PURIFICATION OF URIDINE PHOSPHORYLASE OF RAT LIVER, SULFHYDRYL GROUP MODIFICATION, AND INITIAL VELOCITY AND PRODUCT INHIBITION STUDIES

A Thesis

Presented to

The University of Manitoba

In Partial Fulfillment

of the Requirements for the Degree of

Doctor of Philosophy

by

Arthur Kraut

October 1969

Dedicated to my parents

ACKNOWLEDGMENTS

The author is indebted to Dr. E.W. Yamada for her contribution to my development as a biochemist.

In addition, the author acknowledges, with thanks the always available assistance offered by the staff and the graduate students of the Biochemistry department; but the third eye of Dr. M.C. Blanchaer stands alone.

Appreciation is extended to Dr. T. Pritchard for the use of the radiochromatogram scanner, and to Dr. M. Zimmerman for his generous gift of deoxyglucosylthymine.

The technical assistance of the following people is gratefully acknowledged, without any reservation (in order of appearance): Mr. B. Grift (technician); Miss S. Grant (linguist); Mr. S. Silver (boy scout leader); Miss A. Loadman (flutist); Mr. A. Hasnain (actor); Mr. A. Leung (bartender). Special thanks to Miss B. Odger, Mrs. B. Barnett and Miss C. Hanneson for the part they performed in the actual production of this thesis.

This investigation was supported by research grants to Dr. Yamada from the Medical Research Council of Canada.

PURIFICATION OF URIDINE PHOSPHORYLASE OF RAT LIVER, SULFHYDRYL GROUP MODIFICATION, AND INITIAL VELOCITY AND PRODUCT INHIBITION STUDIES

by

Arthur Kraut

ABSTRACT

This study entailed the purification of uridine phosphorylase (uridine:orthophosphate ribosyltransferase, E.C. 2.4.2.3) from rat liver homogenates. With the aid of heat treatment, DEAE-Sephadex, Sephadex G-200, and hydroxylapatite chromatography, a 1,900-fold purification of the enzyme was achieved. By disc gel electrophoresis, this preparation was shown still to contain two major bands. Certain properties of uridine phosphorylase such as, the pH-activity relationship, molecular weight, transferase activity, and stability were investigated.

The initial velocity patterns of nucleoside cleavage and uridine synthesis, and the product inhibition patterns of uridine cleavage were consistent with a kinetically ordered mechanism which has a ternary complex and in which phosphate and α-ribose-1-phosphate combine with the free enzyme. Uracil inhibited uridine and deoxyuridine synthesis with 50% inhibition occurring at a concentration of about 1 mM.

Aged purified preparations can be substantially reactivated with 2-mercaptoethanol or dithiothreitol. Various studies were done with seven sulfhydryl reagents including time, concentration, and pH studies. The results suggest that uridine phosphorylase has at least one sulfhydryl group at or near its active site.

A model for the mechanism of uridine cleavage is presented.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

Ac - acetate (buffer)

AS - ammonium sulfate (fraction)

Cdr - cytidine; CMP, CDP, CTP, --5'-mono-, di-, and tri-phosphate esters of cytidine

DEAE - diethylaminoethyl

DGT - deoxyglucosylthymine

DTT - dithiothreitol

EDTA - ethylene diamine tetraacetate (sodium salt)

G-G - glycylglycine (buffer)

I_{50} - concentration for 50% inhibition

K_{ia} - dissociation constant for substrate A

K_{ii} - intercept inhibition constant

K_{iq} - dissociation constant for substrate Q

K_{is} - slope inhibition constant

K_m - Michaelis constant (also K_a, K_b, K_p, K_q). Apparent K - constant not determined by replots

KCl - potassium chloride

PCA - perchloric acid

P_i - inorganic phosphate (buffer)

pK - -log equilibrium constant

PNP - purine nucleoside phosphorylase

RER - rapid equilibrium random mechanism

R-1-P - α-ribose-1-phosphate

dR-1-P - α-deoxyribose-1-phosphate

SH - sulfhydryl

2-SH - 2-mercaptoethanol

T-C - Theorell-Chance mechanism

Tdr - thymidine; TMP, TDP, TTP --5'-mono-, di-, and tri-phosphate esters of thymidine

Tdrpase - thymidine phosphorylase

Tris - tris-hydroxymethylaminomethane (buffer)

U - uracil

Udr - deoxyuridine

Ur - uridine; UMP, UDP, UTP --5'-mono, di-, and tri-phosphate esters of uridine

Urpase - uridine phosphorylase

v - initial velocity

V_M - maximum velocity

Sulfhydryl group inhibitors

DTNB - 5,5' dithiobis(2-nitrobenzoic acid) or Ellman's reagent

IA - iodoacetate

INH_2 - iodoacetamide

IOB - o-iodosobenzoate

NEM - N-ethylmaleimide

pMB - p-mercuribenzoate

pMPS - p-mercuriphenylsulfonate

SECTION I

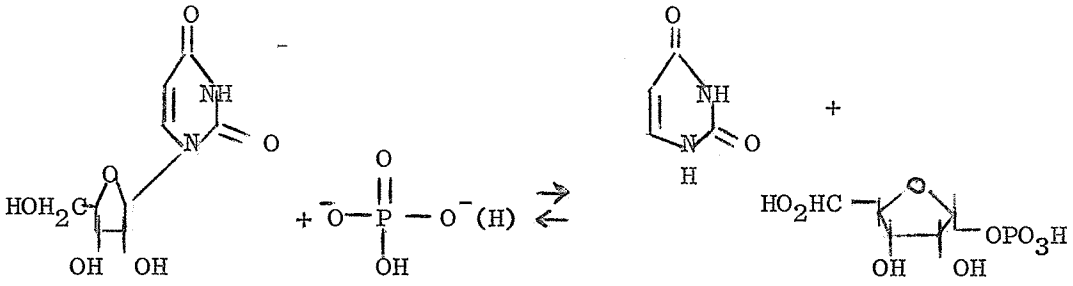
INTRODUCTION

It is well to remember, however, that though these practical benefits of biochemistry have been great, and promise to be greater, they are the result of studies largely undertaken for their own sake, rather than as conscious attempts to cure a disease or to increase a crop.

Fruton and Simmonds
in
'General Biochemistry'

A. ORGANIZATION OF THE THESIS

Uridine phosphorylase (E.C. 2.4.2.3.) catalyzes a reversible transfer of ribose or deoxyribose from certain pyrimidine nucleosides to inorganic phosphate. A typical reaction is illustrated below in which uridine is the nucleoside:



uridine — inorganic phosphate — uracil — α-ribose-1-phosphate

This thesis on uridine phosphorylase is divided into three major sections:

(1) Purification and properties

(2) Sulfhydryl group modification studies

(3) Initial velocity and product inhibition studies

A search through the literature has revealed that only a few papers have been published on these topics. These papers will be discussed in some detail in the appropriate sections, although a synopsis of recent papers on uridine phosphorylase is given below. For the purpose of orientation this section will also include a classification of nucleoside cleaving enzymes, and some comments on the metabolic role of nucleoside phosphorylases.

B. LITERATURE REVIEW

1. Recent (1960-1969) Studies on Uridine Phosphorylase

The latest survey of phosphorylases (1) and nucleoside phosphorylases (2), in particular, was published in 1961. Since this time more detailed studies have been published on nucleoside phosphorylases, i.e. purine nucleoside phosphorylases (3-7), thymidine phosphorylases (8-12), and uridine phosphorylases (7, 13-21).

Up to 1960 the studies of uridine phosphorylase were primarily concerned with the role of this enzyme in metabolism (22-27). The studies appearing since 1960 can be annotated in the following way: purification (7, 13, 14, 28); classification (15); induction and regenerating liver experiments (16-19, 29); enzyme activity with analogues (13, 20, 21, 30-34); kinetics (13, 35). Thus, perhaps five of these papers (13, 14, 19, 26, 35) are of direct importance to this particular study, although comparisons are made with certain points in other papers (22, 25, 27).

2. Classification of Nucleoside Cleaving Enzymes

Since the 1936 studies of Cori and Cori the name 'phosphorylase' has become a generic term for all enzymes that catalyze the cleavage of their organic substrates by a phosphorolytic mechanism (36). These enzymes are the principal ones, if not the only ones, for the cleavage of nucleosides in mammalian tissues. Transferases and hydrolases are the two other major classes of nucleoside cleaving enzymes in living matter.

a) Phosphorylases

i) Uridine phosphorylase, thymidine phosphorylase, and purine nucleoside phosphorylase

Kalckar, in 1947, during his study of purine metabolism (37) in mammalian tissues, was the first to suggest that phosphorolysis of nucleoside linkages may be of universal significance in nucleic acid metabolism. In 1954, Friedkin and Roberts isolated a pyrimidine deoxyribosyl phosphorylase from horse liver, which they named 'thymidine phosphorylase' (38). Two years earlier, however, Paege and Schlenk had studied a pyrimidine ribonucleoside phosphorylase from Escherichia coli (27). They named it 'uridine phosphorylase', but its specificity for ribose-1-phosphate was not established. Later work showed that an enzyme isolated from E. coli had some activity, albeit fairly low, towards deoxyuridine (one-third that of uridine) and thymidine (one-twentieth that of uridine) (15). For the enzyme from mammalian tissues some workers suggested that the name uridine-deoxyuridine phosphorylase would be a more descriptive one (20), since the enzyme has high cleaving activity towards uridine and deoxyuridine. Nevertheless, the systematic name is still uridine:orthophosphate ribosyltransferase.

A classification of nucleoside phosphorylases incorporating information found in the literature up to this time (July, 1969) is shown schematically in Figure 1A. The sub-divisions given for uridine phosphorylase and purine nucleoside phosphorylase should not be considered of universal significance yet, for only a few preparations from different sources have been studied. Still, nucleoside phosphorylases are readily separable into two major groups based on the specificity of the enzyme towards the nitrogenous base (1): those that are active with

purine nucleosides and those that are active with pyrimidine nucleosides. The specificity of the enzymes also appears to be directed towards the N-glycosidic bond of the nitrogenous base since the pyrimidine nucleoside cleaving enzymes catalyze the formation and cleavage of 3-pentosyl purines and the purine nucleoside cleaving enzymes of 9-pentosyl purines (31, 32). Partially purified preparations of uridine phosphorylase can catalyze a reversible reaction with 3-ribosylxanthine (32), the cleavage of 3-ribosyluric acid (15), and the synthesis of 3-ribosyloxallopurinol (31). Thymidine phosphorylase can catalyze a reversible reaction with 3-deoxyribosylxanthine (32), and the synthesis of 3-deoxyribosylallopurinol (31). These findings are consistent with the observation that a pyrimidine ribonucleotide pyrophosphorylase, purified 5,400 from beef erythrocytes, can also accept purines as substrates to form 3-ribosylnucleotides (39).

Pyrimidine nucleoside phosphorylases (Figure 1A) have been separated into a uridine phosphorylase fraction and a thymidine phosphorylase fraction (14, 19). Cytidine phosphorylase activity has not as yet been detected. The separation of these phosphorylases from mammalian tissue extracts was accomplished six years (14) after their separation from extracts of E. coli (40). These phosphorylases were not further purified after being separated and they were probably between 10-20 fold pure relative to the homogenate. In the initial separation from rat liver by DEAE-cellulose chromatography (15), the uridine phosphorylase fraction had significant activity towards three naturally-occurring nucleosides, in the order uridine > deoxyuridine $\gg$ thymidine, at pH 7.4. The thymidine phosphorylase fraction, on the other hand, was highly specific for the deoxyribosyl moiety, and the major substrates

were deoxyuridine and thymidine with uridine being cleaved to a very small extent at pH 7.4 (data for rat liver thymidine phosphorylase was not given). The uridine phosphorylases were also grouped according to their pH optimum for the phosphorolysis of uridine. Some tissue sources have an enzyme with a pH optimum of 8.1 (group A, Table I), while others have a pH optimum of 6.6 (group B, Table I). The latter enzymes were postulated to act by a hydrolytic mechanism with phosphate acting as a stabilizer rather than as a substrate. It is now known, however, that E. coli and guinea pig intestinal enzymes operate phosphorolytically for they have been shown to catalyze reverse reactions with ribose-1-phosphate (31); thermodynamically, a hydrolytic mechanism would not be easily reversed. In addition, the different pH optima found for the uridine phosphorylase fractions suggested to the authors (15) that the enzymes in group A would be found in the nucleus of the cell, while those in group B would be found in the cytoplasm. This relationship between pH optimum and intracellular location was not apparent for the uridine phosphorylases of rat liver (to be discussed below) (19).

Certain features in the chromatograms in the paper by Krenitsky, Mellors, and Barclay (15) bring up the question of whether uridine and thymidine phosphorylases from different sources are separable to the same extent by DEAE-cellulose chromatography (discussed again in section II E 2c).

(1) The relative activity of each enzyme fraction for the substrates varied widely. For example, dog liver uridine phosphorylase had very high cleaving activity towards both uridine and thymidine (thymidine phosphorylase activity was not detected), whereas in mouse intestinal epithelium, uridine phosphorylase had eight times greater

activity towards uridine than towards thymidine.

(2) The relative proportions of both enzymes varied widely. For example, mouse liver had very high thymidine phosphorylase activity and low uridine phosphorylase activity, whereas the reverse was true for mouse intestinal epithelium. In Ehrlich ascites cells, a thymidine phosphorylase fraction was not detected. Of course, it is quite likely that the difference in distribution was due to the nature of the source.

(3) The relative position of the activity peaks for the two enzymes from different sources changed (14, 15).

DEAE-Sephadex chromatography separated a rat liver preparation into three active fractions (19): a cytoplasmic uridine phosphorylase, a cytoplasmic thymidine phosphorylase, and a nuclear uridine phosphorylase (it contained about 10% of the total uridine cleaving activity). Recent work from the same laboratory, however, suggested that the latter enzyme is localized mainly on the plasma membrane (Bose and Yamada, unpublished results). On the basis that they catalyze the same reaction, are separated by chromatography on DEAE-Sephadex, have similar pH optima and substrate specificities, it was suggested that the uridine phosphorylases might be isoenzymes (19).

An anomalous pyrimidine nucleoside phosphorylase, partially purified from fish muscle (7), catalyzed the phosphorolysis of uridine and thymidine to about the same extent, but acted very slowly on deoxyuridine. Perhaps this is an indication of two very specific phosphorylases as found in E. coli; however, these activities were not separable by DEAE-cellulose chromatography (DEAE-Sephadex chromatography was not used).

ii) Differentiation between uridine phosphorylase and thymidine phosphorylase

The information available at the present time points to a number of possible differences between these two enzymes, apart from substrate specificity.

(1) Substrate specificity. Basically, uridine phosphorylase is specific for the pyrimidine moiety of the nucleoside and thymidine phosphorylase for the deoxyribose moiety (15).

(2) Order of addition of substrates to the enzyme (25, 35). This is discussed in the 'Introduction' to section IV.

(3) Transferase activity (11, 35). This is discussed in section I B 2b.

(4) Effect of inhibitors. Deoxyglucosylthymine inhibited the uridine, deoxyuridine, thymidine cleaving activities of a partially purified uridine phosphorylase from Ehrlich ascites cells. It had no effect on the deoxyuridine and thymidine cleaving activities of an extract of thymidine phosphorylase from horse liver, but it produced a 50% inhibition of deoxyuridine cleaving activity of a crude rat liver preparation (33).

2-Thiouracil may inhibit uridine phosphorylase preferentially but the evidence is vague at this time. It has been shown that nucleoside synthesis occurred using a partially purified thymidine phosphorylase preparation from E. coli with 2-thiouracil and deoxyribose-1-phosphate or ribose-1-phosphate (1/250th that of deoxyribose-1-phosphate) (40). Yet with the horse liver enzyme/ribo- and deoxyribonucleosides were formed with 2-thiouracil to the same extent with a partially purified thymidine phosphorylase preparation (41). Uridine phosphorylase from rat liver

could not form the ribonucleoside from 2-thiouracil, yet the latter inhibited the synthesis of uridine (21). It may be that 2-thiouracil is a substrate for uridine phosphorylase only in the presence of deoxyribose-1-phosphate. Another point that has not been checked is the activity of rat liver thymidine phosphorylase for 2-thiouracil with ribose-1-phosphate and deoxyribose-1-phosphate.

Allopurinol did not inhibit the synthesis of uridine by a crude uridine phosphorylase preparation from human leukocytes, but it did inhibit the formation of deoxyuridine by thymidine phosphorylase (42). Again the inhibition of deoxyuridine synthesis by uridine phosphorylase was not reported.

(5) Activity with substrate analogues. Partially purified mammalian uridine phosphorylases catalyzed the cleavage of 3-ribosyluric acid (15), the synthesis of 3-ribosyloxallopurinol (31), and possibly the cleavage of 1-β-D-arabinosyluracil (43 (*E. coli* enzyme)). Experiments were not done to check whether uridine and thymidine phosphorylases could catalyze the cleavage of 3-deoxyribosyluric acid, or the synthesis of 3-deoxyribosyloxallopurinol. Thymidine phosphorylase catalyzed the synthesis of deoxyribosylxanthine at a greater rate than uridine phosphorylase catalyzed the synthesis of ribosylxanthine (32). The synthesis of deoxyribosylxanthine by uridine phosphorylase was not checked. A thymidine phosphorylase preparation (20,000 x g) from human spleen did not catalyze the phosphorolysis of β-D-arabinosylthymine, or of β-D-arabinosyluracil (30).

(6) pH optimum. Uridine phosphorylase has a pH optimum of 8.1 for uridine cleavage and 6.6 for deoxyuridine cleavage (14, 19) whereas thymidine phosphorylase has a pH optimum of 5.9 for the phosphorolysis

of deoxyuridine and thymidine, and one of 7.3 for transferase activity (10, 30).

b) Transferases

There are two possible mechanisms by which a pentosyl transfer can occur (35):

i) Indirect transfer (phosphate dependent)

The initial phosphorolysis of a nucleoside results in the formation of a free pentose-1-phosphate or enzyme-bound pentose-1-phosphate, which then can react with a free base (B) to form a new nucleoside. This is simply the reverse of phosphorolysis, that is,

$$B_1\text{-pentose} + \text{phosphate} \overset{\rightarrow}{\leftarrow} B_1 + \text{pentose-1-phosphate}$$

$$\text{pentose-1-phosphate} + B_2 \overset{\rightarrow}{\leftarrow} B_2\text{-pentose} + \text{phosphate}$$

ii) Direct pentosyl transfer (phosphate independent)

A nucleoside or an enzyme pentosyl intermediate is the pentosyl donor:

$$B_1\text{-pentose} + B_2 \overset{\rightarrow}{\leftarrow} B_2\text{-pentose} + B_1$$

The indirect transfer mechanism should be catalyzed by all phosphorylases under appropriate substrate conditions, however the direct mechanism is not a general one. With purified purine nucleoside phosphorylases and thymidine phosphorylases mechanism (ii) occurs, with the phosphorylase and transferase activities apparently catalyzed by one enzyme (3, 8, 9, 11, 12, 44). Thymidine phosphorylases catalyze deoxyribosyl transfer between pyrimidines while purine nucleoside phosphorylases catalyze ribosyl and deoxyribosyl transfer between purines.

One report with purine nucleoside phosphorylase suggests that the two activities are functions of different but interconvertible molecular species of one enzyme (45). Some of the transferase studies with the purine nucleoside phosphorylase (3, 44) may be invalidated by possible trace amounts of phosphate in the enzyme preparation (5, 35).

Evidence at this time suggests that uridine phosphorylase does not catalyze a ribosyl transfer (30, 33, 35), however this was not checked with a purified preparation that is not contaminated with thymidine phosphorylase activity. In addition, a search of the literature did not reveal any studies on deoxyribosyl transfer with uridine phosphorylase. Conditions which may be important in the study of transferase activity of uridine phosphorylase will be mentioned in the 'Discussion' (section II E 6c). In summary, conclusive evidence for direct transfer in mammalian tissues has been presented for thymidine phosphorylase only. In bacteria, separate enzymes have been found that catalyze a direct ribosyl transfer (46) or a direct deoxyribosyl transfer (47), both being nonspecific for the accepting base.

c) Hydrolases

Purine and pyrimidine ribonucleoside hydrolases have been found in bacteria (48), yeast (49), mung bean (50), and fish muscle (51). Similar enzymes have not yet been shown to occur in mammalian systems (2).

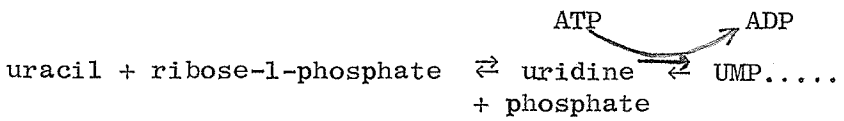
3. Rat Liver as a Source of Uridine Phosphorylase

Friedkin and Kalckar (2) tabulated sources of nucleoside phosphorylases from 1930-1958. Table I gives more recent examples from the

literature on the distribution of uridine phosphorylase, although few new sources are evident (no Urpase was extensively purified, and only in references (15) and (19) has a separation of Urpase from Tdrpase been shown). Results on specific activity and total activity of uridine phosphorylase preparations are scant, and in addition they are difficult to compare for the various authors used different units of activity. Also, in one study (25) enzyme activity was assayed by an indirect ribose transferase reaction (which is assumed in Table I to represent Urpase only). For clarity, the specific activity values and the units used are given in Table II. It is apparent that the specific activity of uridine phosphorylase in liver may not be the highest, but liver is probably the best source in terms of total activity. In addition, much of the metabolic work has been done with rat liver (16-19, 22-25).

4. Role of Uridine Phosphorylase in Metabolism

Besides playing an important role in nucleic acid catabolism by catalyzing the cleavage of nucleoside, uridine phosphorylase is one of the phosphorylases in the 'salvage' (52) or 'reserve' pathway (22) of nucleic acid anabolism. This pathway utilizes the combined action of nucleoside phosphorylases and nucleoside kinases:



At the formation of the nucleotide, the de novo and salvage pathways merge. The interrelationship of these two pathways in the synthesis of nucleic acids was shown schematically in a review by Sugino (53). This article was concerned with metabolism of deoxyribonucleotides;

still very little information was given on any of the phosphorylases. In addition, there is evidence for the direct synthesis of mononucleotides from pyrimidines and phosphoribosylpyrophosphate in beef erythrocytes, mouse liver, calf thymus (39) and mouse leukemic cells (54). This complicates the salvage pathway as shown above, but as yet a pyrimidine ribonucleotide pyrophosphorylase has not been found in normal rat liver (26).

Another de novo pathway, not involving orotic acid but rather free uracil, had been proposed about ten years ago (52, 55). It was discounted, however, after an evaluation of the reversibility of the enzymes which were thought to be involved in this pathway. Briefly, the pathway entailed the decarboxylation of carbamyl-aspartate followed by the action of the hydrolase and dehydrogenase from the pyrimidine catabolic pathway. After uracil had been so synthesized the action of the salvage pathway enzymes would lead to the production of UMP.

The physiological role of the salvage pyrimidine nucleotide pathway is not entirely clear but several possibilities will be discussed. Skold (22) has shown that in some cases there is a direct correlation between the activity of the salvage pathway enzymes and growth. For example, Ehrlich ascites cells, intestinal mucosal cells, and regenerating liver of mouse show high activity of the salvage pathway enzymes and low activity of the de novo pathway as a whole; heart muscle, brain,and liver from mouse, show relatively low phosphorylase and kinase activities. Unfortunately, bone marrow which also has a rapid cell turnover has relatively low enzyme levels, but this may have been due to difficulties during isolation (22). Another point to be explained is why the main increase in uridine phosphorylase activity comes about 12 hours after the main increase in

ribonucleic acid synthesis (22). But a factor pointing to a role of the salvage pathway enzymes in growth is the concomitant decrease in the enzymes involved in the catabolism of pyrimidines in regenerating liver (22, 56); liver is believed to be the only organ to have the catabolic enzymes (26, 52, 57).

Yamada has shown that in regenerating rat liver, the specific activity of nuclear and cytoplasmic uridine phosphorylases were increased by injecting a rat with uridine or cytidine; on the other hand, the specific activity of thymidine phosphorylase was not changed by uridine injection and it was decreased after cytidine injection (19). Cortisol injection also increased uridine and deoxyuridine phosphorylase activities in normal and regenerating liver (16). Perhaps in the future some relationship will be shown to exist between this response to inducers and growth.

