

AN ABSTRACT OF THE THESIS OF

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Title: THE EFFECT OF HEXACHLOROPHENE AND RELATED COMPOUNDS ON  
TORULA YEAST GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND SOME  
OTHER DEHYDROGENASE ENZYMES

Abstract approved: *Redacted for Privacy*  
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Low concentrations of hexachlorophene (HCP) inhibit a number of pyridine nucleotide-linked dehydrogenase enzymes. The  $I_{50}$  HCP concentrations were 105  $\mu\text{M}$  for pig heart isocitrate dehydrogenase (ICD), 65  $\mu\text{M}$  for horse liver alcohol dehydrogenase, 39  $\mu\text{M}$  for torula yeast glucose-6-phosphate dehydrogenase (G6PD), 6.0  $\mu\text{M}$  for beef heart malate dehydrogenase, and 1.6  $\mu\text{M}$  for bovine liver glutamate dehydrogenase (GDH) at the enzyme concentrations tested. HCP exhibited cooperative inhibition of these enzymes since the observed maximum interaction coefficient,  $n'$ , between HCP binding sites ranged between 1.62 and 3.33 but it was not an allosteric effector as evidenced by Hill coefficients for the substrates of approximately 1.0 both in the absence and the presence of HCP. More detailed kinetic analysis showed that HCP in most cases exhibited mixed kinetics, giving average  $K_i$

values with G6PD of 16.6  $\mu\text{M}$  for  $\text{NADP}^+$  and 18.2  $\mu\text{M}$  for glucose-6-phosphate; with ICD of 171  $\mu\text{M}$  for  $\text{NADP}^+$  and 4.0  $\mu\text{M}$  for isocitrate; and with GDH of 0.7  $\mu\text{M}$  for NADH, 1.4  $\mu\text{M}$  for  $\alpha$ -ketoglutarate and 0.5  $\mu\text{M}$  for ammonium acetate, under the conditions studied. The kinetics of inhibition of torula yeast G6PD by other bisphenols, 3,4-TCP and DCP, were similar to those found with HCP. Kinetic analysis suggested that inhibition of G6PD, ICD, and GDH by HCP probably involved binding of the bisphenol to at least two inhibitory sites.

The effect of HCP on torula yeast G6PD was studied further by the methods of density gradient ultracentrifugation, equilibrium dialysis, acrylamide electrophoresis, and difference spectroscopy. The results from these studies suggest that the mechanism for inhibition of dehydrogenases by HCP and related compounds is quite complex, probably involving, at least in the case of torula yeast G6PD: the direct binding of HCP to the enzyme, resulting in inhibition and conformational changes in the enzyme and dissociation of the active dimeric form of the enzyme to its inactive subunits. In some cases the binding of HCP to G6PD not only caused dissociation, but also resulted in the formation of aggregates of higher molecular weight as observed by density gradient ultracentrifugation and electrophoresis. Maximum binding of 52 moles of HCP to each mole of G6PD was found but saturation was not reached even at the highest HCP concentrations that could be tested. While probably not an important mechanism for inhibition, some evidence was obtained suggesting that HCP formed complexes with the G6PD substrates,  $\text{NADP}^+$  and G6P.

The Effect of Hexachlorophene and Related Compounds  
on Torula Yeast Glucose-6-Phosphate Dehydrogenase  
and Some Other Dehydrogenase Enzymes

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DEDICATION

to

my parents:

Mr. Maw-Hsuan Wang and Mrs. Ya-Ru Kim Wang

and to

my major professor:

Dr. Donald Raymond Buhler

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## ABBREVIATIONS AND SYMBOLS

Ac-CoA	acetyl coenzyme A
ADH	alcohol dehydrogenase
AMP	adenosine monophosphate
APS	ammonium persulfate
ATP	adenosine triphosphate
BSA	bovine serum albumin
$^{14}\text{C}$ -HCP	methylene- $^{14}\text{C}$ -hexachlorophene
Cit.	citrate
cpm	counts per minute
DCP	2,2'-methylenebis (4-chlorophenol)
DTE	dithioerythritol
DTT	dithiothreitol
E	enzyme
EDTA	ethylenediaminetetraacetic acid
Fum.	fumarate
GDH	glutamate dehydrogenase
G6P	glucose-6-phosphate
G6PD	glucose-6-phosphate dehydrogenase
Hb	hemoglobin
HCP	hexachlorophene; 2,2'-methylenebis (3,4,6-trichlorophenol)
i	inhibited fraction of enzyme activity ( $i = \frac{v - v_i}{v_i}$ )
I	inhibitor
$I_t$	total inhibitor

ICD	isocitrate dehydrogenase
ip	intraperitoneal
K	the apparent product of n dissociation constants
$K_i$	enzyme-inhibitor dissociation constant
$K_m$	Michaelis-Menten constant
LD50	dose that causes 50 percent lethality
LDH	lactate dehydrogenase
M	molar concentration
Mal.	malate
MCE	mercaptoethanol
mCi	millicurie
NDH	malate dehydrogenase
mM	millimolar concentration
mmole	millimole (1 mmole = $10^{-3}$ mole)
MW	molecular weight
N	total no. of binding sites, or normal concentration depends on where it is used
n	Hill coefficient or interaction coefficient between substrates
n'	Hill coefficient or interaction coefficient between inhibitors
$NAD^+$	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
nmole(s)	nanomole(s) (1 nmole = $10^{-9}$ mole)

No.	number
O.D.	optical density
OAA	oxaloacetate
P	product
6-PGA	6-phosphogluconate
P <sub>i</sub>	inorganic phosphate or phosphate buffer depends on where it is used
pmole(s)	picomole(s) (1 pmole = 10 <sup>-12</sup> mole)
PPP	pentose phosphate pathway
PVP	polyvinylpyrrolidone
rpm	revolution per minute
S	substrate of Svedberg unit (1 S = 1 x 10 <sup>-13</sup> sec), depending on where used
S.D.	standard deviation
SDS	sodium dodecylsulfate
SH	sulfhydryl
Suc.	succinate
TCA	tricarboxylic acid cycle
3,4-TCP	2,2'-methylenebis (3,4-dichlorophenol)
TEA	triethanolamine
TMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane
v	enzyme reaction rate (general term including v <sub>i</sub> and v <sub>0</sub> ) used in Lineweaver-Burk plots, Δ O.D. or Δ A per minute
$\bar{v}$	specific volume (ml/g)
v <sub>i</sub>	reaction rate of the inhibited reaction

$V_m$	maximum velocity
$v_o$	the rate of enzyme reaction without inhibitor
v/v	volume by volume
w/v	weight by volume
m $\mu$	millimicron (1 m $\mu$ = 10 <sup>0</sup> Å = 10 <sup>-7</sup> cm)
$\mu$ M	micromolar concentration
$\mu$ mole(s)	micromole(s) (1 $\mu$ mole = 10 <sup>-6</sup> mole)
$\bar{v}$	no. of moles of ligand bound per mole of protein
$\lambda$	wavelength
$\Delta A$	absorbance difference ( $\Delta$ O.D.)
[ ]	concentration

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INTRODUCTION

Hexachlorophene

Relationship of chemical structure of the chlorinated phenols and  
bisphenols and their antibacterial activities

Phenolic compounds inhibit the growth (bacteriostatic) or kill (bactericidal) bacteria. In many cases, substituted phenols have been shown to possess greater antibacterial properties than phenol itself. The antibacterial characteristics of bisphenols were first reported in 1906 (Bechhold and Ehrlich, 1906). These workers also found that the effectiveness of phenols increases with the number of halogens present in the molecule. The bisphenols followed this same pattern with regard to gram-positive organisms (Gump and Walter, 1968).

Hexachlorophene [HCP; 2,2'-methylenebis-(3,4,6-trichlorophenol); G-11] (Figure 1) was first synthesized by Gump in 1941 from 2,4,5-trichlorophenol and formaldehyde (Gump and Walter, 1968). HCP was found to be one of the most effective bactericides among the bisphenols tested.

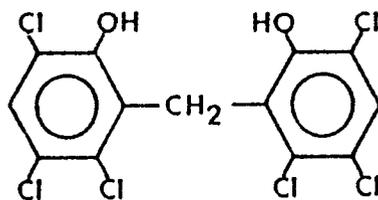


Figure 1. Hexachlorophene, HCP, G-11  
2,2'-methylenebis-(3,4,6-trichlorophenol)

Besides the requirement for halogen, another factor is apparently of importance for the antibacterial activity of bisphenols. Bisphenols must also be linked at the position ortho (2,2'-) to the hydroxyl groups for maximum activity. While the type of the linkage is of minor significance, it should be small enough (carbon to carbon, methylene, sulfur, oxygen, etc.) so that the hydroxyls are not separated too far. A chelated structure or hydrogen bondage between the two hydroxyl groups may be responsible for the high activity of the halogenated 2,2'-bisphenols (Gump and Walter, 1968) and perhaps explain the great difference in the ionization constants of the hydroxyls. The negative logarithms of the dissociation constants (pK values) for the first and second hydroxyls of HCP as determined by Mahler (1954) are  $pK_1$  5.4 and  $pK_2$  10.9.

### Uses

For about 25 years until quite recently, HCP has been an important antibacterial ingredient in a large number of soaps, detergents, emollients, salves, lotions, aerosols, powders, and deodorants. Wide use of HCP in such cosmetic and personal care products resulted from the considerable antibacterial activity of HCP, especially against gram positive bacteria, and the strong binding of the bisphenol to the skin for prolonging action.

In surgical and hospital use, it was incorporated into liquid and solid soaps and used in combination with synthetic detergents. When HCP was used in an aqueous alcoholic solution (in combination with

formaldehyde for surgical instruments), it made possible the cold sterilization of surgical and dental instruments. Recent concern about its toxicity (Kimbrough, 1971, 1973a, 1973b; and Lockhart, 1973), however, has greatly curtailed use of this compound.

In industry, HCP was used in liquid and bar soaps to reduce the possibility of infections, and also used to preserve cutting oils. In veterinary medicine, HCP-containing products were employed in the treatment of mange, ringworm, and liver flukes, and for animal shampoos. HCP has also served in agriculture as a fungicide and pesticide (Johnson, 1971). Comprehensive reviews of the chemical, physical, and bacteriological properties of HCP and its applications are available (Gump and Walter, 1968; Sindar Corporation, 1970; and Armour-Dial, Inc., 1971).

#### Toxicological properties

HCP may enter the body via percutaneous absorption, inhalation, or through absorption via the gastrointestinal tract.

HCP has been reported to be toxic to human and other animals. Poisoning in human was mainly accidental; e.g., patients mistook pHiso-Hex (Winthrop Laboratories, New York), which contains 3% HCP, for milk of magnesia. Anorexia, nausea, vomiting, abdominal cramps, and diarrhea were the major symptoms (see for example, Wear et al., 1962). In some cases (Wear et al., 1962; and Lustig, 1963), death resulted. Recently, however, HCP poisoning in burn patients and suspected deaths among newborn infants has apparently resulted from the percutaneous absorption of HCP from antibacterial lotions or salves (Lockhart, 1973). The

danger of exposure to excessive amounts of HCP has been well documented following the recent deaths of 23 French babies who had been exposed to talcum powder that contained 6% HCP (Lockhart, 1973).

The efficacy of HCP has been investigated in various parts of the world (Gump and Walter, 1968). Data on the toxicity of HCP has been reviewed by Gump (1969), Kimbrough (1971, 1973a, 1973b), and Lockhart (1973). In general, the toxic effects of HCP vary according to the body conditions of the animals, the route of exposure, and the species (Table 1).

#### Mechanisms of toxicity

Up to the present time, the primary basis for toxicity of HCP is not clear. Toxicity could occur via some unknown mechanism(s), or it could result from one or more of the following effects.

1. HCP chelates essential metals. Metal ions play a prominent role in biochemical phenomena, resulting, in part, from their association with both large and small molecules (Gurd and Wilcox, 1956). Reduced levels of essential metals could thus result in disturbances of metabolism. Adams (1958) and Adams and Hobbs (1958) suggested that chelation of HCP with  $\text{Fe}^{+2}$ ,  $\text{Fe}^{+3}$ , and  $\text{Cu}^{+2}$  may be responsible for the antibacterial activity of HCP.

2. HCP binds to proteins and enzymes. Proteins and enzymes are essential to life. HCP binds to proteins or enzymes which could alter their physiological properties and hence cause disorders in all related systems. HCP has been shown to strongly bind to bacterial (Gould et

TABLE 1  
SOME RECENTLY PUBLISHED TOXICITY DATA ON HCP IN ANIMALS

Species	Dose (mg/kg body weight)	Method of treatment	Effect	Reference
cat	20	oral	LD50, survivors showed neurological damage	Hanig <u>et al.</u> , 1973
cattle	30-60	oral	LD50	Udall and Malone, 1970
dog	40-50	oral	lethal	Gump, 1969
rat (Sherman strain adult)	56-66	oral	LD50	Gaines and Kimbrough, 1971
rat (Sherman strain weanling)	120	HCP in peanut oil by stomach tube	LD50	Gaines <u>et al.</u> , 1973
rat (Wistar adults)	22	ip	LD50, signs of intoxication were: general lethargy, posterior paralysis, increased respiration rate, hyperthemia and diarrhea	Nakaue <u>et al.</u> , 1973
rat (Wistar weanling)	40	ip	LD50	Nakaue <u>et al.</u> , 1973

al., 1953; and Joswick et al., 1971), mitochondrial (Caldwell et al., 1972), microsomal (Gandolfi et al., 1974), and erythrocyte and plasma (Flores and Buhler, 1972; and Miller and Buhler, 1974) proteins. Enzymes, including lactate, formate, glucose and butanol dehydrogenase and cytochrome oxidase systems of Bacillus subtilis and Escherichia coli were inhibited by HCP (Gould et al., 1953). Mitochondrial cytochrome oxidase (Caldwell et al., 1972) and liver microsomal mixed-function oxidases and cytochromes P-450 and  $b_5$  (Gandolfi et al., 1974) were also inhibited by HCP. Binding of HCP to proteins is thought to involve hydrogen bonding between the phenolic protons of HCP and the oxygen atom in the peptide linkage of proteins (Haque and Buhler, 1972) or hydrophobic interactions between the bisphenol and the protein (Miller and Buhler, manuscript in preparation).

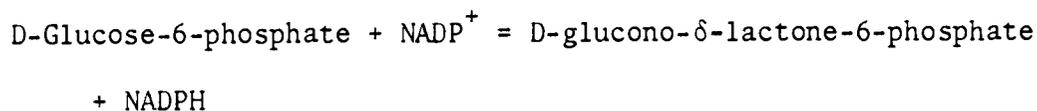
3. HCP effects membrane surfaces. Norman (1960) found that HCP is rapidly bound on the surfaces of plant tissues leading to irreversible injury through leakage of cell solutes. The mode of action of HCP at low concentrations is postulated to be detergent-like, involving adsorption at the cell surface, disruption of membrane permeability causing leakage of cell contents with progressive loss of function, and eventually leading to death of the organisms (Corner et al., 1971; Joswick et al., 1971; and Silvermale et al., 1971). HCP also causes red cell hemolysis (Corner, 1974; Flores and Buhler, 1974; and Miller and Buhler, 1974) and damage to the red cell membrane resulting in ion leakage (Miller and Buhler, 1974). In addition, HCP increases the permeability of mitochondrial and lipid bilayer membranes (Ramberg and Buhler, manuscript in preparation).

4. HCP effects the nervous systems. HCP elicits destruction of preformed myelin, resulting in demyelination of the central nervous system (Lampert et al., 1973; Towfighi et al., 1973; de Jesus, Jr., and Pleasure, 1973; Pleasure et al., 1973; and Martinez et al., 1974).

5. HCP effects the respiration system. HCP is an uncoupler of oxidative phosphorylation (Caldwell et al., 1972; Cammer and Moore, 1972; Nakaue et al., 1972; and Rose et al., 1975). HCP also inhibits both endogenous and exogenous respiration (oxygen uptake) in Bacillus megaterium and inhibits the respiration of isolated B. megaterium membranes and can act on several components of the electron transport chain in the membrane (Frederick et al., 1974). These latter workers believe that the primary lethal action of HCP was respiratory inhibition at sites within the membrane-bound part of the electron transport chain.

#### Glucose-6-Phosphate Dehydrogenase

Glucose-6-phosphate dehydrogenase (G6PD; D-glucose-6-phosphate: NADP<sup>+</sup>-oxidoreductase, EC 1.1.1.49; Zwischenferment) catalyzes the oxidation of D-glucose-6-phosphate (G6P) to D-glucono- $\delta$ -lactone-6-phosphate according to the following equation:



G6PD was discovered in 1931 (Warburg and Christian, 1931) from erythrocyte and fermentating yeast and this enzyme has been demonstrated to

exist in practically all animal tissues and microorganisms (Waller et al., 1957; and Loehr and Waller, 1958). The following literature review on the enzyme only covers the area that is related to this thesis.

### Significance and purification

G6PD is the branch point for glucose utilization via glycolysis or the pentose phosphate pathway (PPP). The PPP (G6PD being the first enzyme) is widely distributed in nature and is considered to be an important source for the organism's supply of: 1) reducing equivalents in the form of NADPH for biosynthesis; e.g., of fatty acids and hence, indirectly, significant to the regulation of lipogenesis (Cohn and Joseph, 1959) or, in erythrocytes, for the regeneration of hemoglobin from methemoglobin; and 2) pentose phosphate; e.g., ribose-5-phosphate, for the synthesis of nucleotides. In bacteria; e.g., Pseudomonas (Kerstens and De Lay, 1968) and Caulobacter (Shedlarski, 1974), G6PD also appears to play an important role in the regulation of the Entner-Doudoroff degradative pathway which diverges from the other routes at D-gluconate-6-phosphate and involves the formation of triose phosphate and pyruvate from hexonic acid (e.g., gluconic acid) phosphate rather than from hexose phosphate (e.g., glucose phosphate) (Entner and Doudoroff, 1952).

Some human and animal tumors contain a high concentration of G6PD (Delbrueck et al., 1959). Deficiencies of the enzyme have been related to drug and food induced anemia and to chronic hemolytic disease (Carson

et al., 1956; Tarlov et al., 1962; and Kirkman et al., 1964). Erythrocytes from individuals with G6PD deficiency are unable to maintain reduced glutathione levels when they are presented with an oxidant stress (Srivastava and Beutler, 1969). Any drug that binds and inhibits G6PD would result in a G6PD deficiency and hence disturb the normal metabolism.

G6PD has been highly purified from many biological systems. Some examples are given in Table 2.

#### Molecular weight and different molecular forms

The molecular weight of the active native G6PD from various sources ranges between 57,000 and 285,000 as summarized in Table 3.

Isoenzymes and multimolecular forms of G6PD are common in mammalian systems (Bonsignore and De Flora, 1972). By using specific method of extraction; e.g., addition of Triton X-100 to the homogenization medium, and freezing and thawing the tissues or applying high frequency while homogenizing, six electrophoretically distinct forms from rat liver and four from rat heart and adipose tissue could be obtained (Lahat et al., 1972).

Lessie and Vander Wyk (1972) reported the finding of two multiple forms of G6PD in partially purified extracts of Pseudomonas multivorans, which differed in pyridine nucleotide specificity at non-saturating values of G6P. The two forms were clearly separated by centrifugation on a linear sucrose density gradient as well as upon electrophoresis.

TABLE 2  
 PURIFICATION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE FROM  
 DIFFERENT BIOLOGICAL SYSTEMS

Sources	Purification factor		Reference	
	Fold	Yield (%)		
Animals	cow-udder	10,244	2.9	Julian <i>et al.</i> , 1961 <sup>a</sup>
	cow-adrenal cortex	2,508	19.9	Criss and McKerns, 1968 <sup>a</sup>
	human-erythrocyte	13,617	3	Kirkman, 1962
	human-erythrocyte	43,500	7.5	Chung and Langdon, 1963a
	human-erythrocyte	63,500	50	Yoshida, 1966
	human-erythrocyte	72,000	32	Cohen and Rosemeyer, 1969a <sup>a</sup>
	human-erythrocyte	86,393	63.5	Rattazzi, 1969
	human-adrenal gland	28	44	McKerns, 1962a
	human-adrenal gland	3,570	13	Squire and Sykes, 1970
	rat-liver	677	7	Matsuda and Yugari, 1967
Bacteria	<u>Escherichia coli</u>	168	36	Sanwal, 1970
	<u>Leuconostoc</u>	125	18.6	Olive and Levy, 1967 <sup>a</sup>
	<u>mesenteroides</u>			
Fungus	<u>Aspergillus niger</u>	67	15	Jagannathan <i>et al.</i> , 1956
	<u>Neurospora crassa</u>	2,474	10.2	Scott and Tatum, 1971 <sup>a</sup>
	<u>Penicillium duponti</u>	550	34	Malcolm and Shepherd, 1972 <sup>a</sup>
Yeast	brewer's yeast	2,535	10	Noltmann <i>et al.</i> , 1961
	torula yeast	475	32	Engel <i>et al.</i> , 1969
	( <u>Candida utilis</u> )	1,038	46	Chilla <i>et al.</i> , 1973 <sup>a</sup>

<sup>a</sup> The purification procedures are also described in Wood (1975).

TABLE 3  
MOLECULAR WEIGHT OF G6PD FROM DIFFERENT SOURCES

Source	Method of determination <sup>a</sup>	Molecular weight	Number of subunits	Conditions and remarks	Reference
Adrenal (bovine)	A and B	284,000	4	[E] = 2-6 mg/ml; 0.1 M acetate, pH 5.5; 20 $\mu$ M NADP <sup>+</sup> ; 10 mM MCE; 1 mM EDTA; 0.15 M KCl; 59,780 rpm, 20°C; 9.45 S (subunit S = 3.70); $\bar{v}$ = 0.737	Singh and Squire, 1974
Erythrocyte (human)	A	190,000	at least 2	[E] = 2.23 mg/ml; 0.15 M P <sub>i</sub> , pH 7.0; 50,740 rpm, 10°C; 7.1 S, $\bar{v}$ = 0.740; NH <sub>2</sub> -terminal a.a. are tyrosine and alanine	Chung and Langdon, 1963a
Erythrocyte (human)	B	240,000	6	[E] = 0.15%; 50 mM acetate, pH 6.0; 1 mM EDTA; 1 mM MCE; 20 $\mu$ M NADP <sup>+</sup> ; 23,150 rpm, 18 hr, 10.1°C; 10.03 S	Yoshida, 1966
<u>Neurospora crassa</u>	B	206,000	4	[E] = 0.120-0.25 mg/ml; 0.1 M Tris, pH 7.4; 1 mM MCE; 2 mM NADP <sup>+</sup> ; 14,000 rpm, 44 hr, 5°C	Scott, 1971
<u>Neurospora crassa</u>	B	63,000	1	[E] = 0.51 mg/ml; 0.1 M Tris, pH 8.5; 0.1 M DDT; 30,000 rpm, 45 hr	Scott, 1971
Liver (rat)	A	110,000	4	[E] = 12.3 mg/ml; 0.1 M Tris, pH 7.2; 10 $\mu$ M NADP <sup>+</sup> ; 51,200 rpm, 16.9°C; 5 S	Matsuda and Yugari, 1967
Liver (rat)	C	60,000 <sup>b</sup> 57,000 <sup>b</sup> 28,000 <sup>b</sup>	2 2	E = 80 $\mu$ g; 0.1 M P <sub>i</sub> , pH 7.0; 0.1 M NaCl; 1 $\mu$ M NADP <sup>+</sup> ; Sephadex G-200 (2 x 38.5 cm)	Yugari and Matsuda, 1967
Yeast (brewers)	B	212,000	4	[E] = 4 mg/ml; 10 mM P <sub>i</sub> , pH 6.8; 0.15 M NaCl; 1.6 mM NADP <sup>+</sup> ; 4,327 rpm, 113 hr 3°C; $\bar{v}$ = 0.739	Yue <u>et al.</u> , 1969
Yeast (torula)	C	110,000 220,000	2 4	Tris 50 mM, pH 7.5; 0.1 M KCl; Sephadex G-150 P <sub>i</sub> , pH 5.8; 20 mM MgSO <sub>4</sub> ; Sephadex G-150	Domagk <u>et al.</u> , 1973
	D	49,000	1	G6PD were preincubated at 37°C with 1% SDS plus 1% MCE; the gel and the phosphate buffer contained 0.1% SDS	

<sup>a</sup> A (sedimentation and diffusion); B (sedimentation equilibrium); C (Sephadex column chromatography); and D (SDS gel electrophoresis).

<sup>b</sup> MW of 60,000 and 57,000 are active dimers and MW of 28,000 is inactive monomer.

G6PD was first separated into multiple forms after starch-gel electrophoresis by Tsao (1960). Beitner and Naor (1972a) reported that G6PD from adipose tissue had four isoenzymes which differed in their subcellular locations. The particle-associated forms were activated by  $Mg^{+2}$  whereas the supernatant isoenzymes were not effected by addition of  $Mg^{+2}$ .

G6PD from Caulobacter crescentus showed three molecular forms which were separable by polyacrylamide gel electrophoresis and by DEAE-Sephadex chromatography but could not be distinguished by sucrose density gradient (5-20%) (Shedlarski, 1974). Shedlarski also reported that the three G6PD bands separated by polyacrylamide gel electrophoresis showed different activities when stained with the standard nitroblue tetrazolium and phenazine methosulfate procedures. Color development occurred first in the fast-migrating band followed by that of the slower band and finally that of the slowest-migrating band. All three bands of activity appeared in the presence of either  $NAD^{+}$  or  $NADP^{+}$  cofactors at non-saturating values of G6P.

G6PD from torula yeast exist as two distinct isoenzymes (Engel et al., 1959; Domagk et al., 1969b; Domschke et al., 1969; and Domschke et al., 1970a). The two enzyme forms are easily separated in the course of the purification procedure (Engel et al., 1969), by fractionation with ammonium sulfate of 75% and 95% saturation (AS 75 and AS 95). The two forms have been shown to differ from each other on the basis of a distinctive inactivation by homologous antibodies, fingerprint

analysis of tryptic peptides, their behavior in both disc-gel electrophoresis and electrofocusing (Domagk et al., 1969b), slightly different inactivation patterns following photooxidation by rose bengal (Domschke et al., 1969), significant difference in  $\alpha$ -helical content (Domschke et al., 1970a), and to a partial extent, their amino acid composition (Engel et al., 1969; and Domagk et al., 1969a). The two isoenzymes are similar in their molecular weights,  $K_m$  values for both substrates, and pH optima.

#### Kinetic mechanism

Soldin and Balinsky (1968) described the kinetic mechanism for red cell G6PD as a sequential ordered mechanism although they were unable to rule out either a rapid-equilibrium random mechanism in which NADPH acts as a dead-end inhibitor by combining with the enzyme-G6P complex or a Theorell-Chance (1951) mechanism. Sanwal (1970), Olive et al. (1971) and Afolayan (1972) also suggested ordered sequential mechanism for G6PD from E. coli, L. mesenteroides, and torula yeast, respectively. The data published by Olive et al. (1971) on L. mesenteroides G6PD definitely ruled out a rapid-equilibrium random mechanism.

#### Michaelis constants

The affinity of G6PD for its substrates expressed as Michaelis constants ( $K_m$ ) have been reported by many authors for G6PD from different sources under different conditions (Table 4). They range between 0.01 to 1 mM for G6P and 2 to 300  $\mu$ M for NADP<sup>+</sup>.

TABLE 4  
MICHAELIS CONSTANTS OF G6PD FROM DIFFERENT SOURCES

Source	Conditions	Michaelis constants		Reference
		$K_m$ , G6P ( $\mu$ M)	$K_m$ , NADP <sup>+</sup> ( $\mu$ M)	
Various	Various	13-1,000	2-300	Noltmann and Kuby, 1963
Bovine adrenal cortex	Tris 100 mM, pH 8.0; 6 mM MgCl <sub>2</sub> ; 0.1 mM G6P and 10 $\mu$ M NADP <sup>+</sup>	42	5.6	Criss and McKerns, 1968
<u>Caulobacter crescentus</u>	Tris 30 mM, pH 7.6; 2 mM G6P and 400 mM NADP <sup>+</sup>	340	29	Shedlarski, 1974
<u>Escherichia coli</u>	Tris 50 mM, pH 7.5; 10 mM MgCl <sub>2</sub> ; 1 mM G6P and 75 $\mu$ M NADP <sup>+</sup>	70	15	Sanwal, 1970
Human erythrocyte	Tris 100 mM, pH 8.0; 7 mM MgCl <sub>2</sub> ; 0.7 mM G6P and 70 $\mu$ M NADP <sup>+</sup>	39	4.4	Yoshida, 1966
<u>Leuconostoc mesenteroides</u>	Tris 33 mM, pH 7.8; 3.3 mM G6P and 160 $\mu$ M NADP <sup>+</sup>	36	7.4	Olive and Levy, 1967
Torula yeast ( <u>Candida utilis</u> )	TEA 93 $\mu$ mole, pH 7.6; 9.3 $\mu$ mole MgCl <sub>2</sub> ; 0.5 $\mu$ mole G6P; 10.25 $\mu$ mole NADP <sup>+</sup> and 0.93 $\mu$ mole EDTA all in 1 ml	270	56	Domagk <u>et al.</u> , 1969b
Yeast	TEA 50 mM, pH 7.5; 0.67 mM G6P and 0.5 mM NADP <sup>+</sup>	69	50	Loehr and Waller, 1974

## Regulation

The metabolic control of G6PD is not well understood. According to the available information, however, once G6PD is produced in the cell there will be at least two categories of regulation. The first is an effect of G6PD enzyme activity; i.e., G6PD is inhibited by external (in regard to the reaction itself) materials, either some other intermediate metabolites or proteins or enzymes; or internally G6PD is controlled by its substrate or product (examples are listed in Table 5). The latter mechanism can be explained by the fact that G6PD is inhibited by its substrate G6P, as well as by its product NADPH (Bonsignore et al., 1969). Secondly, regulation via an effect on substrates or products of G6PD catalyzed reaction; i.e., control of G6PD substrate or product concentrations by other reactions. Fatty acids synthesis and other biosynthesis need NADPH, hence the level of lipogenesis or other activities that require NADPH may control the activity of G6PD (Tepperman and Tepperman, 1963). Also it has been claimed by Clark and Jakoby (1970) that the ration of  $\text{NADP}^+$  to NADPH should be the regulatory mechanism for G6PD from torula yeast. In plants, the activity of the oxidative pentose-phosphate pathway in chloroplasts is regulated by light-dark transitions (Bassham and Kirk, 1968). The pathway is active in the dark and inactive in the light through light inhibition of G6PD activity (Lendzian and Ziegler, 1970). It has been suggested that the regulation is also effected by the ratio of NADPH to  $\text{NADP}^+$  in the chloroplast (Bassham and Kirk, 1968; and Lendzian and Ziegler, 1970).

TABLE 5  
METABOLIC INHIBITORS OF G6PD FROM DIFFERENT SOURCES

Inhibitor	Source of G6PD	Reference
Adenine nucleotide	erythrocyte	Bonsignore <u>et al.</u> , 1963
ATP	torula yeast, etc.	Avigad, 1966
ATP	yeast	Bonsignore <u>et al.</u> , 1966
ATP	<u>Hydrogenomonas</u>	Blackkolb and Schlegel, 1968
ATP	torula yeast	Afolayan, 1972
3',5'-cyclic AMP (activator)	torula yeast	Afolayan, 1972
Erythrose-4-phosphate	torula yeast	Domagk <u>et al.</u> , 1973
Glyceraldehyde-3-phosphate	torula yeast	Domagk <u>et al.</u> , 1973
G6P	human erythrocyte	Bonsignore <u>et al.</u> , 1969, 1971
NADP <sup>+</sup>	human erythrocyte	Luzzatto, 1967
NADPH	yeast	Eger-Neufeldt <u>et al.</u> , 1965
NADPH	<u>Hydrogenomonas</u>	Blackkolb and Schlegel, 1968
NADPH	torula yeast	Domagk <u>et al.</u> , 1969a
NADPH	human erythrocyte	Bonsignore <u>et al.</u> , 1969, 1971
NADPH	<u>Escherichia coli</u>	Sanwal, 1970
NADPH	torula yeast	Afolayan, 1972
Nucleotide mono- and poly phosphate	torula yeast	Domagk <u>et al.</u> , 1969a
Phosphoenol pyruvate	<u>Arthrobacter 7C</u> and	Turnail and Schlegel, 1972
6-Phosphogluconic acid (in the presence of a specific protein)	human erythrocyte	Bonsignore <u>et al.</u> , 1969
Pyridoxal phosphate	torula yeast	Domagk <u>et al.</u> , 1969a
Free fatty acids (Myristic, lauric, and palmitic acids in decreasing order)	rat liver	Yugari and Matsuda, 1967
Long chain fatty acid	erythrocyte	Wieland <u>et al.</u> , 1965
Palmitoyl-CoA, steroyl-CoA, and lauroyl-CoA	yeast	Eger-Neufeldt <u>et al.</u> , 1965
Palmitoyl-CoA	yeast	Taketa and Pogell, 1966
Palmitoyl-CoA	<u>Penicillium duponti</u>	Malcolm and Shepherd, 1972
Palmitoyl-CoA	yeast, rat liver	Kawaguchi and Bloch, 1974
Thyroid hormones (e.g., triiodothyroxine)	cow adrenal-cortex	McKerns, 1962b
Estrone, estradiol, and estriol	bovine adrenal-cortex	McKerns, 1962a, 1963
Dehydroepiandrosterone	rat mammary	Levy, 1963
Steroid hormones (e.g. $\Delta^5$ -3 $\beta$ -hydroxysteroid)	erythrocyte	Marks and Banks, 1960
NADP <sup>+</sup> -glycohydralase (EC 3.2.2.6)	rat liver	Bonsignore <u>et al.</u> , 1968
NADP <sup>+</sup> -pyrophosphatase (EC 3.6.1.9)	rat liver	De Flora, 1968
Inactivating protein (an enzyme)	rat liver	De Flora, 1968

A mechanism of action of many hormones in regulating cell function and replication in their target organs may also depend on activation or inhibition of unique species of G6PD (McKerns, 1964, 1966, 1967, and Table 5).

#### Substrate and coenzyme specificities

G6PD from a number of sources acts on analogs of G6P (Kirkman, 1962; and Yoshida, 1966) with reduced rates and with low affinities in comparison with the natural substrate G6P. The coenzyme specificity can be grouped into four types as shown in Table 6.

#### Dissociation

Free fatty acid (e.g., lauric acid) disaggregates the active rat liver G6PD (MW 57,000 and 60,000) into inactive subunits (MW 28,000) (Yugari and Matsuda, 1967). G6P, 6-PGA, and NADPH catalyze the dissociation of G6PD from erythrocyte into inactive subunits, whereas  $\text{NADP}^+$  in the presence of sulfhydryl compounds and EDTA at slightly alkaline pH, results in re-association of these subunits back to a polymeric, active state (Bonsignore et al., 1969). Palmitoyl-CoA causes dissociation of dimeric torula yeast G6PD enzyme to monomers and binds to the monomeric subunits (Kawaguchi and Block, 1974).

#### Amino acid composition and the active site

The amino acid composition of different G6PD have been reported (Domagk et al., 1969a; Scott and Tatum, 1971; and Cohen and Rosemeyer,

TABLE 6  
COENZYME SPECIFICITY OF G6PD FROM DIFFERENT SOURCES

Specificity	Source	Reference
Strictly specific for NADP <sup>+</sup>	brewers' yeast	Warburg and Christian, 1936 Kornberg and Horecker, 1955 Avigard, 1966
	<u>Escherichia coli</u>	Sanwal, 1970
	NADP-linked G6PD from <u>Acetobacter xylinum</u>	Benziman and Mazover, 1973
	<u>Penicillium duponti</u> torula yeast	Malcolm and Shepherd, 1972 Domagk et al., 1969a
Display maximal activity with NADP <sup>+</sup> and lower variable activity with NAD <sup>+</sup> and some analogs of both NAD <sup>+</sup> and NADP <sup>+</sup> <sup>a</sup>	G6PD from most animal sources	Levy, 1961 Levy, 1963 Yoshida, 1966
	React with both NAD <sup>+</sup> and NADP <sup>+</sup> at comparable rate <sup>b</sup>	<u>Caulobacter crescentus</u>
<u>Hydrogenomonas H 16</u>		Blackkolb and Schlegel, 1968
<u>Leuconostoc mesenteroides</u>		DeMoss et al., 1953 Olive and Levy, 1967 Olive et al., 1971
	<u>Pseudomonas aeruginosa</u>	Lessie and Neidhardt, 1967
	<u>Thiobacillus ferrooxidans</u>	Tabita and Lundgren, 1971
	Strictly specific for NAD <sup>+</sup>	NAD <sup>+</sup> -linked G6PD from <u>Acetobacter xylinum</u>

<sup>a</sup> Most of the G6PD's which display dual pyridine nucleotide specificity consist of a single enzyme capable of reacting both with NAD<sup>+</sup> and NADP<sup>+</sup> (Cheldelin, 1961; Olive and Levy, 1967; Nevaldine and Levy, 1967; and Lessie and Neidhardt, 1967).

<sup>b</sup> In rat adipose tissue, it was reported that different isoenzymes are involved in the NAD<sup>+</sup>- and NADP<sup>+</sup>-linked reactions. The intracellular distribution of the NAD<sup>+</sup>-linked isoenzymes differed from that of NADP<sup>+</sup>-linked isoenzymes (Beitner and Naor, 1972b).

<sup>c</sup> The NAD<sup>+</sup>-linked G6PD of Acetobacter xylinum is a different and separable protein from the NADP<sup>+</sup>-linked G6PD from this same source.

1975). Despite the degree of evolutionary divergence between yeast and human, the amino acid compositions are very similar. The protein contains about 15% basic, 22.6% acidic, 44.1% aliphatic, 9.4% aromatic, 4.1% sulfur-containing, and 4.8% imino acids.

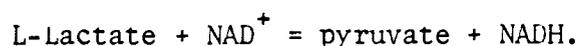
On the basis of chemical modification, the side groups of amino acid histidine (Domschke et al., 1969), tyrosine (Domschke et al., 1970b), arginine (Domagk et al., 1970a), and lysine (Domschke and Domagk, 1969) are shown to be involved in the active sites of G6PD and from torula yeast.

#### Other Dehydrogenases

The dehydrogenases described below are all important in controlling intermediary metabolism. Their relative position in the various pathways are indicated in Figure 2 (White et al., 1968; Barman, 1969; and Lehninger, 1972).

Lactate dehydrogenase (LDH; L-lactate:NAD<sup>+</sup>-oxidoreductase; EC 1.1.1.27).

LDH catalyzes the terminal step of glycolysis by vertebrate muscle and for many other anaerobic, fermentative modes of carbohydrate metabolism:



This reaction is shared, however, by the so-called homolactic fermentation (i.e., a fermentation that yields lactate as the sole product) carried out by a large number of microorganisms.

