADDING F-6-P TO HOMOGENATE
SUPERNATANT
PELLET
F-6-P
G-6-P
PHOSPHOFRUCTOKINASE ACTIVITY
6
5
4
3
2
1

Figure 18

reversible, which raises the possibility that the process might occur in vivo.

5. Interconversion of Hexose-Phosphates in Homogenates, and Formation of Glucose.

In previous experiments we had found that addition of the product fructose-1,6-P_2 (or glucose-6-P) to a homogenate sample, at concentrations of 1 to 2 mM, had nearly the same solubilizing effect on the phosphofructokinase as seen with fructose-6-P. We suspected that these metabolites could be interconverted by enzymes present in the homogenates. Therefore a series of experiments were done to determine the ultimate fate of added hexose-phosphates in liver homogenates.

In the experiments previously described, 0.25 M sucrose was present in all solutions. This however presented a problem in monitoring glucose concentrations because an acidification step with perchloric acid, used as part of the procedure for metabolite measurements, caused hydrolysis of the sucrose and gave spurious results in the glucose determination. We therefore carried out 2 parallel sets of experiments, one with sucrose omitted from the buffers, and another with 0.25 M sucrose present. Since it appeared from the previous experiments that the added hexose phosphates were being converted to glucose, and since we were also interested in the product, fructose-1,6-P_2, we began the experiments by adding fructose-1,6-P_2. (We also measured levels of triose-phosphates.) In order that all intermediates could attain 0.5 to 1 mM levels, the experiments were begun with a 3.5 mM concentration of fructose-1,6-P_2.

As shown in Figure 19, the homogenate rapidly produced fructose-6-P, triose-phosphates (dihydroxyacetone-P, glyceraldehyde-3-P), glucose-6-P, and glucose from the added fructose-1,6-P_2. The fructose-1,6-P_2 level dropped to zero within 20 to 25 minutes, during which time the glucose concentration steadily increased; the glucose concentration leveled off after about 30 minutes, when the glucose-6-P had been depleted.

About 70% of the added fructose-1,6-P_2 could be accounted for as glucose product. The other 30% was presumably converted to free trioses or to lactate. (Identical results were obtained whether or not the liver homogenization buffers contained 0.25 M sucrose.)

The results in Figure 19 indicate that fructose-6-P and glucose-6-P are produced extremely rapidly from added fructose-1,6-P_2. Therefore it is not possible in the studies with added fructose-1,6-P_2 to discriminate between solubilization due to the added fructose-1,6-P_2 and solubilization due to conversion of fructose-1,6-P_2 into fructose-6-P and glucose-6-P. Since fructose-1,6-P_2 is apparently not formed from fructose-6-P in the homogenate, it is possible to definitely conclude that either fructose-6-P or glucose-6-P, or both, are solubilizers, since both result in solubilization of particulate phosphofructokinase when added to homogenates. The phosphoglucose isomerase activity in homogenates is so large that addition of either glucose-6-P or fructose-6-P will almost immediately produce a significant concentration of the other isomer. (Since glucose-6-P is not known to bind to either the catalytic or allosteric sites of phosphofructokinase, we suspect that fructose-6-P is the actual solubilizing metabolite.)

FIGURE 19. CONVERSION OF FRUCTOSE-1,6-P_2 TO GLUCOSE IN HOMOGENATES.

Fructose-1,6-P_2 was added to about 15 ml of pig liver homogenate, at 23°, to give an initial concentration of 3.5 mM. At timed intervals, 1 ml aliquots were withdrawn and diluted in 2 ml of cold (0°) 6 N perchloric acid. Metabolite concentrations were determined as described in Materials and Methods. The abbreviations are: FDP, fructose-1,6-P_2; F-6-P, fructose-6-P; G6P, glucose-6-P; DHAP, dihydroxyacetone-P; G3P, glyceraldehyde-3-P; the (DHAP + G3P)/2 shows the equivalent concentration of hexose in the form of trioses. This experiment was repeated several times in the presence and absence of 0.25 M sucrose in the homogenization buffer, and identical results were obtained for all the metabolites except glucose. Apparently the perchloric acid used in the metabolite determinations hydrolyzes the sucrose to glucose plus fructose, and thus interferes with the glucose determination. Therefore in order to obtain the curve for glucose, 0.25 M sucrose was omitted from the homogenization buffer.

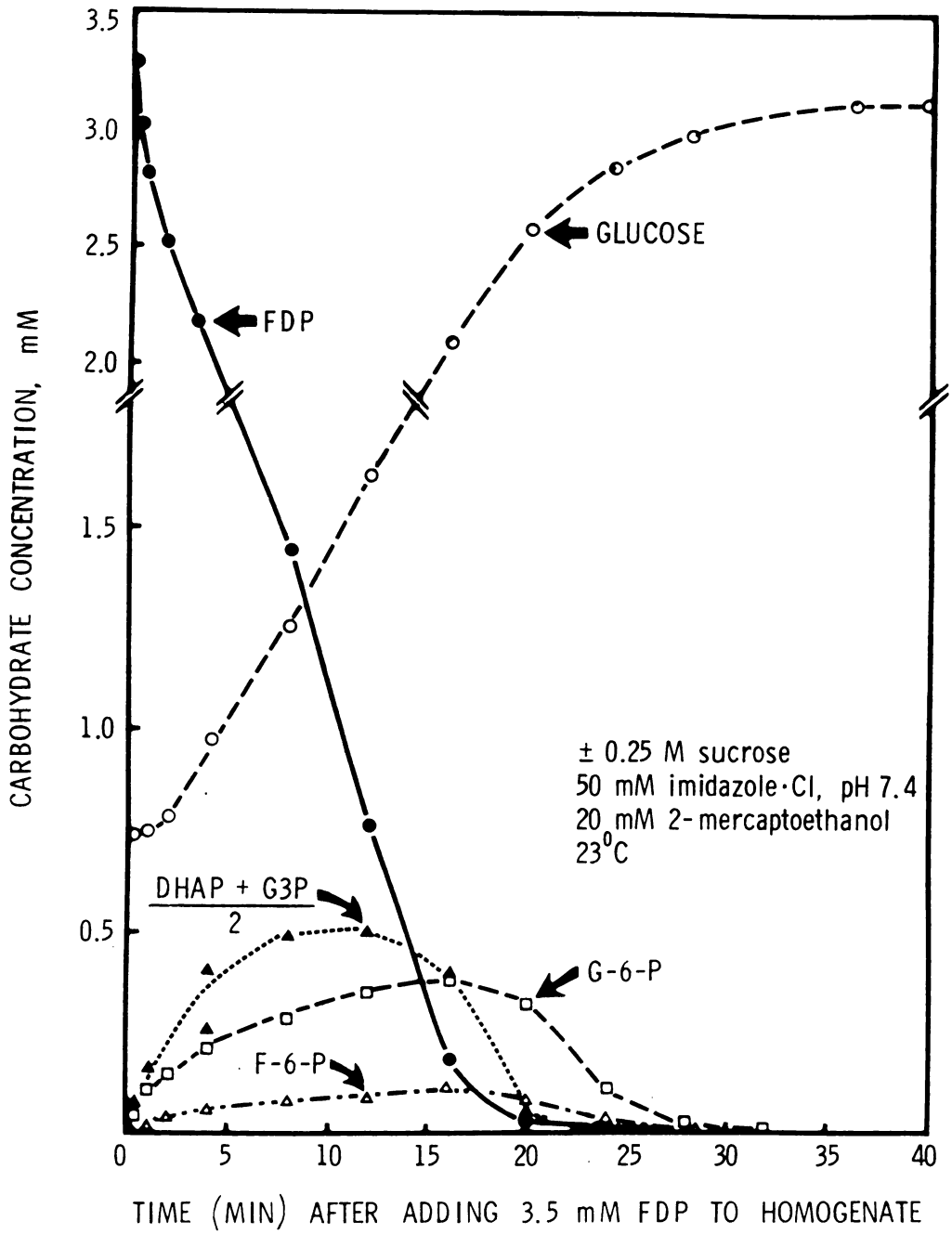

3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
5
10
15
20
25
30
35
40
CARBOHYDRATE CONCENTRATION, mM
TIME (MIN) AFTER ADDING 3.5 mM FDP TO HOMOGENATE
FDP
GLUCOSE
± 0.25 M sucrose
50 mM imidazole·Cl, pH 7.4
20 mM 2-mercaptoethanol
23°C
DHAP + G3P
2
G-6-P
F-6-P

Figure 19

6. Conversion of ATP to ADP and AMP in Homogenates.

Initial tests of adenosine mono-, di-, and triphosphates showed that all yielded solubilization of particulate phosphofructokinase when added to liver homogenates. In view of the extensive phosphatase action on the hexose-phosphates described previously, we suspected that the solubilizing effects of ATP and ADP might be due to the formation of AMP. Therefore we next investigated the fate of added ATP in liver homogenates. As shown in Figure 20, an homogenate initially made 3.4 mM in ATP converts all of it to ADP and AMP in less than 5 minutes. The ADP concentration reached a peak of about 1 mM within about 3 minutes, and then decreased rapidly, reaching almost zero levels within 5 minutes after the addition of 3.4 mM ATP. The AMP reached a maximum concentration of about 2.4 mM within 3 minutes, and decreased slowly over a 2 hr period; the decrease in the second hour was very slight. So it appears that ATP and ADP are degraded very rapidly, presumably by a highly active phosphatase specific for anhydride linkages. AMP is degraded much more slowly, perhaps by a less active phosphatase, specific for phosphomonoesters. AMP might also be convertd to AMP by AMP deaminase, which we would also predict would be less active than the phosphatases for ATP and ADP.

Figure 20 also includes part of the data from a similar experiment with a frozen liver; the AMP was found to be much less stable in the frozen liver homogenate.

FIGURE 20. CONVERSION OF ATP TO ADP AND AMP IN HOMOGENATES.

This experiment was done in the same manner as the experiment described in Figure 19, except that ATP was added to the homogenate (instead of fructose-1,6-P_2), to a concentration of 3.4 m$\underline{M}$.

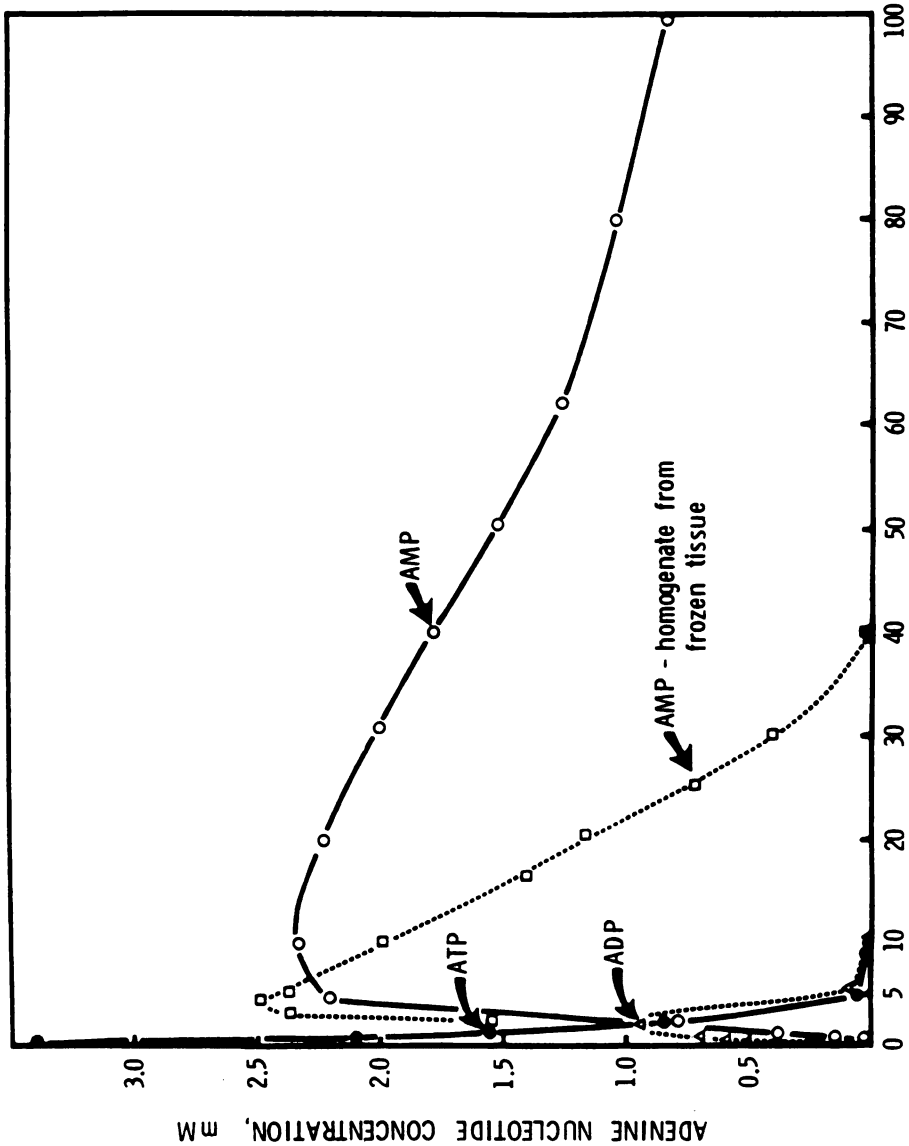

ADENINE NUCLEOTIDE CONCENTRATION, mM
3.0
2.5
2.0
1.5
1.0
0.5
0
5
10
20
30
40
50
60
70
80
90
100
TIME (MIN) AFTER ADDING 3.4 mM ATP TO HOMOGENATE
AMP
AMP - homogenate from frozen tissue
ATP
ADP

Figure 20

7. Specific Solubilization of Particulate Phosphofructokinase by Hexose-Phosphates and Nucleotides.

Addition of a number of hexose-phosphates and certain nucleotides to homogenates was found to affect the relative distribution of the soluble and particulate forms of phosphofructokinase. Detailed studies were carried out with fructose-6-P (Figure 21), fructose-1,6-P_2 (not shown), and AMP (Figure 22). Solubilization constants (defined in the legend to Figure 21), were determined for these and other metabolites, and summarized in Table 5. The experiments shown in Figures 21 and 22 were repeated several times with livers obtained during the fall and winter, and no significant differences were found in the solubilization constants. However the total phosphofructokinase activity in different livers varied as much as 1.8-fold. We are not certain what causes the variation in activity.

a. Solubilization by Fructose-6-P.