Kornberg speculated that reactions such as phosphorolytic ones would have a very useful function in the ebb and flow phase of metabolism (36). In a similar vein, Cohen suggested that the ready reversibility of the phosphorylases allowed these enzymes to function in a pivotal position for possible regulatory control of metabolic processes (1). To elaborate briefly on this point, uridine phosphorylase is a branching enzyme between the salvage pathway and the pathway of pyrimidine catabolism. Results on regenerating liver (22, 56) suggest that the increase in the specific activity of uridine and deoxyuridine phosphorylases 36-72 hours after partial hepatectomy overlaps that of nucleoside kinases (24-48 hours) and, later on, that of the catabolic enzymes (60-96 hours). Since the increase of the kinases and the catabolic enzymes are inversely related, they would presumably control the ultimate

direction of uracil metabolism for they catalyze the rate-limiting steps. Still it may be important to have direct control on uridine phosphorylase in order to make it unidirectional either towards nucleoside synthesis or breakdown, at the appropriate times. This may be especially important in the direction of nucleoside synthesis since a crude rat liver preparation of uridine kinase has been found to require a very high concentration of uridine to saturate it (55), i.e. 0.01 M (but this does not appear to be true for Ehrlich ascites cell preparations (58, 59)). If this is the case for rat liver it would be highly beneficial if uridine phosphorylase could be made to operate only in the direction for nucleoside synthesis and thus to force up the concentration of uridine as quickly as possible in order to get maximum synthesis of UMP. For thymidine phosphorylase, differential inhibition of nucleoside cleavage and formation by thymine has already been shown (10).

Friedkin and Kalckar (2) speculated that nucleoside phosphorylases might be involved in preventing the loss of sugars from the cell by the free diffusion of uncharged compounds. After 'phosphorylase capture' they can be shunted into various pathways. Perhaps the catabolic products of uracil and thymine play a special role, for example, β-alanine, a catabolic product of the pyrimidine pathway, is necessary for the synthesis of anserine. In this connection, it is interesting to note that about 10% of the total labeled uridine (using uniformly tritiated uridine, uridine-5-^{3}H, or uniformly labeled uridine-^{14}C) was found in mouse liver protein (60). The amino acids were analyzed with an autoanalyzer after first hydrolyzing the protein fraction with 6 M hydrochloric acid. Radioactivity was found under aspartic acid, glutamic

acid, and alanine peaks. Perhaps the presence of a uracil-amino acid (uracil-alanine, pK values of 2.5 and 8.5) and its derivatives on acid hydrolysis may have interferred with the above analysis. This pyrimidine-aminoacid has only been found in pea seedlings (61).

In the regression of tumours, Freidkin and Roberts (38) suggested that,

"The synthesis of uncommon nucleosides by the nucleoside phosphorylases may be a primary enzymatic event which eventually results in the known antimetabolic effects of unnatural pyrimidines and purine bases".

In a recent study on thymidine phosphorylase (62) the authors stated that,

"The metabolic functions of the protein containing deoxythymidine phosphorylase and pyrimidine deoxyribosyltransferase activities have not been clarified. The demonstration that the enzymatic activities of this protein can be subjected to substrate inhibition, product inhibition and inhibition by purine bases suggests that it may play an essential role in the intracellular metabolism of pyrimidine deoxynucleosides".

If the above three points are accepted as criteria, one can say that uridine phosphorylase 'may play an essential role in the intracellular metabolism' of pyrimidine ribo- and perhaps deoxyribonucleosides, besides having its basic role of nucleoside cleavage. In the present work we have shown that uridine phosphorylase can be inhibited by substrate and product, and Gallo et al have shown that hypoxanthine and 6-mercaptopurine inhibited the activity of uridine phosphorylase (42).

A quote from a 1961 review of nucleoside phosphorylases (2) would be an apt conclusion to this discussion on the role of these enzymes in metabolism:

"Despite intensive investigation and speculation of biochemists over a period of 25 years, the role of purine and pyrimidine phosphorylases in cellular physiology remains an enigma".

C. PURPOSE OF THE PRESENT STUDY

Perhaps by directing more studies into the properties of a purified uridine phosphorylase, new evidence may be uncovered concerning its role in metabolism. This work is an initial step in this direction.

SECTION II

PURIFICATION AND SOME PROPERTIES OF URIDINE PHOSPHORYLASE

The investigator must be prepared to find his own way through the jungle represented by the protein mixture he has elected to fractionate. The difficulties of any rigid schedule will quickly become apparent under the test of general application.

H. Sober *et al.* (68, p.91)

A. INTRODUCTION

1. Objectives and Literature Review

One of the two main objectives of this study was to produce a homogeneous protein preparation that contains uridine phosphorylase activity. The other major objective was to provide more kinetic information on uridine phosphorylase than at present available from the literature; in many instances this involved detailed analysis. Thus, the purification of uridine phosphorylase is the cornerstone of this thesis, for serious kinetic studies are best pursued with pure enzymes (64, Vol. II, p. 771).

Although the present preparations of uridine phosphorylase are highly purified, they are not homogeneous. At the most, from past work, uridine phosphorylase from Ehrlich ascites cells had been purified up to a 250-fold increase in specific activity (13, 14), and in one of these cases (14) it was shown to be homogeneous by moving boundary electrophoresis. It has also been purified 10-fold from rat liver (26), 20-fold from E. coli (27), and about 8-fold from rabbit bone marrow (63). A brief summary of two of these purification procedures (13, 27) may be found in 'Methods in Enzymology' (65, Vol. VI, p. 189). In a recent paper a method was developed for separating purine nucleoside phosphorylase, thymidine phosphorylase, and uridine phosphorylase activities from each other in E. coli preparations by acid precipitation and chromatography on Dowex-1, but a high degree of purification was not achieved (28). Since most of the methods from the above studies have no actual bearing on the methods used in the present study, they will

not be described here, but certain parts will be referred to in the appropriate places in 'Discussion'.

Table III summarizes some of the purification results of nucleoside cleaving enzymes. Evidently the increase in specific activity reported in this thesis is one of the better ones at this time. Five highly purified uridine phosphorylase preparations were obtained from this work with a purification range of 300-1900 fold relative to the homogenate. For the sake of clarity, it would be useful at this time to give the major studies carried out with these five preparations, for most of these preparations were used for experiments on properties of uridine phosphorylase. The summary is shown in Table IV.

2. Assays for Nucleoside Phosphorylase Activity

The enzyme assay used in this work is a spectrophotometric one that is based on the method of Yamada (19), but there are a number of methods which may be used to estimate nucleoside phosphorylase activity:

(a) Determination of inorganic phosphate in the presence of esterified phosphate (2, 13).

(b) A coupled assay for the estimation of purine nucleoside phosphorylase activity. Hypoxanthine, a product of the phosphorolysis of inosine, is oxidized to uric acid by xanthine oxidase; uric acid is detected spectrophotometrically (3, 37).

(c) Detection of the pentose moiety by using the:

Burton diphenylamine reagent - an estimation of deoxyribose-1-phosphate formation (8).

Thiobarituric acid reaction - an estimation of deoxyribose and deoxyribose-1-phosphate formation (13).

Orcinol reaction - an estimation of ribose and ribose-1-phosphate formation (13, 21, 26, 27).

(d) Spectrophotometric assay

Indirect reading. This is based on the differential absorption of free base and nucleoside in strong alkali (8, 9, 14, 19, 26, 27, 38, 50); the reading is taken after the addition of alkali to the assay tube.

Direct reading. This is based on the differential absorption at 280 mμ between uridine and uracil at pH 7.0 (49), that is, continuous reading with time.

(e) Radioactive assay

Enzyme activity can be estimated by the incorporation of radioactive base into a nucleoside (25, 30), or by the formation of labeled base from labeled nucleoside (21, 22). The separation of nucleoside and base is commonly done by paper chromatography (25) or column chromatography (21).

3. Historical Development of the Spectrophotometric Assay

From the literature, it appears that this type of assay for purine nucleoside phosphorylases, uridine and thymidine cleaving enzymes were developed by different workers.

(a) Carter (in 1951) described two methods for the assay of uridine hydrolase activity from yeast (49).

Direct assay (described above). This method was employed by Carter during his purification procedures.

Indirect assay (described above). This method was employed by Carter for kinetic studies. In one variation of the indirect assay, Canellakis (23) measured the increase in absorption at 285 mμ in 1 M

sodium hydroxide after phosphorolysis by a crude rat liver uridine phosphorylase preparation. In another, Reichard and Skold measured the change in absorption at 300 mμ in alkaline solution after first stopping the reaction with 4 N perchloric acid; protein would also be removed by this method (55).

(b) Friedkin and Roberts developed an assay for thymidine phosphorylase utilizing the marked difference in absorption spectra between thymine and thymidine at 300 mμ in alkaline solution (38). Perchloric acid was used to stop enzymic action.

(c) Kalckar described differential spectrophotometry between purines and their nucleosides (37).

4. Approach to the Problem of Purification

Since the purification section of this thesis is extensive, it would be of value at this time to clearly indicate the approach that was taken to the problem of purification. In this regard the author would like to point out the value of the articles by Schwimmer and Pardee (66), Dixon and Webb (67), and Sober et al (68) in elucidating the problems involved in purification.

The basic assay procedure was already well established (19) and the technique necessary for separating uridine phosphorylase from thymidine phosphorylase was already known (14, 19). In addition the initial steps for obtaining a high-speed supernatant were taken from the work of Yamada (19). The first approach was to briefly examine some of the classical methods for purifying proteins, i.e. calcium phosphate gel fractionation, acetone precipitation, and ammonium sulfate fractionation. The major effort in developing the purification scheme,

however, went into an investigation of the fractionation of proteins by chromatography on columns, i.e. ion-exchange, adsorption, and molecular sieving columns.

As will be pointed out with the data many of the properties of uridine phosphorylase were not studied with the most highly purified preparation from each run. Properties such as stability (13, 14), effect of pH on enzyme activity (13, 19, 22, 26), transferase activity (20, 33) have been studied to some extent at least in the past. It should be noted that for most of these studies either a 10-20-fold pure uridine phosphorylase preparation was used (19, 22, 26, 33), although several experiments were done with a highly purified Ehrlich ascites cell enzyme (13, 14). Thus this present work uses enzyme preparations purified 200-fold or greater, free from thymidine phosphorylase activity, for the first time, in a detailed study of uridine phosphorylase from any source. The information available on the above mentioned properties from past work is confirmed and extended here. In a separate category are the estimate of molecular weight, the effect of small molecular weight thiols on the activity of uridine phosphorylase, and substrate inhibition by uracil since data have not been found in the literature on these points.

B. MATERIALS

Animals

Male albino rats were obtained from Holtzman Company, Wisconsin.

Equipment

The following equipment was used in these studies:

Balances: Top-loading (Mettler); Torque; Sartorius Selecta

Centrifuges: Sorvall RC-2 refrigerated; Spinco (model L)

Beckman (model E)

Chromatography:

paper - Whatman #1

chromatography tanks - Research Specialties Ltd.

columns - Pharmacia Fine Chemicals Ltd.

fraction collector - Gilson Medical Electronics

Colorimeter: Klett-Summerson

Electrophoresis: Canalco, model 12 (disc gel)

Homogenizing and Dialyzing:

homogenizer, teflon pestle (Potter-Elvehjem)

dialyzing tank - Oxford Laboratories (model B)

dialyzing bags - A.H. Thomas Co., Catalogue No. 4465-A2

connecting tubing - Tygon

Micropipettes: Pedersen (1 to 1000 microlitres)

Mineralight: Ultra-Violet Products Inc.

Parafilm: American Can Co.

pH Meter: Radiometer, model 22

Spectrophotometer: Beckman, model DU

Water Bath: Research Specialities Ltd. (Temperature controlled)

Chemicals

Important chemicals that were used in this work and their sources are given below:

Chemicals	Firm
(a) General	
Ammonium sulfate (enzyme grade)	Mann Research Lab.
Dipotassium hydrogen phosphate	Matheson Coleman and Bell Ltd.
Dithiothreitol	P-L Biochemicals
Ethylene diamine tetraacetate (sodium salt)	Sigma Chemical
Glycylglycine	Sigma
2-Mercaptoethanol	Eastman Organic Chemicals
Molecular weight markers	Mann (kit #8109A)
Polyethylene glycol (Carbowax-20M)	Union Carbide
Potassium dihydrogen ortho-phosphate	British Drug Houses Ltd.
Potassium chloride	Baker
Polyvinylpyrrolidone	Mann (Pharmaceutical Grade (40))
Sodium chloride	Baker
Sucrose (enzyme grade)	Mann
Tris-hydroxymethylaminomethane (HCl)	Sigma
Tris-hydroxymethylaminomethane (base)	Sigma
(b) Column Materials	
Bio-Gel agarose (0.5 M)	Bio-Rad Lab.
Blue dextran	Pharmacia
Cellex P	Bio-Rad Lab

Chemicals	Firm
Diethylaminoethyl Sephadex	Pharmacia
Hydroxylapatite	Pharmacia
Polyacrylamide P-300	Bio-Rad Lab
Sephadex G-200	Pharmacia
Sephadex G-200 superfine	Pharmacia

(c) Inhibitors

Deoxyglucosylthymine	Gift from Dr. M. Zimmerman (Merck Sharp and Dohme Research Laboratories, Division of Merck and Co. Inc., Rahway, New Jersey, 07065)
5,5' Dithiobis (2-nitro-benzoic acid)	Calbiochem
Iodoacetic acid	Mann
Iodoacetamide	Calbiochem
o-Iodosobenzoate	Mann
N-ethylmaleimide	Mann
p-Mercuribenzoate (sodium salt)	Calbiochem
p-Mercuriphenylsulfonate (sodium salt)	Sigma

(d) Polyacrylamide gel electrophoresis

Acrylamide (electrophoresis grade)	Eastman Organic Chemicals
Amido black	Canalco
Ammonium persulfate	Canalco
Coomassie brilliant blue R 250	Consolidated Lab.
5,5' Diethylbarbiturate (Barbital)	Fisher Scientific Co.
Glycine	Nutritional Biochem
Kodak Photo-Flo 200	Eastman

Chemicals	Firm
N',N' Methylenebisacrylamide	Eastman
Riboflavin	Eastman
N',N',N',N', Tetramethyl-ethylenediamine	Eastman

(e) Substrates

Deoxyuridine	Sigma
Deoxyribose-1-Phosphate (cyclohexylammonium salt)	Sigma
Ribose-1-Phosphate (dicyclohexylammonium salt)	Sigma
Thymidine	Mann
Uracil	Sigma
Uracil-2-^{14}C (58.0 mC/mmole)	Schwarz Bioresearch Inc.
Uridine	Mann

C. METHODS

1. Enzyme Assays

Deionized redistilled water (occasionally, redistilled water) was used for the preparation of solutions, and phosphate buffers were in the form of their potassium salts. The pH of assay tubes was taken by the method explained in section II C 8. Assays of activity during purification, kinetic, and transfer studies involved different techniques, as explained below.

a) Assays for purification studies

The spectrophotometric method of Yamada (19) for nucleoside cleavage was used throughout the purification work.

i) The following procedure was used in all purification steps except for column chromatography at pH 8.0. The final incubation medium contained the following (in micromoles) in a final volume of 1.5 ml: phosphate buffer at pH 7.5, 150: 2-mercaptoethanol, 7.5; enzyme diluted to 0.5 ml with 0.05 M phosphate buffer, pH 7.0; Ur, Udr, or Tdr at pH 7.0, 5.0. The final pH of the reaction mixture was 7.34. The reaction was started by the addition of the nucleoside. After a ten minute incubation period, the reaction was stopped by the addition of 0.45 ml 2.12 N perchloric acid, and by the introduction of the assay tube immediately into an ice-bucket at 0°. To the controls (one for each assay tube or duplicate determinations), perchloric acid was added first, followed by the addition of the nucleoside after a few minutes (there was no apparent effect on the absorbance readings if the controls were incubated, left at room temperature, or placed in an ice-bucket).

After five minutes in ice, the tubes were centrifuged at 14,800 x g for ten minutes at 2^{o} in a RC-2 centrifuge. From each tube one ml of supernatant was removed and mixed with 0.07 ml of 10 M sodium hydroxide (not standardized). The readings were taken against controls at 290 mμ for Ur and Udr and 295 mμ for Tdr.

ii) This incubation medium was used to assay effluents from columns at pH 8.0 (in micromoles): phosphate buffer, pH 6.85, 30; phosphate buffer pH 7.4, 170; 2-mercaptoethanol, 7.5; enzyme diluted to 1.2 ml with 0.02 M phosphate buffer, pH 8.0; substrate, pH 7.0, 5. The final pH was 7.3 (as read at 37^{o}). The assay technique was the same as in assay (i).

All assays were done in duplicate and usually with Ur, Udr, and Tdr, except when monitoring the column effluents. The enzyme activity in the various aliquots was estimated from one determination and usually with one substrate.

b) Assay for kinetic studies

i) Nucleoside cleavage

This procedure is a modification of assay (ai) and it was used for kinetic assays with enzyme preparation E and reactivated enzyme preparation D. However it could have been used with the other purified preparations and with some of the purer fractions obtained during the purification procedure.

The exact incubation medium used depended on the kinetic experiment so the ingredients and other conditions will be described along with the relevant data. The reaction was started with nucleoside, and after incubation it was stopped with perchloric acid as previously

described. After waiting at least five minutes, 0.140 ml of 10 M sodium hydroxide was added directly to the tubes (except for the absence of enzyme, the control tubes were identical with the assay tubes). After the tubes were shaken, readings were taken in the manner already described. When more than 25 μmoles of phosphate buffer were used or when 150 μmoles of acetate buffer were used, a precipitate formed after the addition of perchloric acid. Nevertheless, the precipitate was not removed by centrifugation since it settled quickly and it did not interfere with the assay results.

Under standard conditions of these assays, the formation of free base was a linear function of time for at least 20 minutes and of protein concentration up to an optical density reading of 0.390 (19).

ii) Nucleoside synthesis

The decrease in absorbance due to the formation of nucleoside was measured (27). The basic incubation medium contained the following in a final volume of 1.5 ml (in micromoles): glycylglycine buffer, pH 8.4, 150; ribose-1-phosphate, pH 7.0, 4.2; uracil, 1.0; enzyme, 10 μlitres. The enzyme was diluted with 0.05 M Tris buffer before it was added to the incubation medium. The final pH was 8.1. The reaction was started by the addition of uracil and the tubes were incubated for 30 or 65 minutes. The rest of the procedure is identical to that described for assay (bi). Any change is described along with the relevant data.

The absorption change between one micromole of uracil and one micromole of uridine at 290 mμ is 4.40; between uracil and deoxyuridine at 290 mμ, 4.70; between thymine and thymidine at 295 mμ, 4.30 (19). The enzyme was not generally added to the control tube for the kinetic

and modification experiments, however, a correction for absorbance due to protein was made where necessary (0.01-0.02 optical density units).

c) Assay for transferase activity

The assay is a modification of the procedures of Gallo et al (10) and de Verdier and Potter (25). Transferase activity was assayed by measuring the conversion of uracil-2-^{14}C to uridine-2-^{14}C. Only qualitative results were obtained, that is, absence or presence of radioactivity in the uridine spot.

i) Direct transfer (phosphate independent assay)

The final incubation medium contained the following in a volume of 0.3 ml (in micromoles): glycylglycine buffer, pH 8.9, 50; Tris buffer, pH 7.2, 4.8; uridine, pH 7.0, 0.2; uracil, 0.05; uracil-2-^{14}C, 0.0086 (0.025 μC); enzyme (in 0.05 M Tris, pH 7.0-1 mM EDTA, pH 7.0-5 mM 2-SH) 5 μl (7.2 μg protein). The final pH of the incubation medium was 8.1. The reaction was started by the addition of uridine. After the assay tubes had been incubated for 30 minutes, the reaction was stopped by placing the tubes in a boiling water bath for four minutes. A control tube, with no enzyme present, was treated in the same way.

The nucleoside and the free base were separated by descending paper chromatography in a solvent system composed of the upper phase from a mixture of ethyl acetate-water-formic acid (12:7:1). The lower or aqueous phase was poured into a 25 ml beaker and placed at the bottom of the developing tank which was covered with upper phase (approx. one centimetre in depth). After 0.2 ml of each sample was applied to Whatman #1 chromatography strips, the paper was pre-equilibrated overnight with the solvent system already in the tank as described. Then the upper phase was slowly poured into the trough and the chromatogram was developed

for 90 minutes. The strips were dried with hot air, and then examined with an ultraviolet lamp to ascertain whether the separation was sufficient for counting in a radiochromatogram scanner.

ii) Indirect transfer (phosphate dependent)

This is the same procedure as in direct transfer except that the incubation medium also contained 0.25 μmoles of phosphate at pH 7.0.

2. Estimation of Protein Concentration

The method of Lowry et al (70) was used for estimating the protein concentration of individual enzyme fractions (duplicate determinations). The protein concentrations of effluents from column chromatography were determined by the method of Warburg and Christian (71) from optical density readings at 260 mμ and 280 mμ. Specific activity is defined as the number of units of enzyme activity per milligram of protein.

3. Unit of Activity

One unit of enzyme(uridine phosphorylase or thymidine phosphorylase) is defined as that amount of enzyme that catalyzes the formation of 1.0 micromole of free base per hour or 1.0 micromole of nucleoside per hour.

4. Preparation of Substrates

a) Nucleosides

Stock solutions (0.1 M) of uridine, deoxyuridine, and thymidine were made by dissolving the appropriate quantities of nucleoside in 10 ml of water. The pH was adjusted to 6.9-7.1.

b) Uracil

A stock solution was made by dissolving 56 mg of uracil in 50 ml of water (0.01 M). No pH adjustment was made.

c) Ribose-1-Phosphate

A stock solution was made by dissolving 92 mg of ribose-1-phosphate in 5 ml of water (0.043 M). The pH was adjusted to 7.0.

d) Phosphate

Stock solutions (1.0 M) of KH_2PO_4 and K_2HPO_4 were prepared. The appropriate pH values were obtained by mixing the two stock solutions in definite ratios. The appropriate concentrations were usually made by subsequent dilution.

5. Purification Procedure

A schematic outline of the program is shown in Figure 1B. The following procedure is a detailed account of the methods used in the purification of preparation C. Although this represents the general scheme followed with other preparations, the schemes for preparations A to E differed in certain respects. These variations are noted at the end of this general account.

All steps were carried out in a cold room held at 4° or in ice buckets at 0°. The pH of the buffers were measured at room temperature and all the buffers used contained 1 mM EDTA except for those used in hydroxylapatite column chromatography. All dialyzing bags were washed with 1% EDTA and then with the buffer to be used in dialysis. Only the final combined fraction from each step was assayed for Ur, Udr, and Tdr cleaving activities. It was apparent after preparation A that at least 50 rat livers should be used for each preparation of purified

enzyme in order to obtain reasonable quantities of activity. Steps 1 to 3 and 7 are based on the methods of Yamada (19). The basic techniques involving chromatography with Sephadex were taken from booklets available from Pharmacia (Canada) Ltd.

a) Procedure for preparation C

Step 1: Preparation of the homogenate. The rats were fed purina rat pellets ad libitum. Eight to ten rats (250-350 grams) were killed by decapitation, usually between 9 A.M. to 1 P.M. Their livers were perfused in situ immediately upon death with ice-cold 0.9% sodium chloride until blanched, then removed, blotted with filter paper, weighed, and cut into fine pieces. The livers were homogenized in a 50 ml glass vessel (immersed in ice-water) with three volumes of 0.25 M sucrose (Potter-Elvehjem apparatus). After about 50 strokes the homogenates were filtered through two layers of cotton gauze (28 x 24 mesh).

Step 2: Preparation of supernatant and nuclear pellet. The homogenate was centrifuged at 900 x g for 20 minutes in an RC-2 refrigerated centrifuge. The pellet was re-homogenized in two volumes of 0.25 M sucrose and centrifuged as above for 10 minutes. Both supernatants were combined and filtered through two layers of cotton gauze. The nuclear pellet was resuspended with homogenization in one volume of 0.05 M P_i, pH 7.0-5 mM 2-SH. The nuclear and supernatant fractions and a few millilitres of homogenate were dialyzed against 20 litres 0.05 M P_i, pH 7.0-10 mM 2-SH for 16 hours in a continuous flow dialyzing apparatus. The nuclear fraction was then frozen at -20^{o}.

Step 3: Preparation of supernatant 2. Dialyzed supernatant 1 was centrifuged at 150,000 x g for one hour in a Spinco ultracentrifuge.

The supernatant was filtered through two layers of cotton gauze or glass wool. The lipid layer was not removed. The pellet was discarded without an assay or protein estimation.

Step 4: Ammonium sulfate fractionation. Enzyme grade ammonium sulfate was added slowly to supernatant 2 under mechanical stirring (glass stirrer) up to 30% saturation (0.7 grams ammonium sulfate per millilitre of solution per 10% saturation). This required about a 90 minute time period. After stirring for another 20 minutes the precipitate (AS_1) was removed by centrifugation at 16,000 x g for 20 minutes. An assay and protein determination was generally done on it. The pH of the supernatant at this point was about 6 (pH paper). Additional solid ammonium sulfate was added until 50% saturation was reached, in the same manner as in the AS_1 preparation. After centrifugation, the supernatant was discarded and the pellet (AS_2) was dissolved in slightly more than the minimal volume of 0.05 M P_i, pH 7.0-5 mM 2-SH. Assays on the fractions from procedures 1-4 were done within a week, if not sooner, and after one cycle of freezing and thawing.

Step 5: Combining and dialyzing of ammonium sulfate fractions. After steps 1-4 were repeated seven times over a two month period with different groups of rat livers, the AS_2 fractions were combined and dialyzed against 13 litres of 0.02 M P_i buffer, pH 8.0-10 mM 2-SH (buffer A) as in step 2.