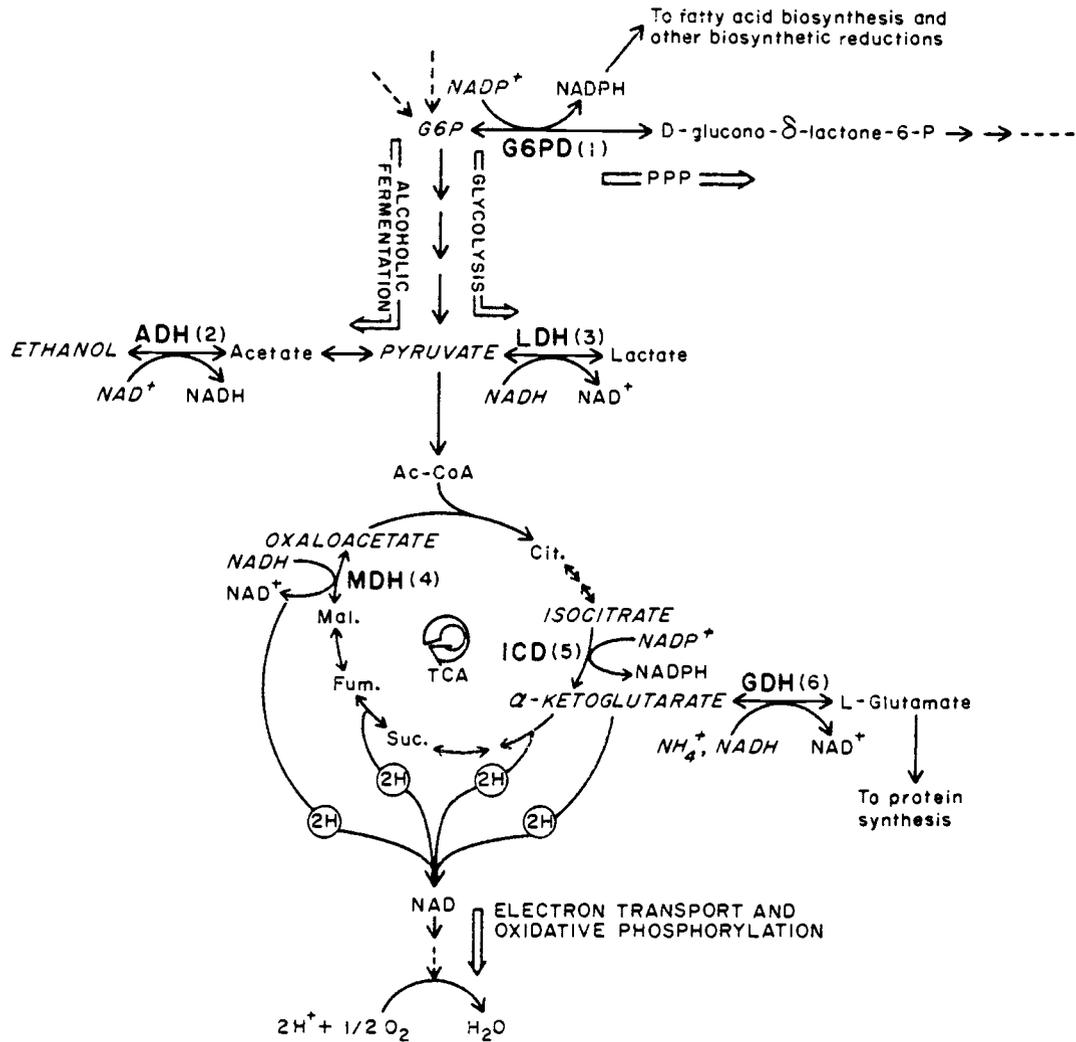


Figure 2. Metabolic pathways involved in the dehydrogenases studied. The enzymes studied are in heavier lettering and the substrates and coenzymes used are in italicized lettering.

Pure LDH from vertebrate sources all appear to be tetramers containing four independent catalytic sites. Vertebrate LDH's can be dissociated into four equal enzymatically inactive subunits of molecular weight 35,000 by protein denaturants such as guanidine and sodium dodecylsulfate (SDS). Two different types of LDH subunits exist that have been called the "M" for skeletal muscle, where it is found as the predominate form) and "H" (for heart muscle, where it predominates) which appear to be controlled by two separate and independent genes. The enzyme is located in the cytosol portion of the cell.

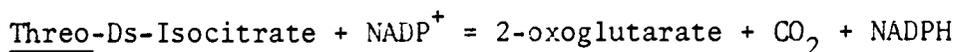
Alcohol dehydrogenase (ADH; alcohol:NAD<sup>+</sup>-oxidoreductase; EC 1.1.1.1).

ADH catalyzes a rate-limiting step in the metabolism of ethanol (Hawkins and Kalant, 1972):



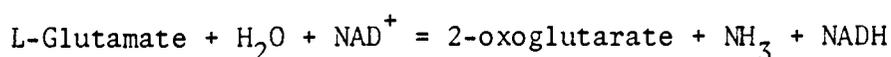
Liver ADH is a dimer of molecular weight 80,000. The identical subunits are composed of 374 amino acids in one chain (Joernvall, 1970) plus two firmly bound zinc atoms. In contrast to animal, yeast ADH is a tetramer with molecular weight of 141,000 (Buehner and Sund, 1969) and contains 4 gram-atoms of zinc per mole of protein.

Isocitrate dehydrogenase (ICD; threo-Ds-isocitrate:NADP<sup>+</sup>-oxidoreductase (decarboxylating); EC 1.1.1.42). ICD catalyzes the oxidative decarboxylation of Ds-isocitrate when in the presence of a divalent cation (Mg<sup>+2</sup> or Mn<sup>+2</sup>) and an oxidant (NAD<sup>+</sup> or NADP<sup>+</sup>):



There are three electrophoretically distinguishable ICD enzyme present in animal tissue (Markert and Møller, 1959; Lowenstein and Smith, 1962; and Bell and Baron, 1964). These enzymes differ in their intracellular localization as well as their cofactor requirement. One enzyme is found exclusively in the mitochondria and requires  $\text{NAD}^+$  as its cofactor (Plaut, 1959; Ernster and Glasky, 1960; and Bell and Baron, 1964). The other two ICD enzymes require  $\text{NADP}^+$  as their cofactor and are found both in the mitochondria and the supernatant fractions (Plaut, 1959; and Bell and Baron, 1964). Pig heart ICD has molecular weight of about 60,000 for the native enzyme and is composed of two hydrodynamically identical subunits (Magar and Robbins, 1969).

Glutamate dehydrogenase (GDH; L-Glutamate: $\text{NAD}^+$ -oxidoreductase (deaminating); EC 1.4.1.2). GDH, a mitochondrial enzyme, catalyzes the reaction:



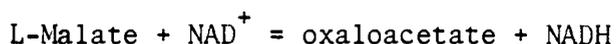
GDH molecules exhibit three levels of structural organization at the quaternary level (Freese and Freese, 1964). As isolated, GDH has molecular weight of 1.0 to 1.3 x 10<sup>6</sup> which then dissociates into enzymatically active subunits of molecular weight 250,000 to 350,000. Further dissociation then occurs into probably identical sub-subunits of molecular weight 40,000 which do not have enzyme activity.

The level of GDH in brain is high (Frieden, 1965) and the presence of GDH is known to play an important role in the central nervous system

(Jasper et al., 1965).

Malate dehydrogenase (MDH; L-malate:NAD<sup>+</sup>-oxidoreductase; EC 1.1.1.37).

MDH catalyzes the NAD<sup>+</sup>-dependent interconversion of L-malate and oxaloacetate:



There are two separable forms of the enzyme in the cells of many organisms, one localized in mitochondria and one found in the cell cytoplasm fractions. Both forms of MDH from mammalian heart muscle appear to be composed of two similar or identical subunits (Dévényi et al., 1966; Murphey et al., 1967; and Gerding and Wolfe, 1969) of molecular weight 35,000 (Murphey et al., 1967). The two stable enzyme forms isolated from the cytoplasm of beef heart (S-MDH a and b) differs in their regulatory properties (Cassman and Vetterlein, 1974).

#### Purpose of this Thesis

Although there are many possible mechanisms that could explain the toxicity of HCP to biological systems as mentioned above, there are still many unknown questions that need to be answered. The research of this thesis is aimed at establishing the extent and mechanism(s) for the inhibition of dehydrogenase enzymes by HCP. G6PD was chosen as the principal enzyme used for studying the mechanism of HCP action. Other dehydrogenases employed were LDH, ICD, ADH, MDH, and GDH. Those enzymes were selected because they are all pyridine nucleotide-linked dehydrogenases and are important in controlling the pentose phosphate pathway,

glycolysis, alcoholic fermentation, the Entner-Doudoroff pathway, fatty acid synthesis, protein synthesis, and electron transport systems. Disclosing the mechanism of HCP action on these dehydrogenases may shed some light on the action of HCP on other enzymes and could thus be very useful in understanding the lethal action of HCP on living organisms.

## MATERIALS AND METHODS

Enzymes and Proteins

Horse liver alcohol dehydrogenase Type IX (alcohol:NAD<sup>+</sup>-oxidoreductase, EC 1.1.1.1), 2.2 units per mg of protein; glucose-6-phosphate dehydrogenase from Candida [Torulopsis] utilis Type XII (D-glucose-6-phosphate:NADP<sup>+</sup>-oxidoreductase, EC 1.1.1.49), 405 units per mg of protein; bovine liver glutamate dehydrogenase Type I (L-glutamate:NAD<sup>+</sup>-oxidoreductase, EC 1.4.1.2), 2.7 units per mg of protein; pig heart isocitrate dehydrogenase Type IV (threo-D<sub>s</sub>-isocitrate:NADP<sup>+</sup>-oxidoreductase, EC 1.1.1.42), 7.3 units per mg of protein, beef heart lactate dehydrogenase Type III (L-lactate:NAD<sup>+</sup>-oxidoreductase, EC 1.1.1.27), 560 units per mg of protein; beef heart malate dehydrogenase (L-malate:NAD<sup>+</sup>-oxidoreductase, EC 1.1.1.37), 2181 units per mg of protein; beef blood hemoglobin (Hb) Type I; beef liver catalase 2x crystallized; and bovine serum albumin (BSA) were obtained from the Sigma Chemical Company, St. Louis, Missouri.

Chemicals

The NAD<sup>+</sup>, NADH, NADP<sup>+</sup>, Tris, iodoacetate, and iodoacetamide used were obtained from Sigma Chemical Company, St. Louis, Missouri. Glycerol and sucrose were from Mallinckrodt Chemical Company, St. Louis, Missouri. High purity CsCl was from Kawecki Berylco Industries, Inc., New York. Aquacide II was from Calbiochem Company, La Jolla, California, and SDS of an electrophoresis purity grade was purchased from

Bio-Rad, Inc., Richmond, California. All other chemicals used were reagent grade from either Sigma or Mallinckrodt.

The chlorinated bisphenols: [3,4-TCP; 2,2'-methylenebis(3,4-dichlorophenol)]; 2,2'-methylenebis(3,5-dichlorophenol); 2,2'-methylenebis(4,6-dichlorophenol); 2,2'-oxybis(tetrachlorophenol); 2,2'-dihydroxy-3,3',5,6,6'-pentachlorodiphenylmethane; 2',4-dihydroxy-2,3,3',5,5',6-hexachlorodiphenylmethane; and HCP were kindly donated by the Givaudan Corporation, Clifton, New Jersey. The U.S.P. grade HCP was twice recrystallized from isopropanol-water to yield a chromatographically pure product, m.p. 165.0-1.65.5°C. Dichlorophene (DCP; 2,2'-methylenebis(4-chlorophenol) was purchased from the Aldrich Chemical Corporation, Milwaukee, Wisconsin, and bithionol [2,2'-thiobis(4,6-dichlorophenol) was obtained from Pfatz and Bauer Company, Flushing, New York.

Methylene-<sup>14</sup>C-hexachlorophene (2.71-3.52 mCi/mole) was obtained from Mallinckrodt-Nuclear Company (St. Louis, Missouri) or New England Nuclear Company (Boston, Massachusetts) and used without further treatment after establishing the purity by thin layer chromatography.

Concentrated liquid scintillator (Spectrofluor) and toluene (methyl-<sup>14</sup>C) were purchased from Amersham/Searle Corporation (Arlington Heights, Illinois).

#### Preparation of HCP Solution

The solubility of HCP in aqueous solutions is very low and for this reason in many previous studies, HCP was dissolved in ethanol. Our results showed, however, that ethanol inhibited G6PD enzyme acti-

vity and that the inhibitory effect increased with increasing preincubation time. Therefore, in most cases for this thesis, HCP was dissolved first in approximately 0.4 N NaOH and then diluted with water or buffer to desired HCP concentration (usually 30  $\mu$ M to 4 mM), resulting in a final NaOH concentration of about 0.01-0.02 N. For the  $I_{50}$  studies, HCP was dissolved in 95% ethanol so as to dissolve more HCP in the solution. Controls where contained the same amount of solvent material were used for all the experiments.

#### Determination of Dehydrogenase Activities

All dehydrogenase assays were carried out in triplicate (except where otherwise indicated) in 1 ml quartz cuvettes or a 0.7 ml rapid sampling cuvette in a Gilford Model 2000 spectrophotometer equipped with a thermostated cell compartment maintained at 30°C (unless otherwise indicated) by Lauda K-2/R constant temperature circulator. A tight fit aluminum holder thermostated by the same bath was utilized to pre-warm the cuvettes and their contents. Enzyme reactions (including most of the kinetic studies) were normally initiated by addition of enzyme which had been stored at 0°C into the prewarmed cuvettes. The amount of enzyme added was adjusted so that the initial rate of reduction or oxidation of pyridine nucleotide was linear with time and proportional to the enzyme concentration. In some cases, the reaction was initiated by addition of substrate after preincubation of the enzyme and other reaction components at 30°C for varying time periods.

All dehydrogenase activities were recorded (10 sec after initiation of the reaction) on a 0-1 O.D. scale recorder (unless otherwise

indicated) as the increase or decrease of absorbance with respect to time at 340 nm. The initial rate of reaction,  $v$  ( $\Delta O.D.$  per min), was determined by drawing tangents to the recorder plot. Mean values were used to prepare Lineweaver-Burk (1934) and Hill (1913) plots.

The components of the mixtures for individual dehydrogenase were as follows (water was used to make up the final volume to 1 ml).

#### Glucose-6-phosphate dehydrogenase (G6PD)

The reaction mixture contained 0.05 M Tris-Cl buffer, pH 7.5; 0.02 M  $MgCl_2$ ; 0.48 mM  $NADP^+$ ; 0.85 mM G6P solution; and the reaction was initiated by adding 0.02 ml of the appropriate enzyme preparation (Worthington Enzyme Manual, Worthington Biochemical Company, 1972--modified). In some cases the enzyme was included in the reaction mixture, and the reaction was initiated by addition of the substrates, G6P or  $NADP^+$ . The enzyme was dissolved in 0.05 M Tris buffer, pH 7.5 containing 0.1 mM  $NADP^+$ . The presence of  $NADP^+$  was important to keep the enzyme stable for a long period of time when frozen. In each assay throughout the entire research, the final enzyme content of the assay mixture was 0.2-0.3  $\mu g$  protein (about 0.1 enzyme unit--one unit will oxidize 1.0  $\mu mole$  of G6P to 6-phosphogluconate per min at pH 7.4 and 25°C in the presence of  $NADP^+$ ).

#### $NADP^+$ -specific isocitrate dehydrogenase (ICD)

The reaction mixture contained 0.05 M Tris-Cl buffer, pH 7.5; 1.3 mM  $MnSO_4$ ; 0.10 mM  $NADP^+$ ; 1.3 mM DL-isocitrate; 0.34 mM EDTA (disodium

salt); 0.10 mM dTE; and 0.02 ml diluted enzyme solution (Cleland et al., 1969). The enzyme was prepared by diluting 0.03 ml of original enzyme to 1.5 ml with 0.25 M Tris-Cl buffer, pH 7.5, containing 0.2 mM DTE. Each assay contained 4  $\mu$ g protein (about 0.03 enzyme unit--one unit will convert 1.0  $\mu$ mole of isocitrate to  $\alpha$ -ketoglutarate per min at pH 7.4 and 37°C). The recorder scale used was 0-0.1 absorbance and the reaction rate was measured at 35-45 sec.

#### Glutamate dehydrogenase (GDH)

The reaction mixture contained 0.034 M TEA buffer, pH 8.0; 2.8 mM EDTA (disodium salt); 0.11 mM NADH; 60 mM ammonium acetate; 6.6 mM  $\alpha$ -ketoglutarate; and 0.02 ml of diluted enzyme solution (Schmidt, 1965). The stock enzyme, in the form of crystalline suspension in ammonium sulfate, was diluted 100 fold with 0.033 M TEA buffer, pH 8.0. The final assay mixture contained about 4  $\mu$ g protein (about 0.011 enzyme unit--one unit oxidizes 1.0  $\mu$ mole L-glutamic acid per min at pH 7.6 and 25°C).

#### Malate dehydrogenase (MDH)

The reaction mixture contained 0.16 M Tris-Cl buffer, pH 8.5; 0.25 mM NADH; 0.40 mM oxaloacetate; and 0.02 ml diluted enzyme (Wedding and Black, 1963). The enzyme stock was diluted 200 fold with 0.02 M phosphate buffer, pH 7.0. The final assay mixture contained 0.02  $\mu$ g protein (about 0.2 enzyme unit--one unit will convert 1.0  $\mu$ mole of oxaloacetate and  $\text{NAD}^+$  to malate and NADH per min at pH 7.5 and 25°C). The recorder scale used was 0-0.5 absorbance.

### Lactate dehydrogeanse (LDH)

The reaction mixture contained 0.05 M glycyglycine buffer, pH 7.5; 2 mM pyruvate; 0.2 mM NADH; and 0.02 ml of diluted enzyme (Stolzenbach, 1966). The stock enzyme was diluted 200 fold with 0.1 M phosphate buffer, pH 7.0. The assay mixture contained about 0.1  $\mu\text{g}$  of protein (about 0.056 enzyme unit--one unit will reduce 1.0  $\mu\text{mole}$  of pyruvate to lactate per min at pH 7.5 and 37°C).

### Alcohol dehydrogenase (ADH)

The reaction mixture contained 0.06 M Tris-Cl buffer, pH 8.8; 5 mM  $\text{MgCl}_2$ ; 0.50 mM  $\text{NAD}^+$ ; 0.20 M ethanol; and 0.02 ml enzyme solution (Kerstens and De Ley, 1966). Enzyme was weighed and dissolved in 0.01 M phosphate buffer, pH 7.0, to adjust enzyme concentrations to about 5.5 mg/ml. Each assay contained about 2.2  $\mu\text{g}$  protein (about  $4.84 \times 10^{-3}$  enzyme unit--one unit will convert 1.0  $\mu\text{mole}$  of ethanol to acetaldehyde per min at pH 8.8 and 25°C).

### Measurement of Other Marker Protein or Enzyme

Hemoglobin (Hb) was determined by its absorbance at 405 or 413.5 nm. Catalase activity was assayed by a photometric method at 250 nm using  $\text{H}_2\text{O}_2$  as substrate (Beers and Sizer, 1952).

### Radioactivity Counting Techniques

All counting were performed in a Packard Tri-Carb liquid scintillation spectrometer Model 3375 (Packard Instrument Company, Inc., Downers

Grove, Illinois) at optimum setting for measuring  $^{14}\text{C}$ . Counting efficiencies were obtained by using the channels ratio method or an external gamma emitting standard (Peng, 1970). Aliquots of samples were mixed with 4 ml Prockop (Prockop and Ebert, 1963) scintillation fluid in small (5 ml capacity) vials placed inside regular 20 ml vials for counting. Membranes used in equilibrium dialysis were blotted dry with Kimwipe paper and then treated the same way as liquid samples for counting.

#### Protein Determination

Protein was determined according to the methods of Lowry et al. (1951) or Schaffner and Weissman (1973) with BSA as a standard.

#### Presentation of Data and Calculation of Inhibition Constants

A Hewlett-Packard 7200A plotter at a remote terminal of Oregon State University's time-shared Control Data Corporation 3300 digital computer was used for producing all lines of Lineweaver-Burk and Hill plots shown in Figures 6, 10-12, 15, and 16. The points were average of 2 to 5 assays. Calculations were performed by interactive programs written by Dr. R. Dyson in FORTRAN and executed on the above computer system (Dyson and Cardenas, 1973). The Michaelis-Menten equation and Hill equation were used as the basis both for the analysis of data and for drawing the lines of the graphs. Computer outputs gave values of  $K_m$  (Michaelis-Menten constant);  $V_m$  (maximum reaction rate); slope of Lineweaver-Burk plot;  $Y_{int}$  (intersection of the line in Lineweaver-Burk plot on Y axis); and  $n$  or  $n'$  (Hill coefficient or interaction

coefficient) of substrate binding sites or inhibitor binding sites, respectively. These constants were obtained for weighted least squares fits to the double reciprocal form of the Michaelis-Menten equation (Lineweaver-Burk plot) or to the logarithmic form of the Hill equation.

Inhibition constants were calculated by using the computer output kinetic values to fit Webb's method (Webb, 1963). Equations (1) through (3):

$$\text{Slope} = \frac{K_m}{V_m} \left( 1 + \frac{[I]}{K_i} \right) \quad (1)$$

$$Y_{\text{int}} = \frac{1}{V_m} \left( 1 + \frac{[I]}{K_i} \right) \quad (2)$$

$$\frac{\text{Slope of the inhibited line}}{\text{Slope of the control line}} = 1 + \frac{[I]}{K_i} \quad (3)$$

were used for the following calculations.

#### Competitive inhibition

Equation (1) was used, where  $K_m$  was obtained from the control (without inhibitor) curve and  $V_m$  from each individual line.

#### Noncompetitive inhibition

Equation (1) or (2) was used, where  $K_m$  was obtained from each individual curve and  $V_m$  was from the control curve.

#### Mixed type inhibition

Equation (1) or (3) was used, where  $K_m$  and  $V_{\text{max}}$  both were obtained

from the control curve.

### Uncompetitive inhibition

Equation (2) was used, where  $Y_{int}$  was obtained from each individual line and  $V_m$  from the uninhibited curve.

### Density Gradient Sedimentation

A Spinco Model L or L2 preparative ultracentrifuge (Beckman Instruments, Inc., Spinco Division, Palo Alto, California) with a swinging bucket rotor (SW-50L, SW-50.1 or SW 65) was used for this study. Gradients were prepared with a linear gradient formed as described by Britten and Roberts (1960) and modified by Martin and Ames (1961). Both sucrose, 5% (w/v, 0.146 M) and 20% (w/v, 0.548 M) or 25% (w/v, 0.73 M); and glycerol, 8.1% (w/v, 0.88M) and 33% (w/v, 3.58 M) or 36% (w/v, 3.91 M), in 0.05 M Tris-HCl buffer, pH 7.5, were used in the study. Linearity of the gradients was tested by adding methyl orange to 25% sucrose solution and determining the absorbance of resulting density gradient fractions photometrically at 505 nm; or by measuring the refractive index reading of the fractionated gradients. Beef liver catalase, BSA, and Hb of known molecular weights were used as markers and were mixed with the samples. The sample mixture (0.2 ml) was applied to the top of the gradient. After centrifugation, the tube was punctured at the bottom, and fractions of 8 to 15 drops each were collected at room temperature and then transferred to an ice bath. Fractions were analyzed for catalase activity, Hb content, protein, radio-

activity, and/or G6PD enzyme activity, depending on the nature of the experiment. The method of Schaffner and Weissman (1973) was used for the protein determination.

#### DEAE-Sephadex Chromatography

A torula yeast sample of about 0.5 mg in 0.4 ml 50 mM Tris-Cl buffer, pH 7.5 (contained 1 mM  $\text{NADP}^+$ ), was applied to a 0.9 x 30 cm column (bed length 27 cm) of DEAE-Sephadex A-25 (Pharmacia, Company) equilibrated with 50 mM Tris-Cl buffer, pH 7.8. The column was eluted with an exponential gradient (0.4 M Tris-Cl buffer, pH 7.8, in the reservoir and 50 mM Tris-Cl buffer, pH 7.8, in the mixing chamber) at a flow rate of 8 ml/hr at 4-5°C. The buffers used all contained 1.3  $\mu\text{M}$   $\text{NADP}^+$ . Fractions (50 drops, about 2.3 ml) were collected in an ISCO fraction collector Model 328 (Instrumentation Specialties Company, Lincoln, Nebraska). Each fraction was read at 230 nm for peptide bond absorption on Cary Model 15 spectrophotometer against the initial collected fraction which served as a reference. The amount of  $\text{NADP}^+$  present and the buffer used made a negligible contribution to the absorbancy at that wavelength. A 0.005 ml (or less by dilution) to 0.01 ml aliquot of the fractions was assayed for G6PD activity.

#### Equilibrium Dialysis

Two methods of conducting the equilibrium dialysis experiments were employed. In the first, dialysis was performed in cylindrical lucite cells constructed according to the design of Myer and Schellman

(1962). The total capacity of the cell is 2 ml which was divided into two equal compartments by a semipermeable membrane of approximately 4 cm<sup>2</sup>. Membranes were cut from Dialyzer tubing (Arthur H. Thomas Company, 1/4 inch wide and 0.002 inch wall thickness) which had been simmered 1 hr or longer in sequence with the following reagents: 50% ethanol twice, 10 mM sodium bicarbonate, 1 mM EDTA solution and redistilled water twice according to McPhie (1971). To one compartment (compartment A) was added 0.85 ml (80.7 pmole) of enzyme solution in 0.05 M Tris-Cl buffer, pH 7.5, and to another compartment (compartment B) was added varying amounts of <sup>14</sup>C-labeled HCP (specific activity 3.52 mCi/mmole) solution of the same volume. For each HCP concentration used, three cells were set with the following two solution combinations: HCP-G6PD, HCP-buffer, and HCP-HCP. The first combination was employed for determining the amount of HCP bound to the enzyme; the second was for calculating the free HCP concentration at equilibrium; and the third was for correcting for losses due to HCP absorption onto the membrane and lucite cell wall. These controls were essential since adsorption of HCP on the lucite cell wall and semipermeable membrane were significant. The Donnan effect was calculated according to Fruton and Simmonds (1961) by equation (4):

$$\frac{[\text{HCP}]_B}{[\text{HCP}]_A} = \frac{\text{protein concentration in compartment A}}{\text{original HCP concentration in compartment B}} + 1 \quad (4)$$

Dialysis was carried out in cold (4-5°C) for a period of 4 days (this period was necessary since the lucite cell wall, membrane, and

G6PD are competing for binding of the  $^{14}\text{C}$ -HCP). At the end of the dialysis period, 0.1 ml aliquots of solution were removed from both compartments at the same time and each was mixed with 4 ml of scintillation fluid in a small vial and counted for  $^{14}\text{C}$  radioactivity as described previously. The molar concentration of free HCP,  $[\text{HCP}]_f$ , and of bound HCP,  $[\text{HCP}]_b$ , were determined from the formulas (5) and (6) shown below:

$$[\text{HCP}]_f = \frac{\text{cpm}_B}{0.1} \times F_1 \times F_2 \times 10^3 \quad (5)$$

$$[\text{HCP}]_b = \frac{\text{cpm}_A}{0.1} \times F_2 \times 10^3 - [\text{HCP}]_f \quad (6)$$

where,

$\text{cpm}_B$  = Net cpm of a 0.1 ml aliquot from B compartment of the HCP-G6PD cell.

$\text{cpm}_A$  = Net cpm of a 0.1 ml aliquot from A compartment of the HCP-G6PD cell.

$F_1 = \frac{\text{Net cpm of a 0.1 ml aliquot from A compartment of HCP-buffer cell}}{\text{Net cpm of a 0.1 ml aliquot from B compartment of HCP-buffer cell}}$

$F_2 = \text{Correction factor for changing cpm to pmoles of HCP}$   
 $= \frac{1}{\text{counting efficiency}} \times \frac{1}{\text{specific activity of } ^{14}\text{C-HCP}}$

After removing aliquots for radioactivity counting, the remaining solution was transferred into 3 inch glass test tubes, and triplicate of 0.02 ml aliquots were assayed for G6PD enzyme activity by using the standard procedure. Enzyme controls were kept under the same conditions without HCP. Protein content was calculated based on the information

from the Sigma Company and an assumed molecular weight of 130,000 for torula yeast G6PD.

In the second method, dialysis was performed by using Dialyzer tubing. Bags made of Dialyzer tubing which had also been treated by the method of McPhie (1971), were filled with 0.2-0.6 ml of G6PD solution (19-57 pmoles of G6PD) in 0.05 M Tris-Cl buffer, pH 7.5, and were suspended in 125 ml of  $^{14}\text{C}$ -HCP in the same buffer (the amount of  $^{14}\text{C}$ -HCP used was not recorded since the amount of the adsorption of  $^{14}\text{C}$ -HCP on the glass container and the amount of  $^{14}\text{C}$ -HCP bound to the dialyzing bags were not calculated). The closed bags were dialyzed in the cold room at 4-5°C with stirring for 4 days. At the end of the dialysis period, 0.1 ml aliquots of the internal and external solutions were counted by liquid scintillation counter to determine the bound and unbound concentrations. Radioactivity counting technique was the same as described previously. External standard ratios were unaffected by the protein concentration employed as compared to buffer only but were slightly increased (i.e., less quenching) in the presence of membrane (no buffer solution). The amount of  $^{14}\text{C}$ -HCP inside the control bags containing buffer only, was used as free  $^{14}\text{C}$ -HCP for calculations. Aliquot of 0.02 ml of the internal solution was also used to assay for G6PD enzyme activity by the standard procedure.

#### Polyacrylamide Gel Electrophoresis

A vertical slab gel apparatus was used (E-C Model 470 vertical gel electrophoresis cell, E-C Apparatus Company). Discontinuous electrophoresis between 0.5 M Tris-Cl buffer, pH 8.9, and 0.04 M Tris-lysine

buffer, pH 8.3 was carried out essentially as described by Beers and Reich (1969). Gels were prepared by mixing 11.2 g of Cyanogum-41 (E-C Apparatus, Company) in 160 ml 0.05 M Tris buffer, pH 8.9. This solution was filtered through glass wool since the Cyanogum-41 was old and some of the powder was not completely dissolved in the buffer. Polymerization was initiated at room temperature by first adding 0.26 ml (0.16%) of TMED and then 0.192 g of APS (0.12%). The mixture was stirred about 10 sec before pouring into the cleaned, dried, and Photo-Flo-coated gel compartment. During polymerization, aluminum foil was used on the back and bottom of the gel maker and a regular 100 watt table lamp was used to shine light from top surface of the gel to help the polymerization. After 3 hr or longer, the apparatus was connected to the power supply and the electrode compartments filled with 0.04 M Tris-glycine buffer, pH 8.3, according to the instructions in the E-C Apparatus manual. Electrophoresis was then carried out at a constant 300 volts for 1 hr to remove the excess APS. After the prerun, the protein samples (in  $\mu\text{g}$  range as indicated in each experiment) which contained about 0.1% bromophenol blue and about 20% sucrose were applied under the buffer solution into each sample well. Experimental runs were made at a constant voltage of 300 volts for 86-110 min. The temperature was maintained at  $6^{\circ}\text{C}$  with the aid of an external cooling system using about 20% ethylene glycol in distilled water. In some cases, experimental conditions were altered somewhat and these are described in the figure legends.

Gels were stained for protein by immersion for 1 to 2 hr in a 0.2%

solution of amido black 10B in methanol-acetic acid-water (5:1:5). Destaining was carried out in the above solvent manually and/or electrically. Manual destaining was used in most cases since loss of minor components has been reported if rapid destaining by electrolytic procedures is used (Gabriel, 1971).

When visualization of G6PD enzyme activity was desired, the sample was diluted 1/25 to 1/100 times before application to the electrophoresis slab. Enzyme activity was detected in the gels with the phenazine methosulfate-nitroblue tetrazolium staining procedure (Beitner and Naor, 1972b). Bands of activity were developed at room temperature in dark under a nitrogen atmosphere.

The running procedure for electrophoresis in the presence of SDS was the same as described above. Preparation of protein samples and the addition of SDS following the method of Weber and Osborn (1969), which included incubation of protein sample with shaking in 1% SDS solution for 2 hr at 37°C. The SDS gel used was 7% Cyanogum-41 contained 0.2% SDS, 0.12% APS, and 0.16% TMED. The molecular weight of the marker proteins used were taken to be 65,000  $\pm$  700 for BSA (Scatchard and Pigliacampi, 1962); 60-65,000 for beef liver catalase (Sund *et al.*, 1967); 40,790  $\pm$  300 for horse liver ADH (Brändén, 1965; and Castellino and Barker, 1968); 36,180  $\pm$  800 for beef heart LDH (Castellino and Barker, 1968); and 15,500 for beef Hb (Weber and Osborn, 1969).

#### Difference Spectra

All test solutions were made up in aqueous buffers prepared ac-

ording to standard procedures (Gomori, 1955). Spectral measurements were carried out in a Cary Model 15 spectrophotometer at 25°C. Difference spectra were obtained by essentially the same procedure described by Wedding et al. (1967) and Henneke and Wedding (1975b). Two cuvettes containing HCP and NADP<sup>+</sup> (or G6P), respectively, were placed side by side in the reference compartment. Two additional cuvettes were prepared in the same manner and placed in the sample compartment and a difference spectrum obtained using the 0 to 0.1 absorbance unit slide wire. The contents of the two sample cuvettes were thoroughly mixed, the cuvettes returned to the sample compartment and a second difference spectrum then obtained. The difference between these two spectra was the difference spectrum finally presented.

## RESULTS

Part A: Kinetic Studies on the Inhibition of Torula Yeast G6PD and  
Some Other Dehydrogenase Enzymes by HCP and Related Compounds

Effect of pH

The pH optimum of torula yeast G6PD was observed to be at pH 8-9 and activity sharply decreased on both sides of these values (Figure 3). The results suggested that ionizable groups in the enzyme-substrate complex with pK's of 6 and 9.9 were required for activity. The pKa's were obtained from plot of  $V/V_0$  versus pH (Figure 3A) following equations (7) and (8) (Appendix I).

$$\log \frac{V/V_0}{1 - V/V_0} + \text{pH} = \text{pK} \quad (\text{at low pH}) \quad (7)$$

$$\log \frac{V/V_0}{1 - V/V_0} + \text{pK} = \text{pH} \quad (\text{at high pH}) \quad (8)$$

Where  $V$  is the observed maximum velocity at a given hydrogen ion concentration and  $V_0$  is the intrinsic maximum velocity. When  $V/V_0 = 0.5$ , equations (7) and (8) are simplified to yield

$$\text{pK} = \text{pH} \quad (9)$$

The observed pK of 6 lies between the values generally thought to be characteristic of carboxyl and imidazole groups in a protein (Cohn and Edsall, 1943) and the pK of 9.9 suggests involvement of a lysine  $\epsilon$ -amino group which was reported to be part of the active site of G6PD (Olive et al., 1971; Domschke and Domagk, 1969).

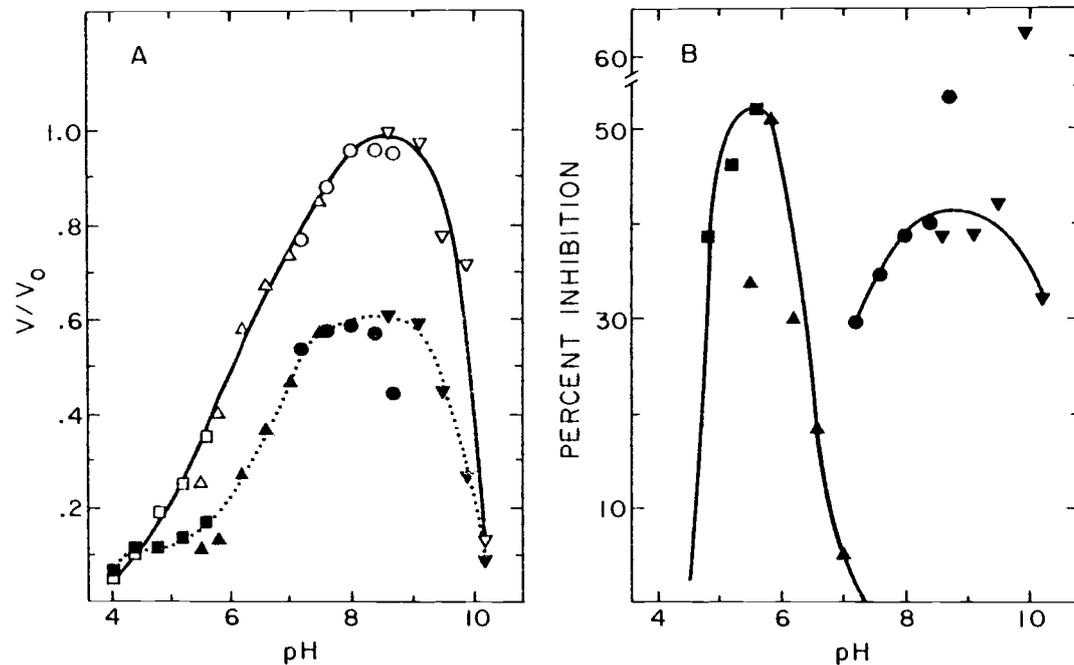


Figure 3. Optimum pH and the effect of pH on the inhibition of torula yeast G6PD by HCP. Buffer used were: acetate (□—□); sodium phosphate (Δ—Δ); Tris-Cl (O—O); and glycine-NaOH (∇—∇). The solid symbols are for the same buffers in the presence of 10.6  $\mu$ M HCP. A. Data obtained with phosphate buffer, both in the absence and presence of HCP, were normalized to the curve at pH 7.5 because of the additional inhibitory effect of the buffer on G6PD. Normalizing factor for the reaction in the absence of HCP is 1.976 and for the reaction in the presence of HCP is 1.314. B. Data were derived from A. Percent inhibition was calculated by using the equation  $(v - v_i)/v \times 100\%$  for each pH value where  $v$  = reaction rate without inhibitor and  $v_i$  = reaction rate in the presence of inhibitor.

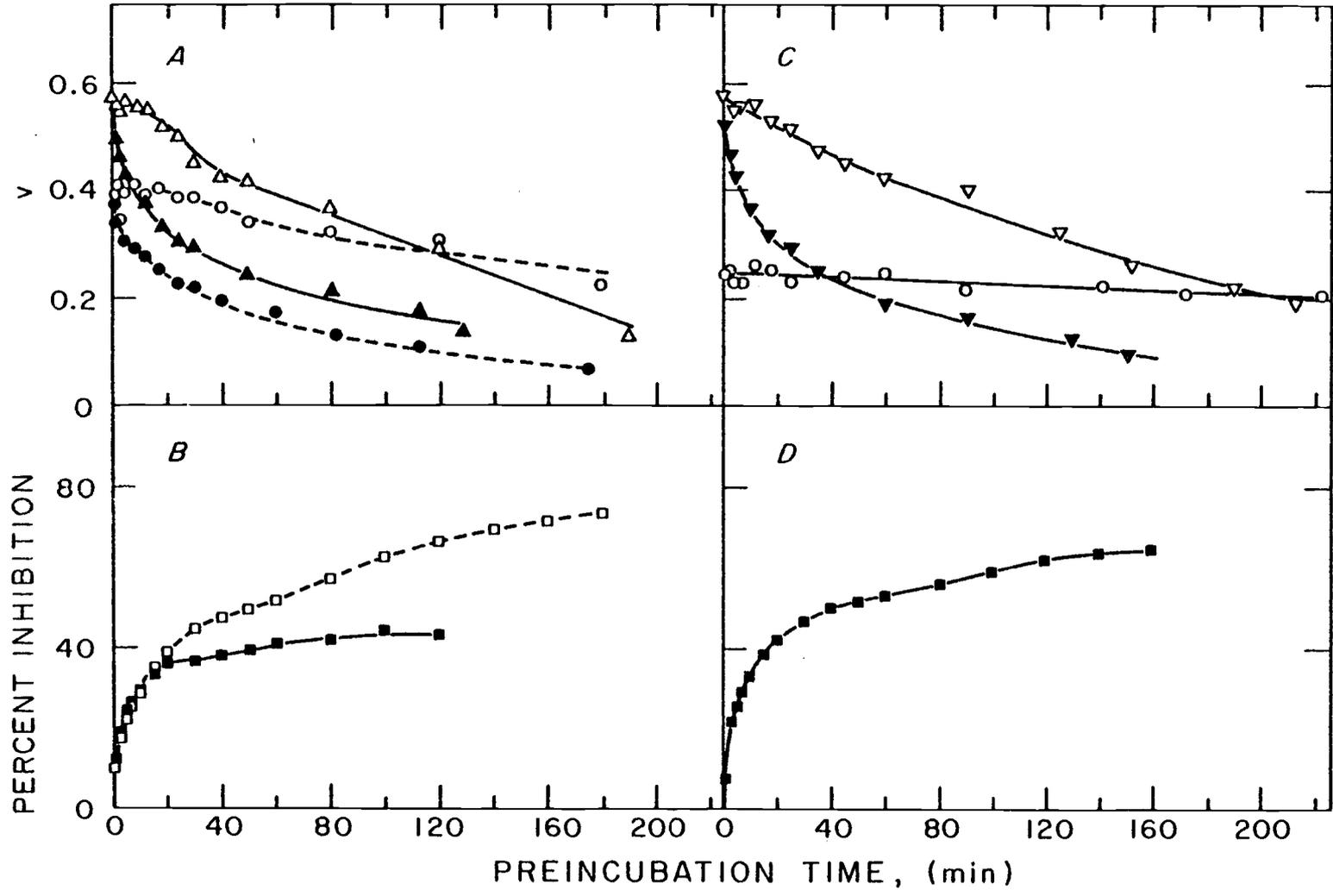
From this pH study, it is obvious that HCP inhibits G6PD at pH 4.5 and above the pH dependence curve for the enzyme in the presence of 10.6  $\mu\text{M}$  HCP was similar to the one without HCP, with pKa's of 6.3 and 9.8, respectively (Figure 3A). Thus it seems that there were two maximum inhibition regions, one at a pH of about 5-6.2 and another around pH 8-10 (Figure 3B). The inhibitory effect of HCP on G6PD and possibly other dehydrogenases was, therefore, pH dependent.