In a homogenate with no added fructose-6-P, 52% of the phosphofructokinase activity sedimented with the 10,000 x g pellet (Figure 21). Upon addition of fructose-6-P to the homogenate aliquots, the fraction of phosphofructokinase activity in the supernatants increased, reaching a maximum of 80% at about 200 μM fructose-6-P. Part of the unsolubilized 20% activity in those pellets could have been due to included supernatant. This residual pellet activity varied from 10 to 25% generally. The solubilization curve for fructose-6-P has a general hyperbolic shape.

Glucose-6-P and fructose-1,6-P_2 gave results very similar to those for fructose-6-P. Since those compounds are interconverted

FIGURE 21. SOLUBILIZATION OF PARTICULATE PHOSPHOFRUCTOKINASE BY FRUCTOSE-6-P.

One ml aliquots of freshly prepared pig liver homogenate were added to 12 ml centrifuge tubes and allowed to stand about 4 minutes, to reach room temperature (23°). Varying amounts of fructose-6-P were then added, the tubes quickly vortexed, centrifuged at 10,000 x g, and particulate and soluble phosphofructokinase activities were determined. The graph shows the total activity (in arbitrary units) in each fraction. The maximal solubilization concentration, $C_{1.0}$, is the lowest concentration of fructose-6-P giving approximately maximum solubilization. The $C_{0.5}$ is the concentration where half of the maximum solubilization occurs.

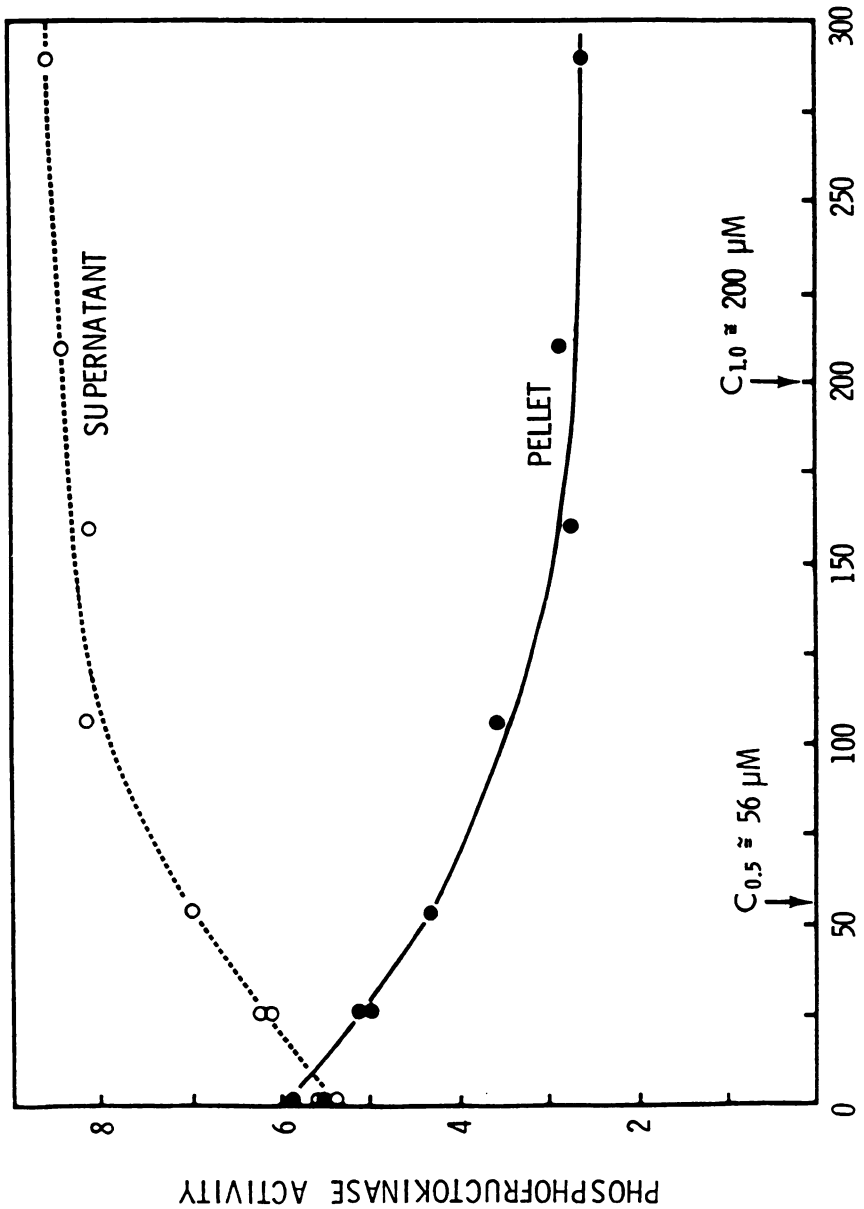

PHOSPHOFRUCTOKINASE ACTIVITY
2
4
6
8
SUPERNATANT
PELLET
$C_{0.5} \cong 56$ µM
$C_{1.0} \cong 200$ µM
0
50
100
150
200
250
300
F-6-P CONCENTRATION IN HOMOGENATE, µM

Figure 21

rapidly in homogenates (Figures 18 and 19), we cannot distinguish between their effects. It seems likely however that at least part of the fructose-1,6-P_2 solubilization is due to formation of fructose-6-P.

b. Solubilization by AMP.

An 8-fold higher concentration of AMP was required to give the same relative amount of solubilization as fructose-6-P (Figure 22). Also the AMP curves are steeper in the low concentration region and level off more gradually. The results with ATP and ADP were similar to those with AMP; this was expected since ATP and ADP are quickly dephosphorylated to yield AMP in homogenates (Figure 20).

c. Solubilization by other Metabolites and Salts.

Complete solubilization of the particulate phosphofructokinase was obtained with 100 mM added NaCl or KCl, and 50 mM potassium phosphate. It should be noted that the homogenates also contained a substantial amount of endogenous salts (estiamted at 1/3 of physiological; about equivalent to 50 mM KCl). GTP (2 mM) also yielded complete solubilization. No solubilization was obtained with 2 mM concentrations of glucose, fructose-1-P, lactate, citrate, or a mixture of dihydroxyacetone-P and glyceraldehyde-3-P. Also no solubilization was obtained with 2 mM concentrations of deoxy-AMP, adenosine, GMP, UTP, or CTP. The observation that fructose-1-P and glucose did not solubilize the enzyme indicates specificity for hexoses phosphorylated at carbon-6.

FIGURE 22. SOLUBILIZATION OF PARTICULATE PHOSPHOFRUCTOKINASE BY AMP.

This experiment was done the same way as described in Figure 21, except AMP was added instead of fructose-6-P. The solubilization concentrations, $C_{0.5}$ and $C_{1.0}$, were determined as described in Figure 21.

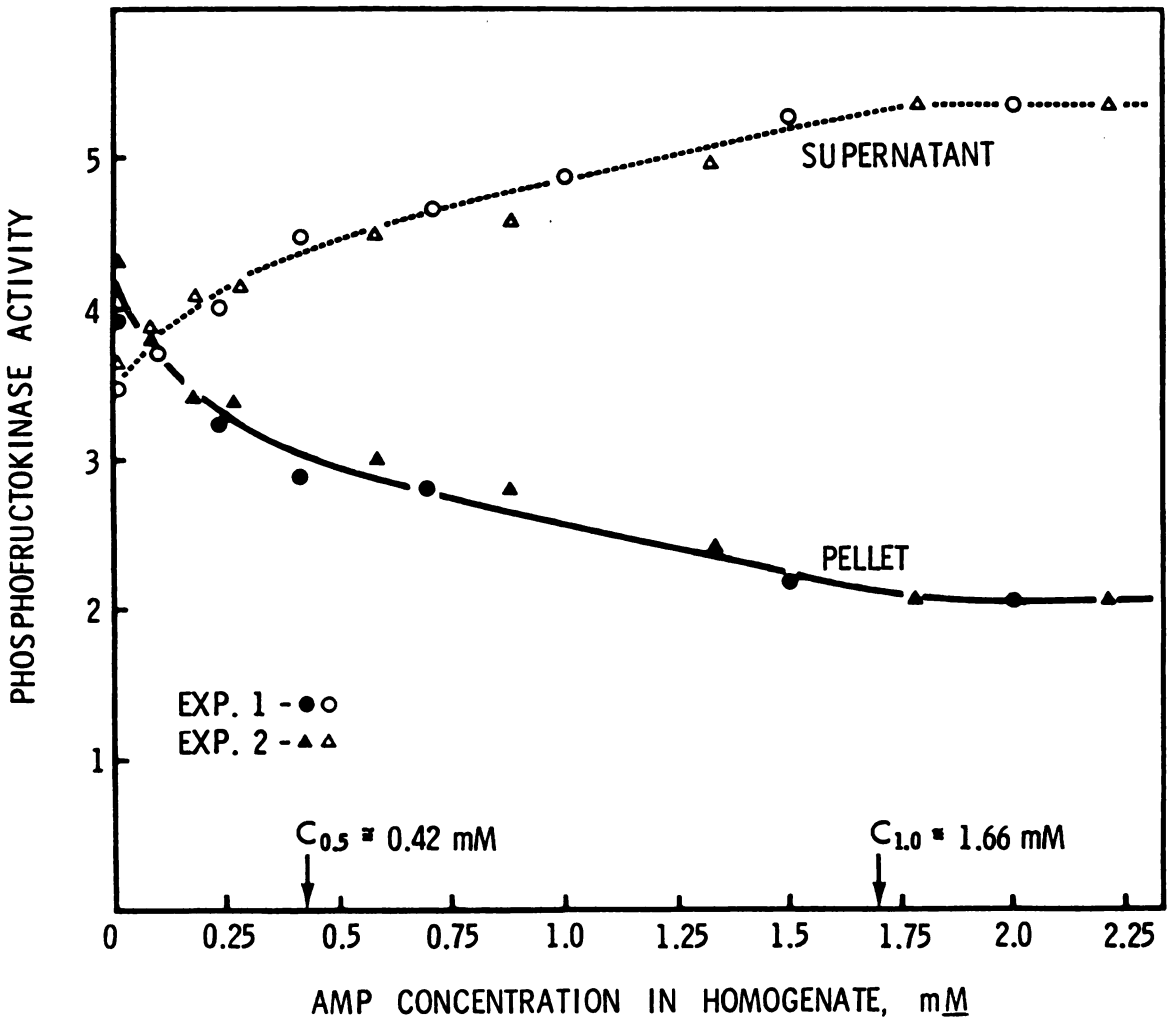

PHOSPHOFRUCTOKINASE ACTIVITY
5
4
3
2
1
SUPERNATANT
PELLET
EXP. 1 - ●○
EXP. 2 - ▲△
$C_{0.5}$ = 0.42 mM
$C_{1.0}$ = 1.66 mM
0
0.25
0.5
0.75
1.0
1.25
1.5
1.75
2.0
2.25
AMP CONCENTRATION IN HOMOGENATE, mM

Figure 22

TABLE 5. APPARENT PHOSPHOFRUCTOKINASE SOLUBILIZATION CONCENTRATIONS FOR VARIOUS COMPOUNDS ADDED TO PIG LIVER HOMOGENATES AT 23°, AND PHYSIOLOGICAL CONCENTRATIONS OF METABOLITES IN RAT LIVER UNDER FED AND FASTED CONDITIONS.

METABOLITE	APPARENT SOLUBILIZATION CONCENTRATION[a], μM		AVERAGE CONCENTRATION IN RAT LIVER[b], nmole/g	
	$C_{0.5}$	$C_{1.0}$	FED	FASTED
FRUCTOSE-6-P	56	220	79	36
FRUCTOSE-1,6-P_2	50	300	25	16
GLUCOSE-6-P	64	255	266	121
AMP	420	1660	234	273

[a]Definitions of $C_{0.5}$ and $C_{1.0}$ are given in the legend to Figure 21. Experimental conditions are described in Materials and Methods.

[b]Data was taken from reference 91. The units of nmole/g are roughly comparable to micromolar units, since the density of liver tissue is in the range of 1-2 g/cm^3.

The finding tht GTP solubilized, but GMP did not seems surprising in view of the solubilization by AMP and ATP. This result was reproducible in 4 separate experiments.

8. Activation of Pig Liver Phosphofructokinase by Fructose-2,6-P_2.

We were next interested in determining whether the recently discovered activator of phosphofructokinase, fructose-2,6-P_2 (97), might have a solubilizing effect on particulate pig liver phosphofructokinase. Since the original studies were done using the rat liver or rabbit muscle phosphofructokinase, our first interest was to test it for activation of the pig liver enzyme.

Fructose-2,6-P_2 was synthesized according to Van Schaftingen and Hers (89), and tested for ability to activate pig liver phosphofructokinase as described in Materials and Methods (3.C.14). As shown in Figure 23, the enzyme was markedly activated, as expected. In the absence of fructose-2,6-P_2, the activity was close to zero because of the low (50 μM) fructose-6-P, high (1 mM) ATP, the neutral pH (which enhances ATP inhibition), and the absence of activating NH_4^+ ions. However in the presence of 0.4 to 0.6 μM concentrations of fructose-2,6-P_2, the enzyme was activated to near V_{max} levels. (The V_{max} was determined by assaying the enzyme in the presence of a high (4 mM) concentration of fructose-6-P.) The concentration of fructose-2,6-P_2 which gave half maximal activation (K_a) was approximatley 0.2 μM. This is in the range of published values (0.05 to 0.1 μM) for the rat liver and rabbit muscle phosphofructokinases (94-96).

FIGURE 23. ACTIVATION OF PIG LIVER PHOSPHOFRUCTOKINASE BY FRUCTOSE-2,6-BISPHOSPHATE.

Fructose-2,6-bisphosphate was synthesized and tested for ability to activate pig liver phosphofructokinase as described in Materials and Methods (3.C.12). The v/V_{max} values were obtained by dividing the reaction rates by the V_{max}, as described previously (3.C.14). The V_{max} was constant for all the assays since they contained the same amount of liver extract. The K_a is the concentration of fructose-2,6-P_2 giving half maximal activation (95). The error bars correspond to ± 5%.