Step 6: First DEAE-Sephadex column, pH 8.0 (preparations B-E). Forty grams of DEAE-Sephadex (A-50) were allowed to swell for 24 hours in buffer A. After the gel was washed with six 2 litre aliquots of buffer A (the fines being removed by suction), the slurry was packed into the column. A layer of Sephadex G-25 (coarse) washed in the same

buffer was added to the top of the bed to protect the gel surface. After the column was equilibrated with two bed volumes of buffer A, the reservoir containing the combined AS_2 fractions was connected to the column. The column was washed with 1 1/2 bed volumes of buffer A; then enzyme activity was eluted with a linear potassium chloride gradient. The mixing chamber was filled with 400 ml of buffer A and the reservoir was filled with 400 ml of 0.4 M KCl in buffer A (buffer B). After all of the deoxyuridine cleaving activity was eluted from the column, the fractions containing more than 36 units each of uridine cleaving activity were combined and concentrated with ammonium sulfate (up to 75% saturation). The deoxyuridine cleaving activity from other fractions was not collected (25% or more of the total). The concentrated fraction was dialyzed immediately against 5 litres of buffer A for nine hours. After an assay and a protein estimation were done, the fraction was frozen at -20°.

Column parameters:

column size	:	2.5 cm X 90 cm
bed volume	:	400 ml
flow rate	:	24 ml per hour
aliquot	:	12 ml
time	:	55 hours (from application of sample to end of concentration).

Step 7: Second DEAE-Sephadex column, pH 8.0 (preparations A-E). In the case of preparation C the sample was applied twelve days after the previous column. Sufficient DEAE-Sephadex was prepared in Step 6 for two columns. After packing the column, about one cm of Sephadex G-25 (coarse) was applied, and the column was equilibrated with one bed volume of buffer A. The dialyzed enzyme fraction was adsorbed on

the column. A linear potassium chloride gradient (as above) was started after one bed volume of buffer A had passed through the column. After most of the deoxyuridine splitting activity had been collected, eight fractions, each containing more than 200 units of uridine splitting activity (fraction #1), and seven fractions containing deoxyuridine splitting activity (fraction #2) were combined and concentrated separately with ammonium sulfate up to 85% saturation. Both fractions were dialyzed immediately against 4 litres of 0.05 M P_i -10 mM 2-SH (buffer C) for 10 hours using the continuous-flow dialyzing apparatus.

Column parameters:

column size	:	2.5 cm x 90 cm
bed volume	:	400 ml
flow rate	:	40-58 ml per hour
aliquot	:	12 ml
time	:	41 hours

Step 8: Third DEAE-Sephadex column, pH 7.0 (preparation C). Twelve grams of DEAE-Sephadex were allowed to swell in 0.02 M P_i, pH 7.1 for 24 hours at 4^{o}. After washing the gel five times with one litre aliquots of the above buffer, the column was packed with the slurry (the fines were removed by suction). A one cm layer of Sephadex G-25 (coarse) washed in the same buffer was added to the top of the bed. After the column was equilibrated with 2 1/2 bed volumes of buffer C, the reservoir holding fraction #1 was connected to the column. The enzyme fraction was adsorbed to the column. A linear potassium chloride gradient was begun after one bed volume of buffer C had passed through the column. The mixing chamber was filled with 400 ml of buffer C and the reservoir was filled with 400 ml of 0.20 M KCl in buffer C. After most of the deoxyuridine cleaving activity was eluted, the fractions

containing more than 150 units of activity (5 fractions) were combined and concentrated with ammonium sulfate (up to 85% saturation). In addition, it was further concentrated a factor of two-fold with Carbowax, over a four hour period. With this particular column a minor deoxyuridine cleaving peak appeared after the major peak and it contained about 15% of the total deoxyuridine cleaving activity. The active fractions of this minor peak were also combined and concentrated with ammonium sulfate.

Column parameters:

column size	:	2.5 cm x 45 cm
bed volume	:	240 ml
flow rate	:	34 ml per hour
aliquot	:	14 ml
time	:	44 hours

Step 9: Sephadex G-200 chromatography, pH 7.3 (preparations A-C). After twenty grams of gel were allowed to swell for three days in 0.02 M P_i, pH 7.3 at 4^{o} it was washed four times with two litre aliquots of the above buffer (the fines were removed by suction). In packing the column, the first few cm were allowed to settle by the force of gravity alone, then the outlet was opened and the pressure head was gradually increased to its final value. After the column was packed with the slurry, a sample applicator was added and the column was equilibrated with two bed volumes of buffer D (0.02 M P_i, pH 7.3-10 mM 2-SH). To check the packing of the column, five ml of 0.2% Blue dextran 2000 in 0.02 M P_i, pH 7.3 were applied to the column. The final fraction from the previous column was applied at one time by pipette; then the outlet was opened and the protein was eluted with buffer D until all of the deoxyuridine cleaving activity was collected. The fractions containing more than

400 units of activity were combined (four tubes), concentrated with Carbowax over a six hour period, and dialyzed for two hours against two litres of 0.02 M P_i -5 mM 2-SH in a continuous-flow dialyzing apparatus (EDTA was not part of the buffer system). After an aliquot of the concentrate had been removed for an enzyme assay and a protein estimation, the remainder was bottled and frozen at -20° for two days.

Column parameters:

column size	:	2.5 cm X 90 cm
bed volume	:	430 ml
flow rate	:	32-35 ml per hour
pressure head	:	21 cm
aliquot	:	12.9 ml
time	:	29 hours

Step 10: Hydroxylapatite chromatography, pH 7.3 (preparations (A-E). After sixty-five grams of adsorbent were allowed to swell for 24 hours in 0.02 M P_i, pH 7.3 at 4° it was washed five times with 500 ml aliquots of this buffer. EDTA was not included in any of the buffer systems with this column for it could interfere with the protein separation by complexing the calcium ions contained in the adsorbent (74). In packing the column, the first few centimetres were allowed to settle by gravity. After the column was packed it was equilibrated with five bed volumes of buffer E (0.02 M P_i -10 mM 2-SH). The ratio of total protein to bed volume in this case was 1.3, but in any case it was not greater than 2.0. The enzyme activity and most of the protein was adsorbed onto the column; then the column was washed with one-half bed volume of buffer E. Elution of the proteins was then carried out with a linear phosphate gradient. The mixing chamber was filled with 300 ml of buffer E and the reservoir was filled with 300 ml of 0.25 M P_i, pH 7.2-10 mM 2-SH. After all of the deoxyuridine cleaving activity was eluted, the fractions

containing more than 150 units of uridine cleaving activity were combined (six tubes) and concentrated over a 12 hour period with Carbowax (the time was longer here because the dialyzing tubing was not repacked for a seven hour period). The concentrate was dialyzed for three hours against two, 500 ml aliquots of 0.05 M Tris, pH 7.4-5 mM 2-SH (no EDTA). The dialyzate was clarified by centrifugation at 11,000 x g for 20 minutes. After an assay and protein estimation were done, the purified enzyme was stored in six sample bottles and frozen at -40°. This fraction was used for kinetic studies.

Column parameters:

column size	:	1.5 cm X 90 cm
bed volume	:	109 ml
flow rate	:	8-12 ml per hour
aliquot	:	8 ml
time	:	48 hours

b) Concentration of protein fractions with Carbowax

This procedure is a modification of the method of Setlow and Lowenstein (72) and Kohn (73). The enzyme fraction was added to a dialyzing bag and immersed in a 500 ml beaker containing Carbowax. The dialysis tubing was packed tightly; its upper end was not tied. About every 45 minutes the wet wax was removed from the surface of the tubing and from the beaker, and then the tubing was repacked in the remaining old wax plus added fresh wax. The above steps were repeated over a 4-6 hour period, after which time the surface of the dialyzing bag was washed thoroughly with water (dialysis, if done, followed directly after this washing). After an enzyme assay and a protein estimation the fraction was applied to the next column. A dialyzing step was omitted with preparations C and D.

c) Variations

(1) 2-Mercaptoethanol. This thiol was added to the column tubes containing substantial enzyme activity in preparations D and E to give a final concentration of about 10 mM. It was thought that this might stabilize the active fractions during the lengthy column steps.

(2) First DEAE-Sephadex column. Different ratios of total protein to bed volume, and different protein concentrations were used, in order to establish the best overloading conditions. This will be discussed in section II D 1d.

(3) Second DEAE-Sephadex column. The basic difference between the columns in preparations A to E was the type of linear gradient used (0.15 M to 0.4 M potassium chloride). This will be discussed in section II D 1f.

(4) Sephadex G-200 column. In preparations D and E, the pH of the column was 8.1 instead of 7.3. In preparations A, B and E, the DEAE-Sephadex, pH 7.3 fraction was applied in two portions, and in preparations A and B the Sephadex G-200 preceded step 8.

(5) Dialysis. The set-up depended upon the volume of sample to be dialyzed. Generally a ratio of 100 to 200 of buffer to sample was used for the various purified fractions, except for supernatant 1 where a lower ratio was used. If more than four litres of buffer were necessary, a continuous-flow dialyzing procedure was used, where the dialyzing tank contained four litres of buffer and the reservoir could contain a maximum of twenty litres.

(6) Heat treatment. This was an additional step for preparation D only. The combined, undialyzed AS_2 fractions were placed in four 50 ml

stainless steel tubes and heated, with stirring, for three minutes at 50° in a water bath. The tubes were then immediately immersed in an ice bucket and stirred for another minute. The precipitate was removed by centrifuging at 12,000 x g for 15 minutes. It was washed with a few millilitres of 0.05 M P_i -5 mM 2-SH. After centrifugation the supernatants were combined and dialyzed for step 6.

6. Molecular Weight Estimation

The molecular weights of uridine phosphorylase and thymidine phosphorylase were estimated by Sephadex G-200 gel filtration (75) of fractions #1 and #2 from preparation B (36-fold pure and 13-fold pure, respectively).

a) Preparation of the column

The preparation of the gel and the packing of the column were done the same way as described in step 9, but the buffer was 0.05 M P_i, pH 7.0-10 mM 2-SH. A sample applicator was not used because the column diameter was too small. The column was equilibrated with three times its bed volume. Blue dextran 2000 (average molecular weight: 2×10^6) was applied first to check the packing of the column.

b) Calibration of the column

The calibrating proteins were obtained from a molecular weight kit:

Protein	Source	Molecular Weight
apoferritin	horse	480,000
γ-globulin	human	160,000
albumin	bovine	67,000
ovalbumin		45,000
chymotrypsinogen	beef pancreas	25,000

Each protein was made up to a concentration of 5 mg/ml in the column buffer, and 0.5 ml of each was chromatographed separately by layering the protein solution, using a micropipette, under a minimal volume of buffer already present at the top of the bed. Uridine phosphorylase (0.2 ml; 9 mg protein) and thymidine phosphorylase (0.2 ml; 11 mg protein) were also eluted separately. Protein was estimated spectrophotometrically by measuring the absorbance at 280 mμ. The two enzymes were also detected by their enzymic activity. The effluent volumes corresponding to the maximum concentration of protein was estimated to the nearest millilitre from the elution diagram by extrapolating both sides of the protein peak to an apex.

Column parameters:

column size	:	1.5 X 30 cm
bed volume	:	41.5 ml
flow rate	:	6.5 ml/hour
aliquot	:	2.0 ml

7. Polyacrylamide Gel Electrophoresis

Only anionic systems and 7% polyacrylamide gels were used. The method of Davis (76) was used for electrophoresis at pH 8.9, and the method of Williams and Reisfeld (77) for electrophoresis at pH 7.5. Both diagnostic and elution experiments were performed. The method of polymerization was the same for both experiments except that 5 mM 2-mercaptoethanol was included in the buffer system for the elution experiments.

a) Preparation of the gel

The ingredients used, and the make up of the gel will not be

explained for it is available in the above two references. The technique itself, however, is slightly different in the present work.

Briefly, the stopper wells were filled with 0.3 ml of 0.25 M sucrose. The gel column (0.5 cm X 6 cm) previously coated with Kodak Photo-Flo solution was attached to the wells. On top of the sucrose, 0.4 ml of 'spacer gel' were allowed to polymerize for about 30 minutes under the influence of fluorescent light. Then the 'separation gel' was added to the column until a bead of gel formed at the end. This was removed with parafilm. The gel was allowed to polymerize, away from strong light for 30 minutes. The sucrose was then drained and the sample, diluted with spacer gel (3-to 20-fold) or with sucrose (two-fold), was added to the top of the spacer gel. If sucrose was used then another layer of spacer gel was added in order to sandwich the sample between spacer gels. If spacer gel was used for dilutions, then, after draining, the column space was washed with this gel to remove traces of sucrose.

b) Analytical disc gel electrophoresis experiments

i) Diagnostic gel electrophoresis

This type of electrophoresis was used to show the banding pattern of preparations C-E, and also that of different fractions obtained from the purification scheme. Normally, electrophoresis was done at room temperature for about 90 minutes with a current of 2 to 2.5 mA/tube. The concentrations of sample that were applied ranged from 0.3 to 13 mg/ml with absolute amounts of 50 to 200 µg. The protein bands were visualized with Coomassie blue (78), although Amido black was used in the initial experiments. The gel samples were stored in 2% trichloroacetic acid at 4^{o}.

ii) Elution gel electrophoresis

Several different procedures were tried for eluting enzyme activity from the gel, but all of them were based on allowing the cut up pieces of gel to stand in buffer. The method described below gave the best recovery of activity, although this was only 7% (this method was only tried once).

Four gel columns were prepared at pH 7.5. The enzyme sample was diluted 1:1 with 40% sucrose and then sandwiched between two layers of spacer gel. Each column contained about 260 μg (3 mg/ml) protein, and 19 units of uridine cleaving activity. Electrophoresis was performed for 3 hours at 4^{o} with a current of 1.75-2.00 mA/tube. The upper and lower reservoir buffers contained 5 mM 2-mercaptoethanol. After development, one gel column was stained with Coomassie blue for a period of 50 minutes, while the other columns were kept at 4^{o}. With the stained column as a guide, each of the other columns were cut into seven pieces with the corresponding ones being combined. The combined gel sections were allowed to stand in 0.06 ml of 0.05 M Tris, pH 7.1-5 mM 2-SH for two hours at 4^{o} (with occasional stirring). An assay was done for uridine and deoxyuridine cleaving activities.

8. pH Study

The kinetic assay procedure was used to measure the effect of pH on nucleoside cleavage and uridine synthesis. A special note should be made on the method of pH measurement. Blank assay tubes (no enzyme present) were first incubated at 37^{o} for several minutes before a reading with the pH meter (equipped with a temperature compensator) was taken. The reading was taken within 10 seconds of its removal from

the water bath. Acetate, phosphate, Tris, and glycylglycine buffers were used to provide a pH range of 4-10 (concentrations are given in section II D 2d).

D. RESULTS

1. Purification of Uridine Phosphorylase

The enzyme was purified on five different occasions with the increase in specific activity of the final fractions being between 300-1,900 fold relative to the homogenate. Besides being due to variations in experimental technique, this wide range is due, to some extent at least, to changes in the procedures used in obtaining the purified preparations. Although these differences in the final degree of purity cannot be clearly assigned to differences in procedure at this time, the changes will be pointed out in the appropriate places. To show these differences more clearly, purification tables of preparations A (Table VI) and D (Table VII) are presented along with preparation C (Table V), albeit in less detail. Since preparation C was the one described in the 'Methods' section, the results will emphasize this particular preparation for the sake of clarity. Also more detailed work was done with this preparation than with most of the other ones (Table IV). In addition, Table VIII presents a list of general data on each of the five enzyme preparations.

a) Homogenate, supernatant 1, and nuclear fraction

Assays and protein estimations are given for dialyzed fractions only. The nucleoside cleaving activities of a sample of homogenate, dialyzed and undialyzed, were never directly compared. Nevertheless, when the difference in activity between undialyzed homogenate and the sum of the activities in (dialyzed supernatant 1 and nuclear fraction) was compared to the difference in activity between dialyzed homogenate

and the sum of the activities in (dialyzed supernatant 1 and nuclear fraction), the difference was negligible (not shown).

The percent recovery of uridine, deoxyuridine, and thymidine cleaving activities from the homogenate in (supernatant 1 plus nuclear fraction) was virtually always in the range of 80 to 120% for each set of rats with the average for each substrate being over 90% (6 to 10 rats/set). The ratios of the specific activities of the homogenate for Ur/Udr, and Ur/Tdr in 36 different sets of rats had a range of 0.32-0.57 (0.44) and 0.60-1.1 (0.75), respectively (the average values are given in parenthesis). Also in 36 sets, the specific activity values for uridine were grouped around 0.07 or 0.09 with a range of 0.065 to 0.100. The range of values for deoxyuridine and thymidine were 0.14-0.21 and 0.074-0.12, respectively.

The percent recovery of the enzyme activity in the nuclear fraction for the seven sets of rats in Table V ranged from 7-14% (Ur), 6-10% (Udr), and 4-9% (Tdr). In five of these sets the order of the percent recovery was Ur $\geq$ Udr $\geq$ Tdr. Higher values for each of the three substrates were frequently obtained in other experiments, but lower values were seldom observed. This is in harmony with the fact that this fraction did not consist of pure nuclei, and therefore variable degrees of cytoplasmic contamination would be expected. The average percent of protein recovered in the nuclear fraction from 36 sets of rats was 43%, and the ratio of the protein estimations of (supernatant 1 plus nuclear fraction / homogenate) has a range of 0.86-1.39 with an average value of 1.03.

b) Supernatant 2

Ultracentrifugation of supernatant 1 resulted in an average percent removal of protein of 54% in preparation C with an absolute range

of 57 to 69% in the different sets of rats in preparation C. In 36 other sets, similar results were obtained. The percent loss of activity for each of the three substrates of the seven sets in preparation C as well as in other sets was not reproducible. The range of the loss for each nucleoside was normally between 2-23%. Nevertheless, there appeared to be a pattern in these losses, albeit a weak one at this time: where the loss in uridine cleaving activity was low, deoxyuridine and thymidine cleaving activities were either both low or both high. If the loss in uridine cleaving activity was high, the loss in deoxyuridine cleaving activity was high, but that of thymidine cleaving activity was low or high. This pattern was obeyed for 11 sets out of 16 and it suggests that the activities of uridine and thymidine phosphorylases may not have been recovered to the same extent in different preparations.

c) Ammonium sulfate fractionation

The increase in specific activity over the previous fraction was rarely more than two-fold for any of the three substrates, but concomitant with this increase was a decrease of volume from supernatant 2 of about 15-fold. The average recovery of activity in the seven, second ammonium sulfate fractions of preparation C (step 4B, Table V), was 76% (Ur), 87% (Udr), and 80% (Tdr) - based on supernatant 2. Similar recoveries were obtained from fourteen other preparations. These AS_2 fractions had a reddish-brown colour; as a result of inadequate perfusing during excision of the liver, hemoglobin may have been present in the homogenate.

The first ammonium sulfate fraction (0-30%) appeared to be enriched in thymidine phosphorylase activity, i.e. high deoxyuridine and thymidine cleaving activities, and little uridine cleaving activity (Table V). Even up to at least 35% saturation with ammonium sulfate,

there was still considerable enrichment of Tdrpase over Urpase in this fraction (not shown). Table V shows three different steps for the second ammonium sulfate fraction (steps 4B, 4C, and 5). The first one (AS_2) represents a summation of the activities from the individual assays on each of the seven sets of rats in preparation C. The values in parenthesis are corrected for enzyme activity used for trial studies, for example concentration of protein solution by Electrophoretic Filter/Concentrator (79). The second assay (AS_2c) is an assay on the undialyzed combined AS_2 fractions, and the third one is an assay on this fraction after dialysis (AS_2cd). The percent change in the activities towards the three nucleosides, before and after dialysis, was small. Most of the other preparations gave a similar result. Table V shows that a significant decrease in uridine cleaving activity between AS_2 and AS_2c occurred with no decrease with the other two substrates. This effect was observed also with preparation E.

The ammonium sulfate fractions of preparations B-E showed a 50% loss of uridine cleaving activity and about a 40% loss of deoxyuridine and thymidine cleaving activities relative to the homogenate. There are at least three reasons for this big loss:

(1) The removal of the nuclear and the 150,000 x g pellets entailed a loss of 10-20% in activity for each pellet.

(2) The ammonium sulfate step itself involved a 10-15% loss in activity.

(3) Storage of the ammonium sulfate fraction for two months at -20° involved at least a 10% loss in activity.

d) First DEAE-Sephadex column, pH 8.0

The possible advantage of using an overloaded DEAE-Sephadex column was discovered accidentally, and this type of column was subsequently

used with five preparations (B-D, E_1, E_2). This procedure looked promising, for example, the recovery of uridine cleaving activity was 80% or better in all preparations used, and for preparation C itself it was 92%. The recoveries of deoxyuridine and thymidine cleaving activities were variable, and they were dependent on the extent of overlap of the uridine and thymidine phosphorylase peaks; the column fractions that were combined were chosen only on the basis of the relative amounts of uridine cleaving activity present. The enzymic activity towards the three nucleosides in the ammonium sulfate concentrate of the selected column fractions was stable to dialysis.

To properly evaluate the results obtained with this column, the increase in specific activity should be related to the increase one gets by using a properly set up column alone as in step 7. Only preparations C and D gave an additional two-fold increase in specific activity. Still, relatively small decreases in the amount of protein in the final concentrated pooled fractions after chromatography could have a beneficial effect on the separation of proteins in succeeding columns, (in preparation E, one gram out of 3.5 grams was removed). Sufficient data is not available to clarify this point.

It was difficult to duplicate the effects of overloading. Both the ratio of (protein applied to bed volume), and the concentration of protein applied, had to be above certain values in order to obtain either breakthrough and/or an incomplete separation of the Urpase peak from the Tdrpase peak. For example, protein to bed volume ratios of 20, together with a concentration of protein above 35 mg/ml generally gave an incomplete separation of the two phosphorylases. Nevertheless, the amount of breakthrough and the degree of overlap were not reproducible when these

two parameters were varied above these values.

e) Second DEAE-Sephadex column pH 8.0

The recovery of uridine cleaving activity in the eluate was 80% or better, and the recovery after concentration with ammonium sulfate and after dialysis was also generally in the same range. In preparation C, the recovery after concentration was 100% (Table V). With preparation D, however, 32% of the uridine cleaving activity and 24% of the deoxyuridine cleaving activity were lost after concentration with ammonium sulfate; with preparation A about 35% of the uridine and deoxyuridine cleaving activities were lost. These losses were exceptional events (2 out of 11 cases). The only difference for procedure D was that Baker Analyzed ammonium sulfate was used instead of Mann (enzyme grade), as in other preparations. The loss with preparation A was probably due to poor technique. An intense reddish-brown colour always appeared with the initial protein material eluted as well as with the uridine phosphorylase peak.

In general, a 3 to 4-fold increase in specific activity for uridine cleavage was achieved with this column, along with a good separation of uridine phosphorylase activity from thymidine phosphorylase activity in 11 different trials - that is, one peak had mainly uridine and deoxyuridine cleaving activities, and the other peak had mainly deoxyuridine and thymidine cleaving activities - as had been previously found by Yamada (19). Nevertheless, as shown in Figure 2 (preparation A, Table VI), there appears to be a certain degree of overlap in this separation for a base line was not reached. The uridine cleaving activity in the common region indicated by dotted lines is 8% or less of the deoxyuridine cleaving activity in the first activity

peak (the enzyme activity was not assayed in detail with both substrates), and no uridine cleaving activity was detected in the upper slope of the thymidine phosphorylase peak on the few occasions when this was checked. The significance of the thymidine cleaving activity present in the uridine phosphorylase peak will be discussed in section II E 2a.

f) Third DEAE-Sephadex column pH 7.1

The recovery of protein and enzyme activity from this column was usually 80% or better, with an increase in specific activity of 2 to 3-fold. The elution profile with preparation C is given in Figure 3. A reddish-brown colour was evident throughout the enzymatically active fractions. With this preparation two cuts were taken of the column eluates as indicated by the horizontal arrows. The minor peak appeared to be identical with the major peak with respect to certain properties, i.e. phosphate dependence, pH optimum for uridine (with 50% of the total activity still present at pH 6.5), and the pH optimum for deoxyuridine. In one experiment, however, deoxyuridine cleaving activity was not detected at pH 7.8, whereas 40% should normally remain at this pH (section II D 2d). Disc gel electrophoresis of these fractions did not reveal any differences in migration on visual inspection.

The minor peak may just be an artifact resulting from experimental conditions. Figure 4 supports this view for the shoulder of the major band (decreasing slope-side) appeared to be dependent on the potassium chloride gradient. As the gradient increased from 0.15 M to 0.4 M the activity peak became more symmetrical; a similar change did not occur in the protein profile (although the width of the band under the activity peak was less with 0.4 M gradient). Since assays for uridine and deoxyuridine were not done a comparison of gradient effects on these two

activities cannot be made.