An inhibition of the enzyme by phosphate ions which had been noticed by Theorell (1935) and later by many others such as Engel *et al.* (1969) was also seen in my studies. The points shown in Figure 3 for phosphate ions have been normalized at pH 7.5 to fit the curve. The normalizing factor for the reaction in the absence of HCP was 1.976 and for the reaction in the presence of HCP was 1.312. Hence, both inhibition by phosphate ions and by HCP were not additive.

#### Preincubation time and magnesium ion effect

The inhibition of torula yeast G6PD by HCP was not instantaneous, but increased with the preincubation time (Figure 4B and D). High  $\text{Mg}^{+2}$  concentrations inhibited enzyme activity (Figure 5A), but a low concentration of  $\text{Mg}^{+2}$  was required for maximum activity (Figure 4A and C). Loss of G6PD activity on preincubation with buffer was faster in the presence of 20 mM  $\text{Mg}^{+2}$  but the enzyme was partially protected from HCP inhibition at longer preincubation times (Figure 4B). Comparison of the concentrations of substrates used (0.483 mM  $\text{NADP}^+$  and 0.851 mM G6P) suggests that the observed  $\text{Mg}^{+2}$  effect may be due to ionic strength.

Figure 4. Effect of preincubation time on torula yeast G6PD and its inhibition by HCP. A. G6PD was incubated with Tris-Cl buffer, pH 7.5, in the absence of  $Mg^{+2}$  but with (●···●) and without (○···○) 1.25  $\mu$ M HCP; and was also incubated with the same buffer in the presence of 20 mM  $Mg^{+2}$  with (▲——▲) and without (△——△) 1.25  $\mu$ M HCP. B. Percent inhibition of G6PD by HCP as a function of time. Data were derived from A, in the presence of  $Mg^{+2}$  (■——■) and in the absence of  $Mg^{+2}$  (□···□). C. G6PD was incubated with glycyglycine buffer, pH 7.5, in the absence of  $Mg^{+2}$  and HCP (○——○); and was also incubated with the same buffer in the presence of 20 mM  $Mg^{+2}$  with (▼——▼), and without (▽——▽) 1.25  $\mu$ M HCP. D. Percent inhibition of G6PD by HCP as a function of time. Data were derived from C, in the presence of 20 mM  $Mg^{+2}$  (■——■).



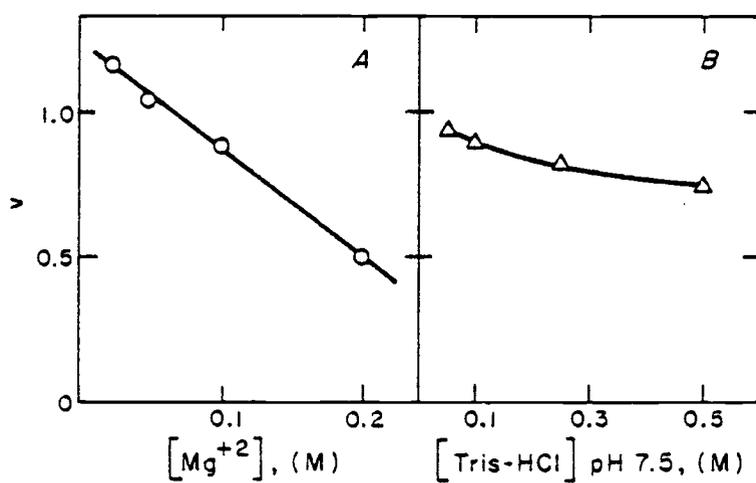


Figure 5. Effect of  $Mg^{+2}$  and Tris on G6PD activity. Standard assay procedure was used except with 3 ml cells and room temperature ( $26^{\circ}C$ ). A. Rate of reaction ( $v$ ) as a function of  $[Mg^{+2}]$ . Buffer used was Tris 0.1 M, pH 7.5. B. Rate of reaction ( $v$ ) as a function of  $[Tris]$ . The  $[Mg^{+2}]$  was fixed at 0.1 M.

The concentration of Tris buffer had little effect on G6PD activity (Figure 5B). At a fixed  $Mg^{+2}$  concentration (0.1 M), a high concentration of Tris buffer slightly reduced enzyme activity.

#### Role of SH groups

The effects of the sulfhydryl reacting reagents, iodoacetate, iodoacetamide, N-ethyl maleimide, and bromopyruvic acid were studied on torula yeast G6PD. Concentrations of iodoacetate or iodoacetamide of 4 mM did not inhibit but slightly activated the enzyme under the assay condition (Table 7). In contrast, 4 mM bromopyruvic acid or N-ethyl maleimide inhibited G6PD activity about 25%. These data suggest that SH groups are not significantly involved in the active site of G6PD but that there may be disulfide bridges which are essential for the enzyme activity as also predicted by Domagk et al. (1969a) for the same enzyme.

No synergistic interaction was observed between HCP and sulfhydryl reagents such as iodoacetamide or iodoacetate at concentrations as high as 4 mM nor could the inhibitory effects of HCP be reversed by addition of 0.3 mM MCE. Moreover, the inhibition pattern of HCP on G6PD (plot of  $v$  versus preincubation time, data not shown) did not change in the presence or absence of iodoacetate.

#### Initial velocity studies

When the concentration of G6P was varied between 0.0426 and 0.851 mM at a constant concentration of  $NADP^+$  (0.483 mM), the rate-substrate concentration response curve was of the classical Michaelis-Menten

TABLE 7  
EFFECT OF SOME SH REAGENTS ON G6PD<sup>a</sup>

Modifiers	Modifier concentration/ (mM)	Percent of control activity
Bromopyruvate	0.4	105.0
	1.0	98.2
	2.0	86.3
	3.0	78.1
	4.0	72.1
Iodoacetate	0.4	105.9
	1.0	105.5
	2.0	113.2
	3.0	105.0
	4.0	107.0
Iodoacetamide	0.4	104.6
	1.0	105.5
	2.0	100.0
	3.0	108.2
	4.0	106.4
N-Ethyl maleimide	0.4	105.5
	1.0	106.4
	2.0	100.5
	3.0	89.5
	4.0	74.4

<sup>a</sup> G6PD (about 1 mg/ml) from torula yeast was dialyzed against 50 mM Tris, pH 7.5, for 5.5 hr at 5°C. The dialyzed enzyme (0.02 ml) was diluted with 50 mM Tris, pH 7.5, to 2.02 ml and 0.02 ml of the diluted solution was used for each assay. Final concentration of enzyme was about 0.076  $\mu$ M.

(1913) type (Figure 6A). A Lineweaver-Burk (1934) plot of these results was linear and extrapolates to an apparent  $K_m$  of  $100 \pm 20 \mu\text{M}$  for G6P (Figure 6C).

Initial velocity studies also indicated a classical hyperbolic response when  $\text{NADP}^+$  was varied in a range of 0.0242 to 0.483 mM at saturating G6P concentration (0.851 mM) (Figure 6B). The double reciprocal plot was also linear and extrapolates to an apparent  $K_m$  of  $45 \pm 20 \mu\text{M}$  for  $\text{NADP}^+$  (Figure 6D). These  $K_m$  values are compatible with those reported by other authors (Table 4).

While enzyme activities were reduced in the presence of HCP (Figure 6A and B), the kinetics were still hyperbolic and there was no evidence for a cooperative binding of substrate molecules to G6PD. This was confirmed by the Hill plots which yielded  $n$  values of 1.0 (Figure 6 insets).

The empirical Hill equation

$$\log \frac{v}{V-v} = n \log[S] - \log K \quad (10)$$

is a form of Michaelis equation and the application of Michaelis-Menten treatment to the derivation of the Hill equation has been given by Atkinson et al. (1965).

#### Inhibition of torula yeast G6PD by HCP

HCP in the micromolar range inhibited torula yeast G6PD (Figure 7A). Under these assay conditions, G6PD was inhibited 60% by 40  $\mu\text{M}$  HCP and almost complete inhibition of the enzyme occurred at 200  $\mu\text{M}$  HCP. The

Figure 6. Substrate saturation curves, Hill plots (inserts) and Lineweaver-Burk plots for torula yeast G6PD in the presence and absence of HCP. A. Variable substrate G6P. Enzyme alone (O—O) and enzyme in the presence of 9.2  $\mu$ M HCP ( $\Delta$ — $\Delta$ ). Filled symbols are corresponding data for Hill plots. B. Variable substrate NADP<sup>+</sup>. Enzyme alone (O—O) and enzyme in the presence of 9.2  $\mu$ M HCP ( $\Delta$ — $\Delta$ ). Filled symbols are corresponding data for Hill plots. C. Lineweaver-Burk plots of the results from A. Enzyme alone (●—●) and with HCP (□—□). D. Lineweaver-Burk plots of the results from B. Enzyme alone (●—●) and with HCP (□—□).

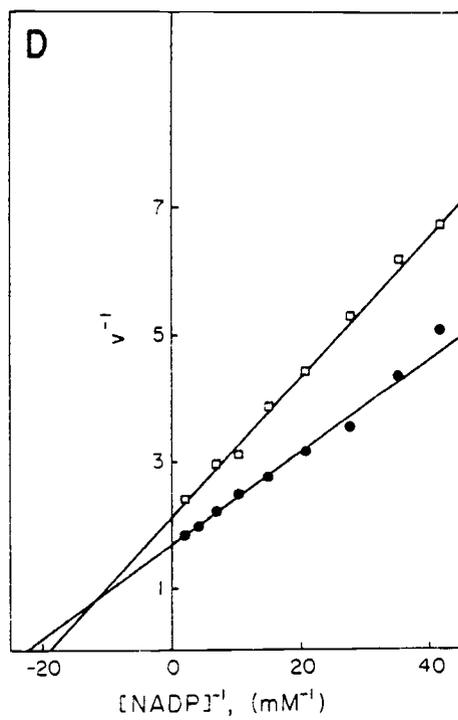
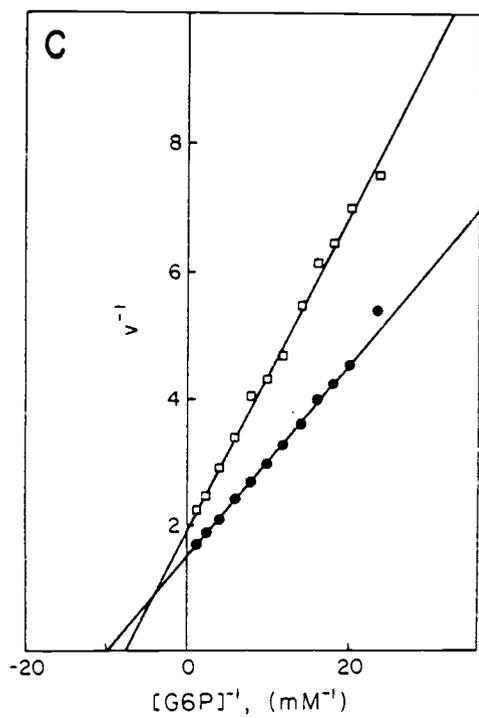
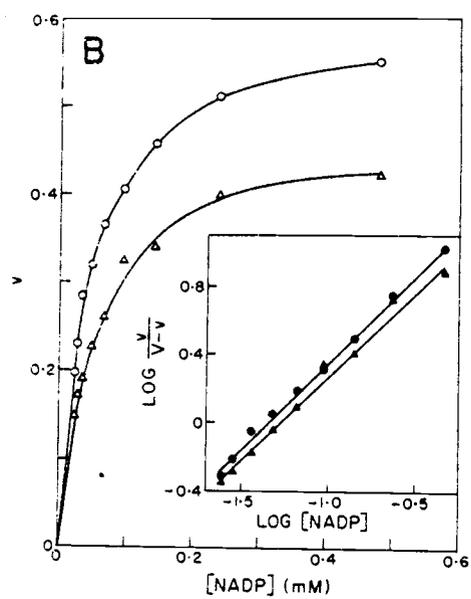
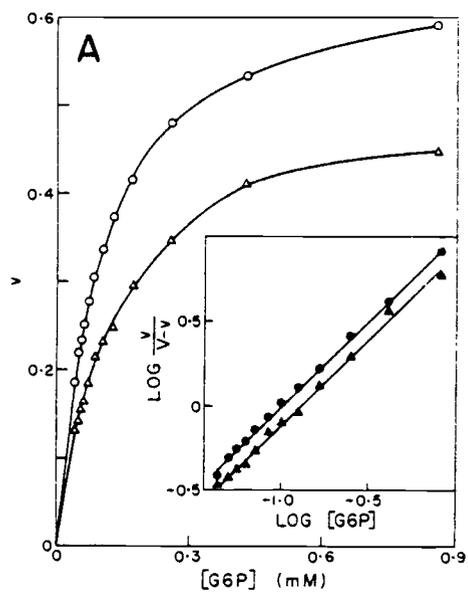
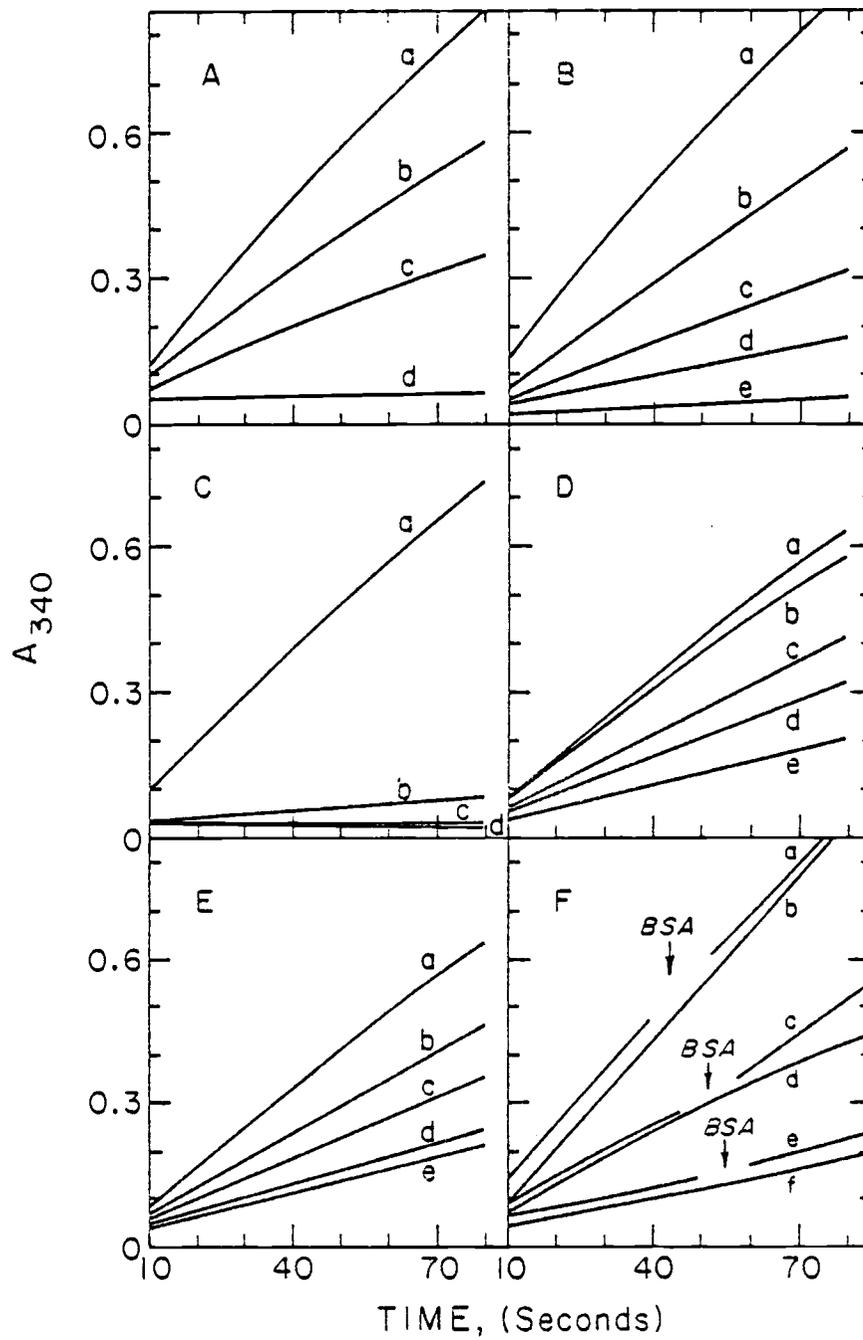


Figure 7. Inhibition of torula yeast G6PD by HCP. Standard assay conditions were employed with HCP as indicated. A. Effect of HCP concentration on G6PD activity. No preincubation was used and the reaction was started by addition of the enzyme. Curve a, enzyme control. Curve b, 20  $\mu\text{M}$  HCP. Curve c, 40  $\mu\text{M}$  HCP. Curve d, 200  $\mu\text{M}$  HCP. B. Effect of preincubation on the inhibition of G6PD by HCP. The reaction was started by addition of  $\text{NADP}^+$  and G6P. Curve a, enzyme preincubated for 15 sec. Curve b, enzyme preincubated with 4  $\mu\text{M}$  HCP for 15 sec. Curve c, enzyme preincubated with 4  $\mu\text{M}$  HCP for 1 min. Curve d, enzyme preincubated with 4  $\mu\text{M}$  HCP for 3 min. Curve e, enzyme preincubated with 4  $\mu\text{M}$  HCP for 5 min. C. Effect of coenzymes on the inhibition of G6PD by HCP. Curve a, enzyme preincubated for 3 min and the reaction initiated by addition of  $\text{NADP}^+$  and G6P. Curve b, enzyme was preincubated with 20  $\mu\text{M}$  HCP and  $\text{NADP}^+$  for 3 min and the reaction started by addition of G6P. Curve c, enzyme was preincubated with 20  $\mu\text{M}$  HCP and G6P for 3 min and the reaction started by addition of  $\text{NADP}^+$ . Curve d, enzyme was preincubated with 20  $\mu\text{M}$  HCP for 3 min and the reaction started by addition of  $\text{NADP}^+$  and G6P. D. Influence of order of addition of  $\text{NADP}^+$  on the inhibition of G6PD by HCP. Various components of the assay mixture were added in the indicated sequence, the mixture preincubated for 3 min (when HCP was present, the inhibitor and enzyme were in contact for 3 min) and the reaction then started by addition of G6P or G6P and  $\text{NADP}^+$ . Curve a, enzyme control. Curve b, preincubation components added in the order  $\text{NADP}^+$ , HCP and enzyme. Curve c, preincubation components added in the order enzyme,  $\text{NADP}^+$  and HCP. Curve d, preincubation components added in the order enzyme, HCP and  $\text{NADP}^+$ . Curve e, enzyme preincubated with 4  $\mu\text{M}$  HCP. E. Influence of order of addition of G6P on the inhibition of G6PD by HCP. Various components of the assay mixture were added in the indicated sequence, the mixture preincubated for 3 min (when HCP was present, the inhibitor and enzyme were in contact for 3 min) and the reaction then started by addition of  $\text{NADP}^+$  or  $\text{NADP}^+$  and G6P. Curve a, enzyme control. Curve b, preincubation components added in the order G6P, HCP and enzyme. Curve c, preincubation components added in the order enzyme, G6P and HCP. Curve d, preincubation components added in the order enzyme, HCP and G6P. Curve e, enzyme preincubated with 4  $\mu\text{M}$  HCP. F. Effect of BSA on the inhibition of G6PD by HCP. BSA (final 0.2 mg/ml) was added at the time indicated. Curves a and b, enzyme control. Curves c and d,  $\text{NADP}^+$  and G6P were added to the reaction mixture containing enzyme and 26  $\mu\text{M}$  HCP to initiate the reaction. Curves e and f,  $\text{NADP}^+$  and G6P were added to the reaction mixture after the enzyme and 5  $\mu\text{M}$  HCP were in contact with each other for 3 min.



inhibitory effect of HCP on G6PD as mentioned before (Figure 4), was markedly dependent on the time in which the bisphenol was in contact with the enzyme. Thus preincubation with HCP resulted in a cumulative loss in activity (Figure 7B).

When G6PD was preincubated for 3 min with 20  $\mu\text{M}$  HCP, activity was completely lost (Figure 7C). Preincubation of the enzyme with HCP in the presence of  $\text{NADP}^+$ , however, partially overcame the inhibitory effect of the bisphenol. No protective effect was shown by G6P under these conditions. At a lower HCP concentration of 4  $\mu\text{M}$ , however, addition of either  $\text{NADP}^+$  or G6P gave partial protection to the enzyme, although  $\text{NADP}^+$  was considerably more effective (Figure 7D and E). The degree of protection of G6PD by both substrates was also very dependent on the order of addition of components of the preincubation mixture. Maximum protection of the enzyme occurred when either substrate was first mixed with HCP and the enzyme added second. Conversely, the greatest inhibition was observed when HCP came in contact with the enzyme prior to addition of either substrate. These results show that both  $\text{NADP}^+$  and G6P offer some protection to torula yeast G6PD against HCP. The substrates may interact with HCP, reducing the effective concentration of the inhibitor since the protective effect was greatest when they were first mixed with the bisphenol.

BSA completely protected torula yeast G6PD against HCP inhibition when it was added to the enzyme prior to addition of the bisphenol (Figure 7F). If the order of addition was changed, so that HCP was mixed with the enzyme before the BSA was introduced, over 50% of the enzyme

activity was lost. When the HCP was in contact with the enzyme for longer time periods, addition of BSA caused only a slight reversal of the inhibition.

#### Effect of HCP on other dehydrogenases

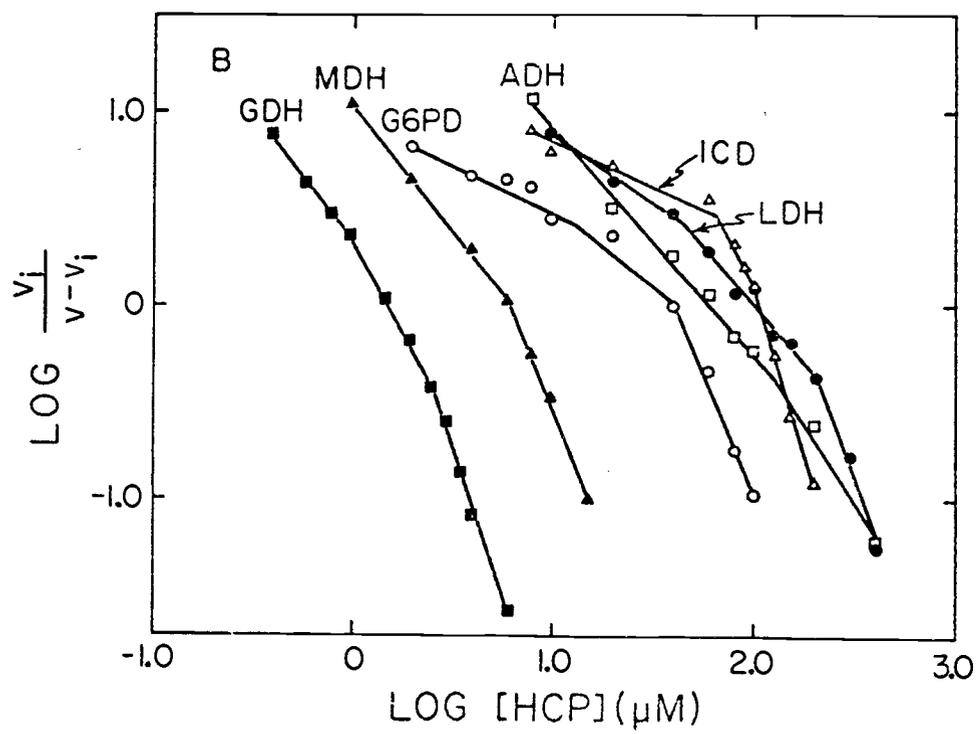
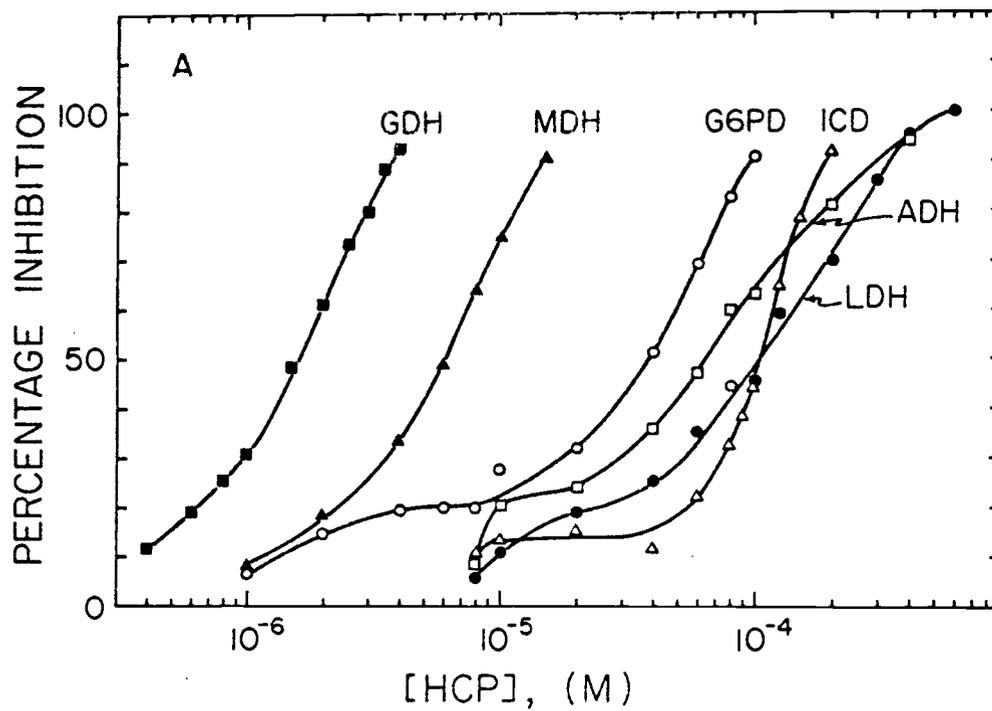
HCP inhibited all of the pyridine nucleotide dehydrogenases tested, yielding  $I_{50}$  values ranging between 1.6  $\mu\text{M}$  for GDH to 110  $\mu\text{M}$  for ICD and LDH (Figure 8A). The extent of inhibition of these enzymes generally also increased upon preincubation with HCP, with ICD being the most sensitive of the six dehydrogenases tested.

The sigmoid shape of the inhibition curves (Figure 8A) suggested the possibility of cooperative interactions between inhibition sites on the enzyme molecule (Monod et al., 1963). These data were plotted according to Jensen and Nester (1966) by equation (11) (for derivation see Segel, 1975).

$$\log \frac{v_i}{v - v_i} = \log K - n' \log [I] \quad (11)$$

where  $v_i$  is the reaction velocity in the presence of inhibitor,  $v$  is the reaction velocity in the absence of inhibitor,  $K$  is the apparent product of  $n$  dissociation constants,  $[I]$  is the inhibitor concentration, and  $n'$  is the interaction coefficient measured between inhibitors. These Hill plots yielded values greater than 1.0 for  $n'$  (Figure 8B and Table 8) indicating that HCP had a cooperative inhibitory effect on the enzymes. The slopes of the plots were not constant, however, and the  $n'$  values for the various enzymes increased with HCP concentration, ultimately reaching values that ranged between 1.62 and 3.33 (the  $n'$  values for

Figure 8. A. Inhibition of various dehydrogenases by HCP. Effect of HCP concentration on the initial reaction rate of bovine liver GDH, beef heart MDH, torula yeast G6PD, pig heart ICD, horse liver ADH and beef heart LDH. B. Hill plots of the data derived from A. The interaction coefficients ( $n'$ ) observed are presented in Table 8.



each linear portion of the Hill plots are given in Table 8). These results suggested that binding of HCP occurred at a minimum of one site during the initial phase of the reaction with the dehydrogenases and to at least three cooperative sites at higher HCP concentrations.

#### Reversibility of HCP inhibition

BSA binds HCP strongly (Miller and Buhler, manuscript in preparation and Figure 22 in part B of this thesis), hence this protein was used to remove HCP from a system which contained a dehydrogenase enzyme and HCP. Addition of BSA to G6PD in the presence of HCP caused a partial reversal of the inhibition (Figure 7Fc). BSA was highly protective to G6PD against subsequent addition of HCP (data not shown).

The presence of BSA also protected GDH and ICD against the inhibitory effects of HCP (Figure 9). In contrast to the observations with

TABLE 8

HILL COEFFICIENT ( $n'$ ) AND  $I_{50}$  VALUES FOR  
THE INHIBITION OF VARIOUS DEHYDROGENASES  
BY HCP (A SUMMARY OF FIGURE 8B)

Enzyme	$n'$ Values	$I_{50}$ ( $\mu\text{M}$ )
GDH	1.32, 1.95, 3.09	1.6
MDH	1.29, 2.59	6.0
G6PD	0.49, 0.79, 2.52	39
ADH	1.18, 1.62	65
ICD	0.45, 2.07, 3.33	105
LDH	0.70, 1.21, 2.86	105

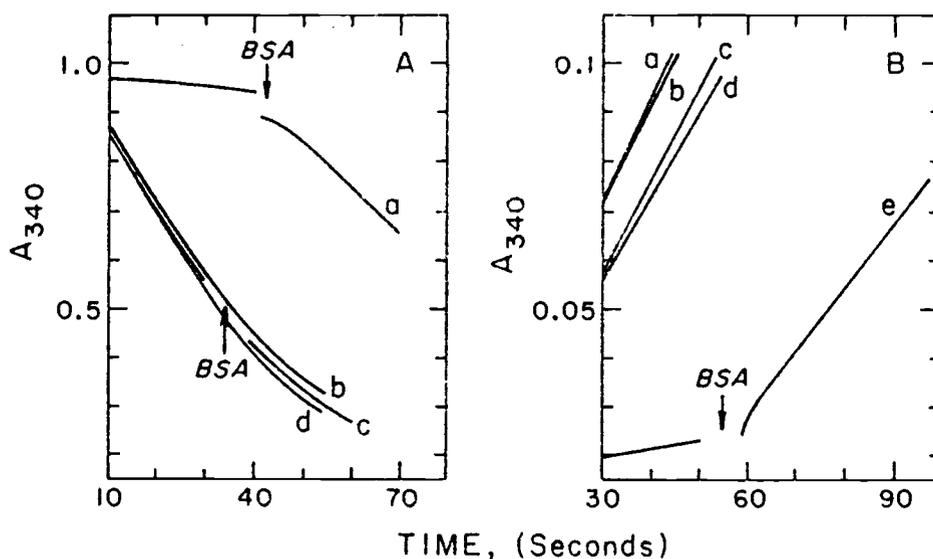


Figure 9. Effect of BSA on the inhibition of bovine liver GDH and pig heart ICD by HCP. A. Reversal of HCP inhibition of GDH by BSA. Various components of the assay mixture were added in the indicated sequence and after the enzyme had been in contact with HCP for the indicated time period, the reaction was initiated by addition of  $\alpha$ -ketoglutarate. The HCP concentration was  $3 \mu\text{M}$  and BSA was added to give a final concentration of  $0.5 \text{ mg/ml}$  as described or indicated. Curve a, preincubation components added in the order enzyme, HCP (enzyme and HCP were in contact for 90 sec prior to initiation of the reaction), NADH, and ammonium acetate; BSA was added after initiation of the reaction at the indicated time. Curve b, preincubation components added in the order enzyme, HCP (enzyme and HCP were in contact for 90 sec prior to initiation of the reaction), BSA, NADH and ammonium acetate. Curve c, preincubation components added in the order enzyme, NADH and ammonium acetate; BSA was added after initiation of the reaction at the indicated time. Curve d, preincubation components added in the order enzyme, BSA, HCP (enzyme and HCP were in contact for 60 sec prior to initiation of the reaction), NADH and ammonium acetate. B. Reversal of HCP inhibition of ICD by BSA. Various components of the assay mixture were added in the indicated sequence and after the enzyme had been in contact with the enzyme for 30 sec, the reaction was initiated by addition of isocitrate. The HCP concentration was  $15 \mu\text{M}$  and BSA was added to give a final concentration of  $0.5 \text{ mg/ml}$  as described or indicated. Curve a, preincubation components added in the order enzyme, BSA, HCP and  $\text{NADP}^+$ . Curve b, preincubation components added in the order enzyme, BSA and  $\text{NADP}^+$ . Curve c, preincubation components added in the order enzyme and  $\text{NADP}^+$ . Curve d, preincubation components added in the order enzyme, HCP, BSA, and  $\text{NADP}^+$ . Curve e, preincubation components added in the order enzyme, HCP and  $\text{NADP}^+$ ; BSA was added after initiation of the reaction at the indicated time.

torula yeast G6PD, however, addition of BSA almost completely restored the activity of the inhibited enzymes.

### Kinetics of HCP inhibition

Dixon and Webb's nomenclature (1964) was used to identify the type of inhibition observed with HCP. The inhibition of G6PD, ICD, and GDH by HCP was reversible (Figure 7F and 9). Lineweaver-Burk plots in the presence of various concentrations of HCP are shown for G6PD (Figure 10A and B), ICD (Figure 11A and B), and GDH (Figure 12). The type of inhibition, the dissociation constant ( $K_i$ ) and the interaction or Hill coefficient ( $n$ ) between dehydrogenase substrates observed are summarized in Table 9. In most cases, inhibition of the three dehydrogenases by HCP followed mixed kinetics. At low HCP concentrations, the inhibited line for G6PD intersected with the control line at the substrate axis in a noncompetitive manner while at higher HCP concentrations the inhibited line intersected with the control line closer to the  $v^{-1}$  axis as observed in the case of mixed-type kinetics (Figure 10A). Thus the type of inhibition of G6PD and ICD appeared to be altered as the concentration of HCP increased. The  $K_i$  values also decreased with increasing HCP concentrations, reflecting a positive cooperative binding (interaction) of HCP with the dehydrogenases. These results are consistent in the finding of  $n'$  values greater than 1.0 (Figure 8B). Although the extent of enzyme inhibition increased at the higher HCP concentrations, there was no indication of a cooperative effect by HCP on substrate binding as evidenced by Hill coefficients ( $n$ ) that varied

Figure 10. Lineweaver-Burk plot for the inhibition of torula yeast G6PD by HCP at various concentrations of substrates. A. [G6P] varied. B. [NADP<sup>+</sup>] varied. Without HCP (●—●); 4 μM HCP (□—□); 9.4 μM HCP (▲—▲); and 16 μM HCP (○—○). C. Replot of slopes. D. Replot of intercepts. [G6P] varied (▼—▼) and [NADP<sup>+</sup>] varied (■—■).

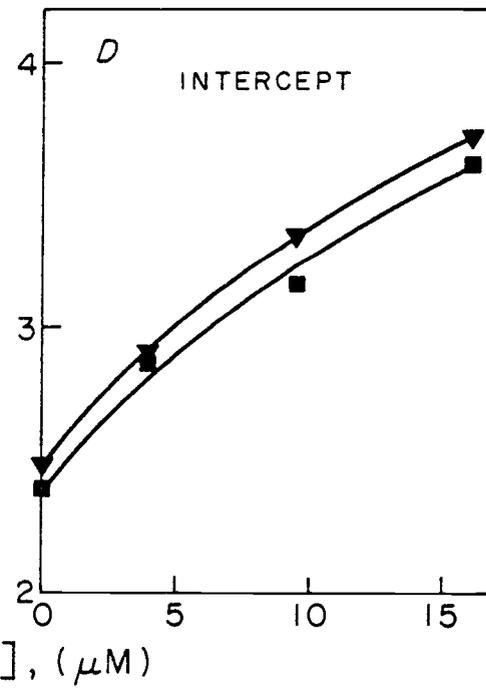
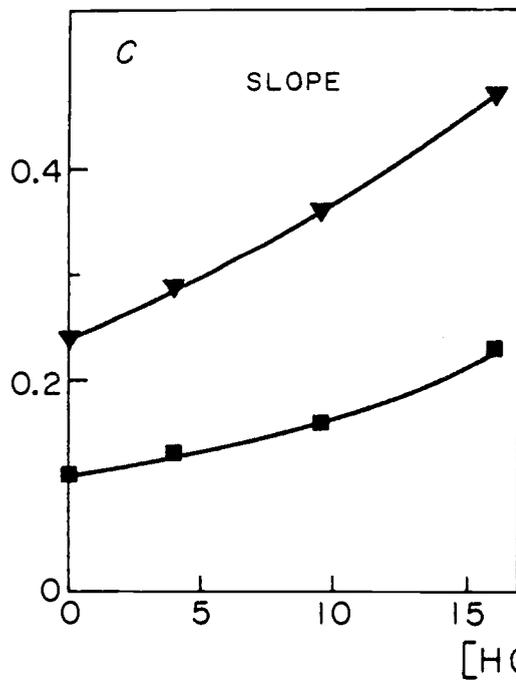
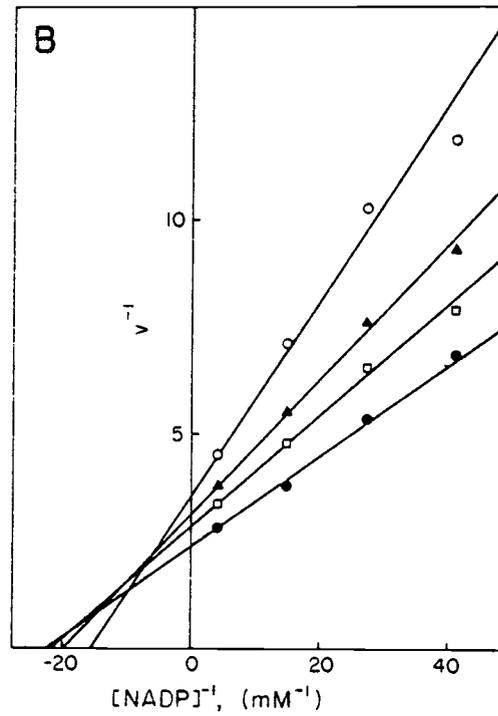
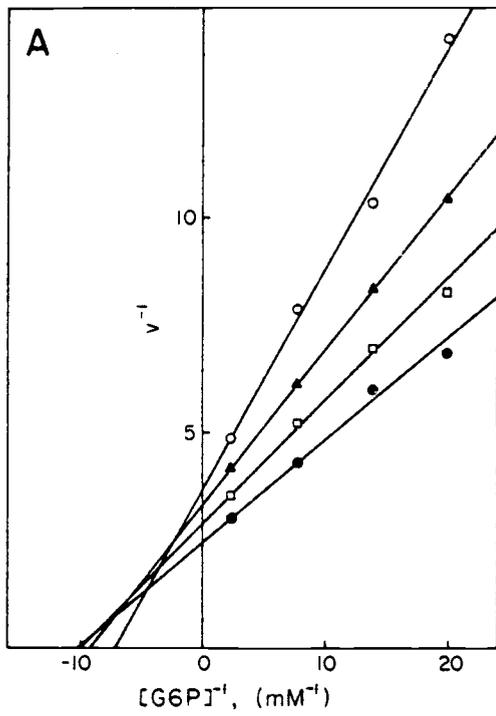


Figure 11. Lineweaver-Burk plots for the inhibition of pig heart ICD by HCP at various concentrations of substrates. A. [Isocitrate] varied. B. [NADP<sup>+</sup>] varied. Without HCP (●—●); 35.1 μM HCP (□—□); and 61.5 μM HCP (▲—▲). C. Replot of slopes. D. Replot of intercepts. [Isocitrate] varied (▼—▼) and [NADP<sup>+</sup>] varied (■—■).