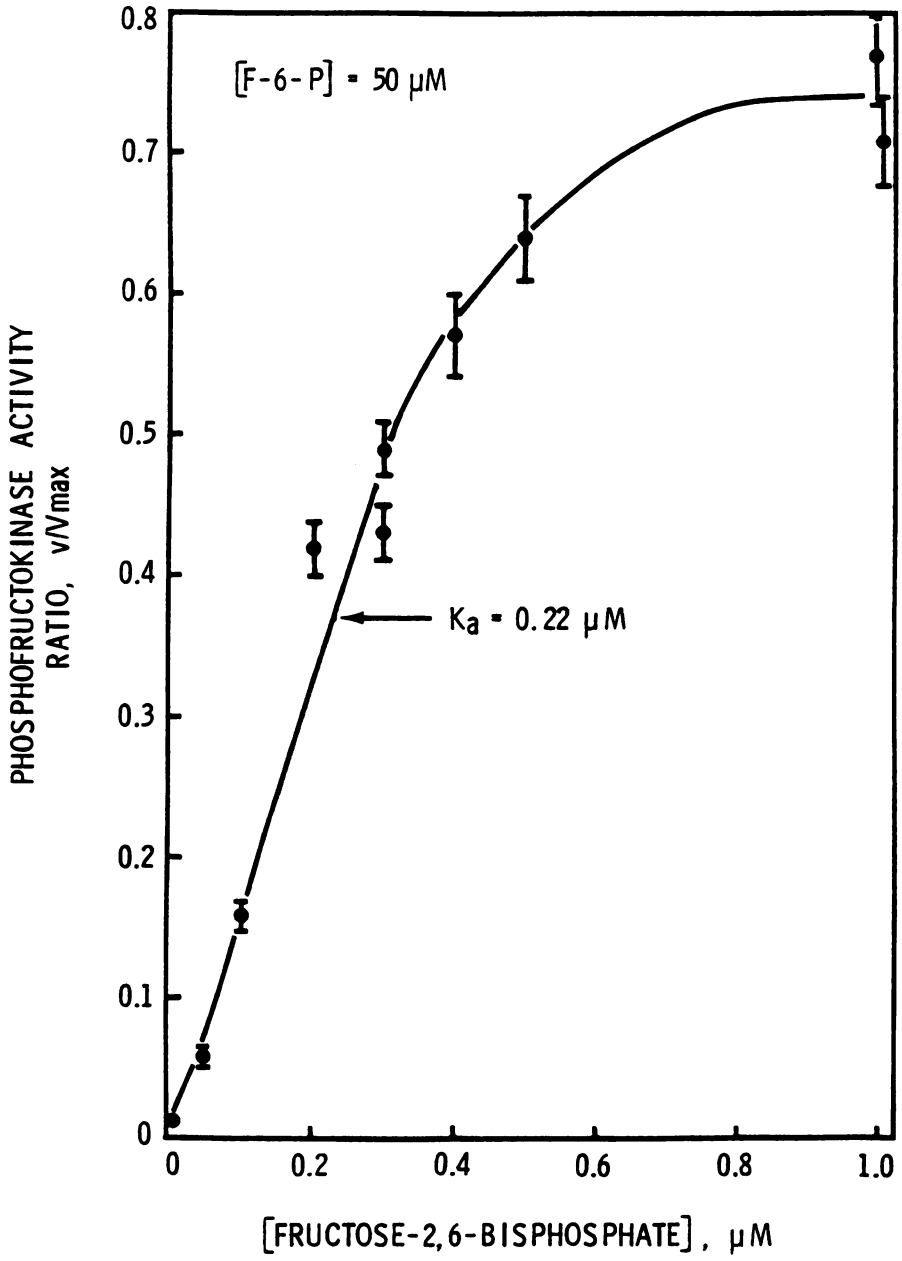

[F-6-P] = 50 μM
Ka = 0.22 μM
PHOSPHOFRUCTOKINASE ACTIVITY RATIO, v/Vmax
[FRUCTOSE-2,6-BISPHOSPHATE], μM
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.2
0.4
0.6
1.0

Figure 23

9. Tests for Effect of Added Fructose-2,6-P_2 on Solubilization of Particulate PFK, in the Presence and Absence of Fructose-6-P and AMP.

After characterizing the activation of pig liver phosphofructokinase by fructose-2,6-P_2, we were next interested in determining whether fructose-2,6-P_2 could solubilize the particulate enzyme. We were also interested in whether fructose-2,6-P_2 might enhance the solubilization effect of fructose-6-P.

When fructose-2,6-P_2 was added to aliquots of homogenate at concentrations of up to 20 μM, we observed no solubilization of particulate phosphofructokinase, even though this concentration is about 100 times higher than that which gives maximal activation of the enzyme.

Since fructose-2,6-P_2 increases the affinity of phosphofructokinase for the substrate fructose-6-P (97), it was of interest to determine whether fructose-2,6-P_2 could enhance the solubilization of the enzyme by fructose-6-P, or perhaps by AMP. To test this possibility, high concentrations (10 to 20 μM) of fructose-2,6-P_2 were added to aliquots of homogenate containing fructose-6-P or AMP at concentrations giving less than the maximum solubilization of the enzyme (10, 25, and 50 μM F-6-P; and 1 mM AMP). However we observed no increase in solubilized enzyme in the fructose-2,6-P_2-treated aliquots, compared to controls (not containing the activator).

Although we did not observe solubilization effects with fructose-2,6-P_2, it is possible that phosphatase activity in the homogenate might have hydrolyzed the added fructose-2,6-P_2 (to fructose-6-P),

thereby preventing us from observing possible effects of fructose-2,6-P_2. Previous workers have shown that in fasted liver, fructose-2,6-P_2 is degraded very rapidly by a specific phosphatase (97). This phosphatase may have been active in our experiments, since the livers were from fasted animals.

10. Subcellular Distribution and Nature of the Particulate Phosphofructokinase.

The next experiments were designed to determine whether the apparent particulate phosphofructokinase was an extremely high molecular weight, self-associated enzyme polymer, or whether it was actually membrane- bound, and if so, to what type of membrane. A survey of phosphofructokinase activity in the various fractions obtained by differential centrifugation is shown in Table 6. About 60% of the activity was in the nuclear fraction, 25% in the microsomal fraction, and 15% in the post- microsomal supernatant (soluble fraction). Hence, about 85% of the enzyme was particulate, and only 15% was actually soluble. It is significant that no phosphofructokinase activity was found in the mitochondrial-lysosomal fraction; this also indicates that phosphofructokinase does not simply bind non-specifically to all types of membranous materials.

In contrast, lactate dehydrogenase, a soluble enzyme, was found mostly (84%) in the soluble fraction, as expected. Some lactate dehydrogenase activity (16%) was found in the nuclear fraction, perhaps as a result of included supernatant, or undisrupted cells.

An explanation for the presence of phosphofructokinase activity in both the nuclear and microsomal fractions (Table 6) consistent with

TABLE 6. PHOSPHOFRUCTOKINASE (PFK) AND LACTATE DEHYDROGENASE (LDH) DISTRIBUTIONS AMONG SUBCELLULAR FRACTIONS OBTAINED BY DIFFERENTIAL CENTRIFUGATION OF PIG LIVER HOMOGENATES.[a]

FRACTION	PERCENT OF TOTAL ACTIVITY[b]	
	PFK	LDH
1,000 x g PELLET nuclei, plasma membranes, heavy mitochondria, erythrocytes, unbroken cells	60	16
25,000 x g PELLET mitochondria, lysosomes	-	-
120,000 x g PELLET microsomes, large proteins	25	-
120,000 x g SUPERNATANT soluble fraction	15	84

[a]Experiments were carried out as described in Materials and Methods. The subcellular components listed are those predominantly found in rat liver under the indicated centrifugation conditions (80).

[b]Total activity is the sum of the activities of the four fractions obtained by differential centrifugation.

binding to a single type membrane, is phosphofructokinase binding to the plasma membranes; enzymes which are localized on the plasma membrane have been shown to exhibit a bimodal distribution of activity between the nuclear and microsomal fractions (92, 93). (Presumably the large plasma membrane fragments formed during homogenization in 0.25 M sucrose sediment with the nuclei, while the smaller plasma membrane fragments sediment with the microsomal fraction.)

11. Sucrose Step-Gradient Flotation of Particulate Phosphofructokinase.

In order to subfractionate the nuclear fraction obtained from the differential centrifugation, a sucrose step-gradient procedure, described in Materials and Methods (3.C.5), was done next. The diagram in Figure 24 shows the appearance of the tubes before and after centrifugation, and the distribution of phosphofructokinase activity in the fractions. We found that 98% of the activity (relative to the initial starting activity) floated to the interface between the 1.6 and 0.25 M sucrose solutions. This indicates that the particulate phosphofructokinase complex has a density less than that of 1.6 M sucrose (density = 1.20 g/cm^3). An additional 20% activity was found in the nuclear pellet. We are not certain whether the 118% recovery is due to removal of inhibition, or activation.

In a similar series of experiments, we found that the particulate phosphofructokinase floated in sucrose concentrations of 50% (w:v) (density = 1.190 g/cm^3), and sedimented in 45% sucrose (density = 1.174 g/cm^3). Therefore the density of the particulate phosphofructokinase must be between these two values.

FIGURE 24. PHOSPHOFRUCTOKINASE DISTRIBUTION IN FRACTIONS OBTAINED BY SUCROSE STEP-GRADIENT FLOTATION OF 1000 x g PELLET.

The experimental procedure is described in Materials and Methods (3.C.5). The diagram shows the appearance of a step-gradient before and after centrifugation. The liquid above and below the floating band contained no phosphofructokinase activity. The total activity recovered from the floating band and pellet was 18% greater than the starting activity. We are not sure whether this could be due to removal of inhibition or due to activation. The predominant subcellular components of the fractions, listed in parentheses, are those found under similar conditions with rat liver (80, 92).

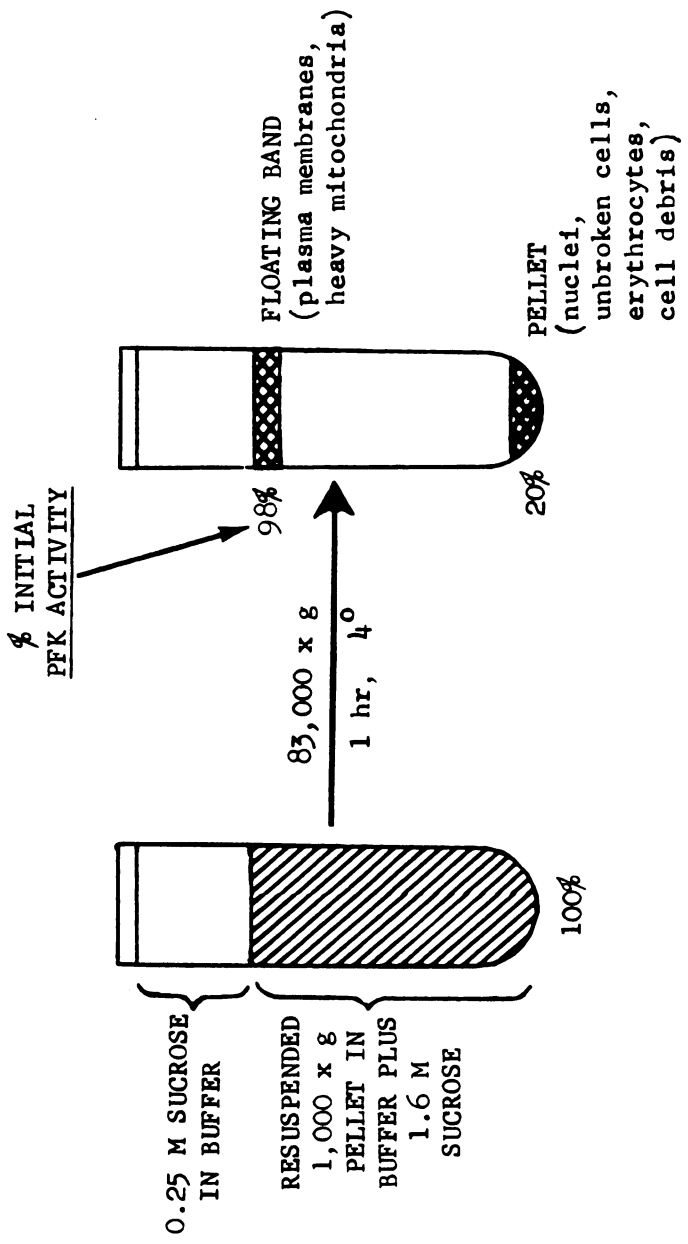

% INITIAL
PFK ACTIVITY
98%
20%
100%
83,000 x g
1 hr, 4°
FLOATING BAND
(plasma membranes,
heavy mitochondria)
PELLET
(nuclei,
unbroken cells,
erythrocytes,
cell debris)
0.25 M SUCROSE
IN BUFFER
RESUSPENDED
1,000 x g
PELLET IN
BUFFER PLUS
1.6 M
SUCROSE

Figure 24

These results show conclusively that: (1) the particulate phosphofructokinase is not an extremely high molecular weight particle; therefore its presence in membrane fractions is not a result of being highly self-associated; (2) that the major portion of the particulate enzyme is not bound to nuclei; and (3) that the particulate phosphofructokinase is membrane-bound. The basis for conclusions one and three is that the density of protein is about 1.33 g/cm^3, so the enzyme must be membrane-bound to float as a particle with a density in the range of 1.18 g/cm^3.

12. Attempts to Reconstitute the Phosphofructokinase-Membrane System.

The floating bands from the sucrose step-gradient experiments were found to contain the major portion of the particulate phosphofructokinase (Figure 24). The major subcellular components in this fraction are nominally the plasma membranes and heavy mitochondria (80), so it seems likely that the phosphofructokinase could be bound to one or both of these fractions. However when the individual membrane fractions are isolated, neither of them contains phosphofructokinase activity. This is most likely due to a gradual dissociation of the enzyme during the repeated centrifugations in the presence of EDTA, which is part of the isolation procedure.

As one approach to determine the membrane to which phosphofructokinase binds, we carried out incubation experiments with mixtures of purified or partially purified pig liver phosphofructokinase and various subcellular membrane fractions, as described in Materials and Methods (3.C.11). Most experiments were done with 0.5 ml incubation

aliquots, containing 0.15 units/ml phosphofructokinase, and 5 to 10 mg/ml membrane protein.