With preparation A and B, steps 8 and 9 were reversed. The present procedure is preferable for a smaller volume of sample could be applied to the Sephadex G-200 column if the anion exchange column were done first. As previously explained in 'Methods', dialysis of the combined concentrated DEAE-Sephadex, pH 7.1 fraction was not performed on preparations C and D, for it was not really essential in preparation for a molecular sieve column. In preparation A there was a 24% loss in uridine cleaving activity, and a 4% loss in deoxyuridine and thymidine cleaving activities on dialysis. In preparation B there was a 17% loss in uridine cleaving activity and an 8% loss in deoxyuridine cleaving activity on dialysis. At present, this cannot be explained.

g) Sephadex G-200 column

The recovery of protein and activity after elution was 90% or better, although for preparation D (Figure 5, Table VII) a low recovery or protein was recorded (1 out of 8 cases). This might have been due to an error in the protein estimation of the final fraction in the preceding column. The activity peaks for deoxyuridine and possibly uridine were fairly symmetrical. The protein profile shows at least four peaks. In all cases the elution profile of Blue dextran indicated tailing (Figure 5B). Again enzymatically active column eluates had a reddish-brown colour. The concentrated uridine phosphorylase fraction was stable to dialysis.

The G-200 columns (bed volumes about 400 ml) were developed at either pH 7-7.3 (preparations A-C) with 0.02 M P_i or 0.05 M P_i, or at pH 8.1 (preparations D and E) with 0.02 M P_i (in all cases 10 mM 2-mercaptoethanol was present). With the two procedures differences in

elution profiles and extent of purification were found. Development with buffer at pH 7.1 produced a definite shoulder in the activity profile which was absent at pH 8.1. In addition, the protein bands were not as sharply defined as at pH 8.1. The average increase in specific activity of the final concentrated pooled fractions for uridine and deoxyuridine was 1.8 with preparations A-C. The increase in specific activity of the final fraction for uridine and deoxyuridine, in the case of preparation D, was 5-fold, but with preparation E it was only 2.6-fold. This point will be taken up in section II E 2b.

h) Hydroxylapatite column

The recovery of uridine and deoxyuridine cleaving activities in the effluent was over 90% in all cases (preparations A-E), but usually only 50% of the protein was eluted after six bed volumes. This was similar to the purification results of Zimmerman (8), where it was found that inactive protein was held back, but that thymidine phosphorylase activity (human spleen) was only partially retarded. Figure 6 (preparation D) shows fairly symmetrical and coincident peaks containing uridine and deoxyuridine cleaving activities. The protein profile shows at least four protein peaks. Other preparations did not give such symmetrical activity peaks, but this is probably due to inaccuracies in the assay (duplicate assay tubes were not done) for the unsymmetrical areas were never in the same places.

The increase in specific activity in the combined concentrated column eluates was generally between 4 and 6-fold, but this increase was greatly dependent on the width of the activity band that was collected. The specific activity for uridine in the best fraction from preparation D (containing 25% of the total activity) was 510 and

this would represent a purification of 7,000-fold. Unfortunately, in order to obtain a reasonable yield, column fractions with a specific activity range of 63 to 510 were combined. The specific activity for deoxyuridine in the most active fraction from preparations A to E was 30, 29, 31, 380,. 42, respectively. In preparation B to E the percent of the total activity present in the four most active fractions was between 70-80%; but the specific activity range for uridine in these four tubes was 40-47 (preparation B), 37-46 (preparation C), 230-510 (preparation D), and 15-42 (preparation E). Although there was no constancy across the activity peak, the specific activity values were much closer for the two most active tubes (given for uridine): 30-32, 46-48, 39-43, 460-510, 36-42, for preparations A to E respectively.

The highest specific activity obtained for uridine cleavage was 144 (Table VIII) which is 40 times greater than that obtained by Canellakis with a partially purified rat liver preparation (26). In preparation E, the main activity band was divided into two cuts and each one was concentrated separately, with fraction E_a showing a 3-fold increase in specific activity, and fraction E_b showing a 13-fold increase; it contained 60% of the total activity (Table VIII). No colour was eluted along with the enzymatically active fractions. There was little loss in activity after concentration in Carbowax and subsequent dialysis (based on the assays of the various column fractions themselves, for with this column, assays were not done on the combined selected fractions before concentration, and on the concentrated fraction before dialysis). Preparation A, which was concentrated with ammonium sulfate (up to 90% saturation), showed a 44% loss in activity after dialysis. An explanation for this will be given under heading (k).

i) Heat treatment

Only preparation D was heat-treated and the maximum increase in specific activity was 1.5 based on the ammonium sulfate (fraction) step (Table VII). The percent decrease in protein was between 32-45%. Four different protein estimations were done, i.e. the heat-treated sample itself, a Tris dialyzate, and the diluted and undiluted phosphate dialyzate, and the estimations varied from 6,600-8,300 mg of protein. Thymidine phosphorylase activity decreased to about the same extent after heat treatment as uridine phosphorylase activity (18% decrease for Ur, 20% for Udr, and 19% for Tdr). This was expected from heat treatment studies on partially purified preparations of thymidine phosphorylase from human spleen (8), and of uridine phosphorylase from Ehrlich ascites cells (13). Also the results of the present study were essentially in agreement with those of Canellakis (26) with rat liver uridine phosphorylase, where a heat treatment step was incorporated into the purification scheme. The increase in purification in that study was 2.3-fold.

j) Specific activity ratios

Three different specific activity ratios for the substrates, Ur, Udr, and Tdr, are given in Table IX for preparation C. The constancy of these ratios was evident after the second DEAE-Sephadex column in which thymidine phosphorylase activity had been removed. Table X summarizes the specific activity ratios of the fractions from steps involving column chromatography of preparations A to E. The ratios were fairly constant. This suggests that the preparations may be enzymatically pure with respect to the presence of other uridine and thymidine phosphorylases (66, p.388) with no evidence of a separation of uridine cleaving activity

from deoxyuridine cleaving activity. In addition, the phosphorolysis of thymidine in all purified preparations appears to be an inherent feature in the action of the uridine phosphorylase isolated in this study (discussed in section II E 2a).

k) Concentration of protein fractions

Ammonium sulfate (80 to 90% saturation) was normally used to precipitate enzyme protein from pooled column fractions when the concentration of protein was above 4 mg/ml in the majority of the individual fractions. Below this concentration Carbowax was used. In preparation A, however, ammonium sulfate (up to 90% saturation) was used to concentrate the selected fractions from the hydroxylapatite column, and the protein concentration at the most was 0.9 mg/ml in the individual fractions. The result was a loss of 44% in enzyme activity. The Carbowax procedure did not result in serious losses, but an unusual result occurred in the protein estimations from the final hydroxylapatite fraction of preparations B, D, and E; a yellow precipitate formed on the addition of the Folin-Ciocalteau reagent. This would suggest that an impurity, probably from Carbowax, was introduced into the dialyzing bag. In preparation D, the Lowry estimation and spectrophotometric determination (absorbance reading at 260 and 280 $m\mu$) gave identical protein estimations. With preparation B the yellow precipitate was formed when 10 μl of sample were used for the estimation but not when 5 μl were used.

Polyvinylpyrrolidone (73) as well as the Electrophoretic Filter/Concentrator (79) were used for concentrating fractions, but the recovery of activity was low (experiments were done at 4^{o}).

1) Other purification steps

Acetone fractionation of AS_2 fractions resulted in about a 90% loss in activity with no increase in specific activity. In calcium phosphate gel fractionation, the total recovery of the enzyme activities towards Ur, Udr, and Tdr was 83%, but to obtain a two-fold increase in specific activity it was necessary to discard about 50% of the total uridine cleaving activity.

Sephadex G-200 superfine and Bio-Gel agarose (0.5 M) were also briefly studied as possible alternative molecular sieving columns. Both columns, run at pH 7.0, gave 90-114% recovery of activity and protein with the activity peaks being more symmetrical than with Sephadex G-200. The protein profiles showed only one broad band rather than several broad bands as with Sephadex G-200, but the increase in specific activity based on the peak tube was slightly greater than with Sephadex G-200. Columns using Cellex P and polyacrylamide P-300 were not successful. After five bed volumes had been collected from the start of the gradient only 50% of the uridine cleaving activity and protein were recovered from the Cellex P column (mixing chamber: 100 ml of 0.02 ml P_i, pH 7.1-1 mM EDTA, pH 7.1-10 mM 2-SH; reservoir: 100 ml of 0.1 M P_i, pH 7.1-1 mM EDTA-10 mM 2-SH). A similar result was obtained when the buffers were at pH 8.0. The flow rate with the polyacrylamide column was close to zero (bed volume 39 ml) after two packing trials; it was abandoned at this point.

2. Properties of Uridine Phosphorylase

a) Enzyme assay

Experiments on the rate of uracil formation as a function of time

and protein concentration were not done on crude fractions containing uridine phosphorylase activity in the present study. Yamada (19) has shown, however, that for a dialyzed homogenate there was linearity with time of incubation for at least 20 minutes, and with protein concentration up to an absorbance reading of 0.390. Similar experiments were done in the present study with highly purified preparations. The change in absorbance during the phosphorolysis of uridine was linear with time for at least 30 minutes; during uridine synthesis, linearity was evident for about 70 minutes (Fig. 7). A similar result was obtained with a partially purified E. coli preparation (27). The phosphorolytic reaction was also linear with added protein over at least a 10-fold range (4.9 to 49 μg) (Fig. 8). It should be noted however that for most of the studies involving uridine synthesis 1-2 μg of protein were frequently used.

Each point on the lines in Figures 7 and 8 (and in subsequent experiments) generally represents the average of two readings. If, however, the duplicates do not agree, both readings are given on the graph. To clearly indicate which fraction from the purification procedure was used in an experiment, the following notation was devised: the most highly purified fraction of a given preparation is denoted by a subscript 'f', for example prep. A_f; any other fraction is denoted by a number which refers to the step in which it was prepared, for example prep. A_4 (ammonium sulfate fraction). The two final fractions in preparation E will be denoted as fraction E_a and fraction E_b (Table VIII).

b) Stability

A comprehensive study of the stability of uridine phorphorylase activity was not done. Nevertheless, general information is given here

so that one can obtain some idea of the stability of the uridine phosphorylase preparations in this work.

i) Storage of enzyme fractions

Preparations B_f, C_f, and D_f were dialyzed against 0.05 M Tris, pH 7.4-5 mM 2-SH. Aliquots were taken and they were frozen at -40^{o}. Preparation E was dialyzed against 0.05 M Tris, pH 7.4-1 mM EDTA-5 mM 2-SH. Preparations C_f and E_b lost 22% of their uridine cleaving activity (assayed at pH 8.1) after 60 days in storage. On the other hand, preparation D_f (1 mg/ml protein) lost 65% of its activity in 18 days of storage. Uridine phosphorylase fractions (20-30 mg/ml protein) from step 6, if stored after concentration with ammonium sulfate at -40^{o}, did not lose any uridine or deoxyuridine cleaving activity after one month, but lost 17% of each activity when stored for two months. Fraction E_a (7 mg/ml protein) lost about 25% of its activity towards uridine and deoxyuridine after three months at -40^{o}. After repeated freezing and thawing, aliquots from fraction E_b lost about 35% of its activity in 20 days in addition to the activity lost by storage alone.

The purified preparations of Pontis et al (13) and Krenitsky et al (14) from Ehrlich ascites cells were much more labile than the present preparations (except for preparation D). Pontis et al were able to maintain the activity of the enzyme for two weeks or more by storing the fraction in 1% bovine serum albumin, and by freezing at -20^{o}. The final enzyme concentration from their purification scheme was 0.06 mg/ml.

ii) Effect of 2-mercaptoethanol and EDTA on storage

2-Mercaptoethanol was used in this study during the purification procedures, for storage, and in most of the assay procedures. EDTA was used in purification and storage of many of the enzyme fractions, although

it was only used for storing one purified preparation (fractions E_a and E_b). EDTA did not appear to have a stabilizing effect on the activity of the stored enzyme at least at this stage of purification (1000-fold). Pontis et al (13) found the same effect with EDTA using a 20 to 40-fold pure uridine phosphorylase fraction. In the summary shown in Table XI it is apparent that 2-mercaptoethanol and EDTA were not commonly used to protect nucleoside cleaving enzymes, even though in references (3, 8, 50) the enzyme preparations were shown to be sensitive to sulfhydryl reagents.

2-Mercaptoethanol did not appear to have any effect on the activity of a fresh preparation of uridine phosphorylase when added directly to the assay medium, and it probably has little effect on an aged preparation of uridine phosphorylase if it were only in contact with the enzyme for 10 minutes and only at a final concentration of 5 mM (results under heading iii). Preparation E_6 lost very little of its activity towards Ur, Udr, and Tdr when dialyzed for 16 hours in the absence of 2-mercaptoethanol. After dialysis for 90 minutes to remove this thiol from fraction E_a, no loss in either uridine or deoxyuridine cleaving activities was evident. Again using preparation E_6 there was no effect on activity by 2-mercaptoethanol over a concentration range of 10 μM to 10 mM during the 10 minute assay incubation period. Pontis et al (13) found that glutathione had no effect on enzyme activity (20 to 40-fold pure preparation), however, there was neither an indication of the age of the enzyme preparation nor of the time involved in the incubation of enzyme and thiol. Nothing can actually be concluded from the present work on the effect of 5-10 mM 2-mercaptoethanol on the stability of uridine phosphorylase on storage, for no preparation was ever stored without this thiol being present. Setlow and Lowenstein (72) found that

freezing crude adenylate deaminase preparations in the absence of 2-mercaptoethanol stabilized the enzyme for at least two months, whereas freezing the enzyme in the presence of the thiol caused inactivation. Nevertheless, during purification and storage of the pure enzyme at 4^{o}, 2-mercaptoethanol (0.1% by volume), glutathione or dithiothreitol was needed for stability. There was no indication of the effect on freezing the pure enzyme with 2-mercaptoethanol present.

Although uridine phosphorylase will be shown in the next section to be a sensitive sulfhydryl enzyme, this does not necessarily mean that a low molecular weight thiol will be needed for storage. Creatine kinase, one of the most sensitive sulfhydryl enzymes known, did not require a thiol for stability (80). It is possible that the disulfide formed on oxidation of 2-mercaptoethanol by dissolved oxygen and catalyzed by substances in the medium, may actually inhibit the activity of the enzyme by forming mixed disulfides with enzyme sulfhydryl groups (64, Vol I, p. 625). Excess oxidized glutathione decreased the activity of phosphofructokinase by about 30% (81). A study on nucleoside monophosphokinases revealed that CMP kinase, UMP kinase, and dCMP kinase activities are differentially inactivated by oxidized glutathione with 50% loss of dCMP kinase activity at a concentration of glutathione of about 24 mM (53).

iii) Inactivation and reactivation experiments

The effect of 2-mercaptoethanol and dithiothreitol on the activity of aged enzyme preparations was discovered by accident near the completion of the studies done for this thesis.

Inactivation. After 24 hours at room temperature, in the presence of 20 mM dithiothreitol, reactivated fraction E_a showed no loss in uridine

or deoxyuridine cleaving activity. After 48 hours, however, the loss of activity with both nucleosides was 27% and after 96 hours it was 82%. After 72 hours a white fluffy material began to form (gel formation or bacterial growth?). On the addition of 13 mM dithiothreitol at 96 hours, there was no clarification and very little reactivation after an incubation period of three hours (the absorbance readings at different time intervals were too variable to give a percent value here). Differential inactivation of the nucleoside cleaving activities was not evident.

Reactivation of aged preparations. Time and concentration reactivation studies at room temperature, with 2-mercaptoethanol and dithiothreitol, were done on aged fraction E_a and E_b (after a three month aging period at -40^{o}). Initially, this was done by adding the thiol directly to the assay tubes, however, with dithiothreitol the absorbance readings were not reproducible when 10 mM or greater concentrations were used. It is interesting to note that with a highly purified purine nucleoside phosphorylase preparation dithiothreitol would sometimes first activate the enzyme and subsequently inhibit it (3). An increase in absorbance was still apparent with uridine phosphorylase, for example, the increase in activity with uridine at pH 8.1 and 7.4 and with deoxyuridine at pH 7.4 was between 50-100%. The increase in activity with 2-mercaptoethanol was more reproducible. The result of a time study is shown in Figure 9A-1. The values are corrected for the decrease in activity of the enzyme in control tubes at different times (control readings decreased by 10-20% for a 5 to a 120 minute preincubation period). With 50 mM 2-mercaptoethanol maximal reactivation is being approached, still the two hour preincubation period does not

appear to be the optimal one (Figure 9A-2).

Reactivation with 10 mM dithiothreitol was repeated on fractions E_a and E_b by first dialyzing to remove 2-mercaptoethanol and then adding dithiothreitol to make a final concentration of 10 mM. Aliquots were removed and assayed at different times. The increase in absorbance on activation for fractions E_a and E_b with uridine and deoxyuridine as substrates (assayed at pH 7.4) is shown in Figure 9B. These readings are not a true reflection of the extent of activation for the absorbance reading of control tubes at different time periods were not taken. Addition of dithiothreitol to give a 10 mM increase in its concentration after 130 minutes did not produce a significant change in absorbance. Fraction E_a did not show a great increase in activity on reactivation probably because it was not aged enough.

This point may be better understood by looking at Table XII where the results of reactivation of preparations C_f to E_f are summarized. The activity towards uridine and deoxyuridine cleavage was increased with the above preparations after a 3 to 9 month storage period, even though in two cases no activity was present before reactivation. The results suggest that the activity of uridine phosphorylase may have been better maintained by activating the preparations at frequent intervals. Fraction E_a was completely reactivated but it did not have far to go. The number of bands and their relative intensity by visual inspection (disc gel electrophoresis) did not differ before and after activation for any of the preparations.

Even though reactivation results of aged enzyme preparations have been found in the literature, few thiol concentration studies (82, 83) and time studies (84) to show optimal conditions were given concomitantly.

For phosphofructokinase (85), 100 mM 2-mercaptoethanol was incubated with the enzyme for 10 minutes at 37°. For malic enzyme, 1 mM dithiothreitol preincubated for 80 minutes at 26°, reactivated the enzyme optimally (84). Mitochondrial nucleoside diphosphokinase was incubated with 100-200 mM 2-mercaptoethanol for 8 minutes. For deoxycytidine kinase, 50 mM 2-mercaptoethanol, preincubated with the enzyme for 60 minutes at 37°, reactivated the enzyme maximally (82). The conditions for maximal reactivation of aged uridine phosphorylase preparations are not yet known, however, it appears that the enzyme should be preincubated with 50 mM 2-mercaptoethanol for at least 180 minutes or with 10 mM dithiothreitol for 120 minutes, at 25°. Such long periods of reactivation are not uncommon. With the citrate cleavage enzyme, it was found necessary to incubate with 5 mM dithiothreitol for as long as 24 hours at 0° for complete activation (86).

c) Homogeneity

Besides the criterion of constancy of specific activity as mentioned with the results of the hydroxylapatite column, two other criteria were used to judge the homogeneity of the purified preparations.

i) Polyacrylamide disc gel electrophoresis

Diagnostic electrophoresis. Preparations C_f-E_f gave two dense bands on disc gel electrophoresis at pH 8.9 as shown in Figure 10, with band A always being the widest and darkest staining band. Preparations D (photos 1 and 2) and fraction E_b also had very faint bands, two above and two below the dense bands. Coomassie blue appeared to bring out the faint bands better (compare photo 1 with 2, and 5 with 6, in Fig. 10) than Amido black when the same amount of protein (50 μg) was applied.

The banding pattern of preparation D at pH 7.5 was similar to that at pH 8.9 except for a narrow light staining band immediately above band A. In preparation C, four additional faint bands appeared after the preparation was 10 weeks old with a concomitant loss of activity (about 50%). Also, the minor band (B) was more heavily stained than in the original preparation. These pattern differences on aging were also apparent in preparation D but not in fraction E_b. With freshly prepared fraction E_a (specific activity 20) three dense bands appeared on electrophoresis, but band A still stained the darkest (not shown).

A definitive concentration study was not done, so there is the possibility that band B was due to aggregation effects. Still, in preparation C_f the protein concentration was 1.3 mg/ml, whereas in preparation D_f and fraction E_b it was between 0.2-0.5 mg/ml, and no differences in the banding pattern were observed. One more fact worth noting is that 200 μg of preparation C (photo 3) did not show the faint bands which appeared with 50 μg of aged preparation C (photos 5 and 6). Furthermore, the banding pattern with 280 μg of preparation D (not shown) did not differ from that with 50 μg (photo 2).

Elution electrophoresis. On elution, 90% of the original uridine and deoxyuridine cleaving activities were lost. Since the absorbance readings were between 0.03 and 0.07 units for uridine and deoxyuridine, it is not possible to present any meaningful results, although it should be added that 90% of the remaining uridine and deoxyuridine cleaving activities was recovered in band A. These results are included in this thesis in order to account for over 75% of preparation D_f (Table IV) that was used in an effort to find better conditions for recovery of activity on elution. Some precautions that were taken were:

(1) to add 2-mercaptoethanol to the buffers

(2) to apply the protein sample in sucrose instead of spacer gel

(3) to run the gel column at 4^{o}

(4) to extend the time of contact between the gel pieces and buffer

(5) to apply larger protein samples.

Few papers have been found in the literature that actually gave the percent recovery of enzyme activity after elution (87-95). In one paper, however, 40% recovery of activity was reported (90). Perhaps modification of the elution procedure would produce a better recovery of activity, although a preparative disc gel technique would provide a better solution (96, 97). Still it should be noted that the two most frequent approaches used in the recent literature for detection of enzyme activity from disc gels do not involve the preparative technique. One basic technique is to allow the cut pieces of gel to stand in buffer for varying time intervals up to 24 hours (88, 89), and sometimes to macerate the pieces afterwards (87) or right away (90, 91), and then to assay for activity. The other technique is to assay for the enzyme directly on the gel or pieces of the gel by adding a specific dye or indicator to the assay medium (92-95).

ii) Ultracentrifugation

Sedimentation velocity analysis with preparation C_f (4 mg/ml protein) at 60,000 rpm. for 90 minutes, resulted in a small band such as seen in the other studies (93, 98). No peak was evident, however, with fraction E_b (5.7 mg/ml protein) under the same conditions. The reason for this discrepancy is not understood. Because the peak was so small it is difficult to say for certain whether it is symmetrical, neverthe-

less, the lack of homogeneity in the purified preparations was evident from electrophoresis alone.

d) pH-Activity study

The pH-activity relationship for uridine and deoxyuridine over a pH range of 4-10 is shown in Figure 11. The pH optima for uridine and deoxyuridine are 8.2 and 6.5 respectively. This is essentially the same as shown by Pontis et al (13) for a partially purified Ehrlich ascites cell preparation; by Yamada (19), and by Canellakis (26) for partially purified rat liver preparations. A greater pH range was used in the present study, and the pH curves appeared to be more symmetrical here. At the pH optimum for each substrate the ratio of Ur/Udr activities was 1.1, which is lower (compare to 1.4) than that obtained for a 15-fold pure preparation of uridine phosphorylase (19). The ratio for the Ehrlich ascites cell preparation was 1.0 (13). Paege and Schlenk found the pH optimum for the phosphorolysis of uridine with a 20-fold pure E. coli uridine phosphorylase preparation to be 7.2 (27), although a more recent paper has reported a value of 7.6 on a 15-fold pure preparation (28).

For the phosphorolysis of nucleosides about 100 mM phosphate was used, in addition to acetate (100 mM), Tris (100 mM), or glycylglycine (167 mM) buffers, in order to obtain the different pH values. Tris inhibited the phosphorolysis of deoxyuridine by about 25%, but it had no effect on the phosphorolysis of uridine. It is interesting to note in this connection that the deoxyribonucleoside cleaving activity of thymidine phosphorylase was also inhibited by Tris (50 mM); more details are given under heading (fii). The loss in activity at pH 5.4 and 9.4

with uridine as the nucleoside was not due to inactivation of the enzyme for when it was preincubated at these pH values for 10 minutes, and then assayed at pH 7.6 or pH 7.9, the normal absorbance readings were recovered (Figure 11).

When phosphate was not included in the incubation medium, 10% of the normal uridine cleaving activity at pH 8.0 was still present. This was not apparent with deoxyuridine as the nucleoside (Figure 11). This difference may be due to experimental error since the absorbance readings were very low, or perhaps to the presence of a uridine hydrolase. Nevertheless, it might also be a reflection of active site differences in uridine phosphorylase for the cleavage of uridine and deoxyuridine. It should be noted that the phosphorolytic and the hydrolytic activities appear to have the same pH optimum.

The pH optimum for the synthesis of uridine appeared to be about 8.5. This is in good agreement with a value of 8.2 for a crude Ehrlich ascites cell preparation (22).

e) Molecular weight estimation

The molecular weights of uridine and thymidine phosphorylases (based on one determination) by Sephadex gel filtration were both estimated to be about 110,000 (Figure 12). These molecular weights are not unreasonable estimates with respect to a value of 80,000 for a purine nucleoside phosphorylase preparation (3) which was estimated by Sephadex G-100 chromatography (Table III), and values of 78,000 and 148,000 for partially purified pyrimidine nucleoside phosphorylases from E. coli and B. stearothermophilus, respectively (estimated by sucrose density gradient centrifugation (99)).