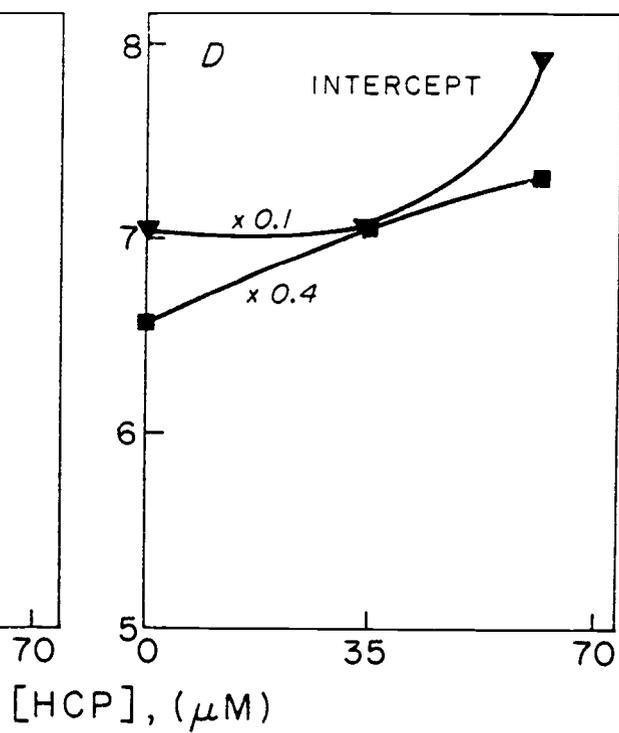
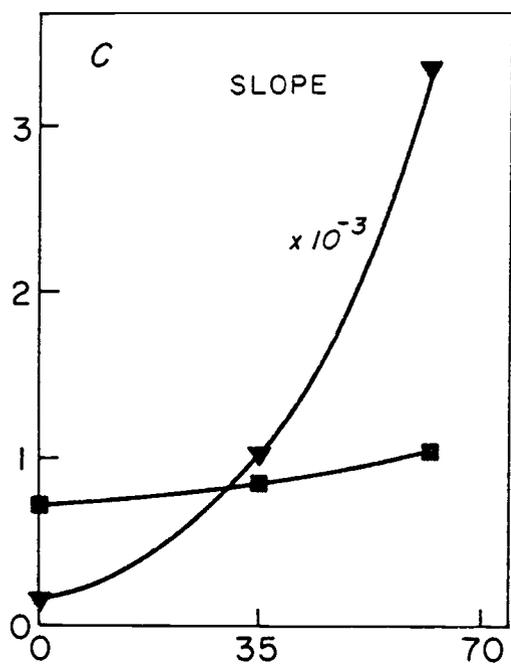
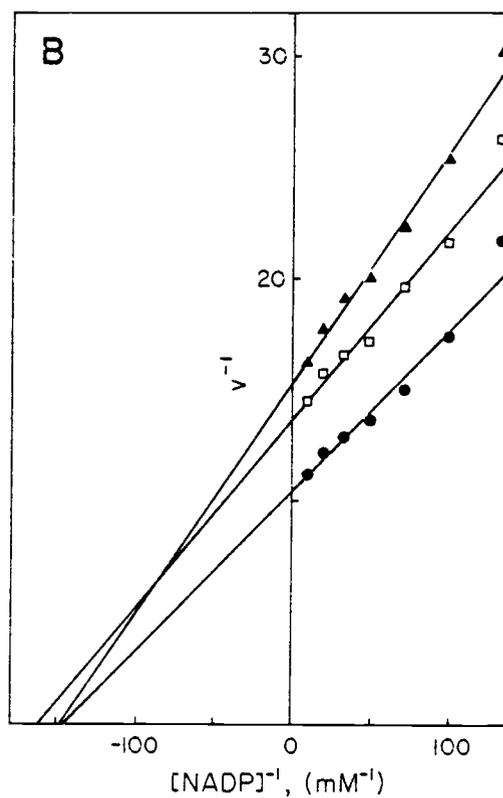
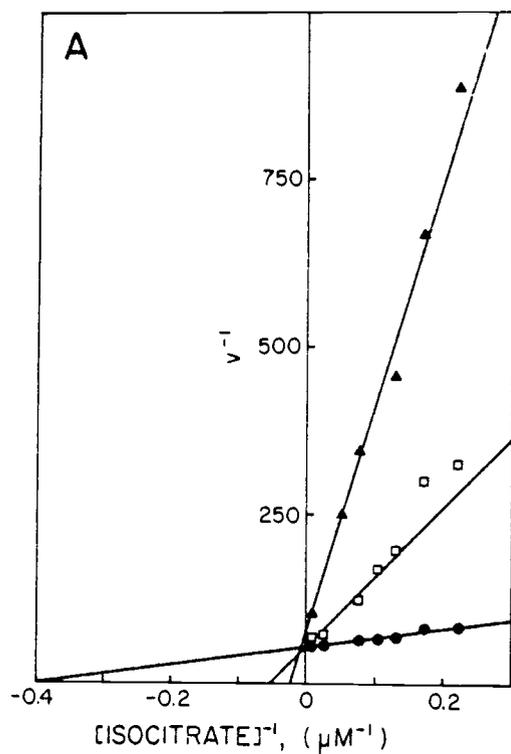


Figure 12. Lineweaver-Burk plots for the inhibition of bovine liver GDH by HCP at various concentrations of substrates. A. [Ammonium acetate] varied. B. [ $\alpha$ -Ketoglutarate] varied. C. [NADH] varied. Without HCP (●—●); 0.67  $\mu$ M HCP (□—□); and 0.86  $\mu$ M HCP (▲—▲).

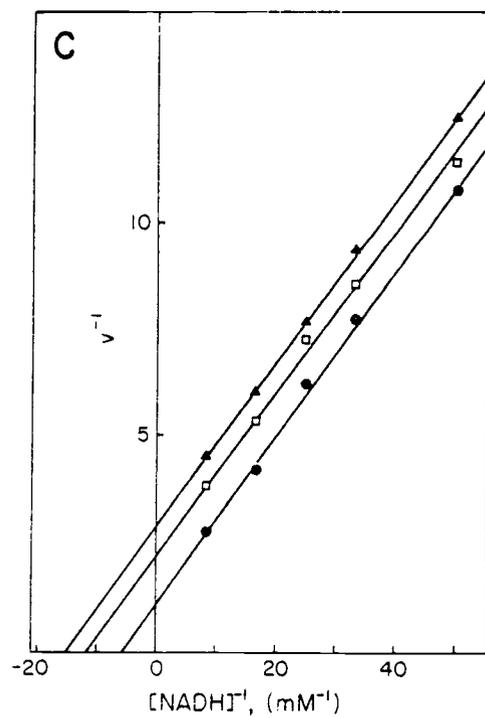
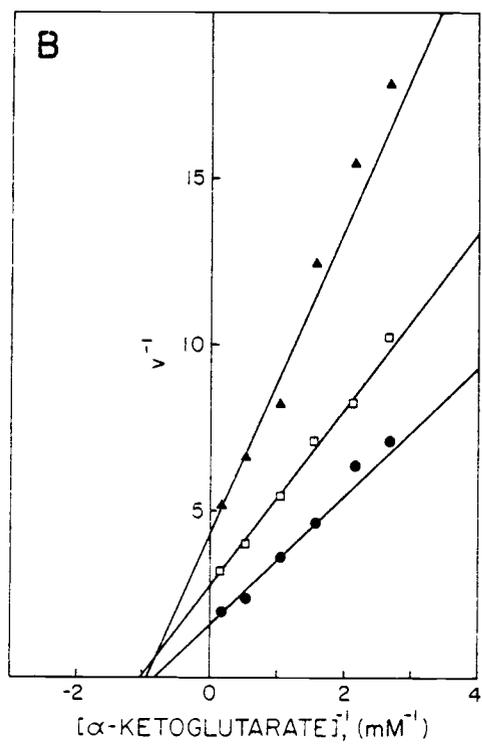
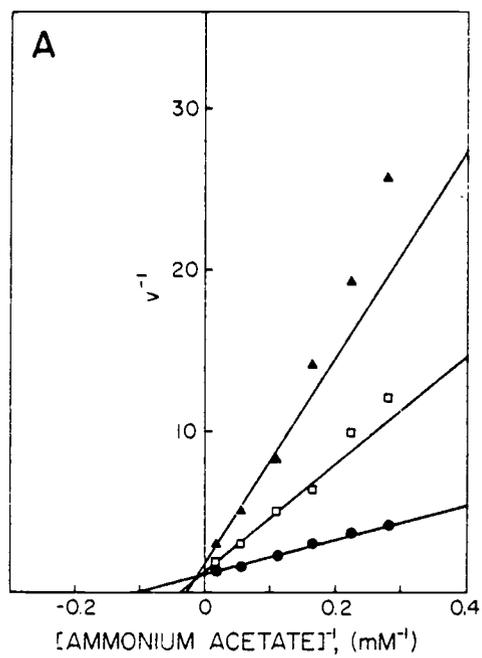


TABLE 9  
 INHIBITION OF GLUTAMATE DEHYDROGENASE, ISOCITRATE DEHYDROGENASE AND  
 GLUCOSE-6-PHOSPHATE DEHYDROGENASE BY HEXACHLOROPHENE<sup>a</sup>

Enzyme	Variable Substrate	[HCP] (μM)	K <sub>m</sub> (μM)	K <sub>i</sub> (μM)	Type of Inhibition	n <sup>b</sup>
Glutamate dehydrogenase	ammonium acetate	0	9,600			0.99
	ammonium acetate	1.18		0.56	mixed	1.04
	ammonium acetate	1.97		0.40	mixed	1.09
	α-ketoglutarate	0	1,250			1.01
	α-ketoglutarate	1.18		1.62	mixed	1.00
	α-ketoglutarate	1.97		1.16	mixed	1.00
	NADH	0	170			1.01
	NADH	0.67		0.86	uncompetitive	0.99
	NADH	0.86		0.54	uncompetitive	1.00
Isocitrate dehydrogenase	isocitrate	0	2.5			0.98
	isocitrate	35.1		5.4	competitive	1.02
	isocitrate	61.5		2.62	mixed	0.99
	NADP <sup>+</sup>	0	6.8			0.85
	NADP <sup>+</sup>	35.1		201	mixed	1.01
	NADP <sup>+</sup>	61.5		140	mixed	1.01
Glucose-6-phosphate dehydrogenase	glucose-6-PO <sub>4</sub>	0	97.2			0.99
	glucose-6-PO <sub>4</sub>	4.0		22.3	noncompetitive	0.99
	glucose-6-PO <sub>4</sub>	9.4		18.4	mixed	1.00
	glucose-6-PO <sub>4</sub>	16.0		13.9	mixed	1.00
	NADP <sup>+</sup>	0	44.1			1.00
	NADP <sup>+</sup>	4.0		17.2	mixed	1.00
	NADP <sup>+</sup>	9.4		19.0	mixed	1.00
	NADP <sup>+</sup>	16.4		13.7	mixed	0.98

<sup>a</sup> Enzymes were assayed under standard conditions.

<sup>b</sup> Interactions or Hill coefficient between substrates.

between 0.97 and 1.00 whether HCP was present or not (Figure 6 and Table 9). These results indicate that HCP does not function as an allosteric effector for torula yeast G6PD, pig heart ICD, and bovine liver GDH. Also the positive slope,  $n = 1.0$ , is interpreted to mean that the substrate binding sites are independent.

The secondary plots of the slopes and intercepts (Plowman, 1972; Segel, 1975) from Figure 10 were not linear functions of HCP concentration but showed slight curvature (Figure 10C and D). Secondary replots of the data in Figure 11 for ICD (Figure 11C and D), however, showed strongly curved slope and intercept curves as HCP concentration was increased, particularly with respect to isocitrate. Similarly, secondary replots of slopes and intercepts from Figure 12 on GDH (Figure 13), yield parabolic curves for both ammonium acetate and  $\alpha$ -ketoglutarate whereas, with respect to NADH, the slope was linear and the intercept curved with increasing HCP concentration. These results suggest, particularly in the case of ICD and GDH, that there may be more than one inhibitory site for HCP on these dehydrogenases.

#### Effect of HCP analogs on G6PD

All of the HCP analogs tested were potent inhibitors of G6PD (Figure 14, Table 10). The  $I_{50}$  values for the various bisphenols ranged between 2.5  $\mu\text{M}$  for 2,2'-oxybis (tetrachlorophenol) to 40  $\mu\text{M}$  for 2,2'-methylenebis (4,6-dichlorophenol) following a general increase in the relative activity of these inhibitors with the degree of chlorination of the aromatic rings. However, there did not appear to be a

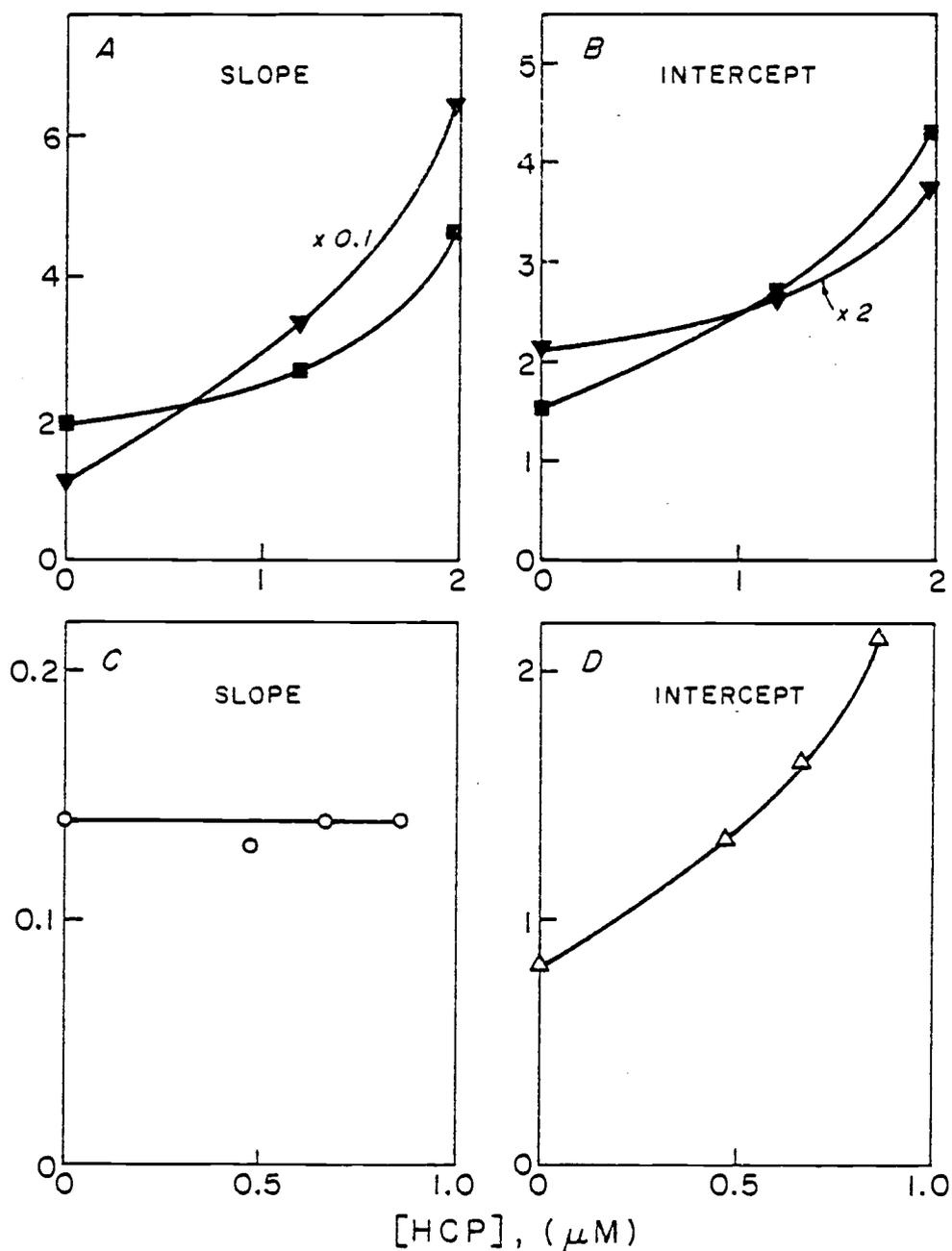


Figure 13. Replots of slopes and intercepts of Lineweaver-Burk plots of HCP inhibition of GDH. A and C. Replots of slopes of Figure 12 [ammonium acetate] varied ( $\nabla$ — $\nabla$ ), [ $\alpha$ -ketoglutarate] varied ( $\blacksquare$ — $\blacksquare$ ), and [NADH] varied (O—O). B and D. Replots of intercepts of Figure 12 [ammonium acetate] varied ( $\nabla$ — $\nabla$ ), [ $\alpha$ -ketoglutarate] varied ( $\blacksquare$ — $\blacksquare$ ), and [NADH] varied ( $\Delta$ — $\Delta$ ).

Figure 14. Inhibition of torula yeast G6PD by various phenols. Standard assay conditions were used except that 42 mM glycylglycine buffer, pH 7.5 was substituted for Tris-HCl buffer. The inhibitors used were dissolved in 0.048 M NaOH. Reactions were initiated by adding G6P and NADP<sup>+</sup> after the enzyme was preincubated with the inhibitor for 40 sec. Inhibitors used were: 2,2'-oxybis (tetrachlorophenol) (●—●); HCP (Δ—Δ); 3,4-TCP (■—■); 2,2'-methylenebis (3,5-dichlorophenol) (○—○); DCP (∇—∇); 2'4-dihydroxy-2,3,3'5,5'6-hexachlorodiphenylmethane (▲—▲); 2,2'-dihydroxy-3,3'5,6,6'-pentachlorophenylmethane (X—X); bithionol (▼—▼); and 2,2'-methylenebis (4,6-dichlorophenol) (◇—◇); 2,4-dinitrophenol (◆—◆). A. Effect of inhibitor concentrations on the reaction rate of torula yeast G6PD. B. Hill plots of the results from A. The maximum interaction coefficients (n') are summarized in Table 10.

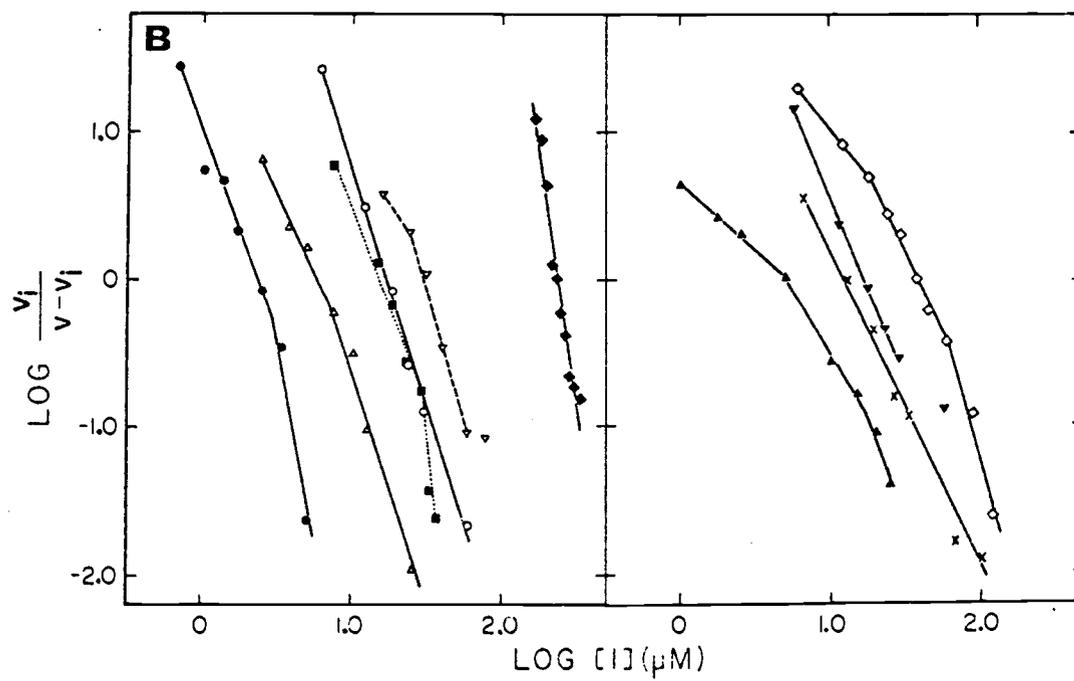
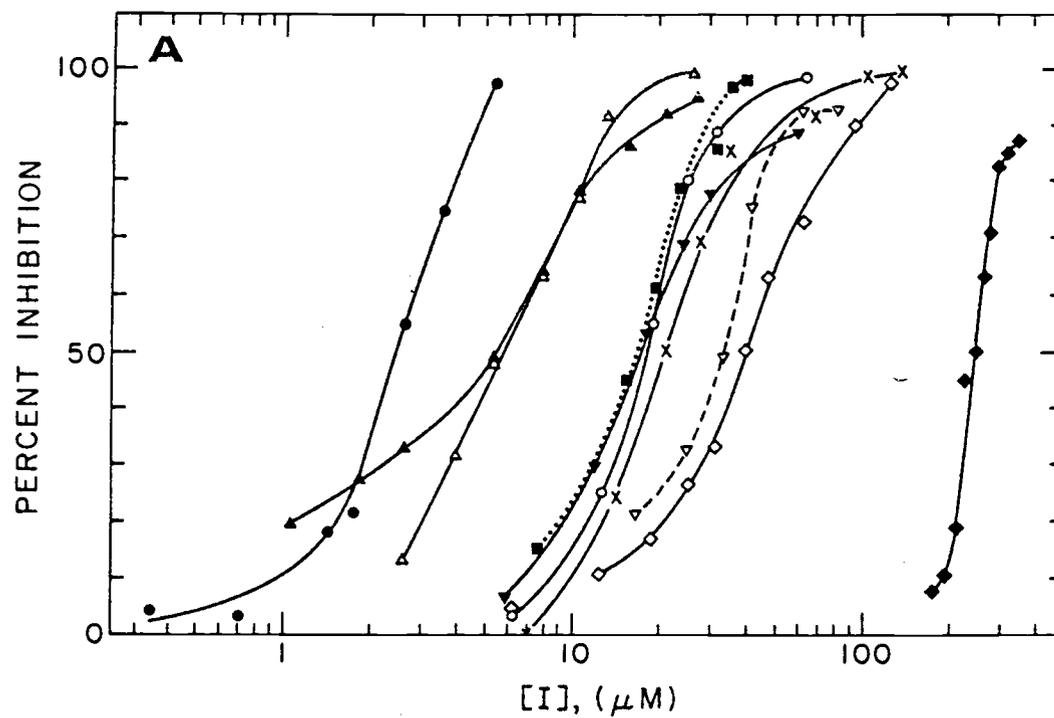


TABLE 10

INHIBITION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE BY HEXACHLOROPHENE AND RELATED COMPOUNDS<sup>a</sup>

Compound	I <sub>50</sub> ( $\mu$ M)	n <sup>b</sup>	pK <sub>a</sub> <sup>c</sup>	Partition Coefficient <sup>c</sup>
2,2'-Oxybis (tetrachlorophenol)	2.5	2.67, 5.24	5.7	8.4
2',4-Dihydroxy-2,3,3',5,5',6-hexachlorodiphenylmethane	5.5	0.89, 1.71, 2.71	8.6	74.6
2,2'-Methylenebis (3,4,5-trichlorophenol); HCP	5.8	2.15, 3.18	5.7	16.1
2,2'-Methylenebis (3,4-dichlorophenol) 3,4-TCP	17	3.01, 4.86	8.1	5.0
2,2'-Thiobis (4,6-dichlorophenol); bithionol	17	2.43	6.1	32.0
2,2'-Methylenebis (3,5-dichlorophenol)	18	3.17	7.6	5.7
2,2'-Dihydroxy-3,3',5,6,6'-pentachlorodiphenylmethane	21	2.12	6.1	120
2,2'-Methylenebis (4-chlorophenol); DCP	34	3.57	9.2	5.0
2,2'-Methylenebis (4,6-dichlorophenol)	40	1.25, 2.23, 3.89	7.0	21.6
2,4-Dinitrophenol	240	7.04	4.0	0

<sup>a</sup> The enzyme was preincubated with the phenols for 40 sec at 30°C prior to addition of G6P and NADP<sup>+</sup>.

<sup>b</sup> Interaction coefficients obtained from different slopes of Hill plot (Figure 14B).

<sup>c</sup> The pK<sub>a</sub> is the dissociation constant of the first bisphenol OH group and the partition coefficient was between hexane and 0.1 M phosphate buffer, pH 6.75 (Nakaue *et al.*, 1972).

good correlation between the inhibition of G6PD and the partition coefficients or pKa's of the various analogs. Previously these physical parameters were found to be closely related to the uncoupling activity of the chlorinated bisphenols (Nakaue et al., 1972).

The percentage inhibition versus inhibitor concentration plots for the various bisphenols were of a sigmoid nature, showing the probable cooperative binding of these phenolic compounds to G6PD (Figure 14A). Hill plots of these data (Figure 14B) provided further evidence for cooperativity, yielding  $n'$  values greater than 1.0 (Table 10). The slope of the Hill plots was not constant but increased with the concentrations of the chlorinated bisphenols. The  $n'$  values for the different bisphenols fell between 2.12 and 5.24 (compared to a value of 7.04 for the phenolic uncoupler, 2,4-dinitrophenol) indicating a minimum binding at two to five cooperative sites, respectively. Inhibitors, unlike substrates, can yield  $n$  values greater than 1.0 in the absence of true cooperative binding. The obvious sigmoidal curves of percent inhibition versus  $[I]$  plot (Figure 14A) suggest that the inhibitors do display cooperative binding at the inhibitor concentrations studied (Segel, 1975).

#### Kinetics of inhibition by HCP analogs

The Lineweaver-Burk plots for G6PD and other chlorinated bisphenols are shown for 3,4-TCP (Figure 15A and B) and DCP (Figure 16A and B). The inhibition kinetics of these HCP analogs (Table 11) are similar to those observed with HCP (Table 9). As previously observed with HCP, the type of inhibition of G6PD appeared to be altered with increasing

Figure 15. Lineweaver-Burk plots for the inhibition of torula yeast G6PD by 3,4-TCP at various concentrations of substrates. A. [G6P] varied. B. [NADP<sup>+</sup>] varied. Without 3,4-TCP (●—●); 13.3 μM 3,4-TCP (□—□); 22.9 μM 3,4-TCP (▲—▲); and 40 μM 3,4-TCP (○—○). C. Replot of slopes. D. Replot of intercepts. [G6P] varied (▼—▼) and [NADP<sup>+</sup>] varied (■—■).

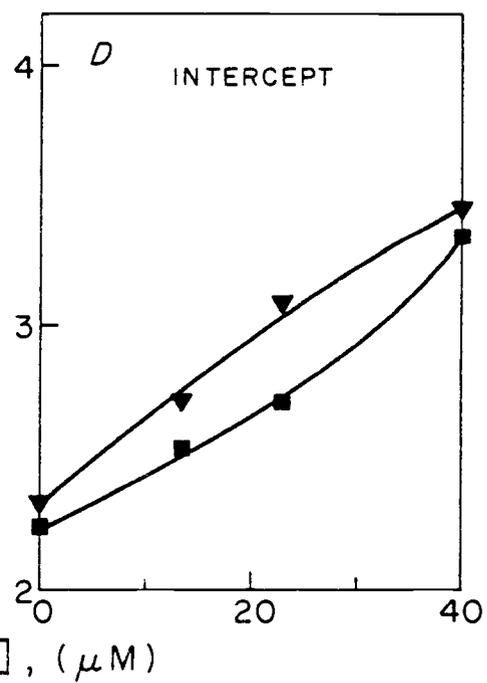
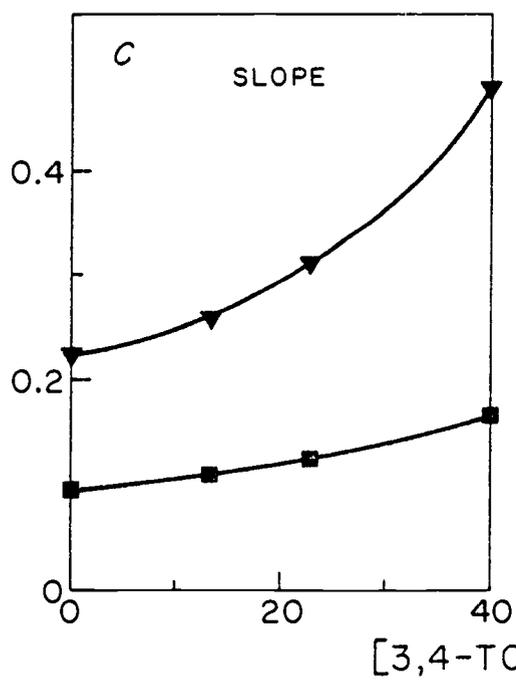
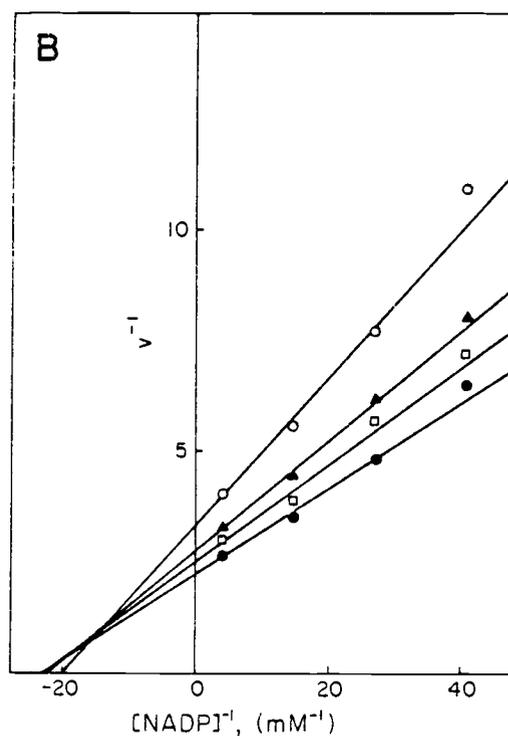
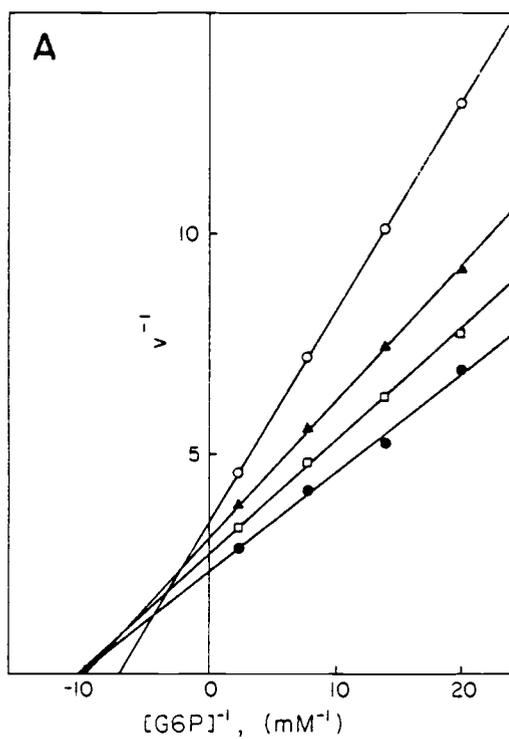


Figure 16. Lineweaver-Burk plots for the inhibition of torula yeast G6PD by DCP at various concentrations of substrates. A. [G6P] varied. B. [NADP<sup>+</sup>] varied. Without DCP (●—●); 66.9 μM DCP (□—□); and 178 μM DCP (▲—▲). C. Replot of slopes. D. Replot of intercepts. [G6P] varied (▼—▼) and [NADP<sup>+</sup>] varied (■—■).

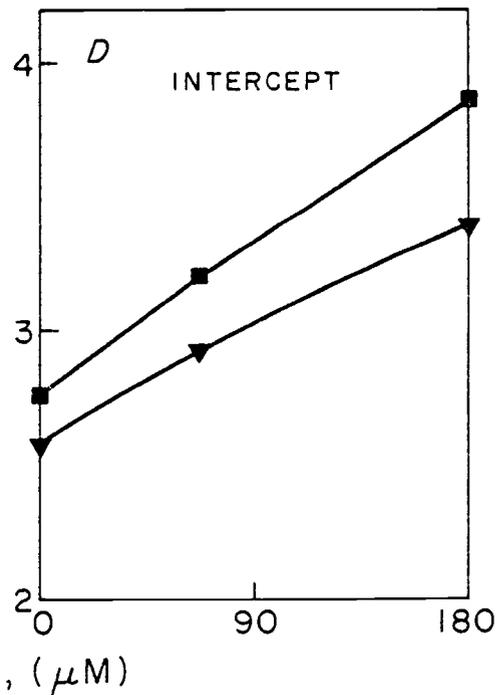
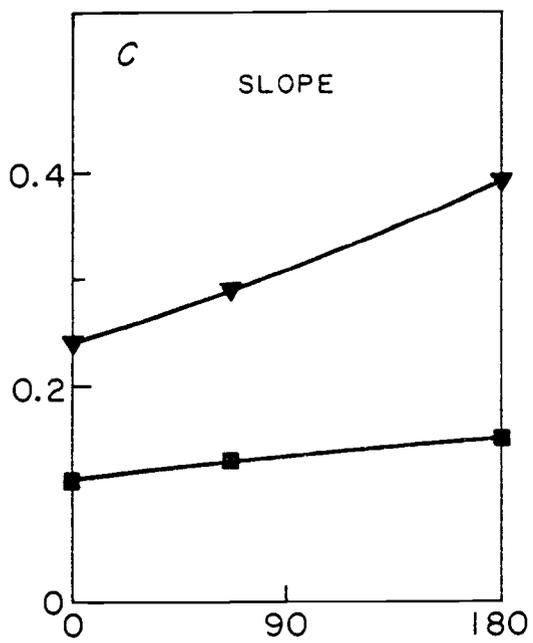
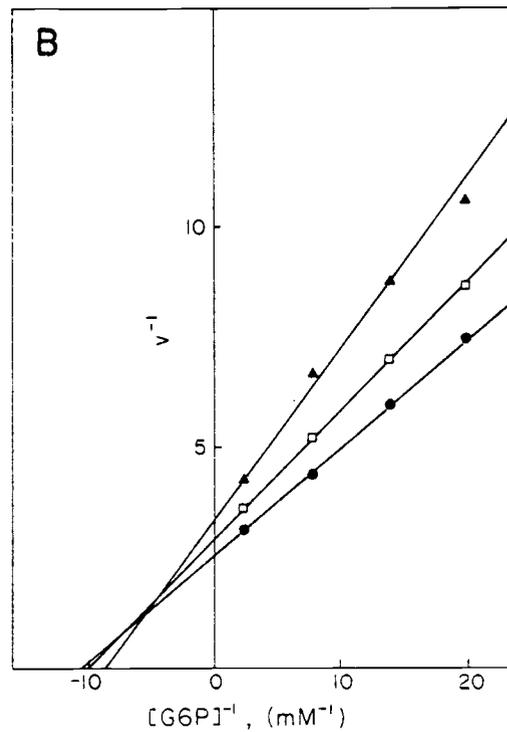
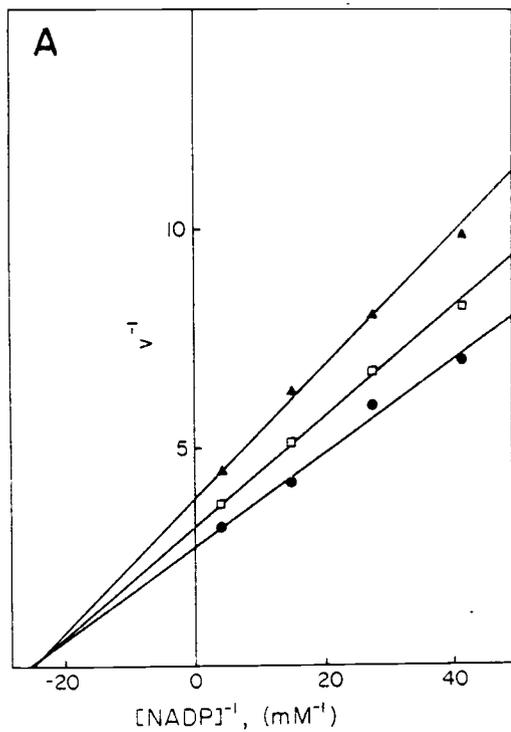


TABLE 11  
INHIBITION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE BY 3,4-TETRACHLOROPHENE AND DICHLOROPHENE<sup>a</sup>

Compound	Concentration ( $\mu\text{M}$ )	Variable substrate	$K_m$ ( $\text{mM}$ )	$K_i$ ( $\mu\text{M}$ )	Hill coefficient (n)	Type of Inhibition
3,4-TCP	0	glucose-6- $\text{P}O_4$	0.0963		1.01	
	13.3	glucose-6- $\text{P}O_4$		80.1	1.00	noncompetitive
	22.9	glucose-6- $\text{P}O_4$		59.1	1.00	mixed
	40.0	glucose-6- $\text{P}O_4$		35.2	1.00	mixed
	0	$\text{NADP}^+$	0.0429		1.16	
	13.3	$\text{NADP}^+$		98.1	0.99	noncompetitive
	22.9	$\text{NADP}^+$		81.8	1.00	mixed
	40.0	$\text{NADP}^+$		55.9	1.01	mixed
DCP	0	glucose-6- $\text{P}O_4$	0.0946		1.00	
	66.9	glucose-6- $\text{P}O_4$		325	1.00	mixed
	178	glucose-6- $\text{P}O_4$		289	0.99	mixed
	0	$\text{NADP}^+$	0.0390		0.99	
	66.9	$\text{NADP}^+$		371	1.00	noncompetitive
	178	$\text{NADP}^+$		418	1.00	noncompetitive

<sup>a</sup> Assayed under standard conditions.

concentrations of 3,4-TCP (Figure 15A and B) and possibly of DCP (Figure 16A). The  $K_i$  values also generally decreased with increasing inhibitor concentration (Table 11), providing further evidence for a cooperative effect of these bisphenol analogs on the enzyme. However, there was no change in the Hill coefficients ( $n$ ) (Table 11) confirming that 3,4-TCP and DCP are not allosteric effectors for the torula yeast enzyme.

Secondary plots of the slopes and intercepts data for G6PD from Figure 15A and B and 16A and B gave curvilinear plots for 3,4-TCP while similar plots for DCP were almost linear (Figures 15C and D and 16C and D). These results are consistent with the binding of 3,4-TCP to at least two separate inhibitory sites on torula G6PD.