In a typical experiment, the control (without membrane) showed 44% of the phosphofructokinase activity in the 27,000 x g pellet. In the mixture containing purified plasma membranes, 64% of the activity sedimented at 27,000 x g. However the nuclei, heavy mitochondria, and major mitochondrial fractions also showed a substantial amount of sedimenting activity, (55, 52, and 54%, respectively). Thus a large amount of non-specific precipitation occured in the reconstituted systems, and the amount of sedimenting activity in any fraction was not significantly above the background levels.

We also observed no enhancement of binding in experiments with 120,000 x g supernatant included in the mixtures, or in experiments with crude pellet and supernatant fractions.

These results suggest that perhaps another cellular component (s), in addition to enzyme and membrane, is required for binding to occur. The other component might be inactivated during the fractionation procedure. Or perhaps the membrane receptors for phosphofructokinase become solubilized during the fractionation. The membrane would then be unable to bind phosphofructokinase, or the solubilized receptors might compete with membrane-bound receptors for the binding site on the enzyme.

13. Analysis for Different Phosphorylated Forms of Phosphofructokinase by DEAE-Cellulose Chromatography.

Previous workers have found that phosphofructokinases from a number of different species and tissues can undergo in vitro phosphorylation

FIGURE 25. DEAE-CELLULOSE CHROMATOGRAPHY OF SOLUBLE PHOSPHOFRUCTOKINASE (PFK), AND PARTICULATE PIG LIVER PHOSPHOFRUCTOKINASE, SOLUBILIZED BY EDTA.

Particulate phosphofructokinase was extracted from pig liver, solubilized with EDTA, and chromatographed on a DEAE-Cellulose column as described in Materials and Methods (3.C.9). Soluble pig liver phosphofructokinase was prechromatographed on a DEAE-cellulose pad, to remove other soluble proteins, and then chromatographed the same was as the particulate enzyme solubilized by EDTA. The graph shows the elution profiles for the two separate experiments.

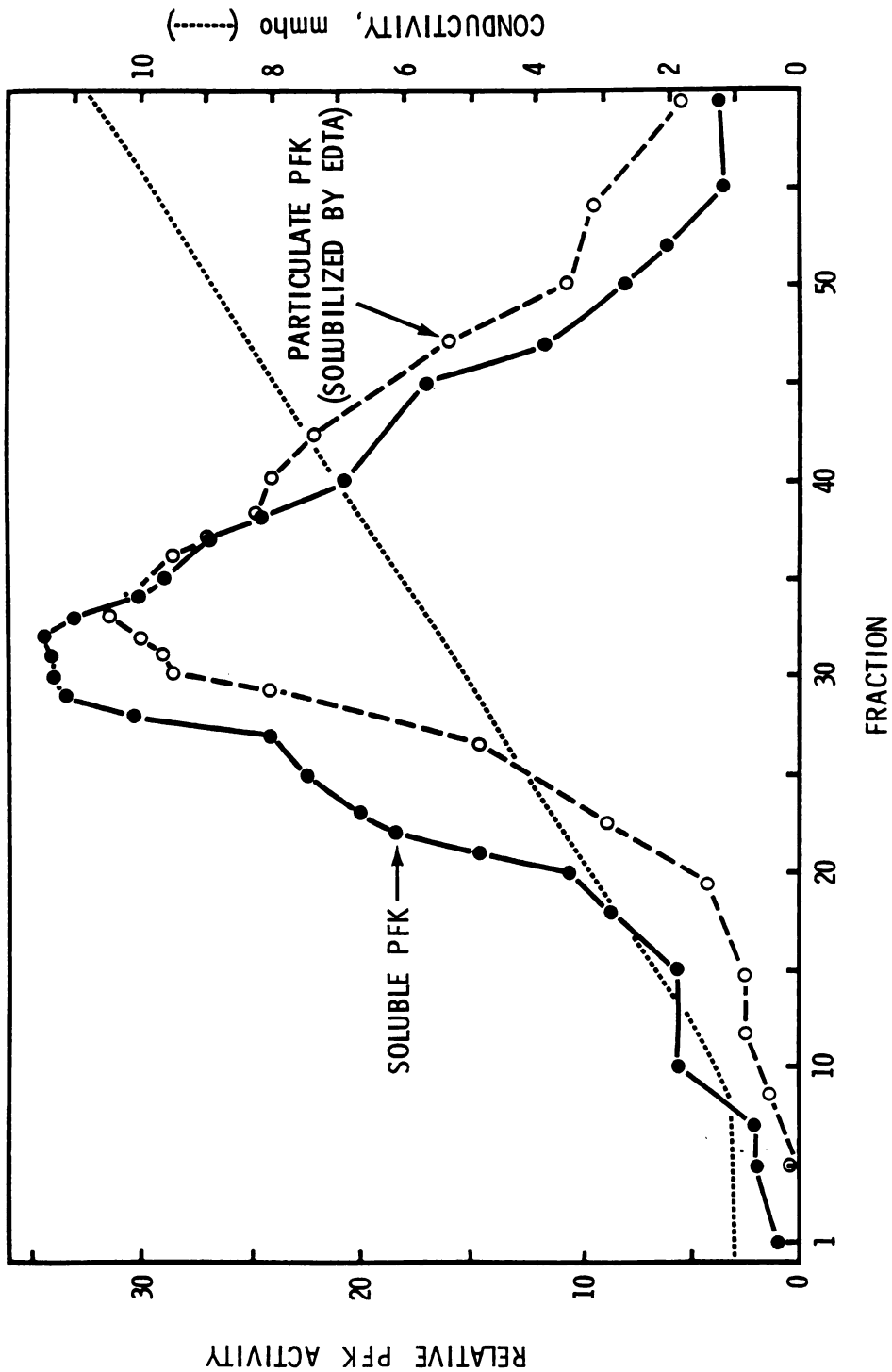

RELATIVE PFK ACTIVITY
0
10
20
30
FRACTION
1
10
20
30
40
50
CONDUCTIVITY, mmho (------)
0
2
4
6
8
10
PARTICULATE PFK
(SOLUBILIZED BY EDTA)
SOLUBLE PFK

Figure 25

and dephosphorylation (98, 99). In rat liver, two different phosphorylated forms of phosphofructokinase have been isolated (100). If pig liver also contains different phosphorylated forms of phosphofructokinase, we should be able to separate them by DEAE-cellulose chromatography. As a preliminary step in the chromatography procedure, the particulate enzyme was extracted and solubilized in a buffer containing 5 mM EDTA (3.C.9). We assumed that the presence of EDTA would not affect the degree of phosphorylation of the enzyme.

Typical results from chromatography experiments are shown in Figure 25. The graph contains the results from two parallel experiments, one with the soluble phosphofructokinase and the other with the EDTA-solubilized particulate enzyme. The elution profiles were found to be nearly identical. Both forms of the enzyme eluted as single broad bands, in the ammonium sulfate concentration range of 18 to 92 mM, with the peaks occuring at 42 and 50 mM, for the soluble and particulate enzymes, respectively. In an attempt to improve the resolution, the chromatography was done at pH 7 (rather than 7.4), which is closer to the isoelectric point of pig liver phosphofructokinase (pI = 5.1). However the phosphofructokinase activity eluted at the beginning of the ammonium sulfate gradient, and thus the resolution was not improved.

14. Isoelectric Focusing of Soluble and Particulate Pig Liver Phosphofructokinase.

Isoelectric focusing was used as another approach to separate potentially different phosphorylated forms of phosphofructokinase. The soluble and particulate forms of the enzyme appear to have similar chromatographic properites on DEAE-cellulose. However if these forms

differ only slightly in charge, the difference would probably be detectable by the more sensitive technique of isoelectric focusing.

The procedure was carried out as described in Materials and Methods (3.C.10). EDTA (5 mM) was included in the homogenization buffer, in order to extract both forms of phosphofructokinase together in the same fraction (3.C.9). The phosphofructokinase extract was chromatographed on a DEAE-cellulose pad, in the presence of EDTA (3.C.9), in order to remove hemoglobin and other soluble proteins which might interfere with the resolution.

In initial experiments, wide range (pH 3 to 10) ampholytes were used. The phosphofructokinase activity was found in one band, in the pH range of 4.8 to 5.0.

Figure 26 shows the results of isoelectric focusing experiments with narrow range (pH 4 to 6) ampholytes. The activity profiles shown in graphs A, B, and C are from three parallel experiments which contained 0.6, 1.0, and 1.5 ml of the DEAE-cellulose pad eluate, respectively. As explained in the legend for Figure 26, the results shown in graph A, (showing a single peak of activity with an isoelectric point of 5.06), are probably the most accurate ; the results shown in graphs B and C, showing lower isoelectric points, were probably artifacts caused by mixing during the fractionation procedure, resulting from the large amounts of coagulated material in these experiments (see legend for Figure 26).

These results suggest that the soluble and particulate forms of phosphofructokinase do not differ in charge or isoelectric point, and therefore probably do not differ in degree of phosphorylation.

FIGURE 26. ISOELECTRIC FOCUSING OF PIG LIVER PHOSPHOFRUCTOKINASE.

Soluble and particulate phosphofructokinase were extracted together in the same fraction, in the presence of 5 mM EDTA (3.C.9.a), and chromatographed on a DEAE-cellulose pad (3.C.9.b). Isoelectric focusing was done at 23°, in a volume of 6.2 ml, with pH 4-6 ampholytes (3.C.10). The columns were allowed to electrofocus for 10 hr, and then 0.15 ml fractions were collected, as described previously (3.C.1). The activity profiles shown in Figures A, B, and C are from three parallel electrofocusing experiments, containing 0.6, 1, and 1.5 ml of the DEAE-cellulose pad eluate, respectively. The isoelectric point (pI) of the peak activity fractions are indicated by the arrows. In the experiments containing larger volumes of the eluate (Figures B and C), there was a large amount of coagulated material, which may have interfered in the fractionation, and may have caused the shift in the activity peaks to lower pH values. In the experiment shown in Figure A, there was only a small amount of coagulated material, which did not interfere in the fractionation procedure; therefore the results shown in Figure A are probably the most accurate.

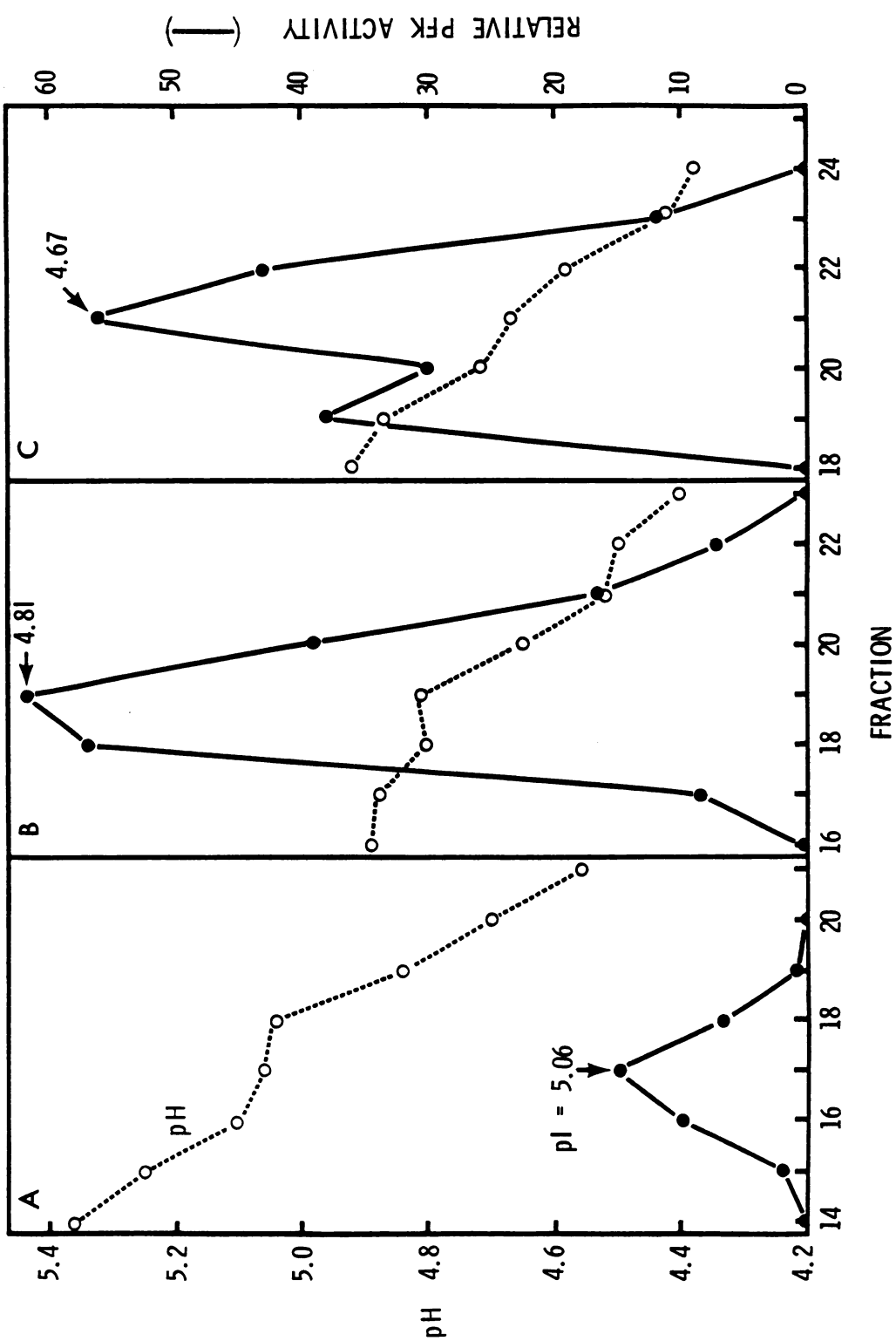


Figure 26

E. DISCUSSION

1. Experimental Effects and Correlation with In Vivo Metabolite Levels.

A new property of pig liver phosphofructokinase has been discovered and characterized. The results show that more than half of the phosphofructokinase in fresh pig liver homogenates is particulate, and that the level of particulate phosphofructokinase is highly sensitive to the levels of certain hexose and nucleotide metabolites. The results are consistent with the formation of particulate phosphofructokinase when the levels of hexose phosphates are low, a condition where the glycolytic rate should be decreased; the results are also consistent with the formation of soluble phosphofructokinase when the hexose phosphate levels are high, condition where the glycolytic rate should be increased. Measurements of metabolite levels and of particulate phosphofructokinase as a function of time show a direct correlation between particulate enzyme and decreased levels of glucose-6-P and fructose-6-P. Flotation experiments provided evidence that the particulate phosphofructokinase is membrane bound, mose likely to the plasma membranes.