The points representing the five standard proteins on Figure 12 do not all have to be on the standard line, and, in addition, the ones that deviate may still be used as long as their behaviour is recognized; for example, ovalbumin, deviates +10%, and γ-globulin, -30% (75). In the present study both of these proteins deviated from the line, but not to the same side of the line as given by Andrews (75). Andrews suggested that his data be taken as the standard relationship for globular proteins. Thus, it would be of value to compare the ratios of elution volume to void volume for the proteins used. The values for the present study are given in brackets: chymotrypsinogen, 2.4 (2.3); ovalbumin, 2.3 (2.0); albumin, 2.0 (1.9); γ-globulin, 1.5 (1.5); apoferritin, 1.2 (1.2). The aggreement is quite good.

f) Transferase activity

i) Transferase experiment

The transferase experiment performed as described in 'Methods', showed that phosphate independent ribosyl transfer did not occur, that is, direct transfer. Phosphate dependent (0.83 mM phosphate) transfer did occur, that is, indirect transfer. The equations for these reactions were given and explained in section I B 2b. Indirect transfer activity simply indicated what had been shown previously with 10-fold or less pure preparations (25, 35), namely, that uridine phosphorylase can catalyze a reversible reaction. The above experiment was done in order to show the connection between the inhibition of phosphorolysis by deoxyglucosylthymine (described below) and transferase activity, for a preparation of uridine phosphorylase free from thymidine phosphorylase activity. To the author's knowledge this was not done before.

ii) Inhibition of activity by deoxyglucosylthymine

Deoxyglucosylthymine inhibited the phosphorolysis of uridine and deoxyuridine as well as the synthesis of uridine (Figure 13). The inhibition of the synthesis of deoxyuridine was not studied. The concentration of deoxyglucosylthymine required for 50% inhibition of the phosphorolysis of uridine at pH 8.1 was 0.10 mM, and for deoxyuridine at pH 6.6, 0.018 mM. Also the I_{50} values for uridine and deoxyuridine at pH 7.3 were 0.11 mM and 0.019 mM, respectively. For uridine synthesis at pH 8.1, the I_{50} value was 0.14 mM. Except for the pH 7.3 studies, as indicated below, each complete experiment was done once. The inhibition produced by deoxyglucosylthymine after a 20 minute preincubation period was the same as after a 5 minute period. Preincubation of phosphate or uridine (assayed at pH 8.1) with the enzyme before adding deoxyglucosylthymine did not reduce the degree of inhibition.

The phosphorolysis of deoxyuridine and thymidine by thymidine phosphorylase (preparation D_{7b}) was not inhibited by a deoxyglucosylthymine concentration of 0.19 mM. In connection with this fraction, it should be noted that it had already lost 50% of its activity towards the two nucleosides after dialysis against 0.05 M Tris-1 mM EDTA (performed just before the inhibition study). The same loss occurred even when 5 mM 2-mercaptoethanol was added to the dialyzing buffer. The inhibitions by deoxyglucosylthymine of uridine phosphorylase and the lack of inhibition against thymidine phosphorylase are in agreement with previous results on crude rat liver and Ehrlich ascites cell enzyme preparations (20, 33).

As indicated in the legend of Figure 13B, the first experiments with uridine and deoxyuridine at pH 7.3 were done on one day. The

experiments at pH 8.1 and 6.6 (Fig. 13A) and the second experiment for uridine at pH 7.3 were done about one week later. The irregular shape of the uridine curve could not be reproduced in the second experiment. The only apparent difference between the two pH 7.3 experiments was that the absorbance reading of the control for the experiment on (8.12.68) was 20% lower than for the experiment on (28.11.68).

g) Substrate inhibition

The synthesis of uridine at pH 8.1 was inhibited by uracil (Fig. 14) with 50% inhibition occurring at a concentration of 1.2 mM uracil. With deoxyribose-1-phosphate as the second substrate, 1.1 mM uracil inhibited the synthesis of deoxyuridine by 65% at pH 6.5 (not shown). Optimal concentrations of the pentose-1-phosphates were used (1.4 mM).

The type of inhibition that was produced is difficult to classify since the Dixon plot (1/v versus uracil) was not obeyed as shown in the insert of Figure 14, that is, the lines did not cross the positive y-axis and they did not intersect in the negative x-quadrant (67, p.80). In passing it is interesting to note that the Dixon plot was obeyed for the inhibition of thymidine synthesis by thymine for thymidine phosphorylase preparations (9, 10). If the velocity values on inhibition were obtained at higher concentrations of uracil, the lines may have intersected in the expected quadrant. This suggests that cooperative effects between two or more uracil sites may have aided the progress of inhibition. Also the concept of overlapping sites for uridine and deoxyuridine is an attractive hypothesis for this situation and it will be discussed in some detail later (sections III D 5b, and III D 8c).

h) Anomalous results after reactivation

These results are sketchy, for the limited amount of enzyme at this late stage made it impossible to do detailed studies; nevertheless the findings were considered to be of some interest and they will be described.

Preparation D_f and fractions E_a and E_b, one to two months after reactivation, gave unexpected absorbance readings for the phosphorolysis of uridine and deoxyuridine at different pH values. Table XIII summarizes the effects with fraction E_b. This fraction had exceptionally low uridine cleaving activity near its optimum pH (compare with Fig. 11) but this activity increased substantially when glycylglycine was incorporated into the buffer system (experiments 3 to 7). No such increase with glycylglycine had been previously recorded. Still there was substantial deoxyuridine cleaving activity near its pH optimum, but there was a marked decrease in the phosphorolysis of deoxyuridine at pH 7.3 (experiments 9 to 11). Nucleoside synthesis with ribose-1-phosphate (with glycylglycine) and deoxyribose-1-phosphate gave 'normal' absorbance readings (experiments 12 and 13). Synthesis of uridine in the absence of glycylglycine was not checked. The results with preparation D_f and fraction E_a were similar, but the effect of glycylglycine was only checked with fraction E_b. Reactivated preparation C_f did not give the above anomalous results. The only difference in procedure that was evident was that this preparation was only thawed twice after its original reactivation, whereas preparation D_f and fractions E_a and E_b were thawed numerous times before all assays were completed.

The reason for the anomalous effects is not known yet. Previous experiments on reactivated samples did not reveal any differences for

the phosphorolysis of uridine and deoxyuridine. Perhaps aging of the reactivated aged samples is an important factor.

E. DISCUSSION

1. Intracellular Localization of Uridine Phosphorylase

Although about 80% of uridine and deoxyuridine cleaving activities were found in the soluble portion of the cell, this does not necessarily mean that these activities are localized in cytoplasmic or soluble fractions in vivo. Thus, if the tissue is homogenized in 0.25 M sucrose, most of the nuclear sap components, i.e. enzymes and low molecular weight chemicals, will occur in the soluble fraction of the cell (56, 100). The soluble space of the cell may be defined as the extraparticulate compartment of the intact cell in which proteins and metabolites are free in solution (56). In addition, cytoplasmic enzymes themselves may be loosely bound to some particulate fraction but released quickly on homogenization in 0.25 M sucrose, as has been found with mitochondrial hexokinase (101) and 5'-nucleotidase (102). Green has concluded that the glycolytic enzymes are bound to the erythrocyte membrane with various degrees of firmness (103). In this connection it is interesting to note that a purine nucleoside phosphorylase has been found to be highly concentrated in the nucleolus of starfish Oocytes (104), and that Bose and Yamada have localized most of the enzyme activity from the crude nuclear fraction as described in the present study, in the plasma membrane (unpublished results from this laboratory).

2. Column Chromatography

a) Thymidine cleaving activity of uridine phosphorylase

The columns gave an overall recovery of activity of 85 to 95% with generally little loss on subsequent concentration and dialysis. The losses that did occur were usually a result of the selection of only certain column

fractions in order to obtain a concentrate of high specific activity.

The DEAE-Sephadex column (step 7) gave a good separation of Urpase from Tdrpase, however a base line was not evident in the chromatogram. This would suggest that the uridine phosphorylase peak was contaminated with a small amount of deoxyuridine and thymidine cleaving activities of thymidine phosphorylase. However, in studies by Yamada (19), where a base line was usually reached, a small but significant thymidine cleaving peak was shown to be present under the uridine cleaving peak. In addition, in the present study, the specific activity ratio Ur/Tdr remained relatively constant for the fractions obtained in the purification procedure after step 7 in the five different preparations (Table X). Also it was observed that if a small amount of thymidine phosphorylase activity contaminated the uridine phosphorylase peak after step 6, it could be removed by the subsequent column step (Table IX). Thus, the above three points suggest that the thymidine cleaving activity remaining with the purified uridine phosphorylase preparations is an inherent part of its catalytic action.

b) Explanations for the variation in the final specific activity

The differences in specific activity in the purified samples (Table VIII) perhaps may be explained by the following four factors, (besides using the argument of technical variation):

(1) overloaded DEAE-Sephadex column

(2) Sephadex G-200 column at pH 8.0

(3) heat treatment step

(4) recombination technique for column fractions

The low final specific activity of preparation A was probably due, for the most part, to the absence of an overloaded DEAE-Sephadex column in

the procedure, and to the fact that fractions collected from the hydroxylapatite column had a wide range of specific activities ranging from 8 to 32. In preparations B and C, although the same steps were used, the range of specific activities of the fractions collected from the hydroxylapatite column for B (from 26 to 47) was greater than for C (from 35 to 46). Still the loss of enzyme activity from preparation B through spilling probably had a greater effect than the above point on its final specific activity.

Perhaps factors (1) to (3) can explain why preparation D had the highest final specific activity of the five preparations. Heat treatment resulted in less than a 2-fold increase in specific activity but about 4 out of 12 grams of protein were eliminated. This could result in a significant effect on the resolution of proteins in subsequent column steps. The Sephadex G-200 column normally gave a 1.8-fold increase in specific activity (2-fold in the most active column fraction) in the final concentrated fraction, but in preparation D, a 5-fold increase occurred (6.6-fold in the most active fraction), and in preparation E, a 2.5-fold increase occurred (3.9-fold in the most active fraction). The only apparent difference between the columns used in preparations D and E and in the other preparations was the change of pH of the buffer from 7.1 to 8.1. It is difficult to assess the contribution of the change of pH to the increase in specific activity since the columns were not repeated often enough, but as already mentioned there were differences in activity and protein profiles in buffers with the above pH values.

One should also consider the volume of sample used, since the separation of proteins on a molecular sieving column is greatly dependent on this volume. In preparation D, the volume of sample applied was 1% of the bed volume whereas in preparation E it was 3%. But when the sample volume was 1% with a column at pH 7.1 the increase in specific activity was still

only 2-fold. Another point to consider in connection with preparations D and E is the recombination technique, for more activity was discarded with D (30%) than with E (15%). In passing, it is interesting to note that Sephadex dextran gels have an aromatic affinity for solutes (105), however for Sephadex G-200 this is not very pronounced.

c) Specific activity ratios

The Ur/Udr specific activity ratio at pH 7.4 from a 10-fold pure rat liver uridine phosphorylase preparation was given as 2.0 by Krenitsky et al (15), however this ratio varied from 8 in E. coli to 0.5 in dog intestinal epithelium (in the present study the ratio was 1.3). Purified uridine phosphorylase from Ehrlich ascites cells gave ratios of 1.1 (13), and 2.0 (14) at pH 7.4. The Ur/Udr ratio indicates the preference of the enzyme for ribose or deoxyribose, whereas the Udr/Tdr ratio indicates the preference for uracil or thymine (15). Thus, from the present study and another study (19), it appears that uridine phosphorylase of rat liver has a greater preference for the ribose moiety from pH 7.2 to 10, and for the deoxyribose moiety from pH 5.1 to 7.2. In addition it has a preference for uracil over thymine from pH 5.5 to 10 (this is only an estimate for a pH-activity curve for thymidine was not done here).

At this point, it would be meaningful to look at Krenitsky's specific activity ratios for his various preparations more closely as given in Table I (15). One explanation that can be given for the variation in the ratios is that uridine phosphorylases from different sources have different substrate specificities. Other explanations were discussed in detail in section I B 2 ai. However another possibility is that the ratios may vary as a result of a pH effect. The pH profiles for Ur, Udr, and Tdr for any

of Krenitsky's sources were not given, and all the assays were done at pH 7.4. The pH optimum for the main substrate was said to be the same in all cases, i.e. 6.6 for uridine cleaving enzymes of group B, 8.1 for uridine cleaving enzymes of group A, and 5.5 for thymidine cleaving activity of thymidine phosphorylase. The literature values for the pH optima for uridine and deoxyuridine for uridine phosphorylase have been reported for rat liver (19) and Ehrlich ascites cells (13), and in both cases they were 6.6 for Udr and 8.1 for Ur; the pH optimum for the thymidine cleaving activity of uridine phosphorylase from rat liver was reported to be 5.7 (19); from Ehrlich ascites cells, 6.8 (14); and from B. stearothermophilus, 7.2 (99), although the evidence that this enzyme also catalyzes the phosphorolysis of uridine and thymidine is not conclusive yet. Perhaps it is possible that the pH profiles of deoxyuridine and thymidine in relation to the pH profile of uridine are different for enzymes from different sources. This could account for the variation in the Ur/Udr and Ur/Tdr ratios.

3. Interpretation of the Disc Gel Patterns

A number of explanations for the significance of the two major bands on disc gel electrophoresis are considered below:

(a) One band is specific for uridine cleavage and the other for deoxyuridine cleavage, or band A has both activities and band B has only one activity.

(b) Both bands have two activities, but band A has the greater activity (1) towards both substrates or (2) towards only one substrate.

(c) Band A contains both uridine and deoxyuridine cleaving activities and band B is due to aggregation effects or to some artifact produced during

electrophoresis.

(d) Only band A has the catalytic activity and band B contains inactive protein (with regard to the activities of uridine phosphorylase).

The information available from the present study cannot definitely point to one interpretation. The elution results can only be considered as a weak support for the fourth contention because the recovery of activity was very low. Nevertheless, it is felt that this explanation is the most likely one at this time; each explanation is considered below:

(a) Band A appears to be at least the major active band. There was enrichment of A over B during purification in preparations C-E. In addition, there was further enrichment in fraction E_b relative to fraction E_a. Also fraction E_b contained 80% of the total uridine and deoxyuridine cleaving activities whereas fraction E_a contained 20% of the total uridine and deoxyuridine cleaving activities from preparation E_f (Table VIII). The specific activity ratios of E_a and E_b were virtually identical (Table X). Thus explanation (a) does not appear likely, and, at the most, band B contains an enzyme with low activity towards one of the nucleosides.

(b) Explanation (b1) cannot be ruled out, but (b2) does not appear likely on the basis of the information given above.

(c) This explanation does not appear likely for the lack of homogeniety was also evident by the inconstancy in specific activity across the activity band in the hydroxylapatite column. Also, variations in procedure for electrophoresis did not produce a marked difference in the patterns (section II D 2 ci).

(d) This is the simplest explanation, but band B may still be more than just an inactive protein. This is discussed under heading 4b.

4. Reactivation of Uridine Phosphorylase

a) Future experiments

The inactivation of uridine phosphorylase with time and its reactivation by 2-mercaptoethanol and dithiothreitol indicate the presence in the protein of one or more reactive thiol groups which are essential for maintaining the protein in an active conformation. It would be interesting to explore the above effect further. An investigation of the following points are considered to be worthwhile in this connection:

(1) The effect of different thiols (mono and di) on reactivation of enzyme activity towards nucleoside cleavage and synthesis.

(2) Aging of the enzyme at different temperatures, pH, and ionic strength conditions.

(3) The formation of disulfide bonds with o-iodosobenzoate and tetrathionate followed by reactivation with 2-mercaptoethanol, dithiothreitol, etc.

(4) The effect on activity of different ratios of reduced and oxidized thiol.

(5) The effect of substrates on aging, that is, protection of sulfhydryl groups.

(6) The effect on molecular weight.

Perhaps a differential effect on uridine and deoxyuridine cleavage will be revealed on reactivation. Such an effect was evident with a nucleoside monophosphokinase preparation, purified up to 147-fold, with substrates dCMP, CMP, UMP (53, 82). If aging and reactivation were simply a matter of a reversible reaction, with an equilibrium between oxidized and reduced

sulfhydryl group conformational protein forms (discussed under heading 4b), one might expect that inhibition with o-iodosobenzoate, to be discussed in section III D 6, should duplicate the effect of aging. In this section on 'Sulfhydryl Group Modification Studies', it will be shown that the activity of uridine phosphorylase is very sensitive to inhibition by o-iodosobenzoate with virtually complete protection against inhibition afforded by uridine. Perhaps storing the enzyme in uridine would decrease the rate of the aging process of the enzyme.

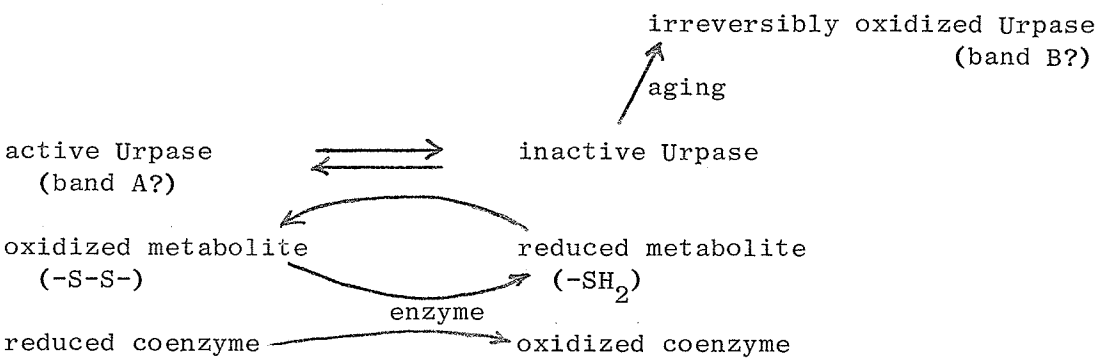
b) Equilibrium between two thiol states

Although more evidence on the importance of sulfhydryl groups for the activity of uridine phosphorylase is given in section III, there will still be no evidence to support or negate the possibility of an equilibrium between an active uridine phosphorylase molecule with reduced SH groups and an inactive uridine phosphorylase molecule with oxidized SH groups. Still this is an interesting possibility, for equilibria between various conformations of proteins in solution is believed to be an important property of proteins (106, 107), and reduced and oxidized thiol states have been postulated (53, 83, 109). Since Sugino et al have found that the three kinase activities of dCMP kinase were differentially inactivated and reactivated (82), as reported above, Sugino suggested (53) that the,

"...redox potential may be one of the factors regulating activities of some enzymes without change in the net amount of protein."

Although Sugino et al did not report on an in vivo enzyme system, they did obtain rapid activation of dCMP kinase with thioredoxin, thioredoxin reductase and $NADPH_2$ (109). Reversibility was not shown. The above speculative discussion can be adequately summarized by showing a generalized version of the sketch given by the above authors. As a tentative explanation for band B, an additional enzyme form is added (irreversibly

oxidized uridine phosphorylase).



This equilibrium between two thiol states will be considered in more detail in connection with a model for the mechanism of action of uridine phosphorylase in section VI B.

5. pH-Activity Study

The phosphate species that is involved as a substrate in phosphorolysis is not known (1). One report gave the concentration of monoanion and dianion as being equal at pH 7.2 (110). This is an interesting point for at this pH the enzyme has equal activities towards uridine and deoxyuridine (Figure 11). Although this may imply that the monoanion is involved in deoxyuridine cleavage and the dianion in uridine cleavage, there are difficulties inherent in this proposal for neither activity is at 50% of its maximum value at this pH, that is, uridine had 66% of its maximum, and deoxyuridine has 74%. One should note however, that the pH curves are rather steep in this region.

The apparent splitting of uridine without adding phosphate to the incubation medium might indicate that the phosphorolytic and hydrolytic activities are an intrinsic feature of uridine phosphorylase. Sucrose phosphorylase isolated from leuconostoc mesenteroides exhibited hydrolytic activity of the order of 1 to 2% of its phosphorolytic activity even after

extensive purification (111). The authors concluded both activities to be an intrinsic property of the enzyme on the basis of: (1) constant ratios of phosphorolytic to hydrolytic activity during purification, (2) the same pH optimum, (3) inhibition by glucose.

It is worthwhile to note that the hydrolytic cleavage of only uridine was not the only difference found in the action of uridine phosphorylase on uridine and deoxyuridine. There was also inhibition of only deoxyuridine cleavage by Tris, and the loss of only uridine cleaving activity under certain conditions as given in section II D 2h. These points will be referred to once more in section III D 5b in connection with overlapping nucleoside sites for uridine phosphorylase.

6. Transferase Activity

Although the present results suggest that uridine phosphorylase does not have direct ribosyl transfer activity, this conclusion has already been predicted by Krenitsky et al (14) based on rather indirect evidence (30, 33). Their reasoning is based on three experimental findings which are given below in order to show the extent of previous work in this area:

(a) Zimmerman found that mouse and rat tumours have low thymidine cleaving activity, but no transferase activity, whereas normal rat liver and human tumours have both activities (30).

(b) Thymidine cleaving activity in rat and mouse tumours was completely inhibited by deoxyglucosylthymine, whereas in normal rat liver and human tumours both activities were not inhibited (30).

(c) At about the same time, it was found that Ur, Udr, and Tdr cleaving activities of a partially purified Ehrlich ascites cell uridine phosphorylase preparation were inhibited by deoxyglucosylthymine; deoxyuridine and

thymidine cleaving activities of a partially purified horse liver thymidine phosphorylase preparation were not inhibited (33).

Thus, the present studies on uridine phosphorylase are more direct. Nevertheless, at this point, further experiments, such as the following ones, should still be done before it is concluded that uridine phosphorylase does not have direct transferase activity:

(a) pH experiment. The activity was only measured at pH 8.1, which is the optimum for the phosphorolysis of uridine, however, the pH optimum for transferase activity may be different. For thymidine phosphorylase the pH optimum for transfer of a deoxyribosyl moiety was 7.3, and for phosphorolysis, 5.9 (10). In addition, perhaps uridine phosphorylase may also be able to catalyze the direct transfer of a deoxyribosyl moiety.

(b) Concentration experiment. The concentration of nucleoside may be important for detecting transferase activity. For thymidine phosphorylase, the transferase mechanism predominated over the phosphorolytic one in the presence of phosphate for the synthesis of nucleosides above a concentration of 5 mM for the nucleoside (11). In the present study a concentration of 0.66 mM was used in the presence and in the absence of phosphate.

7. Substrate Inhibition

a) Comparison with results from the literature

Although no study, up to this time, has been directed towards the inhibition of uridine synthesis by uracil, it is still surprising, in the light of the present results, that in many studies the concentrations of uracil were between 4-10 mM and substrate inhibition was not observed (13, 22, 26, 27). In two other studies an assay for indirect transfer activity was used and the final uracil concentrations were 0.67 mM (25) and 0.17 mM

(35). In one paper (21) it was noted that the activity of a partially purified uridine phosphorylase preparation from rat liver was inhibited by high concentrations of uracil, but a concentration of 6.6 mM was still used. The above results cannot be reconciled with the fact that substrate inhibition was found in the present work above a concentration of 0.7 mM. It should be noted, however, that a purified rat liver preparation was not used for any study from the literature, and in only two cases were rat liver preparations used (21, 26).

b) Comparison with results on substrate inhibition of thymidine phosphorylase

Substrate inhibition of thymidine phosphorylase preparations has been studied in some detail (9, 10) and it would be of interest to compare I_{50} values with the present study. With a highly purified thymidine phosphorylase preparation from rabbit liver (9), inhibition by thymine began at about 3 mM with 50% inhibition at about 8 mM. With a partially purified thymidine phosphorylase preparation from human leukocytes (10), inhibition by thymine of thymidine synthesis resulted in an I_{50} value of about 7 mM; inhibition by uracil of deoxyuridine synthesis resulted in an I_{50} value of about 10 mM. Thus uridine phosphorylase (with an I_{50} value of about 1.1 mM) is more sensitive to substrate inhibition than thymidine phosphorylase, although a rat liver preparation of thymidine phosphorylase has not as yet been examined.

c) Physiological role for substrate inhibition

Substrate inhibition by uracil should be looked at in relation to the numerous effects of other metabolites on the kinases and other enzymes that are involved in ribo-and deoxyribonucleotide metabolism (53). Feedback circuits, however, are very complex and they are beyond the scope of this thesis. Still it was suggested in the 'Introduction' (section I B 4) that at an appropriate concentration of uracil, uridine phosphorylase might be forced

to catalyze an essentially unidirectional reaction, that is, nucleoside synthesis. Furthermore, this may be an important development for producing sufficiently high concentrations of uridine for the efficient catalysis of UMP production by uridine kinase during periods of growth. In the most general terms one could say that the function of substrate inhibition by uracil is,

"the maintenance of cellular economy and the cellular physiological balance at the lower metabolite level" (53).

8. Modifications of the Present Scheme for Purifying Uridine Phosphorylase

While uridine phosphorylase from rat liver has been purified as much as 1,900-fold relative to the homogenate with a recovery of 8% of the initial uridine cleaving activity, a number of ways can be suggested for improving the yield and decreasing the time involved:

(a) Purchase frozen perfused rat livers.