#### Partial analysis of HCP inhibition

The previous kinetic studies demonstrated the inhibition of torula yeast G6PD and other dehydrogenases by HCP and its analogs, but it did not identify the mechanism(s) of inhibition. The kinetic method of Reiner (1959) makes it possible to distinguish between several types of inhibition mechanisms. This procedure involves the plotting of the inhibitor concentration ( $I_t$ ) versus the inhibited fraction of enzyme activity divided by the fraction of activity which is not inhibited,  $i/(1 - i)$ . A plot of this type for the inhibition of G6PD by HCP is shown in Figure 17. According to Reiner, if the substrate inhibitor is the only inhibitor, the plot should yield a straight line passing through the origin. My plots, however, were distinctly curvilinear

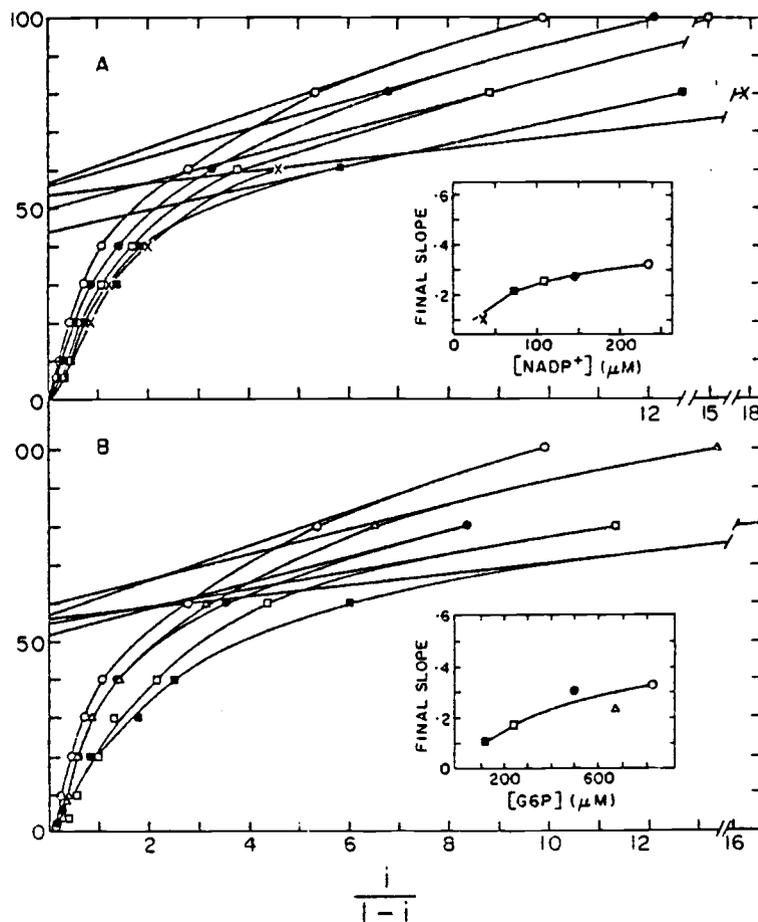


Figure 17. Reiner plots of  $I_t$  versus  $i/(1-i)$  and final slope versus substrate concentration (inserts) for the inhibition of torula yeast G6PD by HCP. The points shown are the means of duplicate assays. A. The  $[G6P]$  was fixed at 851  $\mu M$  and  $[NADP^+]$  was varied: 36.3  $\mu M$  (X—X); 72.6  $\mu M$  (■—■); 109  $\mu M$  (□—□); 145  $\mu M$  (●—●); and 242  $\mu M$  (○—○). B. The  $[NADP^+]$  was fixed at 242  $\mu M$  and  $[G6P]$  was varied: 128  $\mu M$  (■—■); 255  $\mu M$  (□—□); 511  $\mu M$  (●—●); 681  $\mu M$  ( $\Delta$ — $\Delta$ ); and 851  $\mu M$  (○—○).

for lower concentrations of both  $\text{NADP}^+$  and G6P indicating that inhibition by the substrate-inhibitor complex was not the only mechanism for HCP inhibition of G6PD. Similar Reiner plots were obtained for other dehydrogenases but are not shown. Extrapolation of the final linear portion of such plots (Figure 17) to the abscissa intercepted at positive values of  $I_t$  but the intercept values did not change in proportion to the substrate concentration. These results suggest (Reiner, 1959) that formation of a substrate-inhibitor complex was not involved in the inhibition by HCP of G6PD or other dehydrogenases.

#### Part B: Studies on the Mechanism of Inhibition of G6PD by HCP

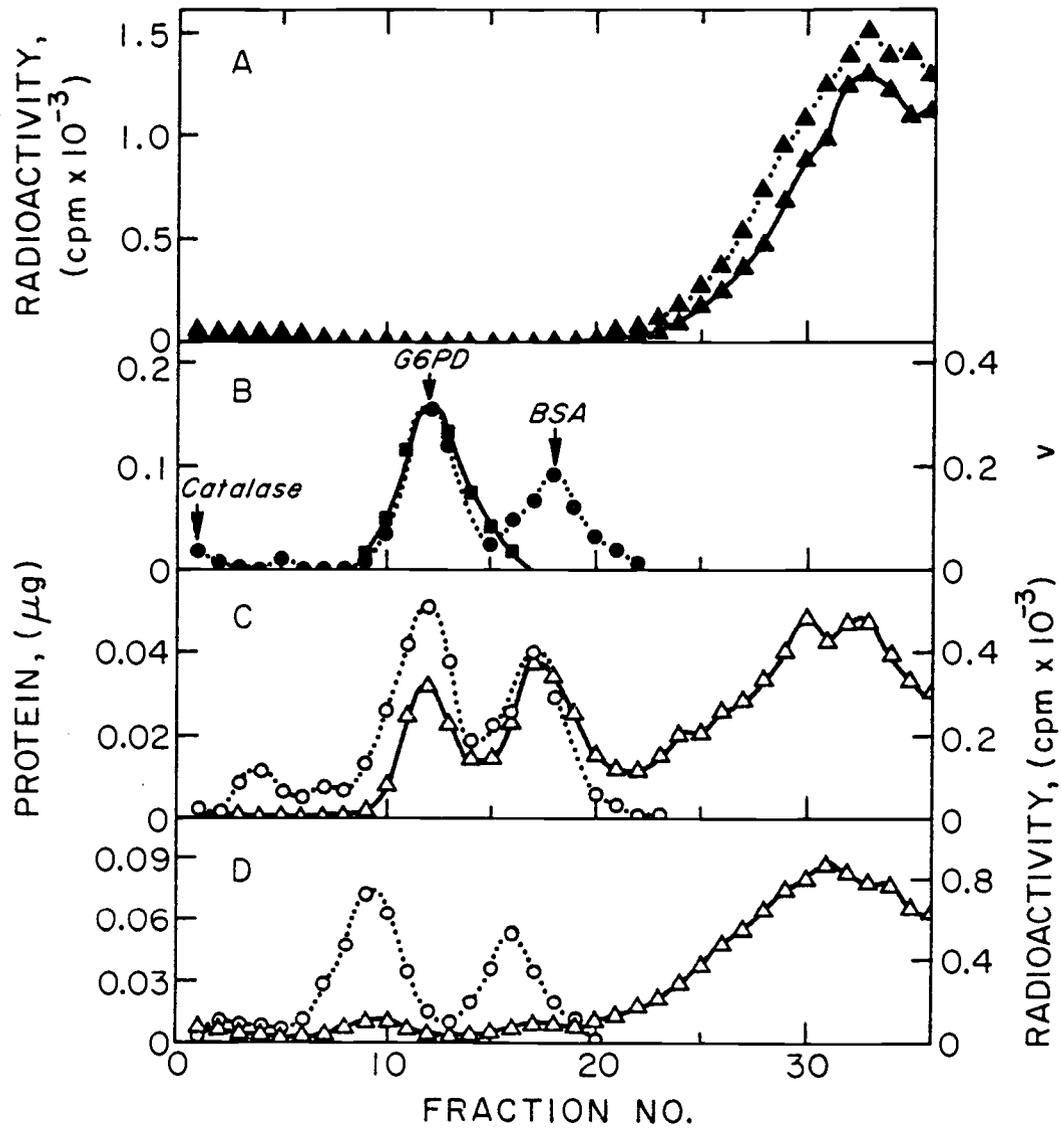
##### Density gradient sedimentation

Sucrose and glycerol were used for density gradient sedimentation studies and the method of Schaffner and Weissmann (1973) was used for protein assay for the fractions. The effect of gradient constituents on protein and G6PD assays are shown in Appendix II.

Torula yeast G6PD gave a single enzymatically active protein peak after fractionation on a sucrose gradient (Figure 18B). The use of the marker proteins catalase (MW 240,000) and BSA (MW 65,000) indicated that the single enzyme activity peak had a molecular weight of approximately 130,000 and was thus the dimeric form of the enzyme. The molecular weight of the G6PD dimer has been reported to be 110,000 (Domagk et al., 1973) and 130,000 (Levy et al., 1966) from torula yeast and mammary gland, respectively.

Figure 18. Characterization of HCP binding to torula yeast G6PD following sucrose density gradient sedimentation. Tubes contained about 0.5 nmole of G6PD and 10 nmoles  $^{14}\text{C}$ -HCP as indicated and/or suitable amounts of BSA and catalase as markers. A. Tube 1 contained  $^{14}\text{C}$ -HCP (radioactivity,  $\blacktriangle\text{---}\blacktriangle$ ). The gradient in tube 2 contained nonradioactive HCP (27  $\mu\text{M}$ ) and  $^{14}\text{C}$ -HCP was added to the top of the tube in the usual manner (radioactivity,  $\blacktriangle\cdots\blacktriangle$ ). B. Tube 3 contained G6PD, BSA and catalase (G6PD enzyme activity,  $\blacksquare\text{---}\blacksquare$ ; and protein,  $\bullet\cdots\bullet$ ). C. Tube 4 contained G6PD and  $^{14}\text{C}$ -HCP (radioactivity,  $\Delta\text{---}\Delta$ ); and protein  $0\cdots 0$ ). D. The gradients of tube 5 contained nonradioactive HCP (27  $\mu\text{M}$ ) and G6PD and  $^{14}\text{C}$ -HCP was added to the top of the tube in the usual manner (radioactivity,  $\Delta\text{---}\Delta$ ; and protein,  $0\cdots 0$ ).

The total volume of the sucrose gradient 5% w/v (0.146 M) and 25% w/v (0.73 M) was 4.6 ml in 0.05 M Tris-Cl buffer, pH 7.5. Centrifugation was for 16 hr using a SW 50.1 swinging bucket rotor at 46,750 rpm and 2°C. Twelve drops were collected in each fraction. Aliquots of the various fractions were removed for determination of G6PD enzyme activity, catalase activity, radioactivity or protein.



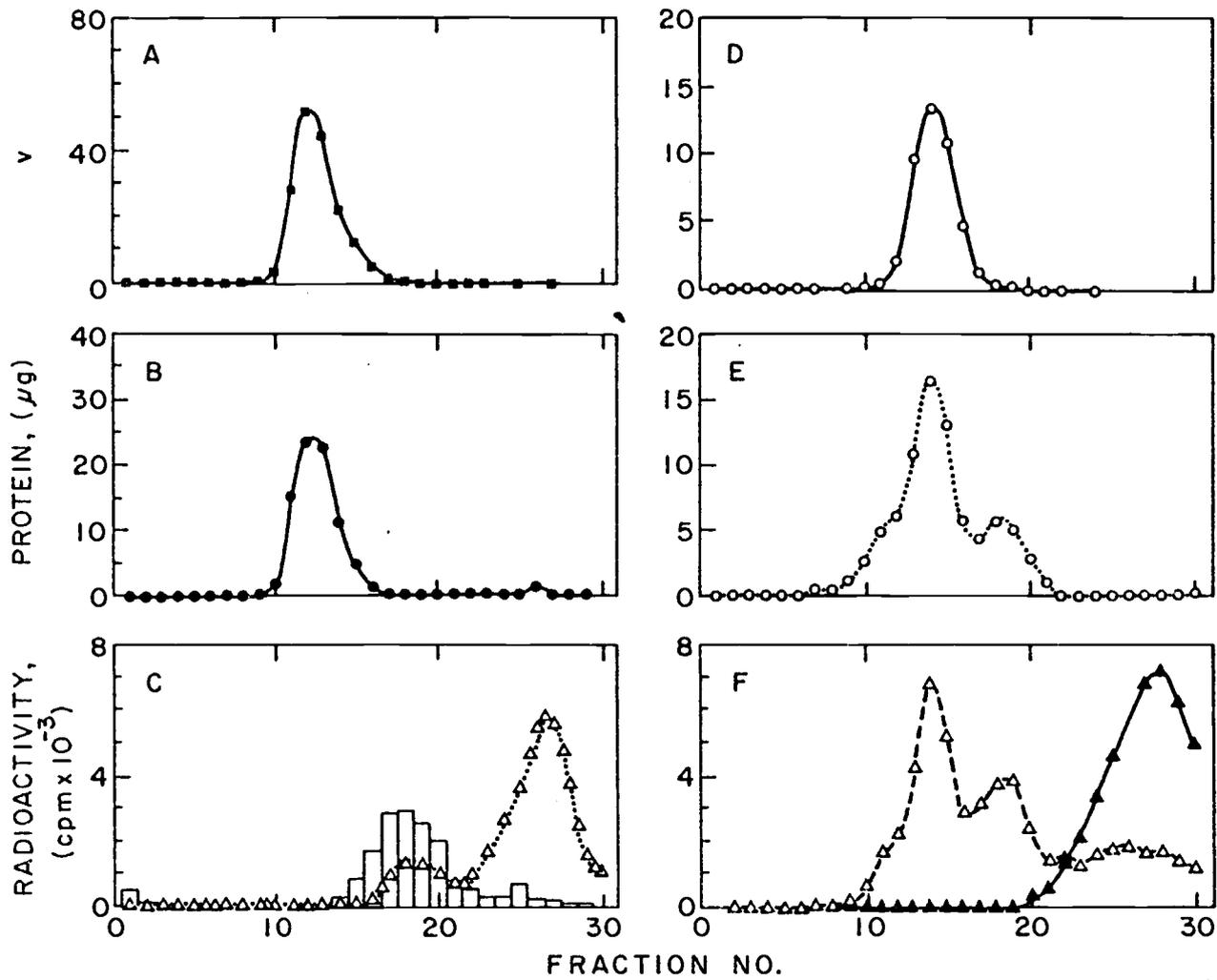
On centrifugation,  $^{14}\text{C}$ -HCP did not sediment in the sucrose gradient but remained at the top of the tubes (Figure 18A). When  $^{14}\text{C}$ -HCP was preincubated with G6PD in a molar ratio of about 13 to 1 and the resulting mixture then centrifuged, radioactivity appeared in two new peaks (Figure 18C). One radioactive peak, also containing protein, migrated in a manner analogous to the native dimeric enzyme. The second radioactive peak also contained protein and sedimented in a manner comparable to that of BSA. These results suggest that HCP causes a partial dissociation of the active dimeric enzyme (MW 130,000) into its inactive monomeric form (Bonsignore et al., 1969) of MW 65,000. The relative proportion of radioactivity in the two peaks varied depending on the particular lot of torula yeast G6PD used and the ratio of HCP to enzyme employed.

The  $^{14}\text{C}$ -HCP bound to G6PD was readily exchangeable. When non-radioactive HCP was included in the gradient used to sediment G6PD following preincubation with  $^{14}\text{C}$ -HCP, the two protein peaks contained considerably reduced amounts of radioactivity and the radioactivity in turn became more evenly distributed throughout the gradient (Figure 18D).

When torula yeast G6PD was exposed to a greater amount of  $^{14}\text{C}$ -HCP (a HCP:G6PD molar ratio of 26), a single peak of reduced enzyme activity was obtained upon sucrose density gradient sedimentation (Figure 19D). Under these circumstances, the enzyme was about 72% inhibited. In addition to the native dimeric enzyme, however, two other protein peaks were found in the presence of HCP; a peak of lower molecular

Figure 19. Sucrose density gradient sedimentation study of HCP binding to torula yeast G6PD. Tubes contained 0.67 nmole G6PD, 17.3 nmoles  $^{14}\text{C}$ -HCP and 1% SDS as indicated. A. G6PD alone (G6PD enzyme activity, ■—■). B. G6PD alone (protein, ●—●). C. G6PD,  $^{14}\text{C}$ -HCP and SDS (radioactivity,  $\Delta\cdots\Delta$ ; estimated protein content, □□□□). D. G6PD and  $^{14}\text{C}$ -HCP (G6PD enzyme activity, □ $\cdots$ □). E. G6PD and  $^{14}\text{C}$ -HCP (protein, O $\cdots$ O). F. G6PD and  $^{14}\text{C}$ -HCP (radioactivity,  $\Delta\text{---}\Delta$ ) and  $^{14}\text{C}$ -HCP alone (radioactivity, ▲—▲).

The gradient was 5-25% w/v sucrose in 0.05 M Tris-Cl buffer, pH 7.5, containing 10  $\mu\text{M}$  NADP $^+$ . Solutions were preincubated at 30°C for 20 min and 0.35 ml samples then applied to the top of the gradient. Tubes were centrifuged at 2°C and 46,750 rpm for 12 hr in a Beckman Model L2 ultracentrifuge with a SW 50.1 swinging bucket rotor. Ten drops were collected in each fraction and a total of 30 fractions collected from each tube.



weight, corresponding to the BSA and Hb protein peak, which presumably represents the G6PD monomers and a small peak of higher molecular weight, perhaps reflecting aggregation of the enzyme of hybridization of the nonequivalent (Chilla and Domagk, 1974) G6PD monomers (Figure 19E). Radioactive HCP was bound to all the components of the mixture (Figure 19F). In the presence of 1% SDS, G6PD was completely dissociated to its monomer which still contained an appreciable amount of bound  $^{14}\text{C}$ -HCP (Figure 19C).

By this density gradient sedimentation technique (Figure 19E), torula yeast G6PD showed the following mean number of moles of HCP bound per 130,000 g of enzyme ( $\pm$  S.D.):  $7.3 \pm 0.8$  (fractions 10-12),  $9.2 \pm 0.6$  (fractions 13-16), and  $17.0 \pm 0.7$  (fractions 18-20). The G6PD monomers thus binds appreciably more HCP per unit weight than the dimeric and aggregated form(s) of the enzyme.

It is possible that the multiple components formed in commercial torula yeast G6PD in the presence of HCP represented impurities or isozymes which migrated as a single peak in the absence of HCP. To test the purity of the torula yeast G6PD used in these studies, the same batch of commercial enzyme as used previously (Figure 19) was subjected to DEAE-Sephadex A-25 chromatography, a procedure known to separate G6PD isozymes (Shedlarski, 1974). It was clear, however, that this lot of commercial G6PD was quite pure already since only one major (97%) enzymatically active peak was detected (Figure 20). The peak fractions 21-26 were combined, concentrated with aquacide II and aliquots of the resulting purified enzyme preparation used

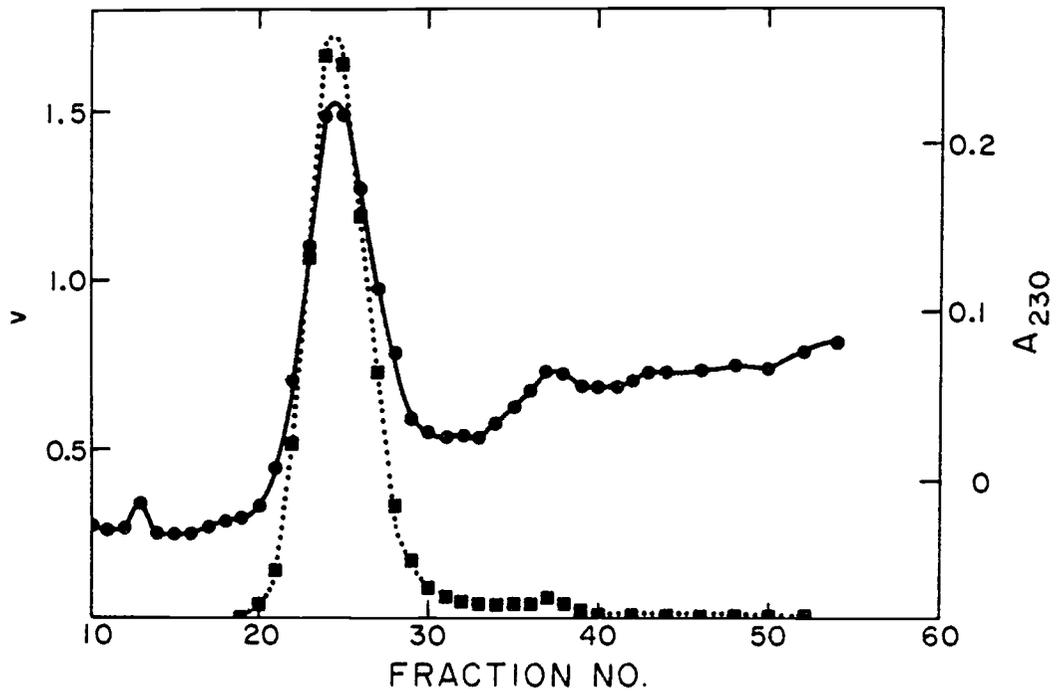


Figure 20. DEAE-Sephadex chromatography. The procedure was described in the Materials and Methods section. G6PD enzyme activity (■····■) and A230 or protein (●—●).

for subsequent studies.

Sucrose density gradient sedimentation of DEAE-Sephadex purified G6PD in the presence of  $^{14}\text{C}$ -HCP at a molar ratio of 36:1 (HCP:G6PD) gave results (Figure 21) very comparable to those found previously with the commercial torula yeast enzyme (Figure 19). A single enzymatically active peak was observed in the presence of HCP (Figure 21D) but enzyme activity was inhibited to about 94% of the control value. Besides the enzymatically active dimer, two additional protein and radioactive (Figure 21E and 21F) peaks were formed in the presence of  $^{14}\text{C}$ -HCP. These results provide further evidence that HCP causes aggregation and dissociation of G6PD. Since the enzyme source was the partially purified enzyme from DEAE-Sephadex chromatography, these results eliminate the possibility of any impurities or isozymes being present in the commercial preparation which could cause the observed complex density gradient patterns produced by HCP.

Under these conditions, the mean binding values ( $\bar{v}$ ) and their S.D. for the three components were:  $6.4 \pm 1.5$  (fractions 10-12),  $13.4 \pm 0.7$  (fractions 13-16), and  $23.7 \pm 0.1$  (fractions 17-20) moles of HCP per 130,000 g of protein. The amount of HCP bound to the various G6PD components was not appreciably changed when the ratio of HCP to G6PD was increased from 26:1 to 36:1.

When torula yeast G6PD and  $^{14}\text{C}$ -HCP were subjected to glycerol density gradient sedimentation, similar results were observed (Figure 22). Formation of aggregates with a molecular weight greater than that of the dimer and the dissociation of dimeric enzyme to monomer

Figure 21. Sucrose density gradient sedimentation study of HCP binding to purified torula yeast G6PD. Tubes contained 0.48 nmole purified (fractions 21-26, Figure 22) G6PD and 17.3 nmoles  $^{14}\text{C}$ -HCP as indicated. A. G6PD alone (G6PD enzyme activity, ■—■). B. G6PD alone (protein, ●—●). C.  $^{14}\text{C}$ -HCP alone (radioactivity, ▲—▲). D. G6PD and  $^{14}\text{C}$ -HCP (G6PD enzyme activity, □····□). E. G6PD and  $^{14}\text{C}$ -HCP (protein, O····O). F. G6PD and  $^{14}\text{C}$ -HCP (radioactivity, Δ---Δ).

The gradient was 5-25% w/v sucrose in 0.05 M Tris-Cl buffer, pH 7.5, containing 10 mM  $\text{NADP}^+$ . Solutions were preincubated at 30°C for 20 min and 0.35 ml samples then applied to the top of the gradient. Tubes were centrifuged at 2°C and 26,750 rpm for 12 hr in a Sorvall Model OTD-2 ultracentrifuge with a SW 50.1 swinging bucket rotor. Ten drops were collected in each fraction and a total of 30 fractions collected from each tube.

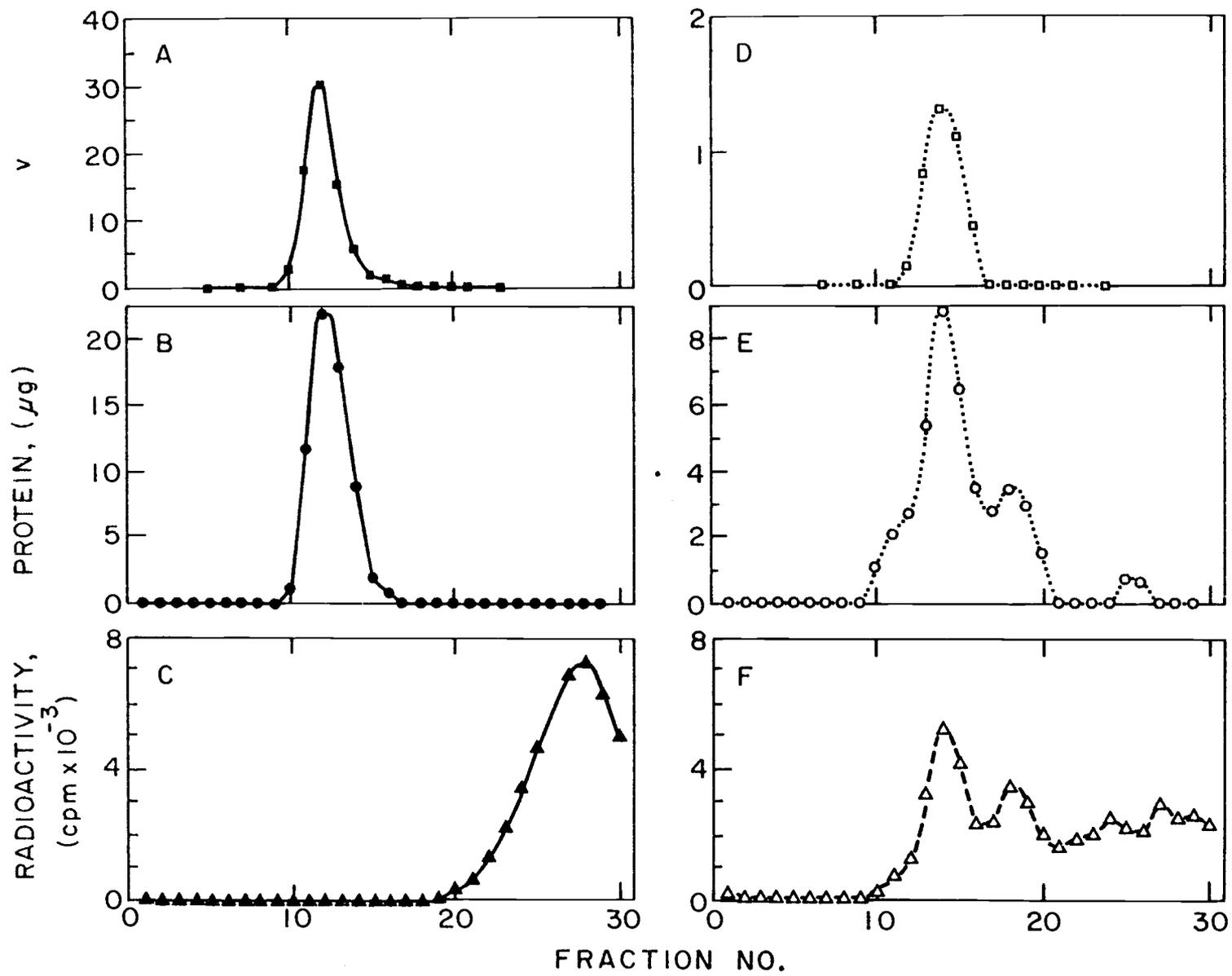
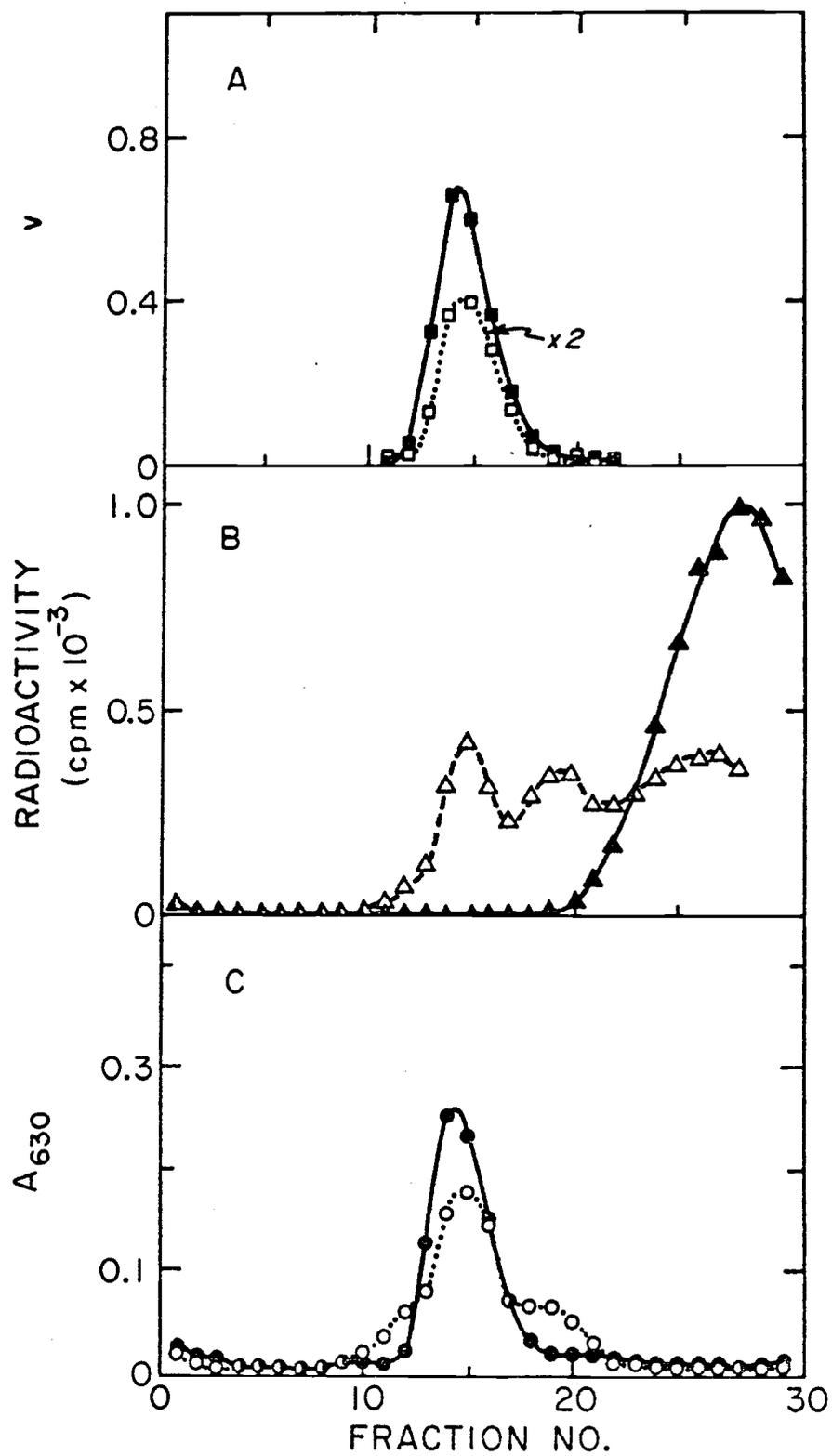


Figure 22. Glycerol density gradient sedimentation study of HCP binding to torula yeast G6PD. About 0.3 nmole G6PD and 9 nmoles  $^{14}\text{C}$ -HCP were used as indicated. A. G6PD enzyme activity (G6PD alone, ■—■; and  $^{14}\text{C}$ HCP and G6PD, □···□). B. Radioactivity ( $^{14}\text{C}$ -HCP alone, ▲—▲); and  $^{14}\text{C}$ -HCP and G6PD, Δ···Δ). C. Protein determination (G6PD alone, ●—●; and  $^{14}\text{C}$ -HCP and G6PD, ○···○).

Glycerol gradient was 8.1-33% w/v in 0.05 M Tris-Cl buffer, pH 7.5, containing 10  $\mu\text{M}$  NADP<sup>+</sup>. The solutions were preincubated at 30°C for 20 min. Samples were centrifuged at 2°C, 50,000 rpm for 12 hr in a Model L2-65 Spinco ultracentrifuge with a SW 50L swinging bucket rotor and immediately fractionated, 10 drops being collected in each fraction.



were consistent with the results of sucrose density gradient sedimentation.

Addition of either G6P and/or  $\text{NADP}^+$  caused a reduction in the binding of  $^{14}\text{C}$ -HCP to both the G6PD dimer as well as the lower molecular weight component (Figure 23). Binding of HCP to G6PD was also reduced in the presence of  $\text{Mg}^{+2}$  and  $\text{K}^+$  ions. No radioactive peaks from  $^{14}\text{C}$ -HCP were found at the G6PD dimer and monomer positions when 53 mM KCl and 21 mM  $\text{MgCl}_2$  were in the incubation mixture and about the same amount of KCl and  $\text{MgCl}_2$  in the gradient (Figure 24F and H). In addition, the inhibition of G6PD activity caused by HCP was reduced in the presence of  $\text{Mg}^{+2}$  as compared to its absence (Figure 24F and 24C).

It has been reported that G6PD from torula yeast exists in two interconvertible species having molecular weights of 110,000 (dimer) and 220,000 (tetramer) and that these tetramers and dimers could be seen upon gel chromatography of G6PD on Sephadex G-150 equilibrated against 20 mM potassium phosphate buffer, pH 5.8, containing 20 mM  $\text{MgSO}_4$  (Domagk et al., 1973). However, I was unable to detect the torula yeast G6PD tetramer in glycerol gradient fractions under similar conditions (Figure 24G).

Effort was made to try to re-activate the enzyme in an aliquot of pooled fractions 12-24 of the tube shown in Figure 24A. This attempt was based on the assumption that a reactivation mixture which contained 0.5 mM  $\text{NADP}^+$ , 0.5 mM EDTA, and 0.2% v/v MCE (Bonsignore et al., 1969) could remove the bound HCP and hence restore some of the enzyme activity

Figure 23. Glycerol density gradient sedimentation study of HCP binding with G6PD from torula yeast. Tubes contained 0.8 nmole torula yeast G6PD and/or 10 nmoles  $^{14}\text{C}$ -HCP. A. G6PD enzyme activity (tube 1: G6PD, ■—■); and tube 2: G6PD and  $^{14}\text{C}$ -HCP, □···□). B. Radioactivity (tube 2: G6PD and  $^{14}\text{C}$ -HCP,  $\Delta$ ··· $\Delta$ ); tube 3:  $^{14}\text{C}$ -HCP,  $\blacktriangle$ — $\blacktriangle$ ; tube 4: G6PD,  $^{14}\text{C}$ -HCP and 8.6  $\mu\text{moles}$  G6P, 0---0; tube 5: G6PD,  $^{14}\text{C}$ -HCP and 2.5  $\mu\text{moles}$   $\text{NADP}^+$ , ●···●) and tube 6: G6PD,  $^{14}\text{C}$ -HCP, 8.6  $\mu\text{moles}$  G6P and 2.5  $\mu\text{moles}$   $\text{NADP}^+$ ,  $\blacktriangle$ --- $\blacktriangle$ ).

Solutions were preincubated at  $30^\circ\text{C}$  for 8 min and then 0.13 ml was added to the top of a 4.6 ml glycerol gradient from 8.1% w/v (0.88 M) to 33% w/v (3.58 M) in 0.04 M glycerol-glycine buffer, pH 7.5. Samples were centrifuged in a Model L-2 Spinco ultracentrifuge with a SW 50.1 swinging bucket rotor at  $4^\circ\text{C}$ , 50,000 rpm for 14.5 hr and immediately fractionated, with 15 drops being collected in each fraction (a total of  $298 \pm 4$  drops were collected in 20 fractions). Aliquots of fractions from tubes 1 and 2 were diluted with glass redistilled water and dialyzed against one liter glass redistilled water at  $4^\circ\text{C}$  for 7 hr with two changes. Aliquots of the dialyzed solutions were assayed for G6PD enzyme activity by the standard procedure except in 0.04 M glycyglycine buffer, pH 7.5. Aliquots of the remaining fractions were used to measure radioactivity.