One key question is whether the results observed could occur at metabolite concentrations which exist in vivo. Although data are not available on the levels of metabolites in pig liver, some indication of order of magnitude can be obtained from results with rat liver, from fasted and fed rats (Table 4). Of special interest is the fact that

the in vivo levels of the hexose-phosphates are higher in fed animals than in fasted animals. Furthermore, fructose-6-P and glucose-6-P vary the most, showing a two-fold increase in fed liver over that in fasted liver. If similar hexose phosphate levels were to occur in pig liver, the fructose-6-P in fed animals would solubilize approximatley 60% of the particulate phosphofructokinase (estimated from Figure 18), while in fasted animals, approximatley 30% of the enzyme would be solubilized.

The glucose-6-P concentration in fed animals would solubilize all the phosphofructokinase, while that in starved animals would solubilize somewhat more than half of the particulate enzyme. However, glucose-6-P is not an allosteric activator of the enzyme and it may not be a true solubilizer; rather, its apparent effect may be due to fructose-6-P produced from the glucose-6-P.

2. The Membrane to which Phosphofructokinase Binds.

The sucrose gradient studies show that phosphofructokinase is associted with a membrane, and that its presence in the particulate fraction is not a result of extreme self-associtition, or association with another high molecular weight macromolecule which would sediment with the particulate fraction. The simplest explanation of the results is that phosphofructokinase binds to the plasma membranes. This requires an explanation for the presence of phosphofructokinase activity in both the nuclear and microsomal fractions. Previous workers have shown (92, 93) that plasma membrane-bound enzymes from liver (92) and other tissues (93), show a bimodal distribution of activity between the nuclear and microsomal fractions. Presumably the plasma membranes

must form either very large or very small fragments when the liver is homogenized in 0.25 M sucrose; thus the plasma membrane fragments sediment with nuclei and microsomes, but not with intermediate sized fractions such as mitochondria and lysosomes.

Although some mitochondria also sediment with the nuclear fraction, it seems unlikely that liver phosphofructokinase binds to mitochondrial membranes, because we observed no phosphofructokinase activity in the major mitochondrial fraction obtained by differential centrifugation. The flotation experiments showed that the major portion of the phosphofructokinase does not bind to the nuclear membrane, because most (80%) of the phosphofructokinase activity floats, while only about 20% is found in the nuclear pellet.

3. Why the Membrane-Binding Could be Significant.

This study documents the phenomenon of liver phosphofructokinase association with the plasma membrane, and shows a clear correlation of increased binding with decreased hexose-phosphate metabolite levels. Now that the phenomenon has been characterized, further studies can be done to determine what possible significance these properties might have in the regulation of phosphofructokinase in vivo. Binding to the membrane certainly might affect the activity of phosphofructokinase, or it might affect the propeties of the membrane. During conditions when hexose phosphate metabolites are elevated, and phosphofructokinase activity would need to be increased, the enzyme is mostly in the soluble form, where it could diffuse freely in the cytoplasm and be more acccessible to its substrates. When hexose phosphate levels are decreased, and the phosphofructokinase activity would need to be

decreased, the enzyme is mostly in the membrane-bound form, and perhaps less accessible to the substrates. This would be especially pronounced if the binding occured at or near the active site of the enzyme, where it could prevent the binding of substrates by a competitive mechanism.

CHAPTER 4

STUDIES ON PIG HEPATOCYTES

A. ABSTRACT

Pig hepatocytes were isolated in order to study the interaction of liver phosphofructokinase with membranes. Hepatocytes were isolated by two methods: treatment of liver slices with collagenase and dispase (104), and a modified collagenase perfusion technique. The tissue slice incubation procedure gave hepatocytes with a high degree of intactness and viability (75 to 90% by trypan blue exclusion); however the yields of viable cells were low (less than 10^5 cells/g liver). The collagenase perfusion technique gave much greater yields (2-5 x 10^6 cells/g liver), although the percent viabilities were more variable, ranging from 30 to 80%.

The phosphofructokinase activity in the isolated hepatocytes was found to be much lower than that in whole pig liver, and it appears that most of the enzyme activity is lost during the hepatocyte isolation procedure. What activity remained was found in the soluble fraction. These results differ markedly from those observed with whole pig liver, where the total units of homogente phosphofructokinase activity is 5 to 15 times greater, and where 50 to 75% sediments at low (1000 x g) centrifugal fields.

In incubation experiments with isolated pig hepatocytes (37° for 20 min), the total phosphofructokinase activity increased 23% in the presence of 40 m$\underline{M}$ glucose, and decreased 15% in the presence of 20 m$\underline{M}$ lactate. Since phosphofructokinase activity was measured at high

fructose-6-P concentration, the changes in activity must be due to changes in the maximum velocity or in the concentration of active enzyme.

B. INTRODUCTION

In the studies previously described in Chapter 3, we found that pig liver phosphofructokinase in whole liver homogenates exhibited a unique, reversible, metabolite-dependent, membrane binding phenomenon. We observed that variations in the concentration of the substrate fructose-6-P, within physiological levels, could regulate the degree of phosphofructokinase membrane binding. This raised the possibility that the membrane binding phenomenon might occur in vivo. Our next interest was to attempt to answer the questions of whether phosphofructokinase might actually bind reversibly to membranes in vivo, and the possible physiological significance. Isolated hepatocytes seemed to be the best system to study such a possibility.

We first considered studying the phosphofructokinase membrane binding phenomenon in hepatocytes from much more convenient sources, such as rabbit or rat liver. However, in studies on whole liver from these animals (described in Chapter 5), we found their liver phosphofructokinases were quite different, and did not display the membrane binding properties we observed with the pig liver enzyme. It was therefore necessary to use isolated pig hepatocyes to study the phosphofructokinase membrane binding.

Isolating hepatocytes from large animals has several difficulties not encountered with smaller animals. Perfusing the entire liver by the portal vein is usually not practical, and therefore perfusion must

be done with a lobe, or a peripheral portion of a lobe, which is usually not as efficient. Also, the large livers have a much greater amount of collagen, making it more difficult to disperse the cells. In spite of the difficulties however, collagenase perfusion techniques have been used successfully, by Clark et al. (102, 103), for isolating hepatocytes from lamb (102), and adult minature pigs (103). Miyazaki et al. (104) reported the isolation of human hepatocytes from liver biopsies, by enzymatic treatment of liver slices with collagenase and dispase. They found that dispase (a neutral protease isolated from Bacillus polymxa 105), although not effective by itself on liver, appearaed to aid the liver dispersal when used subsequently to the collagenase treatment.

For our studies we used hepatocytes isolated by collagenase and dispase treatment of liver slices, and by collagenase perfusion of a peripheral portion of a liver lobe. We then investigated the effects of glycolytic and gluconeogenic incubation conditions on the level and distribution of the hepatocyte phosphofructokinase.

C. MATERIALS AND METHODS

1. Tissues and Reagents.

Most chemicals were obtained from the sources described in the Materials and Methods section of Chapter 3, and were reagent grade. Collagenase (type CLS II) was obtained from Worthington. Porcine insulin (crystalline, 24 I.U./mg), glucagon (crystalline), and dispase (neutral protease, type IX) were obtained from Sigma Chemical Co. Dispase was assayed according to the procedure described by Kunitz (106), with casein as the substrate, and in Hanks buffer plus 20 mM HEPES, pH 7.4. (We found that the presence of Ca^{2+} and Mg^{2+} ions was essential for optimum activity of the enzyme.) A unit of protease activity (107), is the amount causing an increase in absorbance at 280 nm of 0.001 absorbance units per minute.

Pig liver was obtained from the Michigan State University Meats Laboratory, from animals which were starved 12-15 hr before slaughter. About 40 g of a peripheral portion of a lobe was excised (102) within 20 minutes after sacrifice of the animal, and placed on ice for transport to the laboratory. When the collagenase perfusion technique was used, the lobe was immediately perfused with about 200 ml of Ca^{2+} -free perfusion buffer (108), containng 0.1 mM EGTA, at 0°, according to Forsell and Schull (109).

2. Isolation of Pig Hepatocytes.

a. Enzymatic Treatment of Liver Slices.

The basic procedure described by Miyazaki et al. (104) was carried out, with the following modifications. Liver slices were incubated in 6 g aliquots, in 500 ml Erlenmeyer flasks, with 20 ml of buffer. Krebs-Henseleit buffer (110) was used for the collagenase and dispase treatments for most experiments. In several experiments, Dulbecco's modified Eagles medium (serum-free) was used. The flasks were incubated at 37° on a shaker-water bath, shaking at a rate of about 60 cycles/min. The flasks were gassed at 10 min intervals with 5% CO_2/95% O_2, to maintain the pH at 7.4-7.5. For most experiments, the incubation time was 20 min.

b. Collagenase Perfusion.

This procedure was carried out in Dr. Lee Shull's laboratory at Michigan State University, with the help of Mr. James Forsell, who collaborated with us on this section of the project. The method has been previously used for the isolation of bovine hepatocytes (109). This techniques uses the basic perfusion apparatus, buffers, and general technique described by Seglen (108) for isolation of rat hepatocytes, with modifications for perfusing a peripheral end of a lobe of a pig liver. The general procedure is as follows.

(1) Pig liver was obtained from the Michigan State University meats laboratory, from animals which were fasted 15 hr before slaughter. As soon as the liver was removed from the animal, a peripheral end of a lobe (about 60 g) was excised by a straight transverse cut, using a scalpel with a no. 11 blade. The liver specimen was then immediately

perfused with 250-400 ml of ice-cold Ca^{2+} and Mg^{2+} -free saline (108) plus 0.5 mM EGTA. Perfusion was done with a 50 ml syringe, with a plastic tapered tip, fitted into a major blood vessel. This perfusion was continued until the blood was removed, and the liver had a tan color.

(2) The liver specimen was then transported to the laboratory (in the same buffer, at 0°), and trimmed to a weight of about 40 g, by another transverse cut, parallel to the first cut. Subsequent perfusions were done on an apparatus described by Seglen (108), using plastic canula fitted into major blood vessels. During the perfusions, the canula may be moved to other blood vessels, to ensure that the perfusion buffer reaches all parts of the liver. The liver was first perfused at 23°, with 200 ml of Ca^{2+} and Mg^{2+} -free perfusion buffer (108) (without EGTA).

(3) The liver was then perfused at 37°, with 125 ml of collagenase buffer (108), containing 0.6 mg/ml of crude collagenase (Worthington, CLS II). The buffer was equilibrated with 95% O_2/5% CO_2 gas mixture, using a sintered glass aerator. Perfusion was done in a recirculating mode (108) for 50 to 60 min.

(4) After collagenase perfusion, the liver was dispersed into a cell suspension, using the combing technique described by Seglen (108). (Generally, the more efficient the perfusion, the greater proportion of the liver could be made to go into suspension.) Parenchymal cells were then isolated as follows (108). The crude cell suspension was centrifuged in a clincial centrifuge with a swinging bucket rotor, at 50 x g for 3 min. The pellet was gently resuspended in about 50 ml of Krebs-Henseleit buffer (110) at 0°, and centrifuged as before. The

resuspension and centrifugation steps were repeated at least 2 more times, until the supernatant was clear. Percent viability was determined by trypan blue exclusion (108). The isolated parenchymal cells were then suspended in KHB plus 2% bovine fraction V albumin (Sigma), and kept at 0° until beginning experiments. Most experiments were begun promptly after the hepatocytes were isolated, however the cells could be stored at 0° for at least 24 hr without losing viability.

3. Incubation of Hepatocytes.

Incubation experiments were done with 4 or 5 ml aliquots of hepatocyte suspension, in 25 ml Erlenmeyer flasks. hepatocytes were suspended at a concentration of about 5×10^6, in Krebs-Henseleit buffer (110) containing 1.5 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 1-2.5% bovine fraction V albumin. Flasks were kept at 0° until beginning the incubation at 37°. After adding various substrates and hormones to be tested, the flasks were transferred to a shaker-water bath, and incubated at 37°, shaking at a rate of about 60 cycles/min. The flasks were gassed with 5% CO_2/95% O_2 for 1 min while incubating, and stoppered. The flasks were gassed again after the first 10 min of incubation. For most experiments, the incubation time was 20 min. After incubating at 37°, the flasks were placed on ice, and the homogenization and enzyme assay procedures were started promptly.

4. Determination of Phosphofructokinase and Lactate Dehydrogenase Activities in Hepatocytes.

All procedures were done at 0 to 4°. Hepatocytes suspensions, incubated as described previously, were transferred to 12 ml centrifuge

tubes (Sorvall) and centrifuged in the Sorvall SS-34 rotor at 2500 RPM for 3 min. Cell pellets were then suspended in 0.5 ml of homogenization buffer, containing 50 m$\underline{M}$ imidazole-HCl, pH 7.4, 0.25 $\underline{M}$ sucrose, 1 m$\underline{M}$ $MgCl_2$, and 5 m$\underline{M}$ DTT. The suspension was transferred to a 2 ml Teflon-glass homogenizer and homogenized with about 10 strokes of the pestle. Homogenates were then centrifuged in the SS-34 rotor at 8000 RPM (10,000 x g) for 5 min. Supernatants were transferred to separate tubes, and pellets were resuspended in 0.5 ml of homogenization buffer, using a Teflon pestle. Both supernatant and pellet fractions were assayed for phosphofructokinase and lactate dehydrogenase activity as described in the Materials and Methods section of Chapter 3.

D. RESULTS

1. Yields and Viabilities of Pig Hepatocytes Isolated by Enzymatic Treatment of Liver Slices and By Collagenase Perfusion.

Hepatocytes were initially isolated by incubating liver slices in the presence of collagenase and dispase, according to the basic procedure of Miyazaki et al. (104), as described in Materials and Methods. The optimum concentration of dispase was about 8 mg/ml. Yields of hepatocytes from this method were low, in the range of 10^5 cells/g liver. This was markedly lower than the 2 x 10^6 cell/g yields reported by Miyazaki et al. (104) for human hepatocytes. The percent viabilities were usually good, in the range of 75 to 90% (by trypan blue exclusion). In two preparations, we used Dulbecco's modified Eagles medium as the isolation buffer. In both preparations the viabilities were 90%, however the yields were not improved.