(b) Increase the capacity of the homogenizing equipment.

(c) Spin at (54,000 x g) instead of (150,000 x g) in order to make use of larger centrifuge tubes; or use newer larger capacity ultracentrifuges.

(d) Assays on the nuclear fraction and the supernatants as well as detailed assays on columns could be eliminated.

(e) Replace the overloaded DEAE-Sephadex column with the heat treatment step.

(f) Concentrate purified enzyme fractions by Diaflo membranes rather than by Carbowax.

(g) Prepare a homogenous enzyme fraction using preparative disc gel electrophoresis after the hydroxylapatite step or perhaps after an earlier

step.

(h) Monitor columns for activity by using the kinetic assay procedure or the direct reading procedure of Carter (49).

(i) Improve the separation of the uridine phosphorylase isozymes on DEAE-Sephadex (19).

(j) A DEAE-Sephadex column at pH 8.8 or a CM-cellulose at pH 6.5 might be a useful procedure after step 7, for the protein(s) in the second band (B) of the disc gel electrophoresis pattern may be more negatively charged than the proteins in band A.

The working time for the purification of uridine phosphorylase could be reduced to 200 hours by incorporating suggestions a-f into the present purification procedure (Figure 1B).

SECTION III

SULFHYDRYL GROUP MODIFICATION STUDIES

... a program relating SH groups to activity cannot be undertaken lightly.

J.L. Webb (64)

A. INTRODUCTION

1. Literature Review

Phosphorylases, as a class, contain sulfhydryl groups which are essential for enzymic activity, as based on inhibition by sulfhydryl reagents (1), although only 3-phosphoglyceraldehyde dehydrogenase (112) and glycogen phosphorylase B (113) have been investigated in detail. Phosphorolysis in its broadest sense includes any conversion of inorganic phosphate to organic phosphate. If uridine phosphorylase continues the pattern set by other phosphorylases it should have a substantial number of sulfhydryl groups, for example, glycogen phosphorylase B has 18 SH groups (113), and 3-phosphoglyceraldehyde dehydrogenase has 20 SH groups (112).

The role of the sulfhydryl groups in nucleoside phosphorylases has as yet to be determined, and no detailed studies have been found in the literature on any of these enzymes. Table XIV summarizes the results found in the literature on inhibition of nucleoside phosphorylases by sulfhydryl reagents. Most of these inhibition studies were on erythrocyte purine nucleoside phosphorylase (3, 69, 114), and it appears to be a sensitive enzyme towards p-mercuribenzoate (pMB). Although there is some disagreement between workers, the different experimental conditions used make it impossible to project a valid comparison (discussed under section III D 9). No information has been found in the literature on the inhibition of uridine phosphorylase by sulfhydryl reagents.

The writings of Boyer (115) and Webb (64) were very helpful in establishing a theoretical foundation on this subject and in providing

an experimental approach to the study of enzyme sulfhydryl groups.

2. Definition and Classification of Sulfhydryl Enzymes

Boyer defined a sulfhydryl enzyme as one that loses catalytic activity when some or all of its original sulfhydryl groups undergo modification. Uridine phosphorylase was found to obey this general definition. A detailed discussion of the role of sulfhydryl groups in enzymes is not warranted for the present study. Nevertheless, since uridine phosphorylase is a sulfhydryl enzyme, it would be of interest to look at four major ways in which this type of enzyme may be classified, based on the postulations of the original author. The majority of the enzymes listed in Table XV have sulfhydryl groups which are thought to be important in maintaining protein structure. Of the eleven enzymes placed under the first two headings, perhaps only for the ones marked with an asterisk is there sufficient experimental evidence to support an involvement of sulfhydryl groups in the catalytic process or in the binding of substrates.

3. Purpose of the Present Study

This study was not actually concerned with providing unequivocal evidence for the role of sulfhydryl groups, but rather it sought to establish whether uridine phosphorylase is a sulfhydryl enzyme, and to give some information on the sensitivity of its SH groups to sulfhydryl reagents.

4. Approach to the Study of Inhibition

There are at least two general approaches to the study of sulfhydryl groups in an enzyme.

(a) Experiment in detail with a specific reagent which can be shown to react with sulfhydryl groups on the enzyme. This approach appeared to be favoured by Boyer:

"Although the recommendation is often made that various types of reagents be used in order to establish that an enzyme has essential SH groups, careful testing with a more specific reagent is to be preferred ..." (115, p. 569).

(b) Experiment with a number of different types of inhibitors. This approach is favoured by Webb:

"The groups introduced by iodoacetate and p-chloromercuribenzoate are negatively charged, whereas those from iodoacetamide and phenylmercuric acetate are uncharged, and this could well be responsible for some of the differences observed between these inhibitors. This is one reason why many studies with SH reagents would profit from a quantitative comparison of the effects of a large number of inhibitors of different types." (64, Vol II, p. 649).

The present approach was to study the effect of a number of inhibitors on enzyme activity (through changes in absorbance). A direct titration of the SH group(s) was not technically feasible because the enzyme was not available in sufficient quantities, and also the enzyme preparation was not homogeneous. The following studies were the basic ones that were performed with most of the inhibitors:

(1) time study

(2) inhibitor concentration study

(3) reversal of inhibition with 2-mercaptoethanol

(4) protection of sulfhydryl groups by substrates

(5) pH study

5. Inhibitors

The inhibitors that were used fall into five classes based on their reaction with a thiol group:

Mercaptide formation - with p-mercuribenzoate (pMB) and p-mercuriphenylsulfonate (pMPS).

Mixed disulfide formation - with 5,5' dithiobis (2-nitrobenzoic acid) (DTNB).

Oxidation - with o-iodosobenzoate (IOB).

Addition to double bonds - with N-ethylmaleimide (NEM).

Alkylation - with iodoacetate (IA) and iodoacetamide (INH_2).

The chemical reaction of an inhibitor with a thiol group will be illustrated in the appropriate graphs.

The inhibitors listed above were chosen since they are considered to have high specificity for sulfhydryl groups under experimental conditions that are easily reproducible in the laboratory. In addition, these are the common reagents used for the specific reactions listed above. The experimental conditions that are necessary to give the greatest specificity for the thiol group have not been well-defined. Nevertheless, reaction with other groups in a protein often requires higher concentrations of inhibitor, longer reaction times, and higher temperatures than with thiol groups (64). This point will be mentioned again in various parts of the 'Discussion'. Still experimental guidelines have been given by Webb (64) for most of the inhibitors used in this study:

pMB: pH should be around neutrality; use minimum concentration.

DTNB: not given.

IOB: pH about 7 (not much lower); temperature between 15-25°; use minimum concentration; 30 minutes is about minimum time for maximal inhibition.

NEM: pH should be below 7; excess reagent should be avoided.

IA: pH about 7; concentration of inhibitor should be below 5 mM; 60 minutes is minimum for maximum inhibition. Although these points are useful as an experimental guideline "each protein or enzyme must be treated as a special problem" (64, Vol III, p. 337).

6. Enzyme Fraction Used for the Inhibition Study

All of the inhibitor studies shown in the present work were done with fraction E_b (Table IV). Since this fraction was stored in a buffer containing 2-mercaptoethanol, 6.2 mμmoles of this thiol were always introduced into the incubation medium along with protein. This amount corresponds to the maximum amount present and does not exclude losses due to the oxidation of 2-mercaptoethanol during storage or to freezing and thawing. Two recent studies have revealed that metal ions such as the ferric ion (116) and the cupric ion (117) have an important role in catalyzing the oxidation of cysteine. In the absence of a cation the oxidation was very slow (116). Since EDTA was used throughout purification (except during the step involving the hydroxylapatite column) as well as deionized redistilled water, the oxidation of 2-mercaptoethanol by metal ions should be minimal. The above concentration of this thiol in the incubation medium will probably affect the I_{50} values of pMB, pMPS, IOB, DTNB, because these values turn out to be within the range of the thiol concentration.

Ethylene diamine tetraacetic acid (1.3 mμmoles) was also introduced into the incubation medium along with protein. This reagent might affect the I_{50} values of the mercurials by forming a complex with the organic mercurial ion (118; 64, Vol I, p. 744).

B. METHODS

1. Incubation Media

The assay technique is the same one as described under assay (bi) in section II C. In all cases 7.2 μg of sample were used for each assay tube. Enzyme activity was always assayed in the direction of nucleoside cleavage. The final incubation media for the three pH conditions used in these studies are given below.

1 M G-G, pH 8.9	0	0	235[b]
1 M Ac, pH 5.5	150 μl	0	0
1 M Tris, pH 7.4	135[a]	135[a]	150
0.05 M Tris, pH 7.2	200	200	200
Water	650	800	550
Enzyme	5	5	5
Inhibitor	15[a]	15[a]	15[b]
0.05 M P_i, pH 6.6	300	0	0
0.05 M P_i, pH 7.0	0	300	300
0.1 M nucleoside pH 7.0	50	50	50
pH of incubation medium	6.7	7.4	8.2

symbols: a, volumes of the two solutions were always 150 μl;
b, volumes of the two solutions were always 250 μl.

2. Procedure for Inhibition of Phosphorolysis

The inhibitor and the enzyme were preincubated for a specified period of time at 25° as described in the legends of the figures showing concentration studies. The time necessary for maximum inhibition was determined for most of the inhibitors before other studies were done. After the preincubation period, phosphate was added to the incubation medium first, followed by the nucleoside. The general procedure (assay (bi)) was followed after this point. The action of the inhibitor was not stopped prior to addition of the substrates. This could have been

done by adding 2-mercaptoethanol or EDTA to the assay tube. The same order of addition and timing was followed for the blank, except that the enzyme was not present. Control assays, i.e., measurement of activity at different times without inhibitor being present, were usually done; the experiments for which this was not done will be pointed out. The percent activity remaining after inhibition was found by dividing the absorbance reading of the tube containing the inhibited enzyme by the reading of the appropriate control tube.

3. Procedure for Substrate Protection

After one of the substrates (Ur, Udr, or P_i) was preincubated with the enzyme for 5 minutes at 25°, the inhibitor was added to the assay tube. The second preincubation period (given in the legend to the appropriate figure) with the inhibitor present was not necessarily equal to the time for equilibrium of inhibition to be attained - the experiment to determine the time of equilibrium was not always done before the protection study (DTNB, NEM); with iodoacetate and iodoacetamide, the time was very long. After this second preincubation period, the second substrate was added, and the assay tube was immediately incubated at 37° for 10 minutes. The final concentrations of substrates (for any protection experiment) introduced into the assay tube were: Ur (3.3 mM), Udr (3.3 mM), and P_i (10 mM).

'Mixed' nucleoside protection studies, that is, with both nucleosides being present in the final incubation medium, were only done with DTNB as the inhibitor. For example, in one case, after deoxyuridine protection of the enzyme at pH 8.2 an assay was done after the addition of uridine and phosphate.

4. Procedure for Reversal of Inhibition with 2-Mercaptoethanol

The general method was to preincubate the enzyme with inhibitor for a specified period of time; then to add 2-mercaptoethanol and preincubate at 25° for another specified period of time, i.e., the reversal time. After the reversal time had expired the substrates were added, and the remaining activity was assayed in the usual manner. For clarity, the exact times and the concentration of 2-mercaptoethanol that were used are listed below.

Inhibitor	Inhibition time (min)	Final conc of 2-SH (mM)	Reversal time (min)
INH_2	20	5	20
IA	60, 30	50, 5	30, 30
pMB	5	5	15
DTNB	30	5	15
IOB	15	5	15

The only detailed study of different reversal times was done with pMPS. After the enzyme and inhibitor were preincubated together for 1.5 minutes, 2-mercaptoethanol was added to give a final concentration of 5 mM. The enzyme activity that remained was checked at different reversal times from 1 to 3 minutes. Appropriate controls (inhibition without reversal) were done.

5. Preparation of Solutions

As a precaution against deterioration of the inhibitors, fresh solutions were prepared daily. In the case of iodoacetate and iodoacetamide stock solutions were prepared for each assay. Serial dilutions were usually made. During its use, the solution of inhibitor was kept in an ice bucket at 0°. The preparation of stock solutions is summarized below:

Inhibitor (mM)*	Solvent
0.5 pMB	1 M glycylglycine, pH 8.9
0.5 pMPS	1 M glycylglycine, pH 8.9
0.5 DTNB	1 M glycylglycine, pH 8.9
5.0 IOB	1 M potassium hydroxide (to give a final pH of approximately 7) and water
100 NEM	1 M Tris, pH 7.4
500 INH_2	1 M Tris,
500 IA	As with IOB

* best stock solution.

C. RESULTS

Most of the Figures are based on one experiment with each point representing the average of tests done in duplicate. Both readings are given if the duplicates varied widely. The results obtained with each sulfhydryl reagent is given below under separate headings, in the order of increasing I_{50} values. A compendium of these results is given in Table XVI.

1. Inhibition by p-Mercuriphenylsulfonate

Figure 15 gives the results of time, inhibitor concentration, reversal, and protection experiments. Maximum inhibition of uridine cleaving activity at pH 8.2 was reached after 3 minutes of preincubation with 0.65 μM pMPS (Fig. 15A). An inhibitor concentration study with uridine as the nucleoside at pH 8.2 gave an I_{50} value of 0.4 μM (Fig. 15B). The substrates, uridine and phosphate, did not show any protection against inhibition by pMPS (Fig. 15B).

Reversal of inhibition with 5 mM 2-mercaptoethanol was quite marked, with the original 60% inhibition level, that occurred after a 1.5 minute preincubation period of inhibitor plus enzyme, decreasing to 8% (Fig. 15C).

2. Inhibition by p-Mercuribenzoate

A time study was not done. Nevertheless, it was thought that a 5 minute preincubation period probably would be sufficient, as it was with pMPS (Fig. 15A). Figure 16A shows the inhibition of uridine

cleaving activity at pH 8.2 and 7.4. The I_{50} value at pH 8.2 was 0.62 μM and at pH 7.4, 0.85 μM. The curves indicate a rapid loss in activity with an increase in inhibitor concentration, and possibly some stimulation of enzyme activity at low concentrations of inhibitor, at both pH values (17% stimulation at pH 7.4). Figure 16B shows inhibition of deoxyuridine cleaving activity. The I_{50} values at pH 7.4 and 6.7 were both lower than those of uridine, but the four I_{50} values were within a factor of 2.3 of each other (Table XVI).

The concentration-inhibition curves are sigmoidal and steep which is expected of inhibitors that combine tightly with enzyme groups (64, Vol II, p. 771). Thymidine phosphorylase does not appear to be as sensitive to pMB action as uridine phosphorylase, but one purine nucleoside phosphorylase preparation appeared to have a similar sensitivity to this type of modification (Table XIV). Although, at this time, no decrease was observed in the extent of inhibition under the present experimental conditions, the results on protection and reversal of inhibition are inconclusive (Figures 15B, and 16B). For example, a concentration of pMB of about 1 μM gave inhibitions of 50 and 90% for uridine cleavage at pH 8.2, on the same day with the same inhibitor preparation, and both points fitted equally well onto the steep portion of the inhibition curve. Since the protection and reversal studies were done on this portion of the curve, a lowering of the extent of inhibition would probably not be detectable.

When the I_{50} value is very low, as it is in the present study, it may become dependent to a great extent on the enzyme concentration. This will be discussed in section D 4 cii. In line with this possibility was the observation that when the control value for uridine cleavage

at pH 8.2 was 33% greater than for the curves shown in Fig. 16A, the I_{50} value was 80% greater than the previous value. Thus I_{50} values may not be the best way of reporting mercurial inhibitions (and possibly inhibitions with DTNB and IOB). Nevertheless, the method of expressing inhibitions in terms of μmoles of inhibitor per μg of enzyme would not be appropriate, since the enzyme preparation used in this study was not a homogeneous one.

3. Inhibition by 5,5' Dithiobis(2-nitrobenzoic acid)

The equilibrium time appeared to be about 30 minutes when using a concentration of DTNB of 1.3 μM (Fig. 17A). After equilibrium was attained, the I_{50} value for uridine cleavage at pH 8.2 was 1.8 μM (Fig. 17B). The I_{50} value (Ur) at pH 8.2 after a preincubation time of 5 minutes was about three times this value, while the I_{50} value (Udr) at pH 6.7 after 5 minutes was ten times this value (Fig. 17B, Table XVI). This amply illustrates the meaninglessness of comparing I_{50} values for the same or different enzymes if equilibrium times are not used for concentration studies.

The substrates, uridine and phosphate, at pH 8.2 gave complete protection against inhibition by 4.0 μM DTNB after 5 minutes of contact between the protected enzyme and the inhibitor, and substantial protection after 30 minutes (50% reduction in inhibition) (Fig. 17B). In another experiment (not shown) uridine reduced the inhibition from 69 to 13% after 30 minutes of contact between the protected enzyme and the inhibitor. Deoxyuridine and phosphate (assay at pH 6.7) reduced the inhibition from 40 to 20% after a protection period of 5 minutes (Fig. 17B).

This figure also shows the slight reversal of inhibition by 5 mM 2-mercaptoethanol (from 40 to 30%) with 1.3 μM DTNB.

The results of protection against inhibition by DTNB by two different nucleosides ('mixed' protection) is given in Table XVII. The absorbance readings represent a combination of uridine and deoxyuridine cleaving activities. But from the pH-activity curve (Fig. 11) it is known that over 90% of the activity measured at pH 8.2 should represent uridine cleaving activity. At pH 6.7, however, the percent contribution from each nucleoside is uncertain for there is substantial uridine cleaving activity at this pH.

Table XVII shows that at equimolar concentrations of the two nucleosides, deoxyuridine decreased uridine cleaving activity at pH 8.2 by 10% (experiments 1 to 3, in the absence of inhibitor). It protected the enzyme against inhibition as shown by the enzyme's subsequent activity with uridine (experiments 2, 4, 5). Nevertheless, uridine protected the enzyme to a greater extent (experiments 6 and 7). Although uridine itself is cleaved at 50% the rate of deoxyuridine at pH 6.3-6.5, when both nucleosides were incubated together the absorbance reading was less than that of uridine or deoxyuridine alone in the absence of inhibitor (experiments 8 to 11). Preincubation with deoxyuridine decreased the inhibition in the presence of 17 μM DTNB by about 40% (experiments 8, 12, and 14), but when uridine was added after the protection period the absorbance reading was then almost identical with the unprotected case (experiments 12 and 15). Uridine appeared to protect the phosphorolytic activity with uridine at pH 6.5 by 100% (experiments 11, 13, 16), and when deoxyuridine was added after the protection period the absorbance reading changed very little (experiment 17).

At pH 7.3 (where uridine and deoxyuridine cleaving activities are approximately equal) there was about a 10% decrease in the absorbance reading when uridine and deoxyuridine were incubated together, compared to the activity with uridine or deoxyuridine alone. One does not know at this time how much of this absorbance reading was due to uridine cleavage alone.

4. Inhibition by o-Iodosobenzoate

The time study in Figure 18A shows that inhibition with IOB attained equilibrium between 20-30 minutes. The concentration studies in Figures 18B and 18C and the above time study were done on different days. The I_{50} values for uridine cleavage at pH 8.1 and 7.3 and for deoxyuridine cleavage at pH 6.6, appeared to be identical (Figure 18B and 18C). In the case of inhibition at pH 6.6, the absorbance readings were 0.07 units or lower, and in this region of the scale small changes in absorbance result in large errors in percent inhibition. This may account for the odd shape of the inhibition curve.

The results of the protection studies were unusual for this was the only time where the enzyme was protected to different degrees by uridine and phosphate, that is 'differential protection'. At pH 8.1 uridine reduced the inhibition on the phosphorolytic activity with uridine from 42 to 4%, and phosphate reduced it from 35 to 28% (based on two experiments) (Fig. 18B). At pH 7.3 uridine reduced the inhibition of uridine cleavage from 41 to 8%, and phosphate reduced it from 35 to 17% (based on one experiment) (Fig. 18B). At pH 6.6 deoxyuridine reduced the inhibition on the phosphorolytic activity with deoxyuridine

from 63 to 36%, but phosphate did not alter the extent of inhibition (based on one experiment) (Fig. 18C). Cytidine did not protect the enzyme against inhibition of its phosphorolytic activity with uridine (it did not reduce the absorbance reading when incubated together with uridine in the absence of inhibitor).

In the experiment on reversal of inhibition (Fig. 18B), the inhibitor (3.3 μM) and enzyme were preincubated for 15 minutes and then 2-mercaptoethanol was added for another 15 minute preincubation period (as described under section III B 4). Therefore the percent inhibition present after reversal should be compared to the inhibition present after 15 minutes and not after 30 minutes in the control. The inhibition decreased by 10% (from 44 to 34%).

5. Inhibition by N-ethylmaleimide

Although the time required for equilibrium to be attained appeared to be greater than 30 minutes, over 90% of the inactivation produced at equilibrium was obtained after 15 minutes (Fig. 19A). The concentration studies for both uridine and deoxyuridine showed a pronounced pH effect, with the higher pH for each nucleoside having a lower I_{50} value (Figures 19B and 19C). As with pMB, the I_{50} value with uridine was two times that with deoxyuridine at pH 7.4 (Table XVI). Both uridine and phosphate gave significant protection against inhibition of phosphorolysis at pH 8.1, with uridine reducing the inhibition from 40 to 16% and phosphate reducing it from 35 to 14% (Fig. 19B). Deoxyuridine and phosphate reduced the inhibition at pH 6.5 from about 40 to 10% (Fig. 19C). A reversal study was not done. With NEM, the time study was done after the concentration study.

6. Inhibition by Iodoacetamide

Equilibrium for the inhibition of uridine cleaving activity at pH 8.2 was not reached after 80 minutes as shown by Figure 20A. The absorbance reading of the controls in this study showed little difference at 5 and 60 minute preincubation periods without substrates. In the reactivation experiment in section II D 2biii, the decrease in the controls was 10 to 20% after preincubation periods ranging from 5 to 120 minutes.

The concentration study in Figure 20B indicated a marked pH effect on inhibition of uridine cleavage. The I_{50} value at pH 7.3 was three times that at pH 8.1. Uridine reduced the inhibition on the phosphorolytic activity with uridine from 60 to 1% and phosphate reduced it from 50 to 6% (at pH 8.1). Although no reversal of inhibition was noted in one experiment with 6.5 mM iodoacetamide, 2-mercaptoethanol (5 mM) did prevent a further increase in inhibition after it was added to the incubation medium (Fig. 20A).

Because of the low absorbance readings for deoxyuridine cleavage at this time with 7.2 μg protein (two months after the preparation of fraction E_b; note the effect of aging on enzyme activity, section II D 2 bi), and the limited amount of protein still available, a complete inhibition study with deoxyuridine as the substrate, at pH 7.3 and 6.6 was not feasible. Instead experiments on the inhibition of uridine and deoxyuridine cleavage after preincubation periods of 20 and 60 minutes with 6.5 and 13.0 mM of iodoacetamide were done as shown in Table XVIII. The inhibition of uridine cleavage was markedly decreased as the pH was lowered (experiments 1 to 11). For example, at 60 minutes with 6.5 mM

iodoacetamide the inhibition of uridine cleavage was 91% at pH 8.1, 60% at pH 7.3, and 0% at pH 6.6. At pH 7.3 and 6.6, the inhibition of deoxyuridine cleavage was low (experiments 12 to 22).

7. Inhibition by Iodoacetate

Equilibrium for the inhibition of the phosphorolytic activity with uridine at pH 8.2 was not reached at the end of 120 minutes as shown in Figure 21A. Nevertheless, concentration studies were done with this preincubation time (Fig. 21B), mainly for convenience. In addition, iodoacetate and iodoacetamide are reactive and unstable in solution, although at physiological pH and temperature they are relatively stable for several hours. One reaction that the alkylating reagents can undergo in solution is shown below (64, Vol III, p. 97):

$$ICH_2COO^- + H_2O \rightleftharpoons HOCH_2COO^- + H^+ + I^-$$

The I_{50} values for uridine cleavage at pH 7.4 and 8.2, and for deoxyuridine cleavage at pH 6.7 were within a factor of two of each other (Table XVI). After a 60 minute preincubation period both uridine and phosphate offered protection against inhibition by iodoacetate. Uridine reduced the inhibition of the phosphorolytic activity with uridine at pH 8.2 from 74 to 16% (13 mM IA) and phosphate reduced it from about 74 to 27% (10 mM IA) (Fig. 21A). There was no reversal with 5 mM 2-mercaptoethanol (30 minute preincubation period with 13 mM iodoacetate followed by a 30 minute reversal time) (Fig. 21A). In another reversal experiment 50 mM 2-mercaptoethanol (60 minute preincubation period with iodoacetate and 30 minute reversal time), the progression of inhibition was halted and the degree of inhibition itself was reduced from 74 to 53% (Fig. 21A).