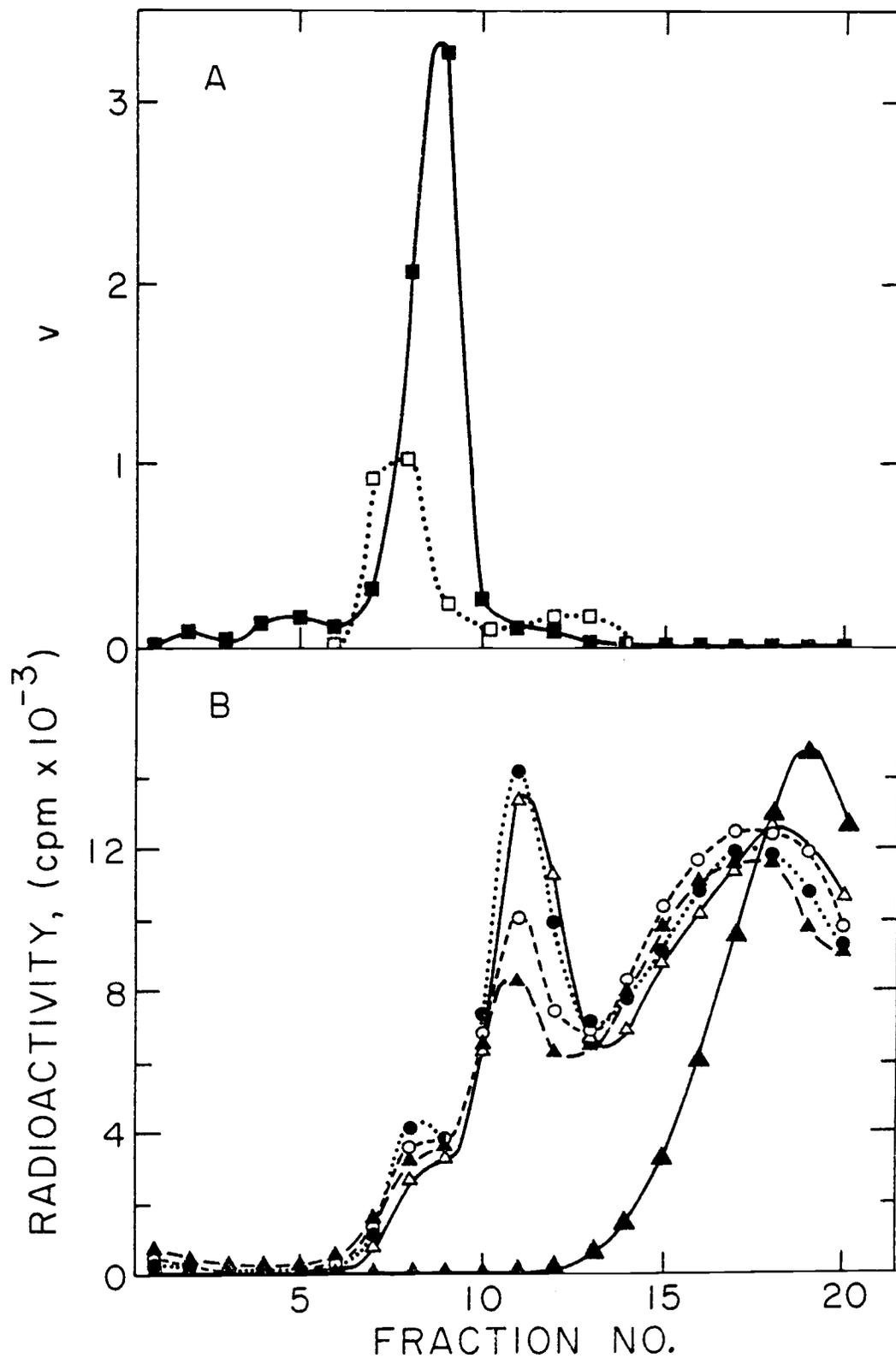
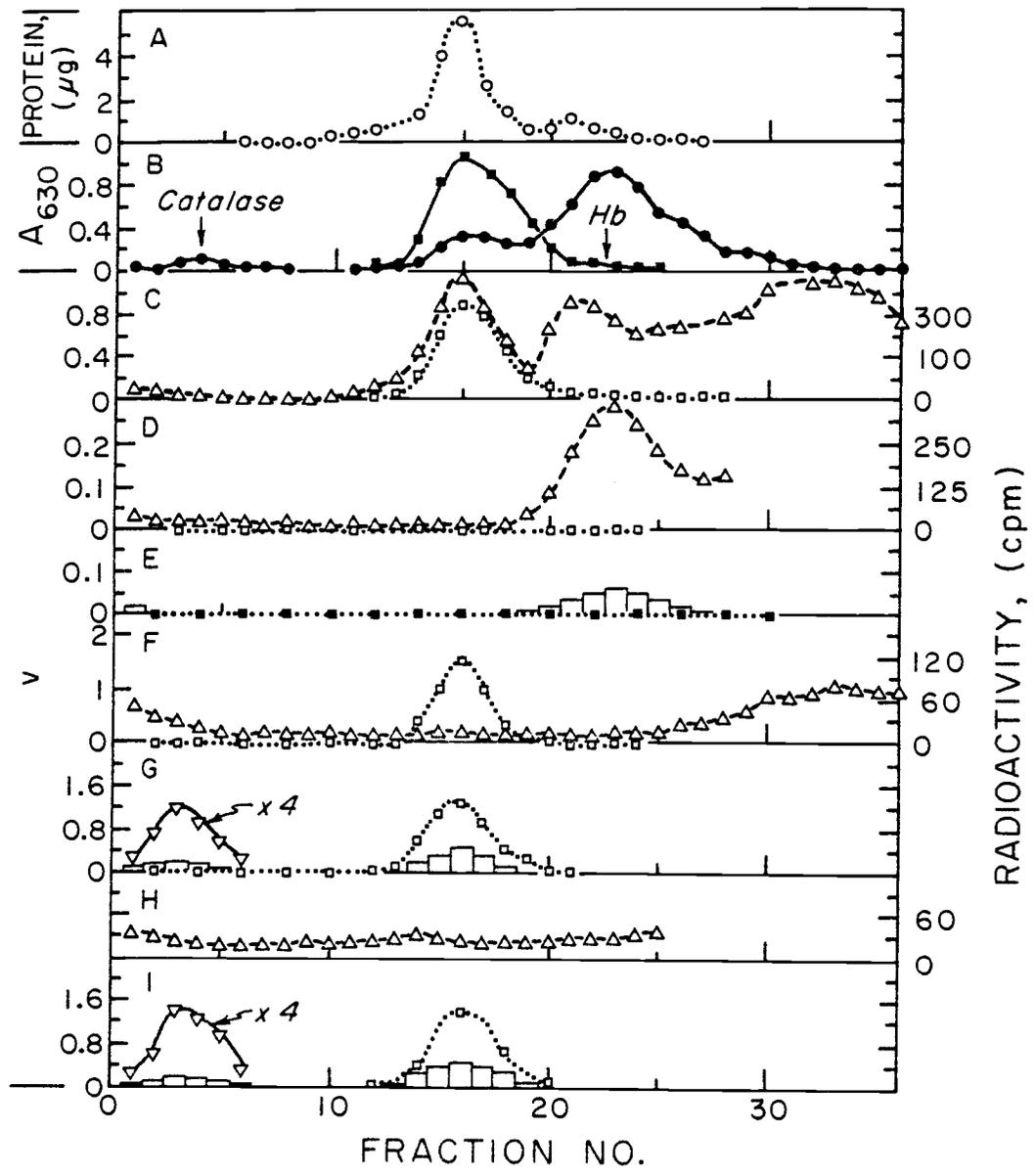


Figure 24. Glycerol density gradient sedimentation study of the influence of metal ions and SDS on the binding of HCP to torula yeast G6PD. Tubes 0.3 nmole G6PD and/or 8 nmoles  $^{14}\text{C}$ -HCP, 22  $\mu\text{g}$  catalase and 0.22 mg beef Hb as indicated. A. G6PD and  $^{14}\text{C}$ -HCP (protein,  $\text{O}\cdots\text{O}$ ). B. G6PD and the marker proteins catalase and Hb (G6PD enzyme activity,  $\blacksquare\text{---}\blacksquare$ ; and protein,  $\bullet\text{---}\bullet$ ). C. G6PD and  $^{14}\text{C}$ -HCP (G6PD enzyme activity,  $\square\cdots\square$ ; and radioactivity,  $\Delta\text{---}\Delta$ ). D. G6PD,  $^{14}\text{C}$ -HCP and 1% SDS (G6PD enzyme activity,  $\square\cdots\square$ ; and radioactivity,  $\Delta\text{---}\Delta$ ). E. G6PD and 1% SDS (G6PD enzyme activity  $\blacksquare\cdots\blacksquare$ ; and estimated protein content,  $\square\text{---}\square$ ). F. G6PD,  $^{14}\text{C}$ -HCP and 21 mM  $\text{MgCl}_2$  in phosphate buffer, pH 5.8, and gradient in the same buffer containing 20 mM  $\text{MgCl}_2$  (G6PD enzyme activity,  $\square\cdots\square$ ; and radioactivity,  $\Delta\text{---}\Delta$ ). G. G6PD, catalase and 21 mM  $\text{MgCl}_2$  in phosphate buffer, pH 5.8, and gradient in the same buffer containing 20 mM  $\text{MgCl}_2$  (G6PD enzyme activity,  $\square\cdots\square$ ; catalase enzyme activity,  $\nabla\text{---}\nabla$ ; and estimated protein content,  $\square\text{---}\square$ ). H. G6PD,  $^{14}\text{C}$ -HCP and 53 mM KCl in phosphate buffer, pH 5.8, and gradient in the same buffer containing 50 mM KCl (radioactivity,  $\Delta\text{---}\Delta$ ). I. G6PD, catalase and 53 mM KCl in phosphate buffer, pH 5.8, and gradient in the same buffer containing 50 mM KCl (G6PD enzyme activity,  $\square\cdots\square$ ; catalase enzyme activity,  $\nabla\text{---}\nabla$ ; and estimated protein content,  $\square\text{---}\square$ ).

Glycerol gradient was 8-36% w/v in 0.05 M Tris-Cl buffer, pH 7.5, or as indicated above. Mixtures were preincubated at  $30^\circ\text{C}$  for 20 min. A Model L2-65 Spinco ultracentrifuge was used with a SW 50L swinging bucket rotor for A-C (tubes 1-3) and a SW 50L swinging bucket rotor for D-I (tubes 4-9). Samples were centrifuged at  $2^\circ\text{C}$ , 50,000 rpm for tubes 1-3 and 46,750 rpm for tubes 4-9 for 16 hr and then immediately fractionated. Fractions contained either 4 or 8 drops. Aliquots of the various fractions were removed for G6PD and catalase assay or counting for radioactivity. Protein was either estimated by dye color intensity on Millipore membrane filter paper or determined quantitatively using BSA as a standard. Hemoglobin was determined by reading the absorbancy at 405 nm.



or that it could reassociate the dissociated inactive monomer (fractions 19-24) to the active dimer (fractions 12-19). After treatment with the reactivation mixture, no restoration of enzyme activity was observed. G6PD activity in fractions 15 and 16, however, were increased about 20%. Further efforts to remove HCP and hence restore G6PD activity in an aliquot of pooled fractions 12 to 24 (Figure 24A) by treatment with PVP were not successful. These results suggest that the dissociation of G6PD by HCP was not readily reversible under the conditions used.

HCP was also found to bind to crystalline BSA (Figure 25A) and freshly prepared rabbit Hb (Figure 25B) following glycerol density gradient sedimentation. The binding of HCP to Hb resulted in an apparent shift towards lighter density of the Hb peak (Figure 25B).

#### Equilibrium dialysis studies

Most equilibrium dialysis experiments with the G6PD from torula yeast and HCP were carried out as described in the first method in the Materials and Methods section. Each dialysis cell contained 0.85 ml of solution. The enzyme concentration in all of the cells was 95 nM (or 80.7 pmole in 0.85 ml) assuming a molecular weight for G6PD of 130,000 (Levy et al., 1966) and the HCP concentration varied over the range of 0.69  $\mu$ M to 47  $\mu$ M.

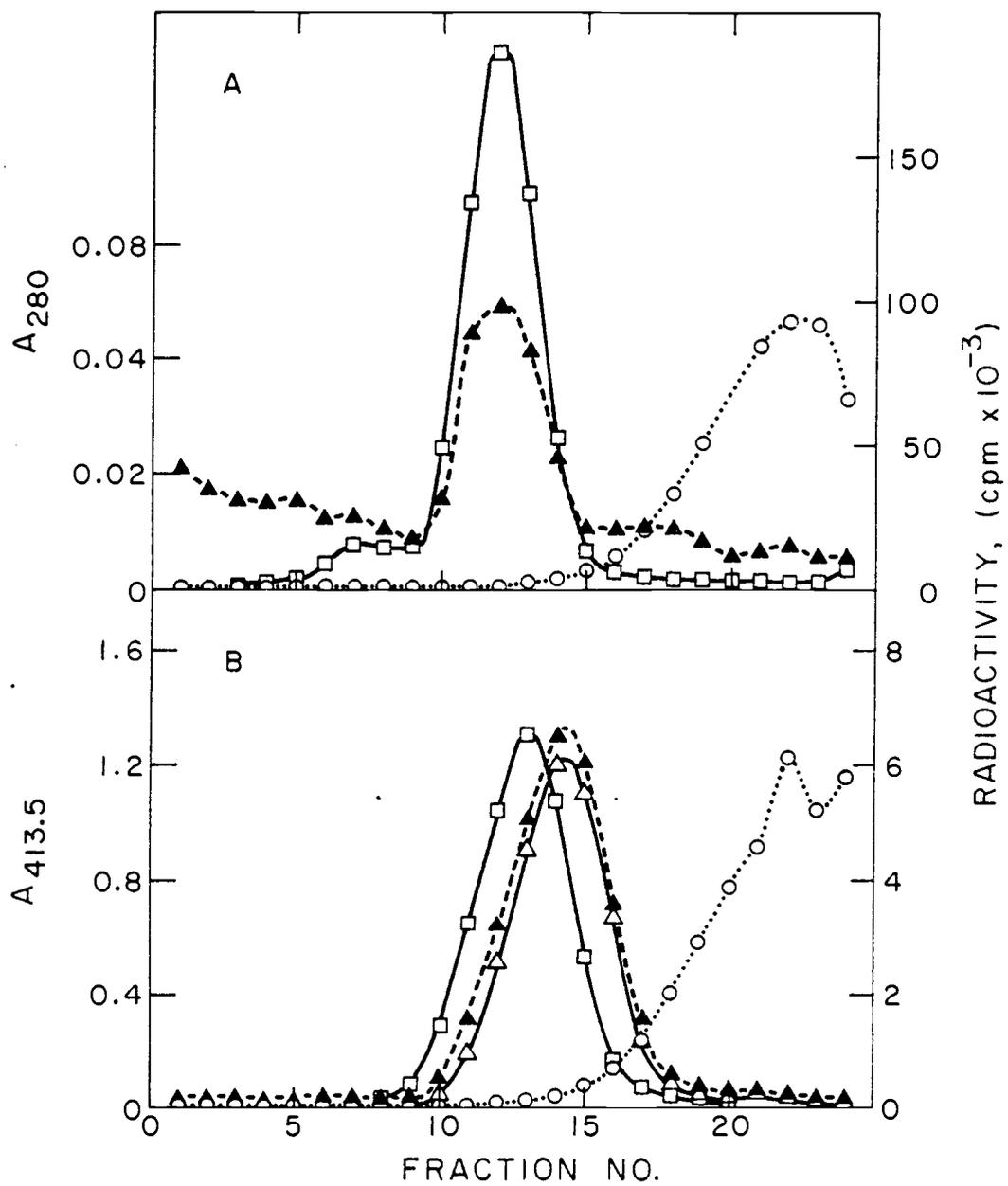
A typical binding curve for lower HCP concentrations at 4°C and for higher HCP concentration at 4°C and 27°C are presented in Figure 26A and B. At 4°C, the binding curve for the lower HCP concentrations

Figure 25. Glycerol density gradient sedimentation study of HCP binding to BSA and Hb from fresh rabbit blood. A. Tube 1 contained 0.3 nmole  $^{14}\text{C}$ -HCP (radioactivity, 0---0); tube 2 contained 7.7 nmoles BSA and 0.3 nmole  $^{14}\text{C}$ -HCP ( $A_{280}$ , ▲---▲); and tube 3 contained 3 nmoles BSA and 0.3 nmole  $^{14}\text{C}$ -HCP (radioactivity, □—□).

The solutions were preincubated at 30°C for 1 hr and then 0.2 ml was added to the top of a 4.6 ml glycerol gradient from 8.1% w/v (0.88 M) to 33% w/v (3.58 M) in a 0.1 M Tris- $\text{PO}_4$  buffer, pH 8.5. Tubes were centrifuged at 2°C, 50,000 rpm for 16 hr in a Model L2-65 Spinco ultracentrifuge with a SW 50L swinging bucket rotor. Following centrifugations, the tubes were immediately fractionated with 12 drops being collected in each fraction. A total of 24 fractions were collected. Whole fractions were counted for radioactivity or read at 280 nm for BSA content.

B. Tube 1 contained 0.16 nmole  $^{14}\text{C}$ -HCP (radioactivity, 0---0); tube 2 contained 0.4 nmole freshly prepared rabbit Hg ( $A_{413.5}$ , □—□); and tube 3 contained 0.4 nmole Hg and 0.16 nmole  $^{14}\text{C}$ -HCP ( $A_{413.5}$ , ▲---▲); and radioactivity, ▲---▲).

The same gradient as A was used except with 0.04 M glycylglycine buffer, pH 7.5. Solutions were preincubated at 30°C for 15 min, and 0.3 ml was applied to the top of the gradients and centrifuged in a SW 50L swinging bucket rotor at 2°C, 50,000 rpm for 15 hr and 15 min (tubes 1 and 2) and with a SW 65 swinging bucket rotor at 2°C, 53,400 rpm for 15 hr and 15 min (tube 3). Immediately after centrifugation, 12 drop fractions were collected from each tube. A total of 24 fractions were collected. Aliquots (0.1 ml) of the fractions were counted for radioactivity. The remainder of the fractions was diluted with 1 ml of water and their absorbancy read at 413.5 nm for Hb content.



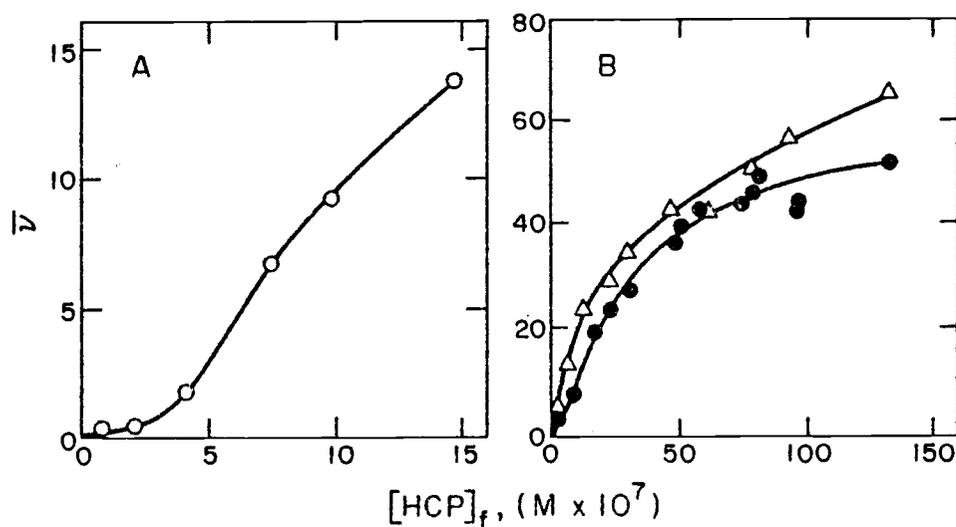


Figure 26. HCP binding isotherm of torula yeast G6PD. A. Equilibrium dialysis at 4°C at low HCP concentrations (O—O). B. Equilibrium dialysis at high HCP concentrations at 4°C (●—●) and 27°C (Δ—Δ). Standard assay procedures were used.

exhibited obvious sigmoidicity (Figure 26A) while the binding curve for higher HCP concentrations also showed slight sigmoidal character with a maximum binding of 52 moles of HCP per mole of G6PD (Figure 26B). At 27°C, however, the binding of HCP was greater and appeared to follow a simple Langmuir isotherm (Figure 26B). In all cases, even at HCP concentrations approaching the HCP solubility limit which has been reported as 21 µg per ml (51.6 µM) in 0.2 M sodium phosphate buffer, pH 7.4 (Brown and Ulsamer, 1975), HCP binding to torula yeast G6PD did not reach a saturation value. It was obvious that the binding of HCP increased with increasing temperature. When the data from the 4°C studies were plotted according to Scatchard (1949), a curve characteristic of positive cooperative-like behavior was observed (Figure 27A). At 27°C, however, a Scatchard plot of the HCP binding data yielded a curve characteristic of either  $n$  identical sites exhibiting negative cooperativity or nonidentical binding sites without cooperativity (Cook, 1972) (Figure 27B). The deviation to be expected for positive cooperativity and negative cooperativity (or dissimilarity of sites or enzyme forms) in Scatchard plots are reported by Cook (1972).

The same binding solutions were tested for G6PD enzyme activity. It was observed that G6PD enzyme activity was inhibited to various extents in the presence of the different HCP concentrations (Figure 28). Hill plots of  $\log v_i / (v - v_i)$  versus  $\log [HCP]_f$  at 4°C for low HCP concentrations gave values for  $n'$  of 0.57 to  $n'$  of 1.85 in the free HCP concentration range of 0.09 µM to 1.0 µM (Figure 29A). At higher free HCP concentrations, similar Hill plots of the limited data possible gave  $n'$  values ranging from 1.26 to 6.67 at 4°C and 1.11 to 5.29 for 27°C

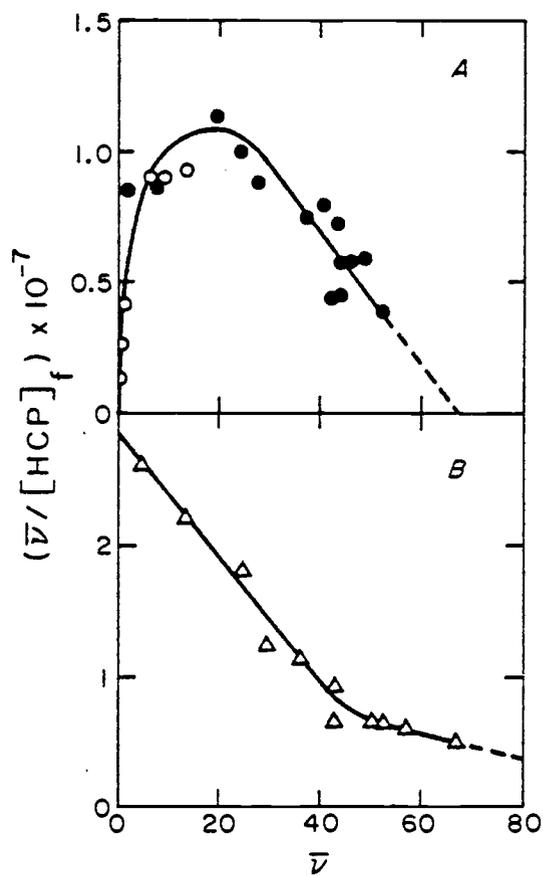


Figure 27. Scatchard plots of HCP binding to torula yeast G6PD. Data were from Figure 26. A. At 4°C (O---O) from Figure 26A and (●—●) from Figure 26B. B. At 27°C (Δ—Δ).

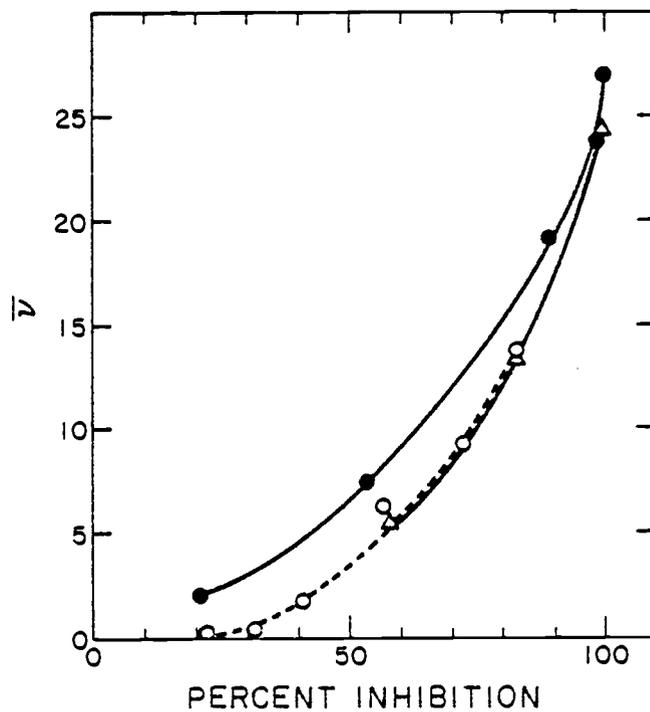


Figure 28. Percent inhibition of torula yeast G6PD by HCP as a function of moles of HCP bound per mole of enzyme. Aliquot of binding solution from Figure 26 were assayed for G6PD activity by the standard assay procedure. The 4°C data for sample of Figure 26A (O---O); the 4°C data for sample of Figure 26B (●—●); and the 27°C data for Figure 26B (Δ—Δ).

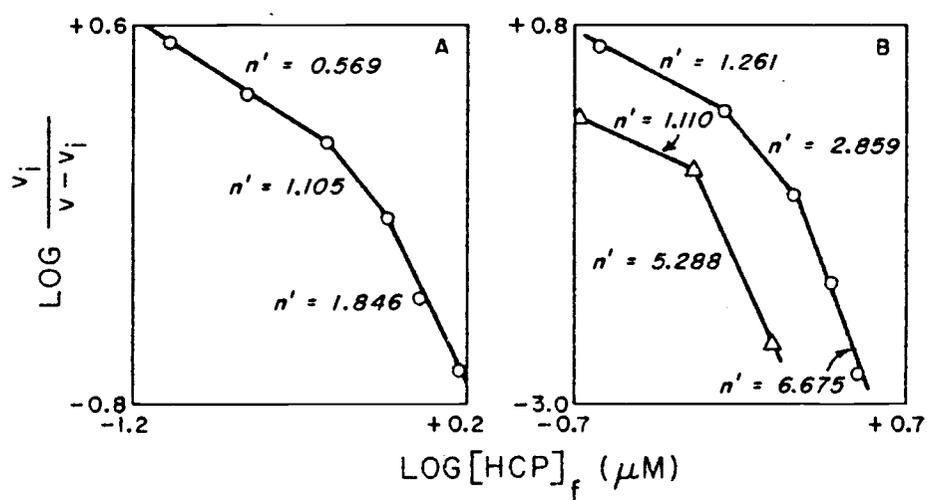


Figure 29. Hill plots of G6PD enzyme activity as a function of HCP concentration. Standard assay procedures were followed. A. At 4°C (O—O). B. At 4°C (●—●) and 27°C (Δ—Δ).

(Figure 29B). Above these free HCP concentrations,  $n'$  values could not be estimated by this method since the enzyme was completely inhibited.

In addition to using enzyme activity data, however, a similar form of Hill plots can also be applied directly to binding data. When the binding is highly cooperative, the following equation (Van Holde, 1971) applies:

$$\frac{\bar{v}}{N - \bar{v}} = K [A]^{n'} \quad (12)$$

where  $\bar{v}$  is the number of moles of ligand bound per mole of enzyme;  $N$  is the maximum number of ligand binding sites per mole of enzyme;  $K$  is dissociation constant of the enzyme-ligand complex;  $[A]$  is free ligand concentration; and  $n'$  is the Hill coefficient. The slope of a graph of  $\log \frac{\bar{v}}{N - \bar{v}}$  versus  $\log [A]$  (the Hill plot) gives the Hill coefficient or  $n'$  value. In the case of HCP binding to G6PD, the true  $N$  value is unknown, therefore, estimated values were used in the following. The maximum number of HCP binding sites at 4°C was assumed to be 68 as determined by extrapolation of the least square fitted line of the major group of points in the Scatchard plot shown in Figure 27A. Under these conditions, the Hill coefficients were 1.68 to 1.17 for the entire free HCP concentration range studied (Figure 30A). Positive cooperative binding at lowest HCP concentrations and probably at the higher concentrations was hence suggested at 4°C. At 27°C, the maximum number of binding sites was assumed to be 118, also determined by extrapolation of the least square fitted line of the highest slope part of the curve in Scatchard plot shown in Figure 27B. The Hill coefficient

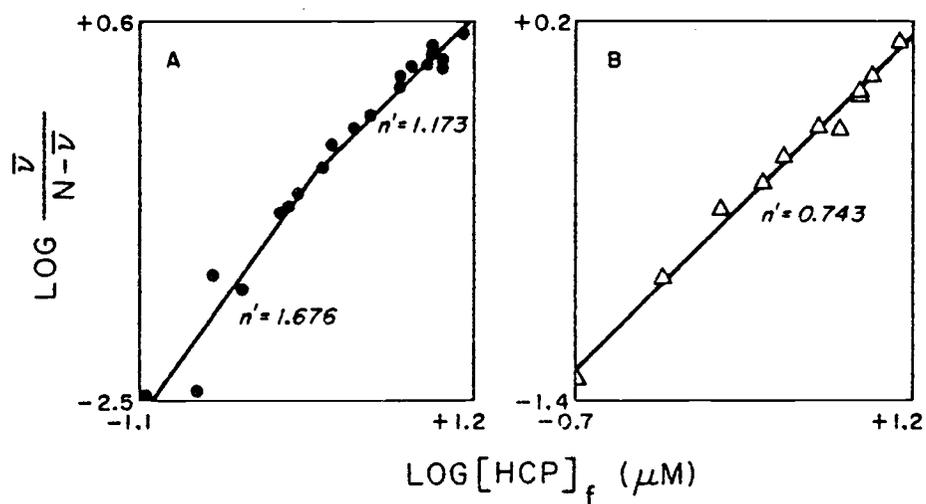


Figure 30. Hill plots of HCP binding to torula yeast G6PD. A. At 4°C, with extrapolated maximum number of HCP binding sites of 68. B. At 27°C, with extrapolated maximum number of HCP binding sites of 118.

was determined to be 0.74 for the entire HCP concentration range (0.26  $\mu\text{M}$  to 13.35  $\mu\text{M}$ ) studied, which suggests that at 27°C, HCP binding sites exhibit negative cooperativity.

At 4°C positive cooperativity of HCP binding to torula yeast G6PD at 4°C at lower HCP concentrations also can be seen when the data were analyzed on the basis of moles of HCP bound per mole of free HCP (Table 12). Under these circumstances, values of about 0.2 to 1.1 were found at 4°C at free HCP concentrations of 0.89  $\mu\text{M}$  to 1.67  $\mu\text{M}$ . The number of moles of HCP bound at 4°C, however, decreased from 1.1 at a free HCP concentration of 0.67  $\mu\text{M}$  to 0.37 at free HCP concentration of 13.35  $\mu\text{M}$ . The ratio of bound to free HCP at 27°C also decreased from 2.5 at a free HCP concentration of 0.2  $\mu\text{M}$  to 0.5 at a free HCP concentration of 13.35  $\mu\text{M}$ . These latter results indicate negative cooperativity in the binding at 4°C and 27°C at higher HCP concentrations.

When the total  $^{14}\text{C}$ -HCP concentration was fixed in a large volume and the G6PD concentration varied inside a dialysis bag from 19 to 57 pmole by using the second method described in the Materials and Methods section, the average moles of HCP bound per mole of G6PD was more or less constant and the enzyme was inhibited to approximately the same extent at all enzyme to inhibitor ratios tested (Table 13).

Binding of HCP to the semipermeable membrane and lucite dialysis cell in the equilibrium dialysis experiments was high, as shown by the values in Table 14 for the experiment described in Figure 26. Although HCP binding to the membrane and cell was appreciable, it was quite reproducible as evidenced by values within 10% of one another

TABLE 12

BINDING OF HCP TO TORULA YEAST G6PD AT VARIOUS FREE HCP CONCENTRATIONS<sup>a</sup>

4°C		27°C	
[HCP] <sub>f</sub> (μM)	[HCP] <sub>b</sub> /[HCP] <sub>f</sub>	[HCP] <sub>f</sub> (μM)	[HCP] <sub>b</sub> /[HCP] <sub>f</sub>
0.09	0.24	0.21	2.50
0.20	0.12	0.62	2.09
0.26	0.80	1.33	1.73
0.41	0.38	2.30	1.21
0.74	0.85	3.00	1.11
0.84	0.83	4.68	0.87
0.99	0.88	6.22	0.65
1.47	0.89	8.03	0.62
1.67	1.08	9.44	0.58
2.38	0.95	7.86	0.62
3.08	0.84	13.3	0.48
4.84	0.72		
5.01	0.76		
5.86	0.70		
7.49	0.55		
7.95	0.55		
9.66	0.42		
8.18	0.57		
9.70	0.44		
13.3	0.37		

<sup>a</sup> Recalculation of the data presented in Figure 26.

TABLE 13  
 BINDING OF HCP TO TORULA YEAST G6PD BY EQUILIBRIUM DIALYSIS<sup>a</sup>

G6PD used (pmole)	<sup>14</sup> C-HCP bound		$\bar{v}$	Percent enzyme inhibition
	(cpm)	(pmole)		
0		0	0	0
19	168.8	29.2	1.54	51.2
28.5	254.7	44.1	1.55	42.8
38.0	352	60.9	1.60	38.2
47.5	520.5	72.8	1.53	42.1
47.5	493	85.3	1.80	41.7
57.0	551.1	95.3	1.67	41.9

<sup>a</sup> Second method described in the Materials and Methods section was used. The loss of <sup>14</sup>C-HCP on the dialysis membrane and glass container was not determined.

in independent triplicate runs. The number of moles of HCP bound per mole of G6PD could be quantitatively affected by the high binding of HCP to the dialysis equipment, but the overall character of the binding data should not be influenced.

In all the binding studies, the Donnan effect was negligibly small.

#### Acrylamide gel electrophoresis

Torula yeast G6PD gave a single subunit band of molecular weight at about 60,000-65,000 as judged by SDS gel electrophoresis using

TABLE 14  
<sup>14</sup>C-HCP BINDING TO MEMBRANE AND LUCITE CELL WALL IN EQUILIBRIUM DIALYSIS<sup>a</sup>

Experiment Number	Concentration of <sup>14</sup> C-HCP <sup>b</sup> (μM)	% Adsorption to the membrane				% Adsorption to the lucite cell				Total <sup>14</sup> C-HCP loss (%)			
		4°C <sup>c</sup>		27°C <sup>c</sup>		4°C <sup>c</sup>		27°C <sup>c</sup>		4°C <sup>c</sup>		27°C <sup>c</sup>	
257 <sup>d</sup>	0.69	6.7	8.7			60.2	64.2			66.9	72.9		
257 <sup>d</sup>	1.51	8.9	--			59.2	--			68.1	--		
263 <sup>d</sup>	2.42	9.4	10.7	6.7	8.9	53.3	54.4	53.3	56.8	62.7	65.1	60.0	65.7
257	2.98	8.5	12.6			56.2	48.8			64.7	61.4		
257	4.74	10.0	12.2			42.3	44.7			52.3	56.9		
263	5.14	8.6		6.1	10.5	40.4	38.1	43.1	42.9	49.0	50.0	49.2	53.4
257	6.29	10.2	15.0			41.2	39.1			51.4	54.1		
257	7.83	10.5	14.5			33.7	29.8			44.2	44.3		
263	8.87	8.7	16.0	7.2	12.2	28.1	31.2	35.0	38.9	36.8	47.2	42.2	51.1
263	12.22	10.3	14.7	8.2	11.5	28.7	26.8	29.3	29.3	39.0	41.5	37.5	40.8
263	13.75	10.8	13.5	9.3	12.2	21.7	20.3	19.5	25.7	32.5	33.8	28.8	37.9
263	18.87	9.6	17.7	9.2	12.1	18.0	19.1	18.3	24.7	27.6	36.8	27.5	36.8
263	20.90	11.8	16.3	--	--	19.0	18.3	--	--	30.8	34.6	--	--
263	24.23	13.4	14.5	9.2	12.1	17.9	23.6	22.8	21.5	31.3	38.1	32.0	33.6
263	28.67	12.7	13.0	10.0	11.8	17.1	21.8	15.5	21.3	29.8	34.8	25.5	33.1
263	30.81	12.1	--	--	--	19.2	--	--	--	31.3	--	--	--
263	36.27	10.0	13.7	9.3	12.2	23.2	25.4	21.3	26.1	33.2	39.1	30.6	38.3
263	40.25	11.1	10.7	--	--	27.9	38.4	--	--	39.0	39.1	--	--
263	47.05	13.2	16.4	9.7	12.2	17.4	17.1	19.2	20.1	30.6	33.5	28.9	32.2

<sup>a</sup> First method described in the Materials and Methods section was used.

<sup>b</sup> <sup>14</sup>C-HCP had specific activity of 3.52 mCi/mmole.

<sup>c</sup> Left column values were for the dialysis that in the right half cell it contained 80.7 pmole of G6PD and the values in the right column were for the dialysis that in the right half cell it contained only buffer as control.

<sup>d</sup> Experiment 257 and Experiment 263 were conducted on the same batch of enzyme but six months apart. The enzyme was kept frozen between these two experiments.

BSA, catalase, ADH, LDH, and Hb as standards (Figure 31). These results are in agreement with the molecular weight estimates for the torula yeast G6PD monomer by Domagk et al. (1973), but differ from the finding of dissimilar subunits of molecular weight 49,000 and 61,000 for this same enzyme (Chilla and Domagk, 1974).

On acrylamide slab gel electrophoresis, G6PD showed one single protein band. Polyacrylamide gel was shown previously not to cause any dissociation of electrophoretically homogenous forms of G6PD (De Flora et al., 1968). After treatment with HCP, however, multiple bands were observed (Figure 32). In addition to a band for the native enzyme, electrophoresis gave one band which moved more rapidly than the G6PD dimer and several bands moving more slowly. The fast moving band occupied a position intermediate between that of the G6PD dimer (MW 130,000) and the BSA standard (MW 65,000) and seemed to be composed of two subbands. The slower moving protein bands were probably high molecular weight aggregates of the torula yeast enzyme similar to the polymer forms of G6PD from rat liver (Holten, 1972). In all the gel electrophoresis studies, BSA was used as a reference. The BSA protein peak migrated faster in the presence of HCP than in its absence.

Dissociation was not observed at a HCP:G6PD ratio of 1 (Figure 33A, well No. 4) nor was it apparent at a HCP:G6PD ratio of 4 (Figure 33A, well No. 5). An altered G6PD electrophoretic pattern started to appear when the HCP:G6PD ratio reached 9 and a marked dissociation was obvious at higher HCP concentrations; i.e., at a HCP:G6PD ratio of 375 (Figure 33A, well No. 8).

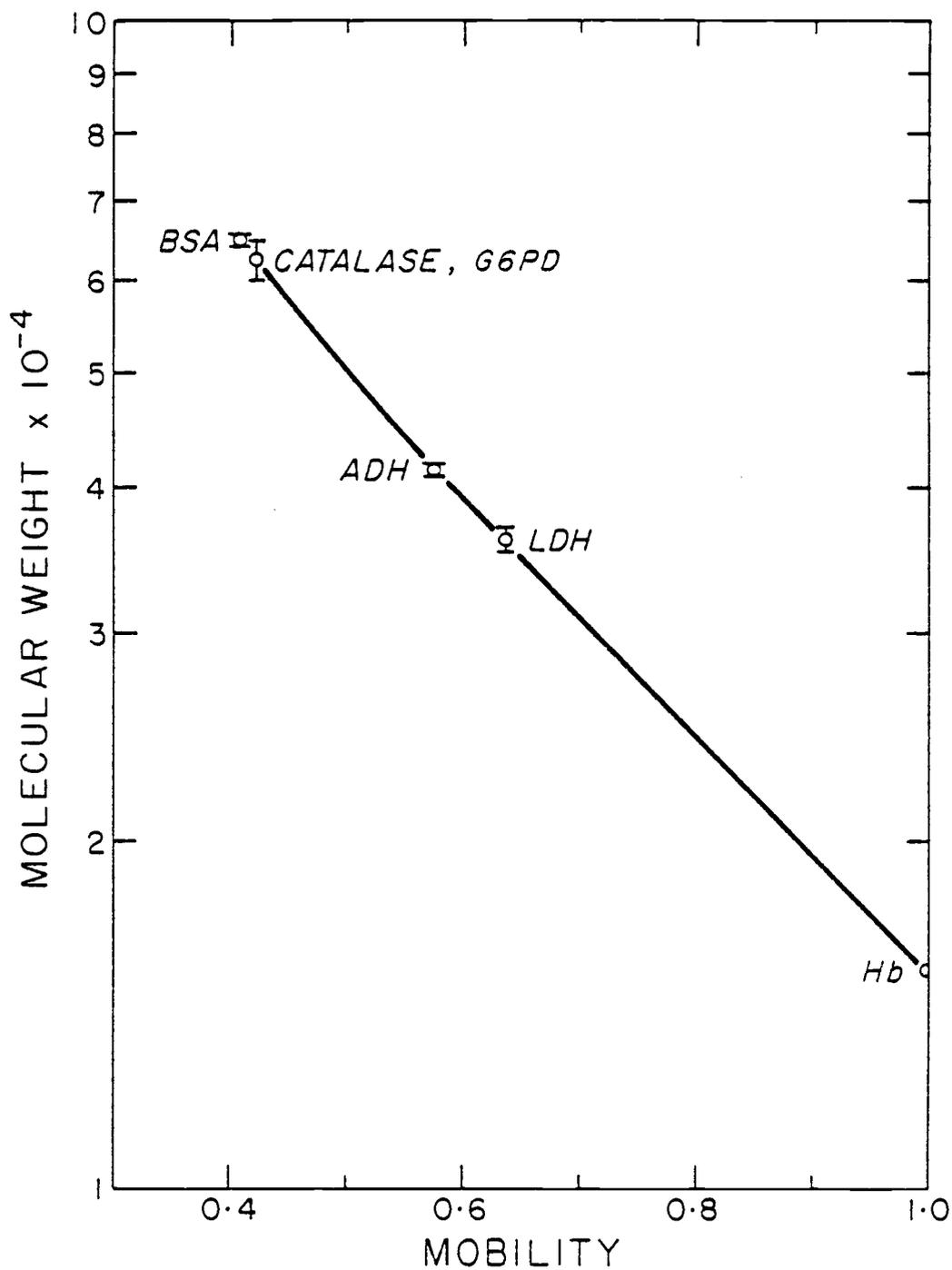


Figure 31. Molecular weight determination of torula yeast G6PD by SDS slab gel electrophoresis.