In order to obtain greater amounts of hepatocytes needed for incubation experiments, we next tried the collagenase perfusion technique, as described in Materials and Methods (4.C.2.b). This method gave much greater yields of parenchymal cells, averaging about 2.5 x 10^6 cells/g liver, and a 40 g lobe section yielded about 10^8 cells. The percent viabilities were more variable however, ranging from 30 to 80%. Repeating the washing and centrifugation steps resulted in higher percent viabilities, at the expense of total yield.

The yields were enhanced when the total amount of collagenase used was increased from 125 to 200 mg.

2. Level and Distribution of Phosphofructokinase and Lactate Dehydrogenase in Isolated Pig Hepatocytes versus Whole Liver.

Once we obtained sufficient quantities of liver parenchymal cells, we were next interested in determining the level and subcellular distribution of phosphofructokinase. As a control, lactate dehydrogenase, a soluble enzyme, was also assayed.

Homogenization was done with a Teflon-glass type homogenizer, and in the presence of isotonic (0.25 M) sucrose, as described in the Materials and Methods section of this chapter. Light microscopic examination of homogenate fractions showed there were no unbroken cells. Homogenates were prepared with 2×10^7 cells and 0.5 ml buffer.

In four different hepatocyte preparations, the average homogenate phosphofurctokinase activity was 0.0165 U/ml. This was about 1/15 of the average value of 0.25 U/ml for whole liver homogenates (1 g tissue/2 ml buffer). The lactate dehydrogenase activity was also lower in hepatocyte homogenates, by about 8-fold. These large differences in activity are much greater than the 1.5 to 2-fold differences predicted by the difference in tissue concentrations. This suggests a large amount of the enzyme activity must be lost during the hepatocyte isolation procedure. Previous workers have also observed losses in constitutive enzyme activities in hepatocytes (108, 102, 112, 113). In some cases (108, 102) the losses were attributed to leakage of protein through the plasma membranes. (Presumably the membranes may have been

slightly damaged.) Other workers (108, 113) have found evidence of protein degradation in hepatocytes, and perhaps this may be the cause of the decrease in certain consitutive enzyme activities (4.E.3).

The distribution of the phosphofructokinase activity in hepatocytes also differed markedly from that in whole liver. The lower remaining enzyme activity in the hepatocytes was found to remain in the supernatant when the homogenate was centrifuged at 10,000 x g for 5 min. This is in marked contrast to results with whole liver, where most of the homogenate phosphofructokinase activity sediments at low (1000 x g) centrifugal fields (Chapter 3). We observed previously that the particulate phosphofructokinase from whole liver became gradualllly solubilized when the particulate material was diluted in a large volume of buffer. This suggested that the binding may be in equilibrium and may depend on the phosphofructokinase concentration. The lower phosphofructokinase activity in the isolated hepatocytes may have caused the enzyme to become solubilized, and may be the reason we observed the enzyme in the soluble fraction.

3. Effect of Glycolytic and Gluconeogenic Incubation Conditions on Phosphofructokinase Activity in Pig Hepatocytes.

We were next intersted in determining whether the level and subcellular distribution of the pig hepatocyte phosphofructokinase might be affected when the cells are incubated under conditions which promote either glycolysis or gluconeogenesis. In order to observe maximum effects, we used relatively high concentrations of substrates together with hormones in the incubation buffers. Incubation experiments and enzyme assay procedures were performed as described in Materials and

Methods (4.C.3). Enzymes were assayed under saturating conditions, to give total activity.

The results of a typical incubation experiment are shown in Table 7. When cells were incubated in the presence of 40 m$\underline{M}$ glucose and 175 $\mu\underline{M}$ insulin, the phosphofructokinase activity increased by about 23% above the control (incubated in Krebs-Henseleit buffer with no additions). In the presence of 5 m$\underline{M}$ glucose, the activity still increased, but only by about 15%. In the presence of 20 m$\underline{M}$ lactate and 100 $\mu\underline{M}$ glucagon, phosphofructokinase activity predictably decreased, by 15%. However, the lactate dehydrogenase activities were found to be unaffected by the glucose or lactate treatments. The total phosphofructokinase activities in these experiments was still only about 10% of that in whole liver homogenates, and as we found in the experiments described in the previous section, the activities were found in the soluble fraction.

Since the phosphofructokinase activites were assayed at saturating conditions, the variations in the activity must have been due to either changes in the V_{max} or in the concentration of active enzyme.

Table 7. Effect of Glycolytic and Gluconeogenic Incubation Conditions on Total Phosphofructokinase Activity in Pig Hepatocytes[a].

ADDITIONS TO BUFFER	TOTAL PHOSPHOFRUCTOKINASE ACTIVITY (mUNITS/10^7 CELLS)
CONTROL (NO ADDITIONS)	3.98
5 mM GLUCOSE	4.58
40 mM GLUCOSE + 175 μM INSULIN	4.88
20 mM LACTATE + 100 μM GLUCAGON	3.39

[a]Hepatocytes were isolated and incubation experiments were carried out as described in Materials and Methods. The incubation time was 20 min. For additional explanation, see text (4.D.3).

E. DISCUSSION

1. Possible Reasons for Differences in Phosphofructokinase Solubility in Hepatocytes versus Whole Liver.

The major differences in our results with isolated pig hepatocytes and those with whole liver (described in Chpater 3) were that the phosphofructokinase activity in hepatocye homogenates was 5 to 15 times lower than in whole liver homogenates, and was soluble, rather than sedimenting at low (100 x g) centrifugal fields. (Obviously there is a large decrease in phosphofructokinase activity during the hepatocyte isolation procedure.)

In previous studies with whole liver, the membrane-bound phosphofructokinase appeared to be in equilibrium with the soluble enzyme, and was gradually solubilized when the membrane fraction was washed repeatedly or diluted in large volumes of buffer. Therefore it seems probable that the solubility of the hepatocyte phosphofructokinase was due to dissociation of the enzyme from the membrane, due to the lower phoshofructokinase concentration.

Our original goal in these studies was to investigate the possible physiological significance of the reversible membrane binding phenomenon of phosphofructokinase. However, the results of the membrane binding study in hepatocytes are inconclusive, since most of the phosphofructokinase appeared to be lost during the hepatocyte isolation procedure. Therefore these results do not exclude the possibility that the

reversible membrane binding phenomenon of pig liver phosphofructokinase might ocur *in vivo*.

Previous studies (Chapter 3) suggested that phosphofructokinase might bind to plasma membranes, and previous workers (102) have suggested that perfusion of liver with crude collagenase might alter the hepatocyte plasma membranes. However, the changes (if any) would probably not affect the inner membrane surface, where we would expect phosphofructokinase would most likely bind.

One possible area for further investigation could invovle determining conditions which could minimize the decrease in phosphofructokinase which occurs during the isolation of pig hepatocytes. Several possibilities will be discussed in a subsequent section.

2. Contrast of Results of Incubation Experiments with Results of other Workers.

The total phosphofructokinase in the incubation experiments (Table 7 and Results) varied in a predictable manner. Under conditions promoting glycolysis (high glucose + insulin) the hepatocyte phosphofructokinase activity increased, while under conditions promoting gluconeogenesis (high lactate + glucagon), the activity decreased. These results seem somewhat similar to those of Pilkis *et al*. (98). Van Schaftingen and Hers (114), Castano *et al*. (115), and Kagimoto and Uyeda (116). In their incubation studies (with *rat* hepatocytes), they found the phosphofructokinase activity regulated by changes in the concentration of fructose-2,6-P_2 (97), a potent activator of the enzyme. Fructose-2,6-P_2 lowers the K_m of phosphofructokinase for fructose-6-P (97), and therefore in order to see an activating effect,

the enzyme must be assayed at sub-saturating conditions. However in our studies, phosphofructokinase was assayed at saturating conditons, where effects of furctose-2,6-P_2 would not be observed. Therefore, the changes in phosphofructokinase activity which we observed were most likely due to changes in the concentration of active enzyme, or in the V_{max}.

Binkley and Richardson (117) observed that in a certain strain of mice, fasting for 24 hr resutls in a marked decrease in total liver phosphofructokinase activity. In mice receiving insulin injections, the total phosphofructokinase activity increased. When the liver extracts from the fasted mice were treated with glutathione or other thiols, the phosphofructokinase activity was restored to normal. Thus it was necessary to omit thiols from the homogenization and assay buffers in order to see the effect. In our studies, we included thiols (DTT and mercaptoethanol) in the buffers, so the effects we observed may be different. However it is also possible that we did not allow enough time for activation to occur. Generally, our observations seem similar to those of Binkley and Richardson (117) in that the phosphofructokinase activity appears to be regulated by changes in the concentration of active enzyme, rather than in the affinity for fructose-6-P.

3. Possible Areas of Future Research on Pig Hepatocyte Phosphofructokinase.

a. Possibilities for Improving Isolation Procedure for Pig Hepatocytes.

Using recently developed techniques for isolating hepatocytes from large animals (102, 103, 109, 111) we were able to obtain reasonable

amounts of pig hepatocytes (about 10^8 cells from 40 g of liver). The viability of the preparations were sometimes high (up to 80%, by trypan blue exclusion); however, occasionally preparations had to be abandoned due to low viability. One area for future research could be to determine how to reproducibly obtain highly viable preparations of pig hepatocytes.

Low hepatocyte viability probably results from cell membrane damage during the mechanical dispersement step after the collagenase perfusion. Adult pig liver appears to contain a high proportion of collagen, and therefore it is especially important to maximize the collagenase activity and perfusion efficiency, in order to obtain lots of dispersed hepatocytes.

In an earlier review on rat hepatocyte isolation, Seglen (108, 131) reported several thorough quantitative studies on variables affecting the dispersal of rat liver into intact hepatocytes. Several of the optimizing conditions described by Seglen for isolating rat hepatocytes might also improve the pig hepatocyte isolation procedure. In addition to giving a review on the development of hepatocyte isolation (108), the paper also gives a detailed description of the perfusion apparatus and isolation procedure. Some key points about the procedure which would probably be important in isolating pig hepatocytes are as follows:

(1) Collagenase requires Ca^{2+} ions for activity, and the optimum concentration was found to be 5 m$\underline{M}$. Mg^{2+} should not be included in the collagenase buffer since it competes with Ca^{2+} and is inhibitory.

(2) The optimum pH for collagenase is about 7.5. Because the liver produces acid during perfusion, the choice of an efficient buffer is important. Seglen found that a bicarbonate/CO_2 buffer system supplemented with HEPES (108) gave the best bufering capacity.

(3) The optimum collagenase concentration was found to be 0.5 mg/ml. (Hyaluronidase was ineffective, and actually was slightly inhibitory.) Seglen used about 5 ml of the collagenase buffer per gram of rat liver. This ratio should probably be increased by at least 2-fold for pig liver.

(4) Perfusion was most efficient using a high flow rate (40 to 60 ml/min). However the perfusion pressure should not distend the tissue, since this might damage cell membranes. Perfusion efficiency was also improved by pre-centrifuging and filtering the collagenase solution to remove undissolved particles, and by including a bubble-trap and filter in the perfusion system.

b. Possibilities for Preventing Decrease in Phosphofructokinase Activity in Pig Hepatocytes during Isolation Procedure.

We found that a large decrease in pig liver phosphofructokinase activity appears to occur during the hepatocyte isolation procedure. For future investigations of the phosphofructokinase membrane binding phenomenon, (which appears to be concentration-dependent), it would be useful to be able to obtain freshly isolated pig hepatocyte preparations with a higher level of phosphofructokinase activity, nearer to the levels found in whole liver.

Previous workers have also observed decreases in other constitutive enzyme activities in isolated hepatocytes. In some cases the decreases

appeared to result from leakage of the enzymes through the cell membranes (102, 108). However the protein leakage was probably due to some of the cells having suffered plasma membrane damage from the isolation procedure. Cells with intact plasma membranes, (judged by trypan blue exclusion), would not be expected to leak proteins.

Isolated hepatocytes have been found to be freely permeable to amino acids (108, 118), and show evidence of protein degradation when incubated in substrate-free media (108, 113). The decrease in phosphofructokinase activity we observed may have also been due to protein degradation. Another possibility is that the phosphofructokinase may have gone to an inactive disulfide form (117). However this does not seem as likely since our hepatocyte extracts contained dithiothreitol, which probably would have been able to re-activate the enzyme (117).

Several investigations have been reported where hepatocyte enzyme activities and metabolic processes have been preserved by including metabolites and hormones in the perfusion and incubation media (108). (For example: hepatocyte glucokinase activity can be preserved by insulin (125); gluconeogenesis from lactate is markedly stimulated by lysine (126), and to a lesser extent by other amino acids (108), NH_4^+, and K^+ ions (127, 132); gluconeogenesis from histidine can only occur in the presence of added methionine (128).

In order to preserve the pig hepatocyte phosphofructokinase activity, we would predict that adding glucose (or fructose) to the perfusion buffer would have the biggest effect. Previous workers (123, 129, 130) found that relatively high (50 mM) concentrations of glucose were required in order to promote glycogen synthesis in isolated rat

hepatocytes. Glucose concentrations of 20 mM were ineffective. Fructose, however, was effective at promoting glycogen synthesis at lower concentrations (20 mM) (129). These results suggest that high concentrations of glucose (or fructose) would be required in order to preserve hepatocyte glycolytic enzyme activities. This is probably more important for pig liver, which must be perfused for a longer period of time with the collagenase buffer compared to rat liver.

Alternatively, supplementing the perfusion media with amino acids might prevent loss of constitutive enzymes from protein degradation. It might also be possible to use a complete cell culture media for the perfusion; however care must be taken to omit compounds that could inhibit collagenase activity, such as Mg^{2+} and sulfhydryl compounds. Perhaps adding vitamins, hormones, and various salts might also help preserve constitutive enzyme activities in pig hepatocytes.

CHAPTER 5

STUDIES ON RAT AND RABBIT LIVER PHOSPHOFRUCTOKINASE

A. ABSTRACT

The subcellular distribution of rat and rabbit liver phosphofructokinases was studied, in order to determine whether rat or rabbit hepatocytes would be useful for studying the phosphofructokinase membrane-binding interaction. The results of this characterization, and some additional properties of rabbit liver phosphofructokinase, are presented.