D. DISCUSSION

1. General Conclusions

Since the phosphorolytic activities of uridine phosphorylase towards uridine and deoxyuridine have been found to be sensitive (and in some cases very sensitive) to the usually accepted sulfhydryl reagents under mild experimental conditions, it is reasonable to conclude that the loss in activity is due to modification of at least one SH group in the enzyme that is at or near the active site. Two SH groups may very well be involved because of the strong inhibition produced by o-iodosobenzoate (section III C 4). No difference was evident between the inhibitions of uridine and deoyxuridine cleaving activities with any of the inhibitors. Nevertheless, this can still not exclude the possibility of the presence of two similar enzymes (as previously discussed in section II E 3) or of overlapping sites on one enzyme (sections III D 5b and III D 8c). Although further suggestions are made in this discussion they are highly speculative.

Table XIX summarizes the inhibitions of the most sensitive mammalian enzymes with the inhibitors used in the present study. Except for the inhibitions by DTNB, the data is compiled from tables given by Webb (64). Thus, at this time, uridine phosphorylase should be classed as one of the most sensitive mammalian enzymes known (under the important restriction, that experimental conditions cannot be properly compared; section III D 9). This table will be referred to again briefly with the discussion on each of the different inhibitors.

Since a titration of sulfhydryl groups was not done, it should be emphasized that we cannot directly relate SH groups to inhibition of

enzyme activity (64, Vol II, p. 799). For example,

(a) the inhibition may run parallel to the SH groups reacted.

(b) the inhibition may develop completely before all the SH groups have reacted.

(c) the inhibition may develop only after a certain number of SH groups have reacted.

2. Time Studies

The time studies were done only at pH 8.1 and only when assaying in the direction of uridine cleavage. Generally, the time necessary to attain equilibrium for the above reaction was used in concentration studies at different pH values with uridine as well as with deoxyuridine (Table XVI). Since one cannot conclude that the equilibrium times will be the same with both nucleosides and at different pH values it is not possible to state that the minimum I_{50} values are always being measured. Nevertheless, these values will at least show differences in the rate of inhibition. Few studies were found in the literature in which time curves were done at different pH values (118, 139) (up to July 1969).

3. Organization of the Discussion

The results with each type of inhibitor will be discussed in order of increasing I_{50} values. Extended generalized discussions on 'protection', 'reversal', etc., will be included with certain of the inhibitors. A comparison of uridine phosphorylase with other sulfhydryl enzymes will conclude this section.

4. Mercurials

a) Protection

The lack of protection afforded by the substrates does not necessarily imply that the thiol radical is not involved in binding of the substrate or in catalysis. Since protection is generally considered to be due to a slowing down of the rate of inhibition rather than to a permanent replacement of inhibitor at the substrate site (64, Vol II, p. 779), the extent of protection would be sensitive to the time of preincubation of inhibitor with protected enzyme and to the relative concentration of inhibitor and protector. Other reasons for the lack of protection were given in section III C 2. The present views on the subject (64) relate the lack of protection by substrates to (a) the substrate not covering the SH group adequately and (b) the substrate having too low a relative affinity for the enzyme. Since the affinity of phosphate for the enzyme (the 'Results' of section IV suggest that phosphate binds to the free enzyme first to give an active complex) appears to be about 1 mM, the latter reason is quite plausible for one substrate at least. Even if protection were observed, as it was with most of the other inhibitors, this still does not necessarily imply that the inhibitor reacted solely at the active site. This point will be discussed under 'Alkylation' (section IV D 8e), for the results of the iodoacetate and iodoacetamide studies are used in discussing possible explanations for protection by substrates.

b) Reversal

Lack of reversal does not necessarily imply that irreversible changes in protein conformation were the primary result of inhibitor

action or that sulfhydryl groups were not involved. Instead it might be that (1) the binding of the sulfhydryl reagent to the enzyme is stronger than to the reverser or (2) the enzyme is chemically altered by the sulfhydryl reagent (64, Vol 1, p. 651). The importance of the preincubation period of inhibitor with enzyme for the detection of reversal of inhibition was clearly shown by the opposite results in the reversal experiments with pMB and pMPS. Webb has pointed out that these two inhibitors show little difference in their effectiveness (64, Vol II, p. 860), and this is in agreement with the results of the present study, that is, with the I_{50} values (Table XVI).

A summary of the findings of many other enzymes shows that complete reversal was obtained in 59% of the cases, partial reversal in 30%, and no reversal in 11% (64, Vol II, p. 825). Because of the effect of time (as well as the concentration of 2-mercaptoethanol mentioned above), future studies done under better experimental conditions will probably show that complete reversal is possible with both mercurials in the case of uridine phosphorylase.

c) Mercurial concentrations

i) Sensitivity of the sulfhydryl group(s)

In a survey on the effect of mercurials on the activity of enzymes, Webb arbritrarily classified an enzyme as being significantly inhibited if it is inhibited 50% or more at a mercurial concentration of 0.01 mM or less, for such enzymes "could usually be considered as having reactive SH groups at or near the active center" (64, Vol II, p. 829). In addition, the time for equilibrium to be attained has been used to classify the SH groups of an enzyme according to their sensitivity to mercurials :

class (a) 1-2 minutes; (b) 5 minutes; (c) 15-20 minutes (64, Vol II, p. 810). By these classifications uridine phosphorylase can be considered to have very sensitive SH groups at or near its active site.

ii) I_{50} values

The I_{50} values themselves would be an accurate indication of the inhibition constant (K_i) for mercurial inhibition if (64, Vol II, p. 860):

(1) the inhibitor is classically noncompetitive (which in most cases it is not).

(2) the inhibition is in zone A of the mutual depletion kinetics system (which it seldom is).

A mutual depletion system (64, Vol I, p. 66) is one in which there is a mutual reduction in the concentration of free enzyme and free inhibitor, that is, Michaelis-Menten kinetics no longer apply. Three possible situations exist and these are listed as zones A, B, and C by Webb depending on the relative concentrations of enzyme and inhibitor, and the dissociation constant of inhibitor from the enzyme. For uridine phosphorylase, sufficient data is not available to characterize the system or the type of inhibition produced, i.e., competitive, noncompetitive, or uncompetitive. Moreover, a homogeneous preparation would be a definite asset in interpreting results with a very sensitive enzyme.

d) Effect of pH

The effects of pH on enzyme inhibition by mercurials are complex and unpredictable, for example, seven enzymes have been found to be inhibited better at low pH and six enzymes better at high pH (64, Vol II, p. 791). If the reactive form of the thiol is the anion ($R'-S^-$) one would have predicted that the rate of inhibition would have increased with pH, but the increase with uridine phosphorylase was very small. In this

connection, the composition of the incubation medium should be considered for,

> "One must assume that in solution in most physiological media the organic mercurials will exist in a variety of complexes, and that this will be an important factor in determining the degree of reaction with proteins and SH groups" (64, Vol II, p. 744).

Thus, there is competition for the mercurial ion by the sulfhydryl anion and a ligand, and there is also competition for the sulfhydryl anion by the mercurial and hydrogen ions (64, Vol II, p. 789). A ligand, in this context, is any anion in the incubation medium. The three different pH systems used in the mercurial study contained different buffers in the incubation medium, that is, pH 6.7: acetate and Tris; pH 7.4: Tris; pH 8.2: glycylglycine and Tris (phosphate was present at all pH values). One might speculate that the competition between various ions reduced to some extent that expected increase in the rate of inhibition of enzyme activity as based on an increase in pH (118).

e) Structural changes?

Since the mercurials can induce structural changes in certain enzymes and cause aggregation or fractionation into subunits in others, the question arises as to whether the inhibitions observed are due primarily to the modification of functional sulfhydryl groups, or secondarily to the structural changes that occurred after modification. The present studies are not complete enough to resolve this question. Nevertheless, since the inhibition curves were steep and the equilibrium time was a few minutes, it appears reasonable to suggest that there was an initial reaction of sulfhydryl group(s) at or near the active site. Then, since reversal of inhibition was not evident after five minutes, structural changes might have occurred in the protein (other explanations

for the lack of reversal were given in section III D 4a).

f) Comparison with other enzymes

Uridine phosphorylase appears to be one of about ten enzymes that are inhibited most strongly by mercurials (Table XIX). About 23 enzymes from other sources are inhibited more strongly than uridine phosphorylase. If the amount of enzyme used in these studies is taken into consideration as well as the two factors mentioned earlier (section III B 5) then uridine phosphorylase might yet be higher up on the list of sensitive enzymes.

5. 5,5' Dithiobis (2-nitrobenzoic acid)

a) Comparison with other enzymes

This inhibitor was introduced by Ellman (145) in 1959, and thus Webb could not have much information to warrant a section for this inhibitor along with other sulfhydryl reagents (64). At least twelve papers have been published between 1967 and 1969 that give some data on inhibition with DTNB (4, 80, 81, 113, 123, 125, 136, 140, 146-149). No paper published before 1967 was found that included a detailed study with DTNB (129). The I_{50} value for uridine phosphorylase was comparable to values obtained with other enzymes, where a comparison of concentrations of DTNB could be made (123, 125, 146, 148, 149). Lower equilibrium times were reported in some cases, for example, glyceraldehyde 3- phosphate dehydrogenase (148) had an equilibrium time of about 15 minutes and transglutaminase (149) had one of about 5 minutes.

Some papers (81, 113, 125, 146, 147, 149) dealt with the differential reactivity of the sulfhydryl groups of an enzyme to DTNB, with enzyme

activity being dependent on certain of these groups. In the case of lactate dehydrogenase (146), activity was completely lost after the titration of one sulfhydryl group per monomer, and in the case of isocitrate dehydrogenase (125) activity was completely lost by the titration of two sulfhydryl groups. Titration studies have yet to be done with uridine phosphorylase.

Besides the formation of a mixed disulfide after reaction with DTNB as shown in Figure 17, there is the possibility that an intramolecular disulfide bond may result as in the reaction of uridine phosphorylase with o-iodosobenzoate (discussed in section III D 6a)(149).

b) Protection

The protection studies suggest that the substrates protected against inhibition by slowing down the rate of inhibition, since there was less protection after a preincubation period of 30 minutes than after 5 minutes. The protection studies with both nucleosides suggest that there may be a common binding site or two overlapping sites for the two nucleosides. Overlapping sites have not been frequently postulated,

> "indeed, the existence of overlapping sites on enzymes may be much more common than has heretofore been appreciated and may in part account for the diverse effects on activity exerted by compounds which apparently compete for the same site (125).

Other effects on the phosphorolytic activity of uridine phosphorylase with uridine and deoxyuridine were previously summarized in section II E 5.

The experiments of Pontis et al (13) support the present finding that uridine inhibited the phosphorolysis of deoxyuridine to a greater extent than deoxyuridine inhibited the phosphorolysis of uridine. In both cases the inhibition was found to be competitive. Also the apparent K_m values for uridine (pH 8.0) and deoxyuridine (pH 6.5) were

almost identical while the inhibition constants for uridine were about ten times smaller than for deoxyuridine at the above pH values. These results suggested to the authors that their highly purified Ehrlich ascites cell preparation might contain two enzymes or "...two different active centra - one for deoxyuridine and one for uridine". The latter possibility appears to be quite close to the idea of overlapping sites.

In order to follow the reasoning that the above authors used, it appears necessary to assume that the values of the constants are a reflection of the affinity of the enzyme for the substrates (although the authors did not use either of the terms affinity or dissociation constant). To make such an assumption one would need more data on the values of the rate constants (section IV A 4a). Also if a comparison were to be made, one would think that the Michaelis constants should be compared at the same pH, that is both at pH 6.5 or 8.0. In this connection it is interesting to observe that with another highly purified Ehrlich ascites cell preparation (14), the apparent K_m for deoxyuridine was between 3 to 14 times the K_m of uridine in the pH range 5.5-8.0.

c) Reversal

Although the reversal with 2-mercaptoethanol was quite small, different experimental conditions should be tried before any definite statements on irreversibility of the inhibition are possible. With lactate dehydrogenase (146), the completely inhibited enzyme could be stored for 24 hours at 2^{o} and still be completely reactivated immediately on addition of 0.5 mM 2-mercaptoethanol. On the other hand, with isocitrate dehydrogenase (125) the activity that remained after inhibition (17%) was only restored to 65% of the original activity after incubation with 2.8 mM 2-mercaptoethanol for 3 days (at 4^{o}).

6. o-Iodosobenzoate

a) Oxidation state of the sulfhydryl group and protection by substrates

Of the seven inhibitors used, o-iodosobenzoate was the only one which did not introduce new groups into the enzyme.

Webb stated that "...what evidence exists for proteins suggests that intramolecular oxidation is dominant" over intermolecular oxidation (64, Vol II, p. 702), in the reaction of IOB with protein SH groups. The oxidation state of the sulfhydryl groups after reaction with IOB will be discussed in some detail now, for the presence of two sulfhydryl groups in the active site might explain the apparent differential protection by uridine and phosphate found in the present study.

The problem concerning the oxidation state of the sulfhydryl groups in proteins after inhibition by IOB has recently been re-investigated (112, 150). Parker and Allison (112), felt that their work strongly suggested that an intramolecular bond was not formed with glyceraldehyde 3-phosphate dehydrogenase, for they found evidence for the modification of only one sulfhydryl group when the inhibitor was tetrathionate or IOB. For example, with IOB as the inhibitor the catalytic SH group may exist as a sulfenic acid derivative:

$$E\begin{matrix}SH\\SH\end{matrix} + IOB \rightleftarrows \underset{\text{sulfenic acid}}{E\begin{matrix}SO^-\\SH\end{matrix}} + (\text{iodobenzoate}) + H^+$$

From a less detailed study with this enzyme, it was concluded that inactivation of the enzyme by IOB was due to the formation of an intrachain disulfide bridge at the active site (112), since two sulfhydryl groups in the active site disappeared after titration with one equivalent IOB per equivalent of enzyme monomer.

Little and O'Brien (150) investigated the effect of various oxidizing reagents on the one thiol group of yeast cytochrome C (R-SH). Their evidence suggested that weaker oxidants such as copper sulfate, o-iodosobenzoate, and potassium ferricyanide caused intermolecular disulfide bond formation under certain conditions (R-S-S-R), whereas stronger oxidants such as N-succinimide, iodine, hydrogen peroxide, and lipid peroxide caused the formation of monomeric oxidation products exclusively,mainly sulfonic acid (RSO_3H). The best conditions for disulfide bond formation with the weaker oxidants were low concentrations of inhibitor and an alkaline pH (to aid dimer formation 0.15 mM cytochrome oxidase was used). With a large molecule such as uridine phosphorylase, and in dilute solution (about 0.04 μM),intermolecular disulfide bond formation is not as probable as intramolecular disulfide bond formation or oxidation of a monothiol to a sulfenic, sulfinic, or sulfonic acid derivative. Also, in the present study, o-iodosobenzoate has a low I_{50} value and the pH range used was between 6.5-8.1. Still, present evidence does not conclusively support either of the above possibilities. Perhaps titration studies in the absence and presence of substrate would clarify the problem to some extent.

One may speculate that the differential protection shown by uridine or deoxyuridine, and phosphate against IOB was caused by the protection of two sulfhydryl groups by uridine directly through binding, whereas phosphate indirectly protected them, possibly by steric hindrance or conformational changes in the active site. Differential protection by substrates is discussed in more detail under 'Alkylation' (section III D 8e). The double bond, the carbonyl oxygens, and the hydrogen from N-1 of uracil are the groups on uridine that may react with sulfhydryl

groups of the enzyme (115).

The lack of change of the I_{50} values with pH is worth studying in more detail, for if verified, it may help to explain the micro-environment of the reactive sulfhydryl group(s). For example, a sulfhydryl group of the enzyme may be hydrogen bonded to other groups and, in this way, it may not be dependent on the ionization of the hydrogen from the sulfhydryl group for reactivity (151). There is some evidence that suggests the involvement of a group other than a sulfhydryl group in the catalytic activity of thymidine phosphorylase (8) and purine nucleoside phosphorylase (5).

b) Reversal

In most cases, according to Webb (64, Vol II, p. 718), partial reversal is possible. Nevertheless, enzymes for which inhibition cannot be reversed have also been reported, including the very sensitive enzyme xanthine oxidase (Table XIX). One likely explanation for irreversibility is

"...progressive secondary inactivation consequent to the protein distortion induced by disulfide bond formation" (64, Vol II, p. 718).

For emphasis, it should be mentioned again that in the present study the conditions used for reversal were probably too mild, for example, glyceraldehyde-3-phosphate dehydrogenase (112) was reactivated 100% with 10 mM dithiothreitol after preincubating with o-iodosobenzoate for 20 minutes at 26°. If one considers, as a working hypothesis, that aging of uridine phosphorylase involves, initially, intramolecular disulfide bond formation, then, as a first approach, the conditions (10 mM dithiothreitol or 50 mM 2-mercaptoethanol for 100 minutes) that were used to reactivate the aged enzyme should be used in future studies of reversal.

c) Comparison with other enzymes

Table XIX indicates that only two other mammalian enzymes were inhibited as strongly by o-iodosobenzoate as uridine phosphorylase was found to be inhibited in the present study. This again illustrates that uridine phosphorylase is a very sensitive sulfhydryl enzyme.

7. N-ethylmaleimide

a) Time and concentration studies

The equilibrium time with uridine phosphorylase may be fairly short (15 minutes), and this is not a common finding for NEM inhibition studies (64, Vol II, p. 343); inhibition times of 40 min, 120 min, and 18 hours have been reported.

Webb stated that the advantages of NEM over other sulfhydryl reagents are three-fold (64, Vol III, p. 342):

(1) probable high selectivity for SH groups

(2) reaction with only certain accessible groups on enzymes

(3) reasonably good penetration into cells...

Thus, a reasonable conclusion at this time is that uridine phosphorylase has at least some freely accessible sulfhydryl groups that are essential for activity. Of course, the present study cannot exclude the presence of other nonessential freely accessible sulfhydryl groups (113, 148).

If the preincubation time of the concentration study is considered in relation to the time study, then the I_{50} values should probably be reduced by about a factor of three in order to take into account the fact that the enzyme was not maximally inactivated with the

concentration used. The new value might be 0.05 mM for uridine cleavage at pH 8.2 (note the decrease in I_{50} values in Fig. 17B at 5 and 30 minutes with DTNB).

b) Effect of pH

Since the rate of inhibition by NEM is strongly dependent on pH, less effective pH systems may be the reason for the high equilibrium times reported in the literature. For example, adenylate kinase required 30 minutes to be maximally inactivated by 0.2 mM at pH 7.5, but at pH 9 a concentration of 0.05 mM gave the same inactivation in five minutes (64, Vol II, p. 342). This would be expected for inhibitions that are due to the reaction of sulfhydryl groups on the enzyme as discussed under section III D 4d. The pH dependency of inhibition was apparent in the present study.

c) Reversal

Although a reversal study was not done, reversal of inhibition would not have been expected. Webb stated that inhibition with N-ethylmaleimide is probably an irreversible reaction (64, Vol II, p. 344). There is the possibility that the irreversibility of inhibition might be due to NEM reacting with a group on the enzyme other than a sulfhydryl group. The terminal amino group of the α-chain of hemoglobin has been found to react with 1.8 mM N-ethylmaleimide at pH 7.15, at 25^{o} after one hour (64, Vol II, p. 343). In a recent work on the specificity of NEM, the authors (152) concluded that high concentrations of NEM and high pH values favoured alkylation of ϵ-amino and imidazole groups, at least in the two proteins used, ribonuclease A, and lysozyme. At the usual conditions for modification of sulfhydryl groups (0.001 M NEM, pH of about 7.0)

side reactions were virtually nonexistent or at the most very slight. The overall conditions used in the present work would probably favour the reaction with sulfhydryl groups, that is, low concentration of NEM and a short preincubation time, even though the pH range was between 6.6 and 8.1. Nonspecificity appears even less likely for uridine phosphorylase when one considers that the above work (152) used a 24 hour preincubation period and an enzyme concentration of 5 mg per ml.

d) Comparison with other enzymes

For emphasis, it should be mentioned again that the inhibition of activity of other enzymes by N-ethylmaleimide as given in Table XIX probably does not indicate the maximum inhibitions possible, for many variables were not considered in doing the inhibition studies, for example, pH and time. Nevertheless, based on the information available, five mammalian enzymes were found in the table given by Webb (64, Vol II, p. 346) that were inhibited to a greater extent than uridine phosphorylase, in addition to eight others that were not from mammalian sources. This again illustrates the sensitivity of the sulfhydryl groups of uridine phosphorylase.

8. Alkylation

a) Time and concentration studies

Webb arbritrarily defined sensitivity to iodoacetate "... as implying a significant inhibition at concentrations below 1 mM since this blocks glycolysis effectively in most cases" (64, Vol III, p. 52). Thus uridine phosphorylase may be considered to be a sensitive enzyme for an I_{50} value of 1.8 mM was attained under the most favourable conditions

used in the present work. Perhaps this conclusion is debatable, but what is probably more important to note is that uridine phosphorylase was inhibited by alkylating reagents under reasonable conditions, since

"...many enzymes which are usually classified as SH enzymes, because of inhibition by other SH reagents, do not react readily with iodoacetate and are not inhibited" (64, Vol III, p. 18).

The best buffer, temperature, and pH conditions for inhibitions may not have used in the present study. With phosphofructokinase (81), 1 mM iodoacetamide inhibited the activity of the enzyme by 25% after five minutes of preincubation in a medium containing 0.05 M glycylglycine buffer (pH 8.0) and 0.2 mM EDTA; but using 0.1 M ammonium bicarbonate buffer (pH 7.6) activity was inhibited by 70% after five minutes (the final degree of inhibition was the same).

The inhibitions with both iodoacetate and iodoacetamide developed slowly, but this was not unusual since maximum inhibition is seldom reached before 30-60 minutes (64, Vol III, p. 51), and the equilibrium time could well be longer; for example, rice alcohol dehydrogenase required more than 120 minutes to attain equilibrium with 1 mM iodoacetate (64, Vol III, p. 50); fumarase was inhibited 60% with 20 mM iodoacetate after 3 hours (139). In the present study complete inhibition was shown only in the case of uridine cleavage at pH 8.2. This could be interpreted as indicating a reaction with a vicinal SH group which reduces the affinity of the substrate for the enzyme or slows the breakdown of the ES complex. Nevertheless, both the nature of the time curve and the effect of pH suggest that higher concentrations and longer times would have given complete inhibition for the other cases as well.

b) Comparison between iodoacetate and iodoacetamide

There was a marked difference in the rate of inhibition between

iodoacetate and iodoacetamide under the same conditions, for example, after 20 minutes at pH 8.2, 6.5 mM iodoacetamide inhibited uridine cleavage by 53% while 6.5 mM iodoacetate inhibited it by only 10%. In considering the effect of iodoacetate and iodoacetamide, two important differences in general reactivity were given by Webb (64, Vol III, p. 271):

(1) Iodoacetamide reacts more rapidly than iodoacetate with SH groups (1.9 times as fast at pH 7.1 and 4 times as fast at pH 6.1) whereas iodoacetate usually reacts more rapidly than iodoacetamide with amino groups.

(2) Following reactions with proteins or enzymes iodoacetate introduces negatively charged groups ($-CH_2COO^-$), while iodoacetamide introduces neutral groups ($-CH_2CONH_2$)...

If the reason for the great difference in reactivity is based solely on charge, it is not easy to understand the type of inhibitions with other inhibitors such as, the mercurials, IOB, and DTNB which are also negatively charged, but yet inhibit at much lower concentrations than iodoacetate. As seen with a neutral alkylating reagent (INH_2), alkylation of uridine phosphorylase was not a rapid reaction, so the fact that alkylation with iodoacetate was slow was not surprising. Nevertheless, since the neutral alkylating reagent inhibited more strongly than the negatively charged one of similar size, under the present experimental conditions, electrostatic effects should play some part in slowing down the inhibition. If one postulates some hindrance to the approach of a small charged molecule to the active site, one must consider the fact that one of the substrates (inorganic phosphate) is also charged. Although the reason for the difference in reactivity is not known yet, one possibility is discussed under the next heading.

The shape of the inhibition-time curves for iodoacetate and iodoacetamide at their lower concentrations are different from all the other time curves in the present study. After an initial fairly rapid reaction, which did not inhibit the enzyme maximally, there was a slower reaction phase. The question is,

> "Are these slower phases due to reaction with less reactive groups on already reacted enzyme, or to secondary inactivation unrelated to further carboxymethylation?" (64, Vol III, p. 51).

The answer to this will have to await a titration study.

c) Effect of pH

The pH effect on inhibition with iodoacetate and iodoacetamide has seldom been reported (64, Vol III, p. 45), although Webb did make a generalization as already mentioned. The present study does not provide sufficient data to make a worthwhile comparison between iodoacetate and iodoacetamide; however, there is an interesting possibility that deserves further study. In a recent work with streptococcal proteinase (153) Gerwin found that the rates of inhibition with chloroacetamide and chloroacetate varied with pH, with chloroacetamide being more reactive at higher pH values and chloroacetate more reactive at lower pH values. An explanation favoured by the author was that some positive grouping(s) in the active site had been lost as the pH was increased, and this group facilitated the removal of the halide anion from the inhibitor. The loss would have a greater effect on the acid derivative than on the amide derivative.