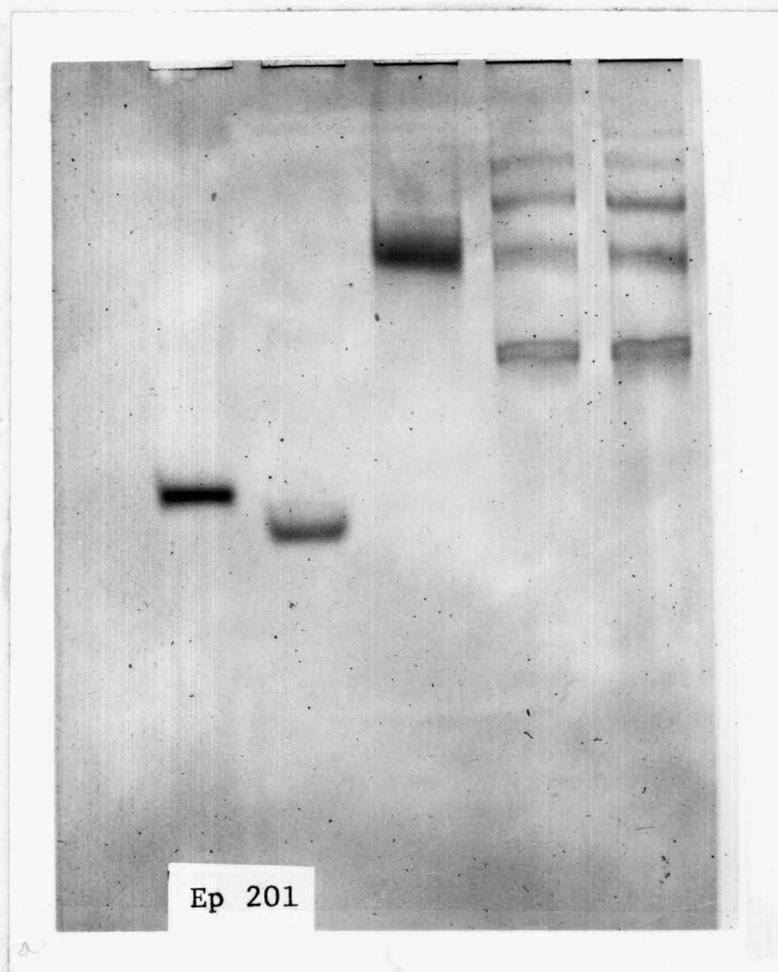
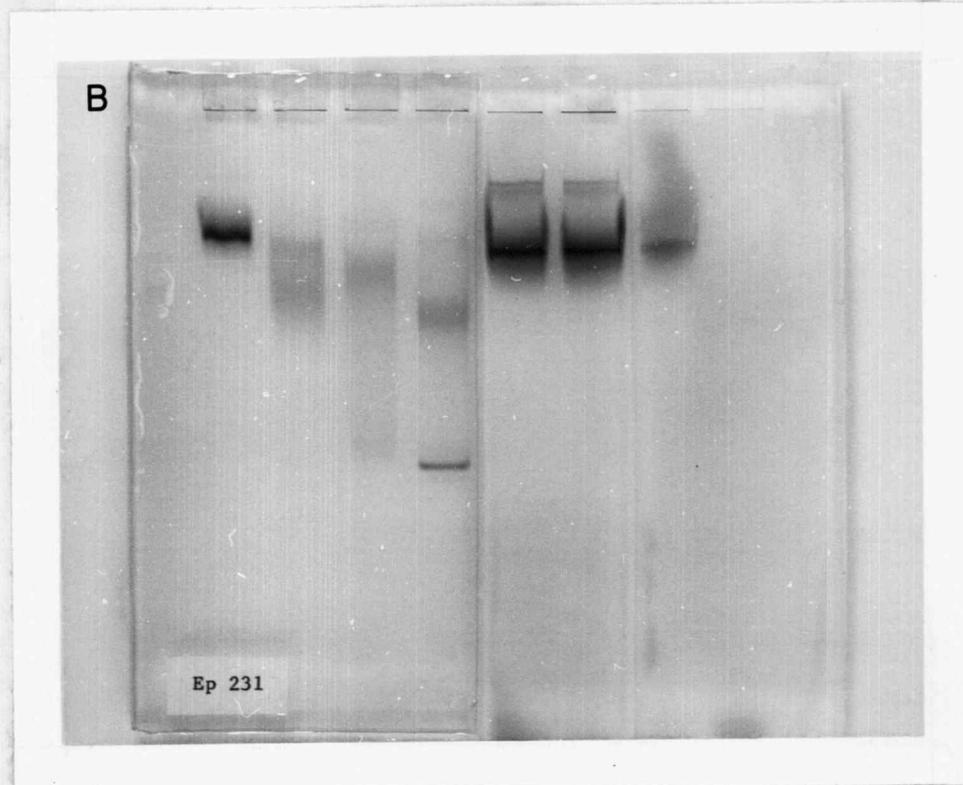
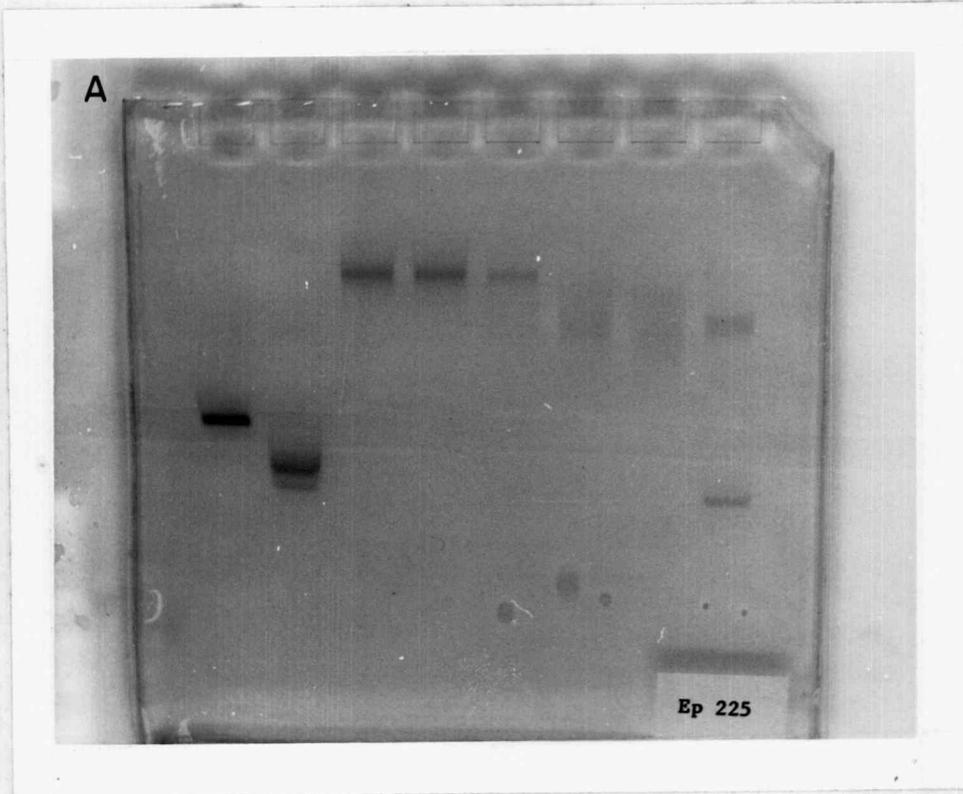


Figure 32. Acrylamide slab gel electrophoresis at pH 8.9. Cyanogum-41 (7%) which contained 0.1% APS, 0.2% TMED and 0.1% Photo-Flor was prerun 30 min at 300 volts. After applying the samples, the gel was run 1.5 hr. The contents of each sample well are listed below, from left to right: (1) 0.12 nmole BSA in 0.05 M Tris buffer, pH 7.5; (2) 0.12 nmole BSA plus 49 nmoles HCP (HCP:BSA molar ratio of 408); (3) 0.18 nmole torula yeast G6PD (suspension in 2.6 M AS, then dialyzed against 125 ml 0.05 M Tris buffer, pH 7.5 for about 2 hr); (4) 0.18 nmole G6PD plus 49 nmoles HCP (HCP:G6PD molar ratio of 272); and (5) duplicate of No. 4.

Figure 33. Acrylamide slab gel electrophoresis at pH 8.9.

A. Cyanogum-41 (7%) which contained 0.12% and 0.16% TMED was prerun 32 min at 300 volts. After the samples were applied, the gel was run 78 min at the same voltage. The contents of each sample well were: 1) 0.12 nmole GSA; 2) 0.12 nmole BSA and 49 nmoles HCP at a HCP:BSA molar ratio of 408; 3) 0.13 nmole torula yeast G6PD (enzyme originally was suspended in AS and it was dialyzed 30 hr against 0.05 M Tris buffer, pH 7.5, before use); 4) 0.13 nmole G6PD plus HCP at a HCP:G6PD molar ratio of 1; 5) 0.13 nmole G6PD plus HCP at a HCP:G6PD molar ratio of 4; 6) 0.13 nmole G6PD plus HCP at a HCP:G6PD ratio of 9; 7) 0.13 nmole G6PD plus HCP at a HCP:G6PD ratio of 23; and 8) 0.13 nmole G6PD plus HCP at a HCP:G6PD ratio of 377. Gels were stained with amino black for proteins.

B. Same gel as in A, except prerun time was 1 hr at 300 volts and the gel running time was 100 min at same voltage. The contents of each well were: 1) 0.15 nmole torula yeast G6PD (the crystalline enzyme was dissolved in 0.05 M Tris buffer, pH 7.5, and dialyzed against the same buffer for 5.5 hr); 2) 0.15 nmole G6PD plus HCP at a HCP:G6PD molar ratio of 25; 3) 0.15 nmole G6PD plus HCP at a HCP:G6PD ratio of 96; 4) 0.15 nmole G6PD plus HCP at a HCP:G6PD ratio of 479; 5-8) same as No. 1-4, except a 1/25 dilution was applied to the gel. No. 1-4 were stained with amino black and No. 5-8 were stained with methosulfate-nitroblue tetrazolium for G6PD enzyme activity.



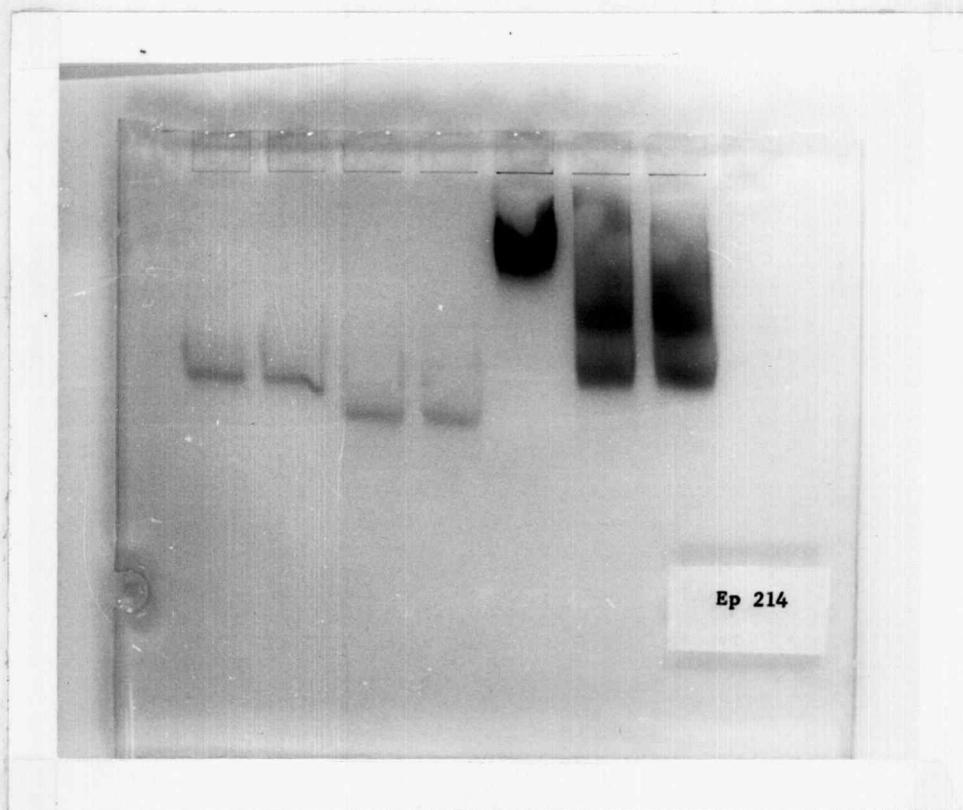


Figure 34. Acrylamide slab gel electrophoresis at pH 7.5. Gel was run at about 100 mA and 150 volts for 75 min. Cyanogum-41 (7%) contained 0.1% TMED in 0.025 M Tris-Cl buffer, pH 7.5. Each sample well contained: 1 and 2) 0.12 nmole BSA; 3 and 4) 0.12 nmole BSA plus 49 nmoles at a HCP: GSA molar ratio of 520; 5) 0.25 nmole torula yeast G6PD; and 6 and 7) 0.25 nmole G6PD plus 49 nmoles HCP at a HCP:G6PD ratio of 196. Gel was stained with amino black for proteins.

The enzyme activity of G6PD on the gel was also tested. In addition to the major G6PD band, two light bands of enzyme activity migrating slower than the main band were formed upon longer incubation of the gel with the reaction mixture (Figure 33B, wells No. 5-8). The enzymatically active slow moving minor band(s) observed in the absence of HCP could result from the presence of isozymes in this batch of torula yeast G6PD similar to those reported by Shedlarski (1974) for G6PD from Caulobacter crescentus.

In the presence of HCP, enzyme activity in all bands decreased in proportion to increasing HCP concentration. No enzyme activity was associated with the extra fast moving protein bands (Figure 33B, wells No. 2-4) that were observed in the presence of HCP (Figure 33B, wells No. 6-8). Presumably these bands were inactive G6PD forms.

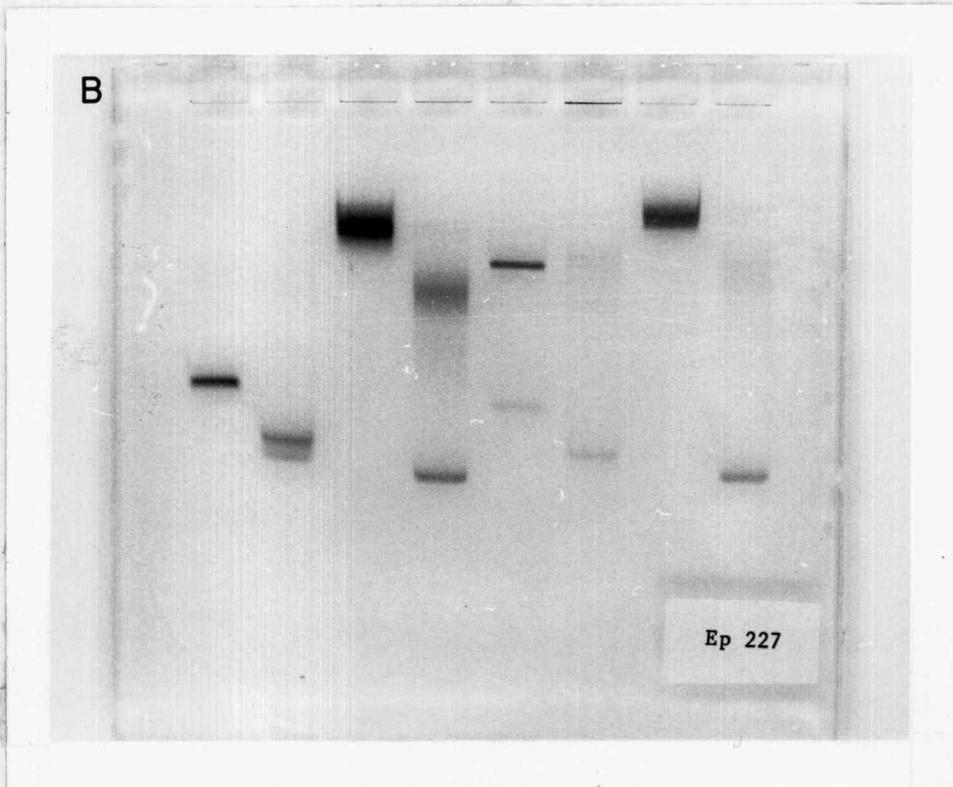
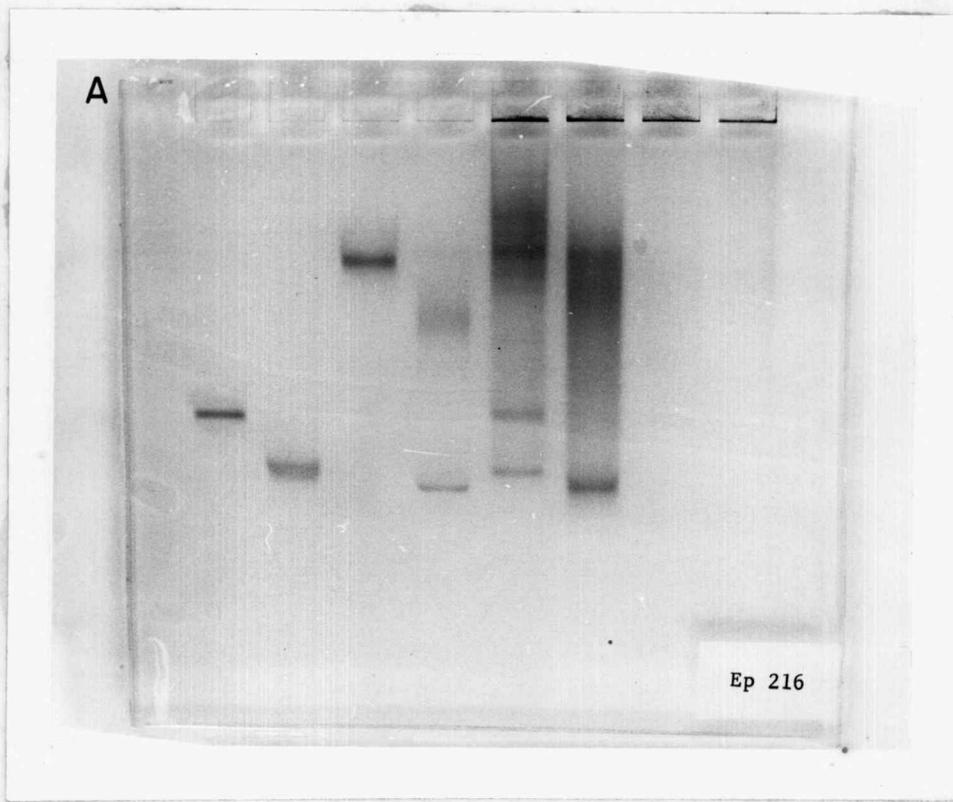
When pH 7.5 buffer was used in the gel instead of pH 8.9 buffer, the relative migration distance of the bands produced after treating torula yeast G6PD with HCP was changed (Figure 34). Two major protein bands with faster mobility were formed from G6PD in the presence of HCP (Figure 34, wells No. 6 and 7).

Besides the binding of HCP to G6PD, BSA and Hb as demonstrated previously by density gradient sedimentation studies (Figures 18, 19, 21-25), HCP also interacts with other proteins such as ICD and catalase (Figure 35A and B) as evidenced by the changing of migration pattern on acrylamide slab gel electrophoresis. In all cases, HCP causes the formation of one or more protein forms which migrate more rapidly than

Figure 35. Acrylamide slab gel electrophoresis at pH 8.9 demonstrating the effect of HCP on BSA, G6PD, ICD, LDH, and catalase.

A. Prerun 80 min, after application of samples the gel was run for 110 min at 300 volts. The contents of each sample well were: 1) 0.12 nmole BSA; 2) 0.12 nmole BSA and plus 49 nmoles HCP at a HCP:BSA molar ratio of 408; 3) 0.26 nmole torula yeast G6PD; 4) 0.26 nmole G6PD plus 49 nmoles HCP at a HCP:G6PD molar ratio of 188; 5) 4.9 nmoles ICD (assuming a molecular weight of 60,000); and 6) 4.9 nmoles ICD and 49 nmoles HCP at a HCP:ICD molar ratio of 10. Gels were stained with amino black.

B. Prerun 45 min, after application of samples, the gel was run for 86 min at 300 volts. The contents of each sample well were: 1) 0.12 nmole BSA; 2) 0.12 nmole BSA plus 49 nmoles HCP at a HCP:BSA molar ratio of 408; 3) 0.13 nmole torula yeast G6PD; 4) 0.13 nmole G6PD plus 49 nmoles HCP at a HCP:G6PD molar ratio of 377; 5) 0.14 nmole LDH; 6) 0.14 nmole LDH plus 49 nmole LDH plus 49 nmoles HCP at a HCP:LDH molar ratio of 350; 7) 0.08 nmole catalase; and 9) 0.04 nmole catalase plus 49 nmoles HCP at a HCP:catalase molar ratio of 613. Gels were stained with amino black.



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the native protein. These results suggest that HCP may induce a dissociation of polymeric proteins.

Interaction of HCP with G6P and NADP<sup>+</sup> as studied by difference spectroscopy

Although the results of previous kinetic studies on torula yeast G6PD failed to provide evidence for interaction of HCP with the enzyme substrates, G6P and NADP<sup>+</sup>, such interaction could still occur. Direct evidence for HCP binding to the G6PD substrates was examined by measurement of the difference spectra before and after HCP was mixed with either G6P or NADP<sup>+</sup>.

The spectrum of HCP is shown in Figure 36 and the extinction coefficients for NADP<sup>+</sup> and HCP at various pH's are presented in Table 15. The difference spectra for NADP<sup>+</sup> and G6P in the presence of HCP indicated hypochromicity at the HCP absorption wavelength (Figures 37 and 38). The nature of the difference spectra obtained seemed to be pH dependent, suggesting that the HCP phenolic groups were involved in the interactions between HCP and both substrates. Further substantiation of an association between HCP and NADP<sup>+</sup> and G6P was obtained by the demonstration of a concentration dependence in the difference spectra obtained at different HCP:NADP<sup>+</sup> and HCP:G6P ratios (Figure 39).

TABLE 15  
 EXTINCTION COEFFICIENTS FOR NADP<sup>+</sup> AND HCP AT  
 VARIOUS pHs IN 0.1 M TRIS BUFFER

Compound	Final concentration (μM)	pH	λ <sub>max</sub>	Absorbance	Molar extinction coefficient x 10 <sup>-3</sup> (cm <sup>2</sup> mole <sup>-1</sup> )
HCP	110.6	7.42	300	0.675	6.10
HCP	110.6	8.17	300.8	0.688	6.22
HCP	110.6	8.84	302.5	0.693	6.27
HCP	110.6	10.19	316.7	0.975	8.82
NADP <sup>+</sup>	53.1	7.40	258.4	0.928	17.5
NADP <sup>+</sup>	53.1	8.15	258.4	0.923	17.4
NADP <sup>+</sup>	53.1	8.80	258.4	0.935	17.6
NADP <sup>+</sup>	53.1	10.18	259.2	0.920	17.3

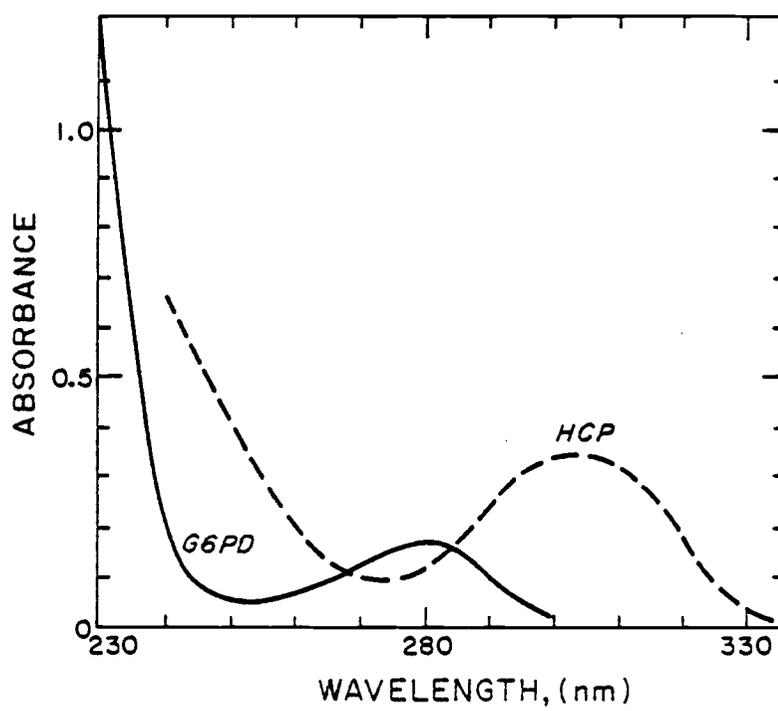


Figure 36. Ultraviolet absorption spectra of torula yeast G6PD and HCP in TrisCl buffer, pH 7.5.

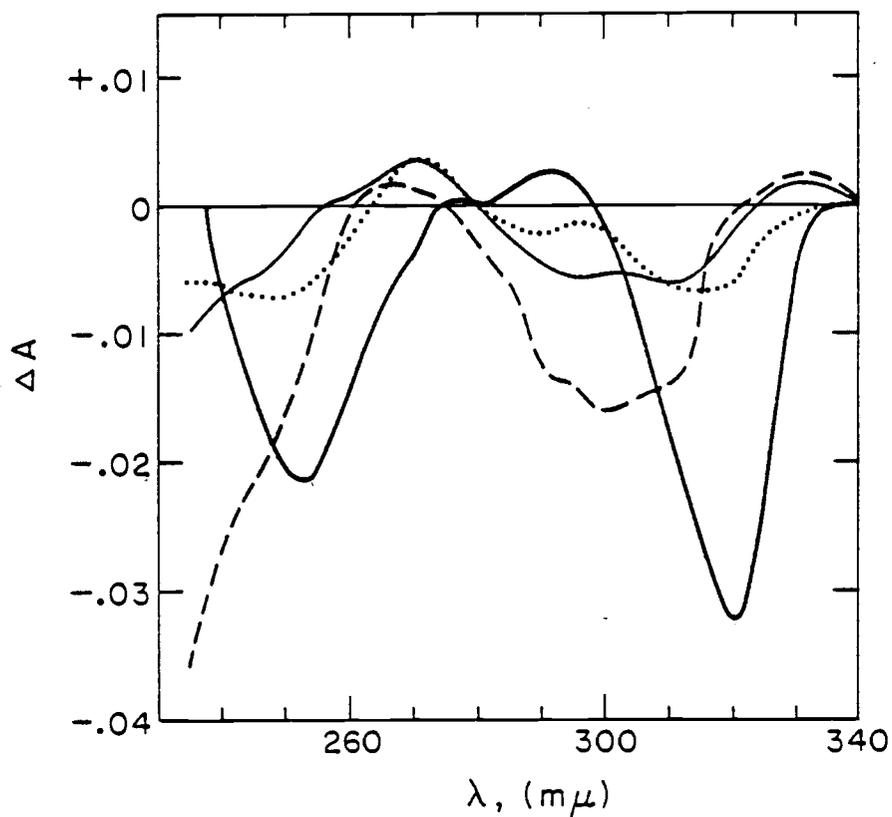


Figure 37. Difference spectra for the interaction of HCP with  $\text{NADP}^+$  at various pHs. Samples contained a final concentration of  $111 \mu\text{M}$  for HCP and  $53.1 \mu\text{M}$  for  $\text{NADP}^+$  before mixing. The experiments employed  $0.1 \text{ M}$  Tris buffer, pH 7.4 (----), 8.2 (—), 8.8 (····), and 10.2 (—).

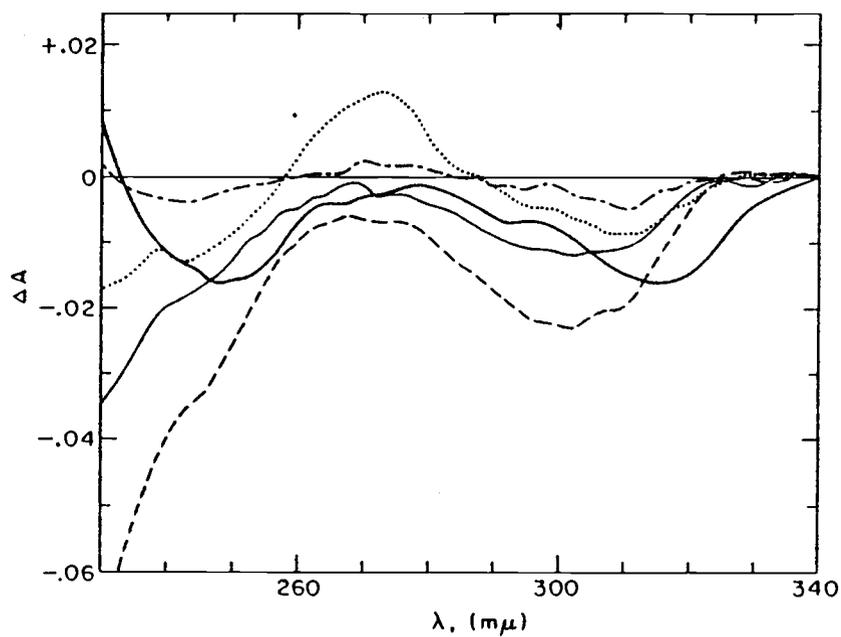


Figure 38. Difference spectra for the interactions of HCP with G6P at various pHs. Samples contained a final concentration of 55.3  $\mu\text{M}$  for HCP and 117  $\mu\text{M}$  for G6P before mixing. The experiments employed the following buffers: 0.1 M Tris, pH 7.4 (----); 0.1 M Tris, pH 8.2 (—); 0.1 M Tris, pH 8.9 (- - - -); 0.1 M glycine-NaOH, pH 8.7 (.....); and 0.1 M glycine-NaOH, pH 9.3 (—).

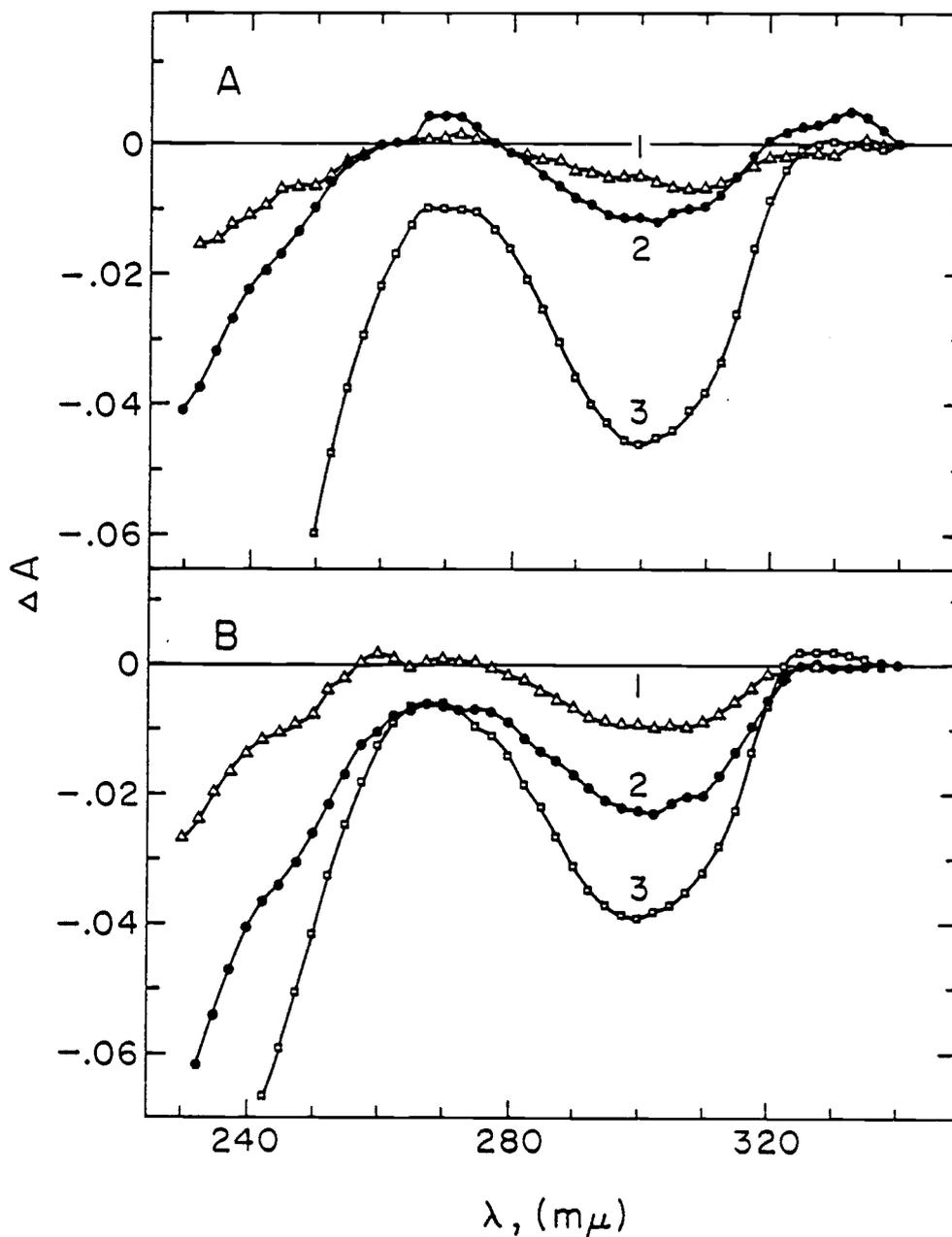


Figure 39. Difference spectra for the interactions of HCP with  $\text{NADP}^+$  and G6P. A. Difference spectra for the interaction of HCP with  $\text{NADP}^+$  at constant ratio of the two concentrations at  $25^\circ\text{C}$ . Buffer was 0.1 M Tris-Cl, pH 7.4. Ratios of HCP: $\text{NADP}^+$  used were 2.1 at [HCP] of 27.6  $\mu\text{M}$  ( $\Delta$ — $\Delta$ ); 55.3  $\mu\text{M}$  ( $\bullet$ — $\bullet$ ); and 111  $\mu\text{M}$  ( $\square$ — $\square$ ). B. Difference spectra for the interaction of HCP with G6P at constant ratio of the two concentrations at  $25^\circ\text{C}$ . Buffer was the same as A. Ratios of HCP:G6P were 0.48 at [HCP] of 27.6  $\mu\text{M}$  ( $\Delta$ — $\Delta$ ); 55.3  $\mu\text{M}$  ( $\bullet$ — $\bullet$ ); and 111  $\mu\text{M}$  ( $\square$ — $\square$ ).

## DISCUSSION

Torula yeast G6PD is most active at a pH range of 7.5-9 (Figure 3A) but activity sharply decreased on the alkaline side of this value. These results are compatible with the observation reported by Domagk et al. (1969a) and Engel et al. (1969) for the yeast enzyme. The reported pH optima for all G6PDs is above 8.0; about 8.5 for the mammalian and yeast enzymes and greater than 9.0 for the Leuconostoc mesenteroides enzyme with  $\text{NADP}^+$  as cofactor (Olive and Levy, 1967). An inhibition of the enzyme by phosphate ions which had been noticed by Theorell (1935) and later by many others such as Engel et al. (1969) was also seen in my study. The effect of phosphate was variable depending upon the concentration and the coenzyme used (Olive and Levy, 1967). For this reason phosphate buffer was not chosen for the studies in this thesis.

Two functional groups of either coenzyme or amino acids at the active site of the enzyme having pK values of about 6 and 9.9 appeared to be involved in the activity of torula yeast G6PD. If the pH affects only the ionization of the enzyme, these pK's suggest that the first group could be the carboxyl groups of glutamic or aspartic acids or the histidine imidazole group in the protein while the second group could be a cysteine sulfhydryl, a lysine  $\epsilon$ -amino or a tyrosine hydroxyl group. Olive et al. (1971) found that the  $\epsilon$ -amino groups of lysine was involved in the binding of G6P to G6PD, but these investigators obtained no evidence for the involvement of either a SH group or a lysine  $\epsilon$ -amino group in  $\text{NADP}^+$  binding. Both histidine (Domschke et al., 1969) and

tyrosine (Domschke et al., 1970b) have been implicated in the mechanism of action of G6PD from torula yeast.

Maximum inhibition of torula yeast G6PD by HCP seems to be bimodal, exhibiting two regions of maximum inhibition at pH 5-6.2 and 8-10 (Figure 3B). The dependent nature of the inhibitory activity on pH cannot be readily explained by the changing degree of dissociation of a single ionizable group. More than one binding site thus may be involved in the inhibitory effect of HCP on torula yeast G6PD. The bimodal pattern of inhibition with pH may reflect changing affinities of binding groups on the enzyme for HCP as the degree of ionization of the bisphenol is altered. The pKa's for HCP are reported to be 5.4 and 10.85 (Mahler, 1954), consequently, at least two different types of bisphenol binding sites may be present on the enzyme. One group of sites may possibly interact with unionized HCP and a second with the monovalent or divalent HCP anion. Alternatively, HCP may inhibit the enzyme by more than one mechanism involving differential pH responses. A similar pH dependence has been found in the inhibition of G6PD by a physiologically important hormone triiodothyroxine that is structurally similar to HCP (McKerns, 1962b).

Inhibition of torula yeast G6PD by HCP was not instantaneous but increased with the preincubation time (Figure 4B and D). This loss of G6PD activity in the presence and absence of  $Mg^{+2}$  is probably not a first-order process since log (percent inhibition or percent of original activity) versus preincubation time plots did not give straight lines (plots not shown). Dissociation of the active G6PD dimer to

inactive subunits also occurs in the presence of HCP (Figures 18, 19, 21-24, 32-35). Kinetic experiments described in this thesis, therefore, were generally conducted without preincubation (i.e., studying the immediate reaction) in order to avoid significant reduction in G6PD activity through dissociation. Palmitoyl-CoA also has been found (Taketa and Pogell, 1966; Kawaguchi and Bloch, 1974) to reduce yeast G6PD activity by both a direct inhibitory effect on the enzyme and a slower inactivation of the enzyme via dissociation to smaller subunits.

Kinetic studies of yeast G6PD have been described by several groups (Noltmann and Kuby, 1963). The  $K_m$  value for  $\text{NADP}^+$  is not influenced significantly by the concentration of G6P present and correspondingly, the  $K_m$  value for G6P is independent of  $\text{NADP}^+$  concentration (Glaser and Brown, 1955). Anderson et al. (1968) have noticed similar results upon the activity of yeast G6PD. These observations simplified experimental design of the studies described in this thesis, since for such a system where the affinity of each substrate for the enzyme is independent of the concentration of the second substrate, true Michaelis constant values may be determined directly by measuring activity as a function of varied concentration of one substrate while maintaining the second substrate at a fixed concentration (Dixon and Webb, 1964).

Torula yeast G6PD exhibits hyperbolic kinetics with its substrates within the substrate concentration range employed (Figure 6A and B). The  $K_m$  values with respect to its substrates were determined to be  $45 \pm 2 \mu\text{M}$  and  $100 \pm 20 \mu\text{M}$  for  $\text{NADP}^+$  and G6P, respectively (Figure 6A and B

and Table 9). These  $K_m$  values are compatible with those reported by other authors for G6PD from other sources (Table 4).

In measurements of enzyme steady-state reaction velocities, it is normally assumed that formation of enzyme-substrate or enzyme-inhibitor complexes do not diminish appreciably the concentration of substrate or inhibitor in solution (Webb, 1963; Dixon and Webb, 1964). It is recognized, however, that the relatively high concentration of some enzyme in vivo (Srere, 1967; Sols and Marco, 1970), or an exceptionally high affinity of an enzyme for a substrate or inhibitor (Goldstein, 1944; Morrison, 1969; and Cha, 1970) may lead to binding of a significant proportion of substrate or inhibitor molecules to the enzyme thus reducing their concentrations. The effects is significant when the ratios  $E_t/K_m$  or  $E_t/K_i$  are greater than 0.01 (Goldstein, 1944; Webb, 1963; and Henderson, 1972). As the ratio increases above 0.01, and analysis based on the Michaelis-Menten equation becomes increasingly invalid (Morrison, 1969; Cha, 1970; and Khoo and Russell, 1970). In this thesis, my  $E_t/K_m$  and  $E_t/K_i$  values were  $4.36 \times 10^{-5}$  and  $1.38 \times 10^{-4}$  for G6PD-substrate complex and G6PD-HCP complex, respectively; values far less than 0.01, hence the Michaelis-Menten equation should be valid for these studies.