In homogenates of fresh rabbit liver, in 0.25 M sucrose, nearly all of the phosphofructokinase activity sediments at 120,000 x g, with the microsomal fraction. In rat liver however, the phosphofructokinase remains in the soluble fraction. Sucrose gradient velocity sedimentation and flotation studies suggested that rabbit liver phosphofructokinase is not actually bound to microsomal membranes. The enzyme also is not bound to particulate glycogen. Rabbit liver phosphofructokinase is therefore probably a large, highly polymerized protein. The enzyme does not dissociate when incubated in the presence of substrates, products, or related metabolites. The protein-protein interaction is also stable in the presenace of 1% Triton X-100 and 0.2 M KCl. We conclude that the rat and rabbit liver phosphofructokinases do not have the same membrane-binding property as the pig liver enzyme.

B. INTRODUCTION

Our initial goal in this study was to investigate the subcellular distribution of phosphofructokinase in rat and rabbit liver, and eventually study the phosphofructokinase-membrane interaction in hepatocytes from these animals, since hepatocytes can be isolated more easily and with greater viability from the rat and rabbit than from the pig. These studies were done before those described in Chapter 4, and it was found that rat and rabbit liver phosphofructokinases do not have the membrane-binding properties that we observed in pig liver. The studies on pig hepatocytes in Chapter 4 were done subsequently.

Initial differential centrifugation studies showed that rabbit liver phosphofructokinase sediments with the microsomal fraction. Further studies were done to determine whether the particulate enzyme was actually bound to microsomal membranes, whether it could be a large, highly polymerized protein, or whether it was associted with particulate glycogen. Other studies were done to determine the effects of substrates, products, and several other treatments on the particulate rabbit liver phosphofructokinase.

C. MATERIALS AND METHODS

Most of the experimental procedures were the same as those described in Chapter 3. Additional procedures will be described in detail.

1. Tissues and Reagents.

All chemicals were reagent grade. Livers were obtained from rats or while New Zealand rabbits. Animals were starved 12 hr before sacrifice. Rabbits were anesthetized by injection of pentobarbital through the ear vein. Livers were placed on ice after removal from the animal. The gall blader was removed carefully in order to avoid contamination from bile.

2. Preparation of Liver Homogenates and Differential Centrifugation.

Livers were washed and homogenized as described previously (3.C.3). The procedure was started within 10 minutes after removing the liver from the animal. The homogenization buffer was also the same as described previously (50 m$\underline{M}$ imidazole·HCl, pH 7.4, 0.25 $\underline{M}$ sucrose, 5 m$\underline{M}$ dithiothreitol). Four ml buffer per gram of tissue was used for homogenization. The standard buffer, used in all procedures after homogenization, contained the same components as the homogenization buffer, plus 3 m$\underline{M}$ $MgCl_2$, and for most experiments with rabbit liver phosphofructokinase, ATP was also included, at a concentration of 5 m$\underline{M}$.

Differential centrifugation was done as described in Chapter 3 (3.C.4) and the legend for Figure 14.

3. Separation of Microsome and Glycogen Fractions.

The 120,000 x g pellet from the differential centrifugation procedure consisted of two layers; a hard clear pellet at the bottom of the tube (identified as glycogen by previous workers (80)), and a reddish layer of material on top, containing the microsomes. The reddish pellet was removed with a few ml of buffer (without ATP), and the clear glycogen pellets were resuspended in buffer using a Dounce homogenizer. The microsomal fractions were combined and resuspended in a Teflon-glass homogenizer, with additional buffer, and centrifuged again at 120,000 x g as before. The washed microsomal pellet was then resuspended in the standard buffer (with our without ATP, depending on experiment), using 0.25 ml buffer per initial gram of liver.

4. Sucrose Step-Gradient Flotation Procedure.

Aliquots of the washed microsome fraction were mixed with concentrated (2.4 M) sucrose (in standard buffer) to give a final sucrose concentration of 1.6 or 1.9 M. Aliquots (2 to 4 ml) of this suspension were then transferred to 1/2 x 2" cellulose nitrate tubes (Beckman), overlayed with buffer, and centrifuged in the SW 50.1 rotor at 45,000 RPM for 1 to 2 hr at 4°. After centrifugation, the upper liquid layers were removed with a Pasteur pipet; floating material at the heavy sucrose interface was collected with a wide-bore pipet and dispersed in 2 to 3 volumes of buffer. The clear solution below the floating

material was collected separately, and the pellet was resuspended in 1 ml of buffer, in a Teflon-glass homogenizer.

5. Determination of Particulate and Soluble Rabbit Liver PFK Activities in Incubation Mixtures.

One ml aliquots of the washed microsome suspension (without ATP) were placed in 1/2 x 2" cellulose nitrate tubes. Tubes were then incubated for various amounts of time in the presence of various compounds (metabolites, salt, detergent). Tubes were then centrifuged in the SW 50.1 rotor at 45,000 RPM for 20 min, at 4 or 20°. Supernatants were then collected with Pasteur pipets and pellets were resuspended in 1 ml of buffer. Supernatant and pellet fractions were then assayed for soluble and particulate phosphofructokinase activity, respectively, as described previously (3.C.7).

6. Sucrose Density Gradient Centrifugation.

Linear 15 to 40% (w/v) sucrose gradients (in the standard buffer) were formed in 1/2 x 2" cellulose nitrate tubes. Aliquots of washed microsome suspension were diluted with an equal volume of standard buffer with sucrose omitted. (Dilution of the sucrose concentration was necessary to prevent the microsomes from sedimenting while standing at 1 x g.) Aliquots (100, 200, or 300 µl) of the diluted microsome suspension were layered on top of the sucrose gradients and centrifuged in the SW 50.1 rotor at 47,000 RPM (200,000 x g) for 75 minutes, at 4°. About 35 fractions (5 drops each) were then collected by puncturing the tube. Phosphofructokinase activity in the fractions was assayed using the standard procedure, and active fractions were combined. The

presence of microsomes was monitored by measuring the ultraviolet absorbance at 295 nm. Although microsomes absorb more strongly at lower UV wavelengths, the ATP in the buffer also absorbs in the UV. However ATP does not absorb significantly at 295 nm or higher.

D. RESULTS

1. Relative Distribution of Rat and Rabbit Liver PFK in Fractions Obtained by Differential Centrifugation.

Liver from white New Zealand Rabbits was fractionated by differential centrifugation as described in Materials and Methods. The total phosphofructokinase activity in each fraction was determined by multiplying the specific activity by the volume of the fraction. The results of a typical fractionation, shown in Table 8, indicate almost all of the phosphofructokinase activity is in the 120,000 x g (microsomal) pellet. The total recovery of activity was greater than that in the starting homogenate, giving a percent recovery greater than 100%. The 5% activity in the 10,000 x g pellet was eluted with one buffer wash, showing this activity was probably due to some included supernatant. Most of the 23% activity in the 120,000 x g (post-microsomal) supernatant was found to sediment when this fraction was centrifuged again at 120,000 x g for an additional 0.5 to 1 hr. In 4 different experiments with whole rabbit livers (average weight = 74 g), the homogenates contained about 54 units of phosphofructokinase. All or most of this activity sedimented at 120,000 x g. The specific activity of the microsomal particulate enzyme was about 70 mU per mg protein. (Protein was measured according to the Lowry procedure (29), using bovine serum albumin as a standard.)

In a differential centrifugation experiment with fresh rat liver, virtually all of the phosphofructokinase activity was found in the soluble fraction (120,000 x g supernatant).

In another experiment, the differential centrifugation procedure was done with frozen rabbit liver. Although some of the phosphofructokinase activity (22%) was found in the microsomal fraction, most of the

TABLE 8. RELATAIVE DISTRIBUTION OF RABBIT LIVER PHOSPHOFRUCTOKINASE IN FRACTIONS OBTAINED BY DIFFERENTIAL CENTRIFUGATION.

FRACTION	PERCENT OF INITIAL HOMOGENATE PFK ACTIVITY[a]	
	FRESH LIVER	FROZEN
10,000 x g PELLET	5[b]	0
120,000 x g PELLET	102[c]	22
120,000 x g SUPERNATANT	23[d]	62

[a]The initial homogenate activity was normalized to 100%.

[b]Most of this activity was removed in the first wash.

[c]This fraction apparently contained more activity than the starting homogenate, hence the recovery is greater than 100%.

[d]At least half of this activity was found to sediment if the fraction was re-centrifuged at 120,000 x g for an additional 0.5 to 1 hr.

activity was in the post-microsomal supernatant. Also the percent recovery in frozen tissue was lower (84%) suggesting perhaps some inactivation had occured. These results show the importance of using fresh tissue when studying the subcelluar distribution of the enzyme.

2. Location of Rabbit Liver PFK Activity in Subfractions of the 120,000 x g Pellet.

The 120,000 x g pellet obtained from differential centrifugation contained two separate layers, a reddish material on top, and a hard transparent pellet underneath it. Previous workers have found that the transparent material is made up of glycogen and the reddish material contains the microsomes (80). When the two layers were collected separately and assayed, as described in Materials and Methods (5.C.3), all of the phosphofructokinase activity was found in the top layer, while the clear pellet contained no significant activity. This shows that rabbit liver phosphofructokinase is associated with the microsomal fraction, and is not bound to glycogen.

3. Sucrose Step-Gradient Centrifugation of Rabbit Liver Particulate Phosphofructokinase.

Aliquots of washed rabbit liver microsome fraction were made 1.6 or 1.9 M in sucrose, overlayed with buffer, and centrifuged, as described in Materials and Methods (5.C.4). In these experiments, most of the microsomal material floated, and formed a reddish turbid band at the interface between the buffer and the heavy sucrose. The liquid below the turbid band still had a slight turbidity, which remained in solution even when centrifuged for 2 hr. The turbid band and the liquid below it were collected separately and assayed as described previously. All of the phosphofructokinase activity was found in the clarified liquid, while the turbid band contained only a trace of activity, probably carried over from the liquid phase below it. This same result was obtained with both 1.6 and 1.9 M sucrose in the initial suspension, and

in the presence and absence of 5 mM ATP. This shows that rabbit liver particulate PFK must have a density equal or greater than that of 1.9 M sucrose (density = 1.246 g/cm^3). These results differ from those with the pig liver particulate PFK, which floated in a sucrose concentration of 1.46 M (density = 1.190 g/cm^3).

4. Stabilization of Rabbit Liver Particulate PFK Activity by ATP.

We observed that the phosphofructokinase activity in the rabbit liver microsomal fraction gradually decreases during storage at 0 to 4° (Figure 27). In a preliminary experiment we found that adding ATP to the buffer prevented inactivation of the enzyme. Figure 27 shows the effect of storing the enzyme in the presence and absence of 5 mM ATP (at 0-4°) on the stability of the microsomal phosphofructokinase. In the absence of ATP, the phosphofructokinase gradually decreased over several days, and little or no activity remained after 8 days. In the presence of 5 mM ATP, the activity still decreased, but at a much slower rate; after 8 days the activity stabilized at 42% of the initial activity. This 42% activity remained stable for 4 days, and then gradually decreased.

In another experiment (Figure 27), an aliquot of washed microsomes was incubated without ATP for 4 days, at which time the activity had decreased to 22%. ATP was then added, to a concentration of 5 mM. As shown in Figure 27, some reactivation had occured, bringing the activity to 34%. This activity was stable for several days.

Previous workers (119) have found that the purified rabbit liver phosphofructokinase undergoes a transition from an active to a less active form when incubated for 2 min (at 25°) in the absence of ATP.

FIGURE 27. STABILIZATION OF RABBIT LIVER PARTICULATE PHOSPHOFRUCTOKINASE ACTIVITY BY ATP.

Rabbit liver microsomes were prepared as described in Materials and methods (5.C.2, 3). Incubations were done at 4°. The microsome suspension, in the standard buffer with no ATP, was divided into 2 aliquots. ATP (0.1 M stock solution) was then added to one of the aliquots to give a final concentration of 5 mM. Phosphofructokinase activity was determined at the indicated time intervals (3.C.6). After 4 days incubation at 4°, the control aliquot (without ATP) was divided in half, and ATP was added to one of the halves, to a concentration of 5 mM.

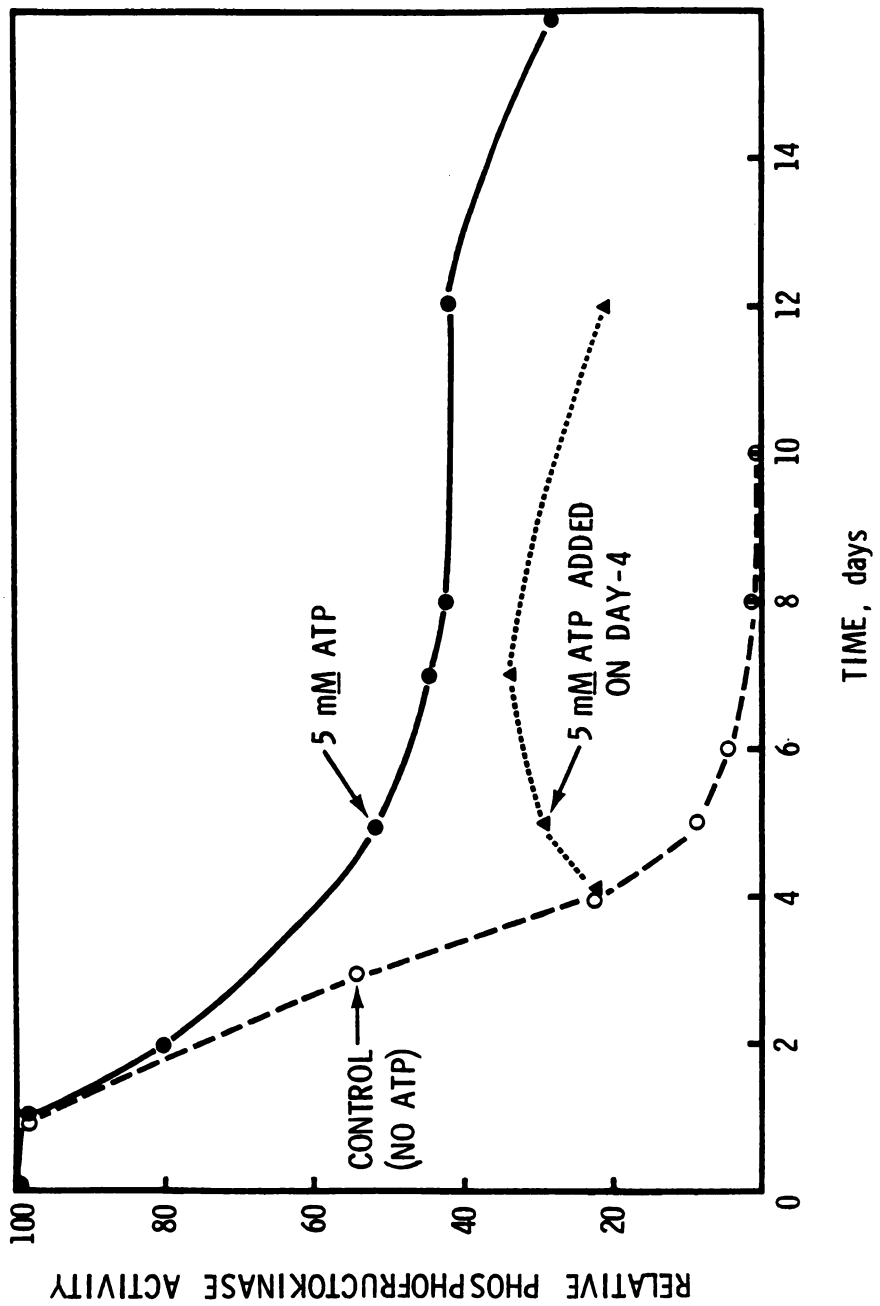

RELATIVE PHOSPHOFRUCTOKINASE ACTIVITY
100
80
60
40
20
0
2
4
6
8
10
12
14
TIME, days
5 mM ATP
5 mM ATP ADDED ON DAY-4
CONTROL (NO ATP)

Figure 27

Adding ATP to the incubation mixture resulted in reactivation of the enzyme, showing the process is reversible. The degree of association of both forms of the enzyme appeared to be unchanged, and thus the effect was attributed to a reversible conformational change of the enzyme.