The observed hyperbolic nature of the substrate saturation curve of torula yeast G6PD (Figure 6A and B) is consistent with observations (Afolayan, 1972) for the same enzyme with  $\text{NADP}^+$  concentrations of 1 to 3 mM. They differ, however, from results with G6PD from Pseudomonas aeruginosa (Lessie and Neidhardt, 1967) and Leuconostoc mesenteroides

(DeMoss et al., 1953) which have dual responses to  $\text{NADP}^+$ . My results also differ from those found with G6PD in some red cell genetic variants which have sigmoidal responses with their substrates (Luzzatto, 1967; and Afolayan and Luzzatto, 1971). Afolayan (1972) attributed the lack of a sigmoidal rate concentration response curve for torula yeast G6PD to the enzyme either being a modulator-dependent regulatory enzyme (Sanwall, 1970) or to its displaying positive cooperativity at much lower  $\text{NADP}^+$  concentrations than employed.

The results of the present studies show that HCP and related chlorinated compounds are effective inhibitors of G6PD from torula yeast. The action of the chlorinated bisphenols is not confined to G6PD since HCP also inhibits five other pyridine nucleotide-dependent dehydrogenases (ADH, GDH, ICD, LDH, and MDH) studied (Figure 8).

There are many ways to analyze inhibitor-enzyme interaction (Chignell, 1972). In this thesis I have employed kinetic analysis, density gradient ultracentrifugation, equilibrium dialysis, electrophoresis, and difference spectroscopy. From kinetic analysis, the Hill coefficients for the substrates of GDH, ICD, and G6PD were found to be approximately 1.0 (Table 9) both in the absence and presence of HCP, indicating that the cooperativity of the substrate binding was not increased by this inhibitor. HCP, therefore, does not function as an allosteric effector of these enzymes. In this regard, the inhibitory behavior observed here is similar to that found with ATP on G6PD from Arthrobacter strain 7C (Turnail and Schlegel, 1972).

The nomenclature "allosteric effect" used in this thesis was proposed by Monod et al. (1965), and it is defined as indirect interactions between topographically distinct binding sites mediated by the protein molecule through conformational transitions. Heterotropic interactions occur between dissimilar ligand molecules and are typified by the effect of activators and inhibitors on the turnover number or apparent affinity (or both) of the enzyme for its substrates(s). In the case of the inhibition of G6PD, GDH, and ICD by HCP, kinetic data suggest, as previously mentioned, that there were no heterotropic interactions between HCP and the substrates of these enzymes. This conclusion was evidenced by the consistency in the Hill coefficient values of 1.0 for the dehydrogenase substrates in the absence and presence of HCP (Table 9).

Inhibition of torula yeast G6PD by HCP could not be prevented nor reversed by sulfhydryl reagents such as DTE and MCE. It thus appears that the inhibitory action of HCP and related bisphenols on dehydrogenase enzymes does not occur through SH groups. This doesn't necessarily mean that HCP failed to bind to these enzymes or even to SH groups. The bisphenol, however, did not interact with essential SH group which could influence the activity of these enzymes. Lebowitz (reported by Gould et al., 1953) previously found that urease, a very sensitive sulfhydryl enzyme, was not inhibited by HCP even at very high concentrations of the bisphenol. Taketa and Pogell (1966) also ruled out essential SH groups as the site of inhibition of yeast G6PD by palmitoyl-CoA.

In addition, there have been no reports concerning the involvement of SH groups in the active site of G6PD. Domagk et al. (1969a), however, have predicted that disulfide bridges may be required for the activity of yeast G6PD.

Inhibition of G6PD by HCP generally appears to follow mixed kinetics according to the definition of Dixon and Webb (1964) as evidenced by plots of the reciprocal of initial reaction velocity versus reciprocal of molar concentration of HCP (Lineweaver-Burk plots) (Figure 10A and B). More detailed characterization of the type of inhibition may be obtained from replots of the slopes and intercepts of the reciprocal plots (Cleland, 1963; Plowman, 1972; and Segel, 1975). Such secondary plots for G6PD (Figure 10C and D) are slightly curvilinear, parabolic for the slope and hyperbolic for the intercept with respect to both substrates. Following a more complete designation (Cleland, 1963; and Plowman, 1972), inhibition of G6PD by HCP thus may be classified as slope-parabolic, intercept-hyperbolic, noncompetitive inhibition. Such nonlinear secondary plots are indicative of complex inhibition involving at least two inhibitory mechanisms of the enzyme reaction (Kosow, 1974; and Segel, 1975). A substrate depletion mechanism due to complex formation between HCP and one or both substrates probably can be eliminated since the reaction mixtures generally contained an approximately 20-fold excess of substrate over HCP. Moreover, addition near saturating concentrations of substrates failed to significantly reverse the inhibition of G6PD by HCP. In fact, addition of the substrates partially protected G6PD against HCP inhibition (Figure 7D and E). The

source of curvature in the slope and intercept plots is most likely due to the presence of at least two inhibitory sites on the enzyme molecules (Gunnarsson et al., 1974; Kosow, 1974; Henneke and Wedding, 1975b; and Segel, 1975). Henneke and Wedding (1975b), for example, have recently shown that two phenol molecules bind to two separate inhibitory sites on porcine heart MDH. One molecule is bound as phenol, the other as a charge transfer complex of phenol with  $\text{NAD}^+$ . There could thus be two or more HCP inhibition sites on each G6PD molecule; or possibly, as in the case of MDH and phenol (Henneke and Wedding, 1975b), there may be one HCP inhibitory site on the enzyme and a substrate-inhibitor complex, formed by interaction between HCP with  $\text{NAD}^+$  and/or G6P, binding to a second inhibitory site (Webb, 1963; and Segel, 1975). My kinetic analysis (Figure 17), however, did not provide support for inhibition through formation of a HCP-substrate complex. In either case, HCP or the HCP-substrate complex then causes conformational changes in the enzyme or even its dissociation to an inactive form.

Reiner (1959) has formulated a kinetic approach to distinguish between different types of inhibition, particularly those involving complexes between the inhibitor and substrate. Analysis of the inhibition of G6PD by HCP according to Reiner yielded a hyperbolic plot of  $I_t$  versus  $\frac{i}{1-i}$  with an initial linear portion and a linear final portion (Figure 17). A plot of this type is characteristic (Reiner, 1959) of mechanisms either involving: 1) the inhibitor interacting directly with the enzyme; or 2) inhibition by formation of a substrate-inhibitor complex which inhibits the enzyme directly and also depletes the

effective concentration of substrate. To distinguish between these two possibilities, the final linear portions of the  $I_t$  versus  $\frac{i}{1-i}$  plots were extrapolated to the abscissa, giving positive values of  $I_t$  (Figure 17). The  $I_t$  intercept values, however, did not increase in proportion to substrate concentration. Such results have been interpreted by Reiner (1959) as evidence that a substrate-inhibitor complex mechanism is not involved. The Reiner method has been employed previously with MDH to identify inhibitory mechanisms involving substrate-inhibitor complexes (Wedding and Black, 1963; and Henneke and Wedding, 1975b).

My kinetic analysis of the inhibition of G6PD by HCP, therefore, suggests that inhibition of the enzyme most likely occurs through the binding of HCP at a minimum of two inhibitory sites on the enzyme. Since G6P and especially  $\text{NADP}^+$  protect against HCP inhibition and also reduce the binding of HCP to G6PD, the substrate binding sites may be the loci for HCP attachment to the enzyme.

Inhibition of ICD by HCP is competitive with respect to isocitrate at low HCP concentration but shows mixed kinetics at higher HCP concentration and with respect to  $\text{NADP}^+$  (Figure 11A and B and Table 9). The inhibition kinetics for GDH are more complex, being of the mixed type in relation to ammonium acetate and  $\alpha$ -ketoglutarate but uncompetitive with respect to NADH (Figure 12 and Table 9). Secondary plots of the slopes and intercepts from the reciprocal plots for HCP inhibition of these enzymes are generally parabolic in nature, although some linear curves were found with certain substrate (Figures 11C and D and 13). Analysis according to Reiner (1959) eliminated the possibility of a

significant contribution by a HCP-substrate complex to the inhibitory process. The finding of parabolic plots of the slopes and/or intercepts versus  $[I]$  as well as the Reiner analysis also suggests that HCP binds inhibitorily to at least two sites per molecule of ICD or GDH.

Homotropic interactions occur between identical ligand molecules and are expressed by a sigmoidal saturation curve of the enzyme with the ligand. In my study, the inhibition of G6PD and other dehydrogenases (ADH, GDH, ICD, LDH, and MDH) by HCP and its analogs show sigmoid rate curves as a function of bisphenol concentration. These results suggest the possibility of cooperative interaction between inhibitor molecules.

The sigmoidal relationship between velocity and inhibitor concentration can be examined further by the use of the Hill equation (11) (Jensen and Nester, 1966; Segel, 1975). The slopes ( $n'$  values) of the resulting Hill plots (Figures 8B and 14B) were generally greater than 1.0 indicating that HCP and related compounds interact cooperatively. The maximum interaction coefficient ( $n'$  value) for HCP with G6PD was 2.52 (Table 8) suggesting that the minimum number of inhibitor binding sites per enzyme molecule was three (Baskey et al., 1964; Jensen and Nester, 1966; Changeux et al., 1968; Davidoff and Carr, 1973; and Segel, 1975). Similar results with HCP were obtained for other dehydrogenases (Table 8) but other chlorinated bisphenols yielded  $n'$  values as high as 5.24 with G6PD (Table 10), implying that there could be as many as five inhibitor sites on this enzyme.

The slopes of my Hill plots were not constant but increased with

rather sharp transitions as the concentration of chlorinated bisphenol increased. Such results might be explained by the presence of multiple inhibitory sites with different affinities for the bisphenol inhibitor on the enzyme (Segel, 1975) or the enzyme changing from a state of low affinity to states of higher affinity for the inhibitor (Koshland et al., 1966). It is also possible that the changing Hill coefficient with different inhibitors concentrations resulted from dissociation-association processes of the enzymes. Slopes of Hill plots that vary with inhibitor concentration have been observed previously by Caskey et al. (1964) for purine ribonucleotide inhibitors of glutamine phosphoribosylpyrophosphate amido-transferase; by Jensen and Nester (1966) for prephenate inhibition of 3-deoxy-D-arabino-heptulosonate 7-phosphate; and by Davidoff and Carr (1973) for the inhibition of pyruvate kinase by triethyltin.

Several structurally similar analogs of HCP also were found to cause inhibition of G6PD. There was no good correlation between the inhibition of G6PD by these analogs and their partition coefficients or pKa's. The number and type of substituents, however, appear to be major factors in increasing the inhibitory effectiveness of the chlorinated bisphenols (Tables 10 and 11).

Inhibition of G6PD by 3,4-TCP and DCP was also inhibitor concentration dependent (Figures 15A and B and 16A and B; and Tables 10 and 11). The overall outlook was of the mixed type, but at higher bisphenol concentrations, inhibition tended to be close to competitive whereas at lower concentrations the inhibition approached noncompetitive.

When the slopes and intercepts of the reciprocal plots were re-plotted against 3,4-TCP and DCP concentrations (Cleland, 1963; Plowman, 1972; and Segel, 1975), generally parabolic curves were obtained with the former compound while the latter gave essentially linear plots (Figures 15C and D and 16C and D). Reiner (1959) plots indicated that these bisphenols interacted directly with the enzyme. These results suggest that 3,4-TCP like HCP, also binds with G6PD at a minimum of two inhibitory sites. DCP, which is much less active as an inhibitor of G6PD, appears to follow simple linear kinetics (Cleland, 1963; and Segel, 1975).

The effect of HCP on torula yeast G6PD was also studied by sucrose and glycerol density gradient sedimentation (Figures 17, 18, and 20-24). The results of these studies lead to the conclusion that HCP causes dissociation of the active dimeric form of G6PD to its inactive monomeric form. Catalase, BSA, and Hb were used as markers in identifying the MW of the G6PD dimer and monomer (Figures 18 and 24). The torula yeast G6PD monomer always banded at the position corresponding to Hb and BSA, which suggests a MW of about 65,000 for the monomer form. Treatment of G6PD with SDS resulted in the dissociation of the native dimer to an enzymatically inactive monomer with a MW similar to that of BSA; i.e., about 65,000 (Figure 19C). Kawaguchi and Bloch (1974) have shown that palmitoyl-CoA inhibits torula yeast G6PD and also deactivates the enzyme by promoting dissociation of the native dimer to an inactive monomer.

Besides dissociation, aggregation to higher molecular weight forms

sometimes was clearly seen in the density gradient sedimentation of G6PD in the presence of HCP (Figure 19E and F, Figure 21E and F, and Figure 22B and C). Since torula yeast G6PD has nonequivalent monomers of 49,000 and 61,000 molecular weight (Chilla and Domagk, 1974), dissociation of the enzyme dimer and reassociation of monomers could produce various molecular weight forms. The aggregated forms showed relatively low enzyme activity. It is possible that the aggregated enzyme possessed different kinetics and hence failed to show enzyme activity under the initial velocity assay condition. In most of these studies, the HCP:G6PD molar ratio were greater than 20. Higher MW polymeric forms of the enzyme were detected at a HCP:G6PD ratio of 26 while a ratio of 9 produced distinct differences in the slab gel electrophoresis pattern which were presumably due to formation of polymeric forms. Aggregation of torula yeast G6PD dimer to a tetramer has been reported to occur at low pH and high  $Mg^{+2}$  concentration Domagk et al., 1973).

HCP binds to all forms of torula yeast G6PD. Following sucrose density gradient sedimentation at a HCP:G6PD molar ratio of 26, the moles of HCP bound per 130,000 g of enzyme was 7 for the polymeric peak, 9 for the dimer peak, and 17 for the monomer peak. Dissociation of the active G6PD dimer to the enzymatically inactive monomer results in greater HCP binding to the G6PD monomer. Under the conditions of this experiment, the enzyme activity was 72% inhibited which corresponds to  $\bar{v} = 9$  in equilibrium dialysis study (Figure 28). These results

suggest that while 2-3 HCP binding sites affect G6PD enzyme activity, additional nonspecific binding of the bisphenol also occurs. When the HCP:G6PD ratio was increased to 36 (Figure 21), the moles of HCP bound per 130,000 g of G6PD were 6, 13, and 24 for the polymeric, dimeric, and monomeric forms, respectively. In this latter experiment, the enzyme was inhibited 94% which corresponds to  $\bar{v} = 24$  in the equilibrium dialysis study (Figure 28). At higher HCP:G6PD molar ratios the equilibrium thus tends to favor the dissociated monomeric form. Consequently, inhibition of torula yeast G6PD by HCP could in part proceed via dissociation of the active dimer form of the enzyme to its enzymatically inactive monomer form. Kawaguchi and Bloch (1974) also suggested that the inhibition of torula yeast G6PD by palmitoyl-CoA was through a mechanism involving conversion of the active G6PD dimer to its enzymatically inactive monomer form.

In the sucrose density gradient studies (Figures 19 and 20), the position of the G6PD dimer (at fraction 12) shifted (to fraction 14) with a corresponding reduction in enzyme activity upon exposure of the enzyme to HCP. A similar change in the buoyant density was not seen in the glycerol density gradient ultracentrifugation studies (Figures 22 and 24). These changes may be due to conformational changes in the enzyme induced by HCP. Such conformational changes in a protein are often associated with changes in protein stability.

Binding of HCP to free G6PD was studied by the method of equilibrium dialysis (Figures 26-30). The maximum number of moles of HCP bound to each mole of G6PD was assumed to be 68 at 4°C and 118 at 27°C

as determined by extrapolation of the least square fitted line of the highest slope part of the Scatchard plot (Figure 27) under the conditions employed. The Scatchard plots are not linear suggesting more than one type of binding site. A positive cooperative-like behavior at 4°C and negative cooperativity at 27°C were observed as judged by the shape of the slope of the curve. These results were similar to those found in the binding of cytidine triphosphate to the aspartate transcarbamylase of *E. coli* at 23°C, (Cook, 1972). Hill plots ( $\log \frac{\bar{v}}{n - \bar{v}}$  vs  $\log [\text{HCP}]_f$ ) of these data indicate a decrease in  $n'$  value from 1.7 to 1.2 at 4°C and a relatively constant value of 0.74 at 27°C (Figure 29). Presumably under conditions where maximum binding occurs (68 moles HCP per mole G6PD at 4°C), the enzyme has almost completely dissociated to its monomeric form (Figure 26 and 27).

The large number of binding sites plus the fact that the saturation binding was not reached seems to suggest that a large amount of nonspecific HCP binding to the enzyme occurs. Such additional low affinity binding could be explained by the proposal of Haque and Buhler (1972) that binding of HCP to protein is through hydrogen bonding between the phenolic protons of HCP and the oxygen atoms in the peptide bonds. In addition, ionic binding of the HCP anion may occur. Such binding would be affected by the ionic composition of the environment and could thus explain the disappearance of the  $^{14}\text{C}$ -HCP binding peak in the presence of 20 mM  $\text{MgCl}_2$  or 53 mM KCl in the density gradient sedimentation studies (Figure 24 F-I). The chelating character of HCP (Adams, 1958; and Adams and Hobbs, 1958) may also be responsible for

the reduced binding of HCP to G6PD when these ions are present.

A small number of higher affinity binding sites for HCP also must be present in G6PD as evidenced by the initial steep portion of the Scatchard plot (Figure 27). Specific HCP binding to critical sites on the enzyme also is to be anticipated from kinetic analysis of the inhibition data. Furthermore, a plot of  $\bar{v}$  versus percent inhibition (Figure 28) shows that 50% enzyme inhibition occurs at low HCP:G6PD ratios of 3-7.

The effect of HCP on G6PD was also examined by polyacrylamide slab gel electrophoresis (Figures 31-35). In general, the electrophoretic pattern shows aggregation (Figure 32) and dissociation (Figures 31-35) of the enzyme in the presence of HCP. The MW of the G6PD subunit was determined to be 60-65,000 from SDS polyacrylamide slab gel electrophoresis (Figure 31). This value is in good agreement with the MW of the G6PD monomer as estimated by density gradient sedimentation.

Electrophoresis of G6PD at pH 8.9 in the absence of SDS showed a single major band of enzyme activity at low HCP:G6PD ratios but enzyme activity was completely inhibited at higher ratios (Figure 33B). One or two broad, diffusely staining protein bands were formed in the presence of HCP. These bands migrate somewhat more rapidly than does G6PD in the absence of HCP. A small, faster moving protein band which lacked enzyme activity was formed at higher HCP:G6PD ratios. This latter, sharply defined band was probably the G6PD monomer since it migrated in a manner similar to BSA. Domagk et al. (1973) has shown, however, that torula yeast G6PD monomers are not identical and are

separated upon electrophoresis. Aggregation of rat liver G6PD dimer (MS 130,000) has been reported by Holten (1972) to yield components with MW's of 210,000, 280,000, 370,000, and additional bands between 370,000 and 500,000 on polyacrylamide disc gel electrophoresis.

Electrophoresis showed a distinct effect on the G6PD pattern by a HCP:G6PD molar ratio of 4 but dissociation became quite obvious at a ratio of 9 (Figure 33). At HCP:G6PD ratio greater than 300, dissociation of the enzyme was extensive.

In pH 7.5 buffer, more than one fast moving protein band also was detected after treating G6PD with HCP (Figure 34). Under these conditions, the two fast moving protein bands were of approximately equal size and stained similarly.

HCP also affected ICD and LDH (Figures 35A and B) as judged by the different electrophoretic patterns found after treatment of these enzymes with HCP. In addition, catalase which has a subunit of MW about 60-65,000, gave a fast moving protein band after treatment with HCP (Figure 35B, well No. 8) which migrated in a manner similar to BSA. These three proteins thus could be other examples of dissociation in the presence of HCP.

Because of the low solubility of HCP in aqueous solutions, the interaction of HCP with the substrates  $\text{NADP}^+$  and G6P could only be studied by difference spectroscopy (Figures 37-39). These experiments showed that HCP caused a reduction in HCP absorption at 300 nm when either  $\text{NADP}^+$  or G6P was present in the system. Such hypochromicity hence gave support to the formation of complexes between HCP and the two substrates. Formation of charge transfer complexes between various

phenols and  $\text{NAD}^+$  have recently been described by Henneke and Wedding (1975a, 1975b). Furthermore, such interactions have been considered to be partially responsible for the inhibition of MDH by phenols (Henneke and Wedding, 1975b). In the case of torula yeast G6PD, however, my kinetic analysis indicates that inhibition via a HCP-substrate complex is not significant.

## CONCLUSIONS

### Molecular Weight of Torula Yeast G6PD and Its Subunits

The molecular weight of the native torula yeast G6PD was determined to be 130,000 by sedimentation equilibrium ultracentrifugation (Appendix III). The active enzyme exists as a dimer but the inactive subunit had a molecular weight of 60-65,000 as determined by SDS polyacrylamide slab gel electrophoresis and sucrose density gradient sedimentation.

### Inhibition Kinetics

HCP inhibits pig heart ICD, horse liver ADH, torula yeast G6PD, beef heart MDH, and bovine liver GDH with  $I_{50}$  values ranging from 1.6-105  $\mu\text{M}$  under the enzyme concentrations studied. The interaction coefficient  $n'$  between HCP binding sites on these dehydrogenases ranged between 1.62 and 3.33 suggested positive cooperativity. In most cases HCP exhibited mixed kinetics giving average  $K_i$  values with G6PD of 16.6  $\mu\text{M}$  for  $\text{NADP}^+$  and 18.2  $\mu\text{M}$  for G6P; with ICD of 171  $\mu\text{M}$  for  $\text{NADP}^+$  and 4.0  $\mu\text{M}$  for isocitrate; and with GDH of 0.7  $\mu\text{M}$  for NADH, 1.4  $\mu\text{M}$  for  $\alpha$ -ketoglutarate and 0.5  $\mu\text{M}$  for ammonium acetate. The inhibition kinetics for torula yeast G6PD by other bisphenols, 3,4-TCP and DCP, were similar to those found with HCP. The inhibitory effect of various HCP analogs increased with the degree of chlorination of the aromatic rings. Secondary plots of the slopes and intercepts of the reciprocal plots versus inhibitor concentration were generally para-

bolic for HCP with respect to G6PD, ICD, and GDH as well as for 3,4-TCP with G6PD. Such parabolic slope and intercept plots are normally interpreted in terms of a minimum of two inhibitor molecules interacting with the enzyme.

Kinetic analysis according to Reiner (1959) suggested that the mechanism for HCP inhibition of G6PD was through the direct binding of the inhibitor to the enzyme and that inhibition via a HCP-substrate complex was relatively unimportant. Although kinetic studies by themselves cannot unequivocally establish the mechanism for an enzyme reaction, other physical evidence supports an inhibitory mechanism involving the strong binding of HCP to the dehydrogenase.

#### Dissociation and Aggregation of G6PD by HCP

Through sucrose and glycerol density gradient sedimentation and polyacrylamide slab gel electrophoresis studies, it was shown that HCP causes dissociation and aggregation of torula yeast G6PD to its inactive monomers or to polymeric forms with unknown enzymatic activity.

#### Binding of HCP to G6PD

Equilibrium dialysis data suggests that HCP binds to torula yeast G6PD at more than one type of sites. Certain high affinity binding sites are probably directly related to enzyme activity, since binding to these sites (3-7 moles HCP per mole G6PD of assumed MW 130,000) caused about 50% inhibition of the enzyme. Binding to nonspecific, low affinity sites also occurs with maximum binding of 52 moles of HCP to each mole of G6PD being found at 4°C. Saturation binding of HCP was not

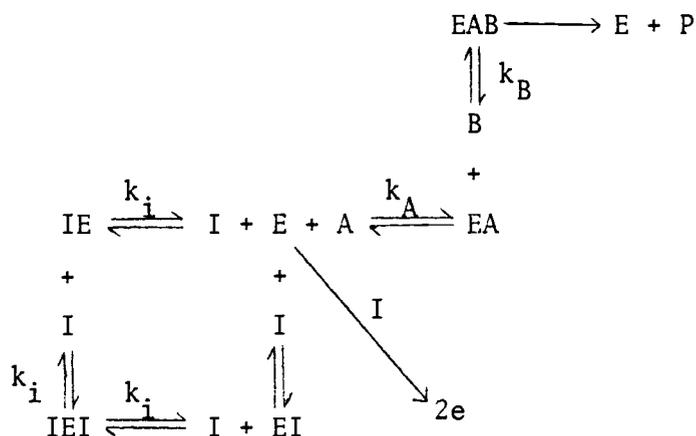
reached, however, even at the highest HCP concentration that could be tested. Extrapolation of the Hill plot of the binding data suggested that HCP could bind to a maximum of at least 68 sites at 4°C. Under these conditions, the enzyme is probably almost completely dissociated to its monomeric form.

### Binding of HCP to G6PD Substrates

Evidence for the association of HCP with  $\text{NADP}^+$  or G6P was obtained from measurements of difference spectra. These studies indicate the probable formation of charge transfer complexes between HCP and both  $\text{NADP}^+$  and G6P and suggest that this interaction is pH and concentration dependent. While it is possible that such complexes could be important in HCP inhibition of G6PD, no kinetic evidence for the involvement of such inhibitor-substrate complexes was obtained.

### Scheme for the Inhibition of G6PD from Torula Yeast by HCP

Based on the above conclusion, the following scheme for the inhibition of G6PD from torula yeast by HCP can be formulated:



where A and B represent the substrates  $\text{NADP}^+$  and G6P, respectively; P is the corresponding reaction products, NADPH and D-glucono- $\delta$ -lactone-6-phosphate; I is the inhibitor HCP; and e is the inactive subunit of the enzyme. The above scheme involves a sequential mechanism (Afolayan, 1972).

From the results of my studies as shown in the above scheme, the enzyme could be inhibited by direct binding of HCP to G6PD to form an inactive EI complex which then could react with a second molecule of HCP to form an inactive IEI complex.

In addition to binding to these inhibitory sites, HCP also binds to a large number of nonspecific sites on G6PD.

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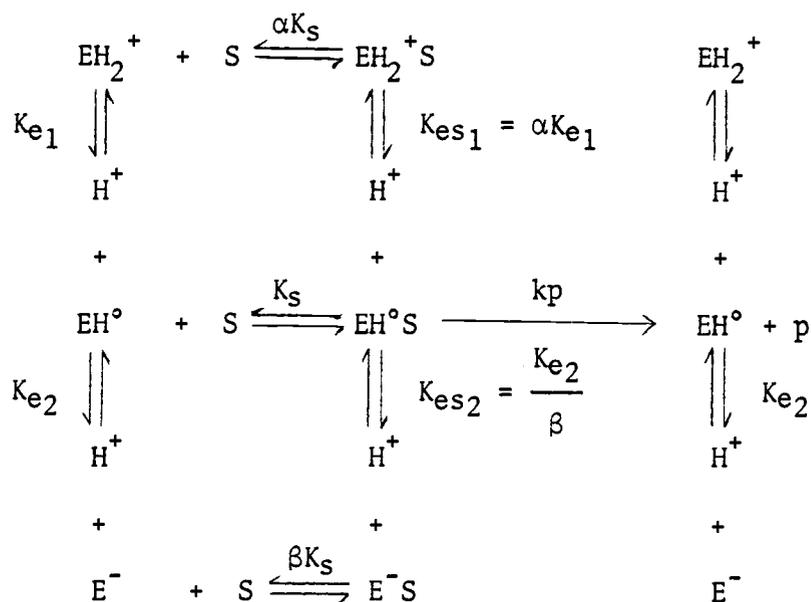
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## APPENDICES

## APPENDIX I

Derivation of Equation (7) and (8)

Assuming an enzyme has many ionizable groups that bind or release protons under the influence of the pH value in the surroundings to form various species such as  $\text{EH}_2^+$ ,  $\text{EH}^\circ$ , and  $\text{E}^-$ , the following scheme can be expressed (Segel, 1975):



$$\frac{v}{V_{\max}} = \frac{[s]}{K_s \left(1 + \frac{[\text{H}^+]}{\text{Ke}_1} + \frac{\text{Ke}_2}{[\text{H}^+]}\right) + [S] \left(1 + \frac{[\text{H}^+]}{\text{Kes}_1} + \frac{\text{Kes}_2}{[\text{H}^+]}\right)} \quad (13)$$

The derivation of equation (13) was given by Segel (1975).

Rearranging equation (13), we have

$$\frac{1}{v} = \frac{K_s \left(1 + \frac{[\text{H}^+]}{\text{Ke}_1} + \frac{\text{Ke}_2}{[\text{H}^+]}\right)}{V_{\max}} \cdot \frac{1}{[S]} + \left(1 + \frac{[\text{H}^+]}{\text{Kes}_1} + \frac{\text{Kes}_2}{[\text{H}^+]}\right) \frac{1}{V_{\max}} \quad (14)$$

Comparing equation (14) with the regular Michaelis-Menten equation,

$$\frac{1}{v} = \frac{K_s}{V_{\max}} \cdot \frac{1}{[S]} + \frac{1}{V_{\max}} \quad (15)$$

we get

$$\frac{1}{V_{\max_{\text{app}}}} = \frac{1}{V_{\max}} \left( 1 + \frac{[H^+]}{K_{es_1}} + \frac{K_{es_2}}{[H^+]} \right)$$

or

$$V_{\max_{\text{app}}} = \frac{V_{\max}}{1 + \frac{[H^+]}{K_{es_1}} + \frac{K_{es_2}}{[H^+]}}$$

At low pH, decreasing  $[H^+]$  increases the reaction rate by promoting the formation of  $EH^{\circ}$  and  $EH^{\circ}S$ . In this region the  $\frac{[H^+]}{K_{es_1}}$  term can be ignored. Then we have:

$$V_{\max_{\text{app}}} = \frac{V_{\max}}{1 + \frac{K_{es_2}}{[H^+]}} \quad (16)$$

Rearranging equation (15) and changing to new symbols of  $V$ ,  $V_0$ , and  $K$  for  $V_{\max_{\text{app}}}$ ,  $V_{\max}$ , and  $K_{es_2}$ , respectively, and solving for  $[H^+]$ , we get,

$$[H^+] = K \frac{V}{V-V_0} \quad \text{or}$$

$$pH = pK - \log \frac{V/V_0}{1-V/V_0} \quad \text{or} \quad \log \frac{V/V_0}{1-V/V_0} + pH = pK \quad (7)$$

At high pH, decreasing  $[H^+]$  reduces the reaction rate by increasing the dissociation of  $EH^\circ$  and  $EH^\circ S$ . In this region the  $\frac{K_{es2}}{[H^+]}$  term can be neglected. Then we have:

$$V_{\max_{app}} = \frac{V_{\max}}{1 + \frac{[H^+]}{K_{es1}}}$$

Solving for  $[H^+]$  and using symbols of  $V$ ,  $V_o$ , and  $K$  for  $V_{\max_{app}}$ ,  $V_{\max}$ , and  $K_{es1}$ , respectively, we get:

$$pH = pK - \log \frac{1 - V/V_o}{V/V_o} \quad \text{or} \quad \log \frac{V/V_o}{1 - V/V_o} + pK = pH \quad (8)$$

## APPENDIX II

Effect of Gradient Constituents on Protein and G6PD Assays

Within a protein range of 0-20  $\mu\text{g/ml}$ , the presence of sucrose of 0.17-1.7% final concentration tended to produce lower absorbance readings than the true values in the Lowry protein assay (Figure 40A). Gerhardt and Beevers (1968) reported that the effect of sucrose on the Lowry assay is protein concentration-dependent.

A final CsCl concentration of 0.26-2.6% gives a large precipitate in the Lowry protein assay. Glycerol and ammonium sulfate also seriously influence the Lowry assay (Figure 40B and C). In addition, HCP reacts strongly with the Folin reagent in a quantitative manner (Figure 41).

Due to the above observations, the Lowry protein procedure could not be used directly to determine protein in gradient sedimentation studies. Consequently, fractions from glycerol and sucrose density gradients were assayed for protein by the procedure of Schaffner and Weissman (1973), a micro method not effected by the presence of Tris buffer, glycerol, sucrose, or HCP.

Addition of sucrose and glycerol to final concentrations of 1.4 w/v and 1.3 w/v, respectively, in the reaction mixture did not influence G6PD activity. Therefore, fractions from sucrose and glycerol density gradient sedimentations could be tested directly for enzyme activity. Glycerol was reported to be an excellent stabilizing agent, particularly at 20%, for mammary G6PD (Levy, 1963).

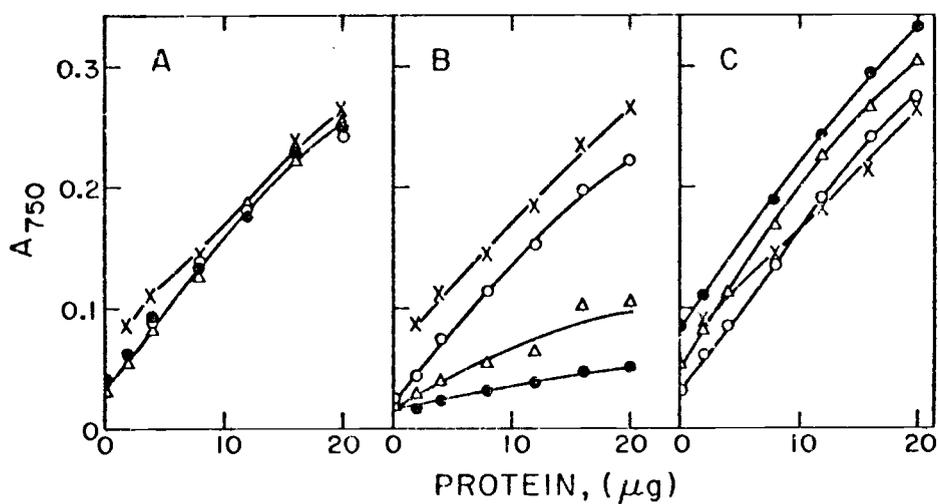


Figure 40. Effect of sucrose, ammonium sulfate, and glycerol on Lowry protein assay. A. Sucrose zero addition (control (x ---- x); 0.14% (O—O); 0.7% (Δ—Δ); and 1.4% (●—●)). B. Ammonium sulfate: zero addition (control, (x ---- x); 0.29% (O—O); 1.45% (Δ—Δ); and 2.9% (●—●)). C. Glycerol: zero addition (control, (x ---- x); 0.65% (Δ—Δ); 0.13% (O—O); and 1.3% (●—●)).

The normal procedure of Lowry et al. (1951) was followed using BSA as the reference protein. The final 1.3 ml contained 0.05 ml of the chemicals under test.

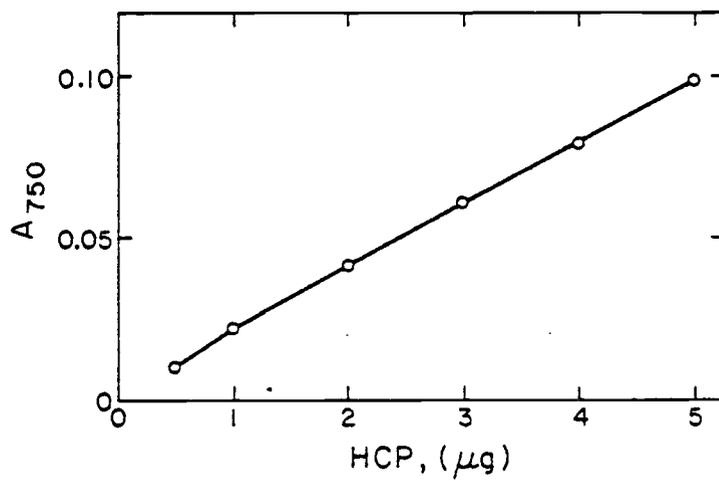


Figure 41. The effect of HCP in the protein assay procedure of Lowry et al. (1951). Different amount of HCP was used instead of protein.

Density gradients with CsCl could not be used in these studies since this chemical inhibited G6PD severely (Figure 42). Based on the effect of high  $Mg^{+2}$  concentrations on G6PD activity (Figure 5A), the inhibitory effect of CsCl on the enzyme could be due to a high ionic strength.

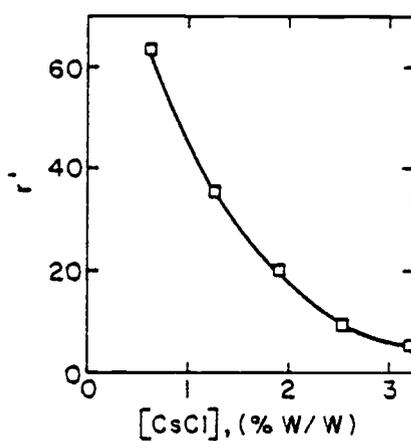


Figure 42. Effect of CsCl on G6PD activity. Standard G6PD assay procedure was employed except 1.10 ml of the CsCl solution was added into the reaction mixture (final volume was 1.05 ml). Fractional residual activity,  $r'$ , was calculated as the rate with inhibitor divided by the rate in the absence of inhibitor.

## APPENDIX III

Sedimentation Equilibrium

Sedimentation equilibrium experiments were performed according to the method of Yphantis (Yphantis, 1964; van Holde, 1967). This technique also known as the "meniscus depletion method" or the "high-speed method," used a six-channel short-column equilibrium type centerpiece (Beckman Instruments, Technical Bulletin E-TB-015B, 1964) on a Model E analytical ultracentrifuge (Beckman Instruments). In the Yphantis (1964) method, equation (17) was used for determination of the molecular weight (M).

$$\ln \frac{c(r)}{c(a)} = \frac{M (1-\bar{v}\rho) \omega^2}{RT} \left( \frac{r^2 - a^2}{2} \right) \quad (17)$$

where M is the molecular weight,  $\bar{v}$  the partial specific volume of the solute,  $\rho$  the solution density, R the gas constant ( $8.314 \times 10^7$  ergs/° mole), T the absolute temperature,  $c(r)$  the concentration at point r, and  $c(a)$  the concentration at meniscus a. In this method, the centrifuge is operated at such high speed that the meniscus concentration is negligible and the absolute concentration at any point is equal to the concentration difference between that point and the meniscus. In the experiment, initial protein concentrations varied from about 0.6 to 1.0 mg/ml and a column height of about 3 mm was used (0.1 ml of solution). An interference optical system was employed to observe the protein distribution. The centrifugations were run at speeds and temperature as indicated in each experiment.

A Kodak Type 77 A filter was placed directly above the light source to isolate monochromatic light and the interference fringe patterns were recorded on Kodak Type II-G spectroscopic glass plates. A "baseline" photograph was normally taken before the centrifuge reached running speed and was used to correct for fringe displacements caused by aberrations in the optical system. Another photograph was taken at about 20 hr or as indicated after initiation of the experiment and at 4-6 hr intervals thereafter until the protein distribution attained equilibrium.

The fringe displacements resulting from the protein concentration were measured on Nikon Model 6C microcomparator following the method of Yphantis (1964). The values of  $\ln c(r)$  and  $(r^2 - a^2)$  were calculated for each set of data points. Then  $\ln c(r)$  was plotted as a function of  $(r^2 - a^2)$ . The slope of this plot was used for determining the molecular weight. Linear molecular weight  $[M (L)]$  was produced by a least-square linear fit to the points and Quad molecular weight  $[M (Q)]$  fit a sectioned quadratic to a selected portion of the data. All the data were analyzed with the aid of a Control Data Corporation 3300 computer (Dyson, 1969).

The partial specific volume ( $\bar{v}$ ) for G6PD was taken as 0.739 for temperature near 3°C and 0.744 for approximately 20°C (Yue et al., 1967, 1969), and the density of the solution was assumed to be equal to the density of the solvent (assuming 1.0).

All the work on analytical ultracentrifugation was kindly carried out by S. Roxby, M. Drury, or Dr. R. Dyson.