Our results show that ATP also prevents a complete inactivation of the enzyme during long term storage. The slight activation we observed when 5 mM ATP was added after a 4 day incubation (at 0-4°) may have been due to conversion of the remaining less active form of the enzyme to the active form.

5. Effect of Metabolites, Detergent, and KCl on Particulate Rabbit Liver Phosphofructokinase.

The next question we asked was whether the particulate rabbit liver phosphofructokinase could be solubilized by treatments which solubilize the particulate pig liver enzyme. Solubilization experiments were done with 1 ml aliquots of the washed microsome fraction, as described in Materials and Methods (5.C.5). ATP was not included in the standard buffer. The treatments tested for solubilization activity were: 6 mM concentrations of F-6-P, F-1,6-P_2, G-6-P, AMP, ADP, and ITP, 9 mM ATP, 1% Triton X-100, and 0.2 M KCl. The control contained no added compounds. After incubating at 0 or 23° for time periods of 5, 30, or 60 minutes, the suspensions were centrifuged at 190,000 x g for 15 min. In all tests, including the control, all of the phosphofructokinase activity was found in the pellet fractions, and therefore no solubilization had occured from any of the treatments. Metabolite determinations (3.C.8) on the supernatant fractions showed that the added

metabolites were not depleted during the incubations. In the suspension treated with Triton X-100, some solubilization of microsomal material had occured, shown by an increase in turbidity of the supernatant and a decrease in pellet size. However all of the phosphofructokinase activity was still found in the pellet. These results differ markedly from the results with the pig liver phosphofructokianse (3.D.1, 2, 3), where we found the enzyme was rapidly solubilized by many of these treatments (Table 4.)

6. Sucrose Gradient Sedimentation Velocity of Rabbit Liver particulate Phosphofructokinase.

A major question we wanted to answer was whether the phosphofructokinase in the microsomal fraction was bound to the microsomal membranes, or whether the enzyme was polymerized to form a high molecular weight particle. The sucrose step-gradient flotation experiments showed that phosphofructokinase did not float with most of the microsomal fraction in heavy suscrose, suggesting that the enzyme was not bound to microsomes.

Another approach we next used was sucrose density gradient sedimentation velocity. Aliquots of the washed microsome fraction were applied on 10 to 40% sucrose gradients and centrifuged as described in Materials and Methods (5.D.3). The results of a typical experiment are shown in Figure 28; after centrifugation a single diffusely turbid band was observed, about 3/4 of the way into the gradient. This band corresponded to the 295 nm absorbance peak (Figure 28), and was attributed to the major microsomal fraction, since it was the only turbid band observed. However the phosphofructokinase activity was found in a peak

FIGURE 28. SEPARATION OF RABBIT LIVER PARTICULATE PHOSPHOFRUCTOKINASE FROM MICROSOMES, BY SUCROSE GRADIENT VELOCITY SEDIMENTATION.

Rabbit liver microsomes were prepared as described previously (5.C.3). Sucrose gradients were run, fractionated, and UV absorbance and phosphofructokinase activity were determined as described in Materials and Methods (5.C.6). The experiment was run at 23°, with a 100 µl aliquot of the diluted microsomes.

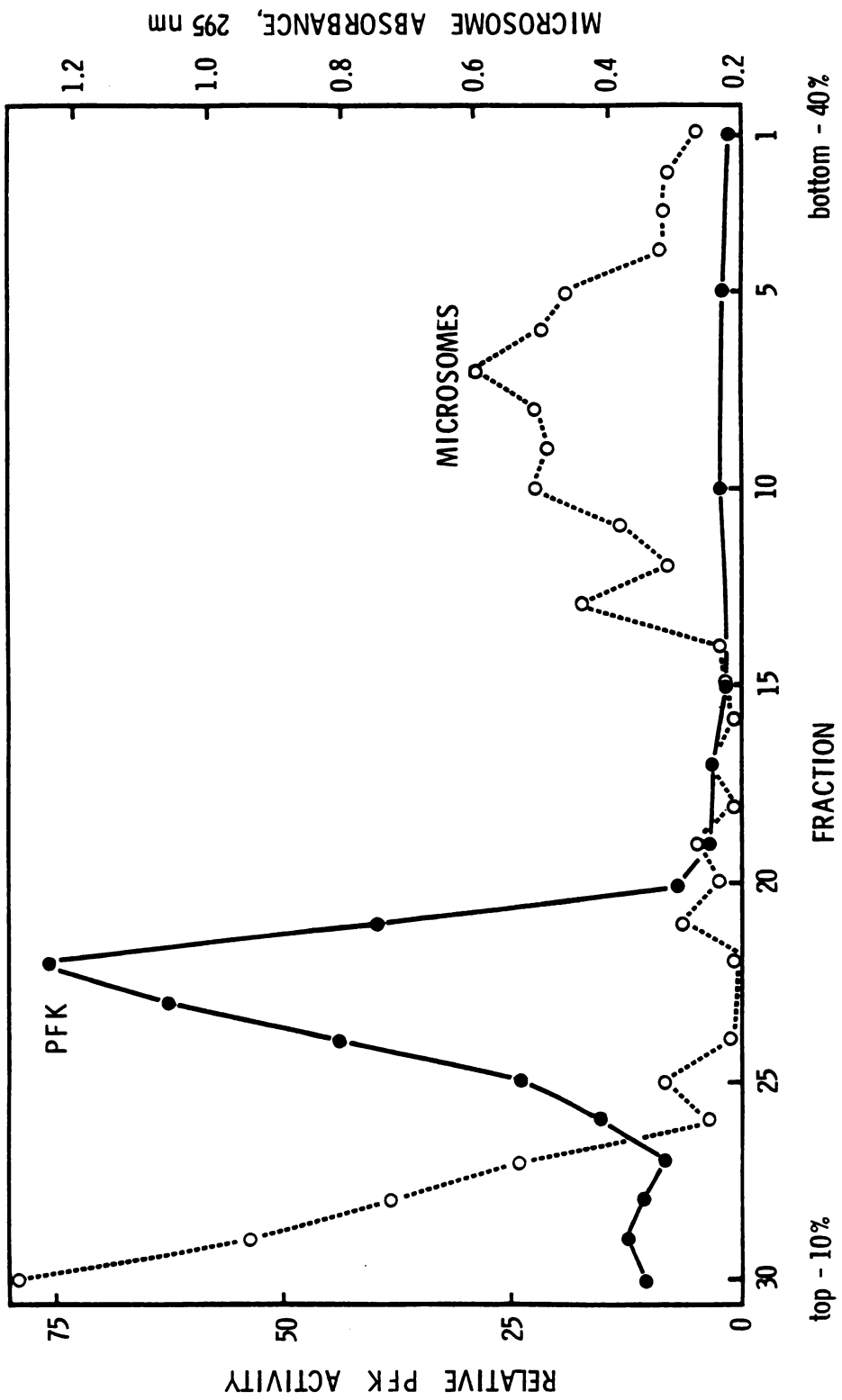

RELATIVE PFK ACTIVITY
MICROSOME ABSORBANCE, 295 nm
PFK
MICROSOMES
FRACTION
top - 10%
bottom - 40%
0
25
50
75
0.2
0.4
0.6
0.8
1.0
1.2
1
5
10
15
20
25
30

Figure 28

in the top 1/4 of the gradient. These results, together with those from the flotation experiments, suggest that particulate rabbit liver phosphofructokinase is not bound to microsomal membranes, and is therefore probably a large, highly polymerized protein, which co-sediments with the microsomes during centrifugation in the standard buffer. The sedimentation rates of microsomes and polymerized phosphofructokinase must be comparable in aqueous solution. However on a sucrose gradient they can be separated, because of the greater resolution.

E. DISCUSSION

1. Comparison of the Subcellular Distribution and of Conditions which Solubilize Particulate Rabbit and Pig Liver Phosphofructokinases.

Rabbit liver phosphofructokinase pellets at a lower centrifugal field (120,000 x g) than most globular proteins; however it does not appear to have the membrane-bindng proerties which we observed with pig liver phosphofructokinase (Chapter 3). The fast sedimenting property of the rabbit liver enzyme is most likely due to self-association. The degree of association is surprisingly unaffected by incubating the enzyme in the presence of substrates, products, and related metabolites, since the sedimentation of the enzyme was unaffected by these treatments. The enzyme also resists dissociation in the presence of 1% Triton X-100 and 0.2 M KCl. Both these treatments solubilize the membrane-bound pig liver phosphofructokinase. Apparently the protein-protein bonds in particulate rabbit liver phosphofructokinase are more resistant to dissociation than the protein-membrane bonds of particulate pig liver phosphofructokinase.

2. Marked Differences in Several Properties of Liver Phosphofructokinases from Rat, Rabbit, and Pig.

Although mammalian liver phosphofructokinases have similar catalytic properties, they differ markedly in their subcellular distribution,

and also several other properties. Several properties showing marked differences are summarized in Table 9.

Our initial interest in studying the rat and rabbit liver phosphofructokinases was in the possibility of using rat or rabbit liver as a system for investigating the phosphofructokinase membrane-binding phenomenon. However we found that the rat, rabbit and pig show marked differences in the subcellular distribution of liver phosphofructokinase. Pig liver phosphofructokinase was found predominantly in the nuclear fraction, and appeared to be bound to the plasma membranes (Chapter 3). However the rat liver enzyme was found in the soluble fraction. We found rabbit liver phosphofructokinase in the microsomal fraction. However it was not bound to microsomes (or glycogen) and appeared to be highly polymerized.

Mammalian liver phosphofructokinases also differ in sensitivity to oxidation. Pig liver phosphofructokinase is unique in that it is highly sensitive to oxidation, and must be kept in the presence of high (100 m$\underline{M}$) concentrations of reducing agents (mercaptoethanol or dithiothreitol) to retain activity (37, 38). In contrast, the rat and rabbit liver enzymes are resistant to oxidation, and can be purified and stored in the absence of added reducing agents without losing activity (119, 135).

Most mammalian phosphofructokinases consist of a single type of subunit, of molecular weight 75 to 85 thousand, which associates to form tetramers (124). The tetramers themselves can associate to varying degrees, depending on the species. In sedimentation studies, previous workers have found rabbit liver phosphofructokinase has a molecular weight of 365 to 450 thousand (119). Previous studies in our

TABLE 9. MAJOR DIFFERENCES IN PROPERTIES OF LIVER PHOSPHOFRUCTOKINASES FROM RAT, RABBIT, AND PIG.

PROPERTY OF LIVER PHOSPHOFRUCTOKINASE	RAT	RABBIT	PIG
MOLECULAR WEIGHT (from sedimentation studies)		365,000 - 450,000[a]	5,000,000 to 10,000,000[b]
REDUCING AGENT REQUIRED TO MAINTAIN ACTIVITY	none required[c]	none required[a]	100 mM 2-mercaptoethanol or dithiothreitol[d,e]
ATP REQUIRED TO MAINTAIN ACTIVITY	none required[c]	5 - 10 mM[a,f]	0.1 mM[d,e]
METABOLITES REQUIRED FOR SOLUBILITY OF PURIFIED ENZYME	none required[c]	none required[a]	ATP, fructose-6-P fructose-1,6-P_2[d,e]
RELATIVE CENTRIFUGAL FIELD WHERE MOST OF ACTIVITY SEDIMENTS (from differential centrifugation studies)	soluble[f]	120,000 x g[f] (sediments with microsomes, but not bound to microsomes or glycogen)	500 - 900 x g[g] (appears to bind to plasma membranes)

[a]Ramaiah, A., and Tejwani, G.P. (1973) Eur. J. Biochem. 39:183-92.

[b]Trujillo, J.L., and Deal, W.C., Jr. (1977) Biochemistry 16:3098-104.

[c]Dunaway, G.A., and Weber, G. (1974) Arch. Biochem. Biophys. 162:620-8.

[d]Massey, T.H., and Deal, W.C., Jr. (1973) J. Biol. Chem. 248:56.

[e]Massey, T.H., and Deal, W.C., Jr. (1975) Methods Enzymol. 42:99.

[f]This work (5.D.1-4)

[g]This work (3.D.10, 11)

laboratory (120) have shown the pig liver enzyme has a much higher molecular weight, of 5 to 10 million. In electron microscopic studies, other workers (124) have found the pig liver phosphofructokinase can form large linear chains, in the presence of 10 mM fructose-6-P or ATP.

Rabbit liver phosphofructokinase is unique in that it requires high concentrations of ATP (5 to 10 mM) in order to retain its active conformation (119, 134). The rat and pig liver enzymes do not require high concentrations of ATP for stability (37, 38, 133). Purified pig liver phosphofructokinase is solubilized and stabilized by 0.1 mM concentrations of ATP (37, 38).

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