Since the pH optimum for deoxyuridine is 6.5 as compared to 8.2 for uridine, it appears reasonable to suggest that the microenvironment for deoxyuridine is more positive than for uridine. To speculate further,

if one assumes again that uridine and deoxyuridine occupy overlapping sites and that the sulfhydryl groups, per se, at pH 6.5 would be less ionized than at pH 8.2, a positive charge strategically placed next to the nonoverlapping SH group in the deoxyuridine site might aid in the ionization of the SH group itself (147). If the sulfhydryl anion is necessary for maximum activity this may be the reason for the different pH optima for the cleavage of uridine and deoxyuridine.

The pK_a values of the sulfhydryl groups of uridine phosphorylase are not known yet. But the values for SH groups depend on the micro-environment, and they could range from 7.2 to 10.2 (64, Vol II, p. 638). Thus, the difference in pH optimum would not rule out SH groups somehow being involved in the mechanisms for uridine and deoxyuridine cleavage. In addition, pK_a values for essential SH groups from different enzymes have been postulated to be 6 (147), 7.3 (123), and greater than 8 (147, 153). To account for a pK_a of 6, the authors postulated "... that a nucleophile in the region of the SH group pulls the proton away from the sulfur atom" (147).

It is not possible to conclude that only SH groups were reacting at different pH values. With fumarase, at pH 6.5, two methionyl residues and one histidyl residue were modified (139, 154). With ribonuclease, at pH 5.5, one histidyl residue was modified, while at pH 8.5, one lysyl residue was modified (64, Vol III, p. 32). An argument, albeit a weak one, in favour of SH groups being involved, at least in the case of uridine cleavage, is that iodoacetamide was a more potent inhibitor than iodoacetate (64, Vol III,p. 271).

d) Reversal

The lack of good reversal of inhibition with iodoacetate and

iodoacetamide was not unexpected: "If the inhibition arises from carboxymethylation of an enzyme group, one would expect the inhibition to be very slowly or completely irreversible upon removal of the iodoacetate", (64, Vol III, p. 48). Some reversal did occur with 50 mM 2-mercaptoethanol, and this suggests that this thiol can compete under the right conditions with enzyme SH groups for iodoacetate.

e) Mechanisms for protection by substrates

Protection of enzymes against inhibition by iodoacetate and presumably iodoacetamide is common (64, Vol III, p. 46). The protections given by uridine and phosphate against inhibition by both alkylating reagents were better than with most of the other inhibitors (Table XVI).

Protection by more than one substrate has been reported in the literature, for example, ethanol and NAD protected yeast alcohol dehydrogenase to the same extent against inhibition by o-iodosobenzoate and iodoacetamide (155); adenosine triphosphate and fructose-1-phosphate protected the same two essential SH groups of phosphofructokinase against DTNB (81); inosine diphosphate, bicarbonate, and phosphoenolpyruvate, gave good but not equal protection against NEM, although at K_m concentrations of the substrates, inosine diphosphate was by far the best protector (123). In the case of phosphofructokinase, the authors suggested that the most likely explanation for protection was that a conformational change occurred in the protein rather than an actual covering up of SH groups by the substrates. With alcohol dehydrogenase (64, Vol III, p. 27), the inhibition was thought to be dynamically competitive with respect to the substrates with the SH groups being at or near the active site.

Four mechanisms were listed by Webb by which a substrate can protect an enzyme against inhibition, besides having the sulfhydryl group(s)

involved in binding or catalysis (64, Vol III, p. 47). Perhaps by using the protection results obtained with iodoacetate and iodoacetamide some of these mechanisms may be eliminated. Protection will again be discussed in section IV D 3e in relation to the order of binding of substrates to the enzyme.

i) Protection of one sulfhydryl group

(1) Steric protection of a vicinal SH group in the active site by the substrates does not appear likely for uridine and phosphate are quite different in structure and size. Equal protection would not be expected.

(2) A sulfhydryl group, at or near the active site, that is sequestered by a conformational change after a substrate binds to the enzyme is a more probable explanation. If both substrates bind to the free enzyme, however, different active site conformational changes would, as a first approach, be expected, yet these different conformations would have to give equal protection to the SH group.

(3) Electrostatic repulsion of the negatively charged iodoacetate by substrates is not probable, for uridine is not a charged molecule, yet it protected uridine phosphorylase as well as the phosphate anion. Also there is excellent protection against inhibition by iodoacetamide with both substrates.

(4) A sulfhydryl group covered or masked, as shown by Webb, implies the existence of two positive or two negative centers on the substrates. This is not possible in the present case. If a different mode for covering or masking the SH group is postulated, one would still not expect one SH group to be protected to the same extent by the different chemical structures of uridine and phosphate.

ii) Protection of two sulfhydryl groups

If there is more than one SH group at or near the active site, the question arises as to how each substrate could protect the SH groups to about the same extent in most cases (according to the protection studies). To this author, there are at least two major approaches that can be taken, based on the above discussion (although they are both highly speculative):

(1) Perhaps the binding sites for uridine and phosphate are so situated that after conformational changes in the active site after binding, both can protect the same SH groups, if they are strategically placed relative to the substrates. This explanation does not entail a sulfhydryl group being involved in catalysis or binding. It is apparent that there would have to be quite a fortuitous arrangement of nonessential SH groups in or near the active site, especially so when one considers that both nucleosides and phosphate have the chemical potential to undergo different types of interactions with sulfhydryl groups (64, Vol I, p. 642; 115).

(2) At least one sulfhydryl group is involved in the binding of each substrate (possibly two for nucleoside binding). In addition there is a conformation change so that after one substrate binds, the other SH group(s) in and near the active site can be protected. This proposal is incorporated into the model presented for the mechanism of uridine phosphorylase in section VI B.

Both proposals can also account for substrate protection found with DTNB, NEM, and IOB, nevertheless, differential protection against inhibition with o-iodosobenzoate may perhaps be better explained by proposal (2): uridine protected two SH groups directly through binding,

and then either by sterical hindrance or by a conformational change prevents reaction of the SH group necessary for phosphate binding or for catalysis. When phosphate is the protector, it can bind to, or in some other way protect, one essential SH group, but it can only indirectly protect the two other essential SH groups (perhaps by conformational changes or steric hindrance). Because of its structure (size and shape) this indirect protection by phosphate may not be very effective. The protection of two sulfhydryl groups of isocitrate dehydrogenase by isocitrate plus manganese sulfate or TPNH plus manganese sulfate was explained by postulating direct protection of a mechanistically essential SH group, and conformational protection of the other one (125).

f) Comparison of inhibition of activity with other enzymes

Table XIX shows that uridine phosphorylase is far from being one of the more reactive enzymes with iodoacetate. Many mammalian enzymes were listed by Webb which showed about 50% inhibition in the 1 mM range, with a few having an I_{50} value below this range. Thus, uridine phosphorylase may be considered to be one of a large group of enzymes that are inhibited significantly in the 1 mM range.

9. Overall Comparison of Uridine Phosphorylase with Other Sulfhydryl Enzymes

Few enzymes are as sensitive as uridine phosphorylase is to all of the inhibitors tested in this study (Table XIX). Nevertheless, an erroneous impression of sensitivity could result if uridine phosphorylase were graded in relation to other enzymes, since experimental conditions from other studies are not always comparable:

Such factors as pH, temperature, period of incubation with the inhibitor, purity of enzyme, and presence of cofactors and coenzymes vary greatly and indeed are quite often not even stated...the inhibitions may represent some intermediate inhibition on the way to a maximal inhibition (64, Vol III, p. 18).

Based on present data (and bearing the above quotation in mind), however, only creatine kinase appeared to be inhibited to a greater extent than uridine phosphorylase, with all of the inhibitors, than any other mammalian enzyme. Excluding the fact that glyceraldehyde-3-phosphate dehydrogenase appears to have the most reactive sulfhydryl group known at present to iodoacetate, this enzyme, isocitrate dehydrogenase, and uridine phosphorylase rank close together in overall sensitivity to sulfhydryl reagents.

Glyceraldehyde-3-phosphate dehydrogenase is considered to have a sulfhydryl group that is involved in catalysis (120), and isocitrate dehydrogenase is postulated to have one or two sulfhydryl groups that are involved in the catalytic process (125). On the other hand, the two sensitive sulfhydryl groups in creatine kinase are considered to be involved in maintaining the structure of the active site (80). Thus, with uridine phosphorylase one should only generalize at this time, and state that the sulfhydryl group(s) may be involved in: (a) binding of substrates, (b) the catalytic process itself, or (c) maintaining the conformation of the active site. It still may simply be, however, that uridine phosphorylase has non-participating sulfhydryl groups at or near the active site,

"...when it is considered that most enzymes contain 5-30 SH groups per molecule and that statistically one would expect one or more of these to be near the active centre" (64, Vol II, p. 648).

10. Future Work

The results in this section suggest that further experiments on sulfhydryl groups could provide an insight into the mechanism of catalysis of uridine phosphorylase. The following studies should give some information in this direction.

(a) Titration studies under various experimental conditions might relate the loss in activity and the modification of sulfhydryl groups in one of the ways suggested in section III D 1.

(b) pH versus pK_m and pH versus log V_M could provide additional support for the involvement of sulfhydryl groups (and other groups) in the enzyme mechanism as in the case of purine nucleoside phosphorylase (5).

(c) More detailed pH, reversal, and protection studies especially in connection with point 1 might further elucidate different types of sulfhydryl groups.

(d) Inhibition studies with substituted inhibitors should aid in mapping the environment around the sulfhydryl group(s) (139).

SECTION IV

INITIAL VELOCITY AND PRODUCT INHIBITION STUDIES

Kinetics, often viewed with awe, has power but limitations, which are often little recognized. Enzyme mechanisms are not proved by kinetics alone, but any mechanism must meet kinetic tests.

P.D. Boyer (156, p. 35)

A. INTRODUCTION

1. Literature Review

a) Initial velocity and product inhibition

Detailed initial velocity and product inhibition studies have not been reported as yet for any preparation of uridine phosphorylase. Apparent Michaelis constants have been given in a number of papers (13, 14, 16, 21) but each value appeared to be based on one experimental line. In one paper a table was given of apparent K_m values for uridine and deoxyuridine between pH 5.5 and 8.0 (14). It has been reported that uracil inhibited the phosphorolysis of nucleosides but this finding was not investigated in any detail (14).

Detailed initial velocity studies have been carried out with partially purified thymidine phosphorylase preparations (10, 11), while initial velocity and product inhibition studies have been carried out on a highly purified preparation of purine nucleoside phosphorylase (4) (sources are given in Table III).

b) Mechanisms proposed for uridine phosphorylase

Two different authors have proposed a mechanism for uridine phosphorylase in which the nucleoside binds to the free enzyme (25, 35). This mechanism is contrary to the mechanism proposed on the basis of the results from the present study. De Verdier and Potter (25) investigated transferase activity present in rat liver supernatant (114,000 x g), Dunning hepatoma, and other organs of the rat by measuring the incorporation of thymine-2-^{14}C into Udr, Tdr, Ur, and other nucleosides; and uracil-2-^{14}C into Ur and Udr. The following three steps were proposed

as an explanation for the presence of two activities in one enzyme, that is, deoxyribonucleoside phosphorylase and trans N-deoxyribosylase activities. In addition, the authors speculated that the two activities suggested the presence of thymidine phosphorylase. They also appeared to suggest that reactions 1 to 3 could explain their results on ribosyl transferring activity (the term ribose transferring enzyme was used):

1) $E + Bdr \rightleftarrows EBdr$

2) $EBdr \rightleftarrows Edr + B$

3) $Edr + P_i \rightleftarrows E + drP_i$

where E: enzyme; Bdr: deoxyribonucleoside; B: base.

The conclusion that at least two enzymes were involved, one for deoxyriboside exchange and one for riboside exchange was "...based on the distribution in the different organs and hepatoma and the effect of arsenate, pH, and fluorouracil" (25). The three reactions given above, however, appear to be based solely on the results with arsenate. The ribosyl exchange reaction in Dunning hepatoma was inhibited by arsenate ions, while the deoxyribosyl exchange in rat liver supernatant was not inhibited. Arsenate probably exerts its effect by forming a pentose-1-arsenate which subsequently breaks down spontaneously to the free sugar. Thus the pentose intermediate which is necessary for reversal of phosphorolysis is removed. The authors postulated that if reactions (1) and (2) were more rapid than reaction (3), arsenate would only have a small effect on pentose exchange. In this way, 'thymidine phosphorylase' can act as a phosphorylase through reactions (1) to (3), and as a transferase through reactions (1) and (2). On the other hand, 'uridine phosphorylase' can act only as a phosphorylase.

Although de Verdier and Potter did not appear to have identified the ribose transferring activity with the presence of a uridine phosphorylase, some of the difficulties which may be inherent in their mechanisms should perhaps still be pointed out.

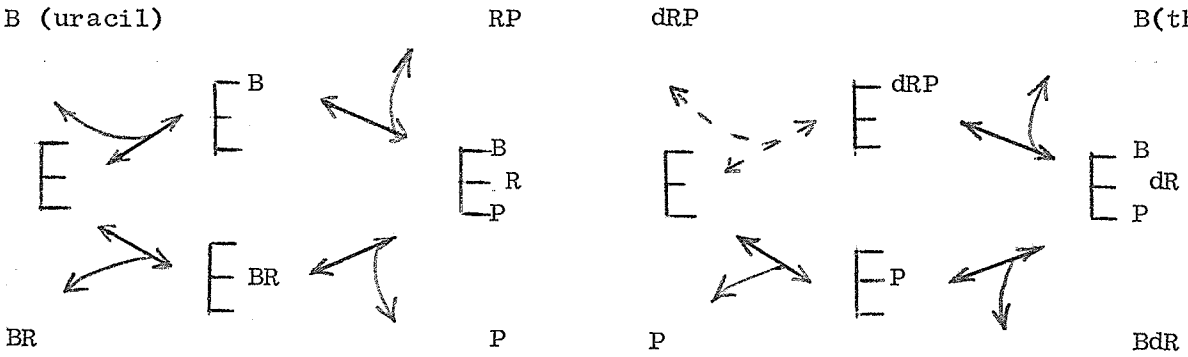
(1) Since uridine phosphorylase cleaves ribo- and deoxyribonucleosides, and thus forms Edr and Er complexes, both ribosyl and deoxyribosyl exchange should occur, especially at the pH used by de Verdier and Potter (pH 7.0) (Fig. 11). To explain the total lack of inhibition of deoxyribosyl transfer with arsenate, one could say that the above two activities of uridine phosphorylase themselves may be affected differently by arsenate, rather than interpreting the results solely to favour the presence of two enzymes. Differences in the stability of the deoxyribose-1-arsenate complexes might be considered in this connection. The greater stability of the deoxyribose complex is consistent with the observation that deoxyribosyl transfer activity of a thymidine phosphorylase preparation was stimulated more by arsenate than ribosyl transfer activity of a uridine phosphorylase preparation (35). This paper is discussed in more detail below.

(2) It appears odd that onlyhepatoma supernatant was used for ribose exchange and only liver supernatant for deoxyribose exchange. The authors may have been investigating a special case, for Krenitsky has suggested that the Dunning hepatoma contains mainly uridine phosphorylase activity while normal rat liver contains both uridine and thymidine phosphorylase activities (14).

(4) The two step reaction for the transfer of a pentose unit to a free base (reactions 1 and 2) and the three reactions for the transfer of a pentose unit to inorganic phosphate do not appear to be

able to explain the stereochemistry that is involved. In the former case, the β-configuration of the glycosidic linkage must be conserved, and in the latter case, the α-configuration of the sugar phosphate must be formed after the reaction (8).

In a more recent study on partially purified uridine and thymidine phosphorylase preparations from guinea pig and rabbit small intestine, respectively, Krenitsky postulated a different mechanism for each enzyme. A transferase assay was used which involved the incorporation of uracil -2-^{14}C into uridine and thymine-2-^{14}C into thymidine. It should also be noted that the uridine and thymidine phosphorylases from each source were separated from each other by DEAE-cellulose chromatography.



Thus, in the uridine phosphorylase mechanism the ribonucleoside (BR) binds to the enzyme (E) first, while in the thymidine phosphorylase mechanism, phosphate (P) binds first and the deoxyribonucleoside second (BdR). The broken lines indicate the steps that are not involved in pentosyl transfer, although they would normally be involved in nucleoside synthesis from thymine and deoxyribose-1-phosphate (dRP). The above mechanisms were based on the three observations that are listed below.

Urpase	Tdrpase
(1) Sephadex chromatography eliminated pentosyl transfer in the absence of added phosphate.	The same experiment did not eliminate all of the transfer activity.
(2) High concentrations of phosphate inhibited both pentosyl transfer and nucleoside synthesis.	High concentrations of phosphate only inhibited nucleoside synthesis.
(3) Stoichiometric amounts of phosphate were required for the optimal rate of pentosyl transfer.	Less than stoichiometric amounts were required.

Krenitsky did not discuss his data in detail with regard to the order of binding of substrates and release of products. The mechanisms will be discussed here in order to establish the relevance of the kinetic study in this thesis. Still, it should be borne in mind that the interpretation presented here may not necessarily coincide with the explanation that Krenitsky might have had in mind.

(1) Krenitsky suggested that even after gel filtration, trace amounts of phosphate might still be associated with his enzyme preparations. Then, if phosphate combined with one of the enzymes first, only trace amounts would be necessary for transfer to occur (discussed in more detail under point 3). Thus, phosphate is the first substrate to bind to thymidine phosphorylase and the second substrate to bind to uridine phosphorylase. An experiment was not done to ascertain whether or not trace amounts of phosphate were actually associated with the enzyme. After gel filtration of a highly purified purine nucleoside phosphorylase preparation with ^{32}P-orthophosphate, however, no radioactivity was found to be associated with the enzyme. Therefore, this raises the possibility that trace amounts of phosphate were not present

in Krenitsky's preparations. In addition, a slow ribosyl transfer reaction was observed after gel filtration, and phosphate was shown to bind to the enzyme first in a predominantly ordered Bi Bi mechanism (see section IV A 4b). This paper is described in more detail under the next heading. Also, point 1 alone does not appear to establish the order in which the products are released.

(2) The inhibition results appear to support the mechanisms as given above. By mass action the high concentration of phosphate would curtail nucleoside synthesis for both enzymes. But with thymidine phosphorylase, direct transfer of deoxyribose would not be inhibited, for as shown by the dotted lines, phosphate and deoxyribose-1-phosphate were postulated not to compete for the free enzyme (deoxyribose-1-phosphate does not bind to the free enzyme for the transfer mechanism). In this way the availability of the EdR-1-P intermediate is assured and it is not subject to the mass action effects of phosphate. Although the assumption symbolized by the dotted lines is an interesting one it is also highly speculative. Nevertheless, evidence that appears partially to favour it will be given with the discussion of point 3.

(3) The explanation of this result is probably related to the one given under point 1; that is, for a mechanism in which phosphate binds first (Tdrpase) only catalytic amounts of phosphate would be necessary to produce the ternary complex - the essential intermediate for the transfer mechanism. With phosphate and ribose-1-phosphate as free intermediates, ribosyl exchange would not be possible until the nucleoside or base has first combined with the enzyme. Thus, one molecule of phosphate or ribose-1-phosphate would be needed for every molecule of nucleoside or base, that is, stoichiometric amounts should be

necessary. Since Krenitsky used the results of the phosphate titration experiment for one of the major points in support of his mechanism, it is unfortunate that he only presented data on ribosyl transfer with one concentration of uridine and uracil, and on deoxyribosyl transfer with one concentration of thymine and thymidine. Since deoxyribosyl transfer by uridine phosphorylase was not studied, one wonders whether any of the results discussed above indicate differences between ribosyl and deoxyribosyl transfer themselves, rather than differences in the mechanism of catalysis between Urpase and Tdrpase. The pH of the incubation media also might have influenced the results (ribosyl transfer: pH 6.5; deoxyribosyl transfer: pH 7.2). The reason for choosing these pH values was not given. As mentioned in section II E 6, the pH optima for phosphorylase and transferase activities are different for thymidine phosphorylase. The pH-activity relationships for uridine phosphorylase do not appear to have been studied.

The effect of arsenate on ribosyl and deoxyribosyl transfer is of interest, even though Krenitsky did not use the results to support his mechanisms. There was a marked stimulation of ribosyl and deoxyribosyl transfer activities by Urpase and Tdrpase, respectively, at high arsenate concentrations. In his paper on the mechanism of action of purine nucleoside phosphorylase (described under the next heading), Krenitsky suggested that the lack of ribosyl transfer in the presence of arsenate indicated a free ribose-1-phosphate intermediate (6). This explanation does not appear to have been used in a consistent manner as can be seen by the mechanisms proposed for Urpase and Tdrpase (given above). For the latter enzymes Krenitsky suggested that the pentose-1-arsenate esters were sufficiently stable to serve as intermediates in

pentosyl transfer (35). The connection between the results of de Verdier and Potter (described above) on the effect of arsenate and the results of Krenitsky is not clear, since in the former case, Urpase and Tdrpase were not separated from each other. In addition, de Verdier and Potter introduced arsenate into an assay tube in which phosphorolysis was already in progress, while Krenitsky used a phosphate free incubation medium to study stimulation of transfer activity.

Gallo and Breitman have proposed that thymidine phosphorylase has one binding site for deoxyuridine and another for deoxyribose-1-phosphate (11). Experiments on the synthesis of thymidine from deoxyuridine or deoxyribose-1-phosphate were performed on a partially purified thymidine phosphorylase preparation from human leukocytes (Table III). Although the amount of contamination of this preparation by uridine phosphorylase activity was not given (10, 11), "deoxythymidine synthesis by uridine phosphorylase is insignificant compared to the activity of deoxythymidine phosphorylase" (10). The lack of competition between the above two deoxyribosyl donors in the synthesis of thymidine indicated that the enzyme had either separate sites for deoxyuridine and deoxyribose-1-phosphate, or that two enzymes were involved (evidence was presented by the authors that strongly suggested that two enzymes were not involved). Neither of these interpretations, however, according to the authors, are easily reconciled with the mechanism proposed by Krenitsky for thymidine phosphorylase. In addition, Gallo and Breitman found that about 30% of the direct deoxyribosyl transfer activity between deoxyuridine and thymine probably took place through a free deoxyribose -1-phosphate intermediate. The remainder of the activity might have been due to an intermediate in which deoxyribose-1-phosphate was tightly

bound to the enzyme. This finding favours to some extent the assumption that Krenitsky made in point 2, namely, that deoxyribose-1-phosphate did not bind to the free enzyme in the transfer mechanism.

Perhaps when the results for both Urpase and Tdrpase are considered together, the proposed mechanisms for the binding of substrates and the release of products become more feasible. Nevertheless, another study on the order of the mechanism of uridine phosphorylase would be of value, especially if another approach were used, for example, that approach using initial velocity and product inhibition studies.

c) Mechanisms proposed for purine nucleoside phosphorylases

To complete this discussion on nucleoside phosphorylases, three different ordered mechanisms for purine nucleoside phosphorylase will be described below (2, 6, 157). The data will not be discussed, although the major points that were used to arrive at the mechanisms will be given.

(1) From an initial velocity and a product inhibition study (4 patterns) of a highly purified human erythrocyte purine nucleoside phosphorylase preparation, it was concluded that the predominant mechanism was ordered Bi Bi with the nucleoside being the first substrate to bind to the enzyme and the purine base the last product to leave (4). Binding studies (gel filtration) did not give any evidence for the formation of a ribosylated enzyme or the formation of a tightly bound complex of phosphate or ribose-1-phosphate with the enzyme. The product inhibition results will be discussed in more detail in section IV D 3h.

(2) From the following results, evidence was obtained which favoured an ordered Bi Bi mechanism with phosphate the first substrate

to bind to the enzyme and pentose-1-phosphate the last product to leave. A crude preparation of purine nucleoside phosphorylase from pathological human skin was used in this study (157).

- An initial velocity study suggested a sequential mechanism.

- A comparison of several reaction parameters (maximum velocity and kinetic constants), obtained by substituting for each substrate an analogous one, suggested either an ordered mechanism with a single ternary complex or a random mechanism with the formation of two ternary complexes (rapid equilibrium random).

- The results on the effect of reaction products on the initial rates (this did not entail product inhibition patterns as described by Cleland (168)), favoured an ordered Bi Bi mechanism as described above.

(3) Krenitsky concluded that the following results favoured an iso ordered Bi Bi mechanism (an ordered mechanism that involves the isomerization of the free enzyme and the initial combination of the purine base with one of the forms of the free enzyme). A commercial crystalline purine nucleoside phosphorylase preparation from calf spleen was used for this study (6).

- Initial velocity studies and one conclusive product inhibition pattern (three other patterns each had only a control and one line in the presence of inhibitor) suggested that either the purine base added second in an ordered mechanism or first in an iso ordered mechanism.

- Inorganic phosphate was required in stoichiometric amounts for optimal ribosyl exchange. This suggested that phosphate should bind to the enzyme second, as discussed above.

- Arsenate could not substitute for phosphate in the exchange between hypoxanthine and inosine. This suggested that a free ribose-1-phosphate intermediate was involved in ribosyl exchange (as discussed above),

and that ribose-1-phosphate would be the second substrate to bind to the enzyme (in the direction of nucleoside synthesis).

- Several points from earlier papers by two other authors were used to support the mechanism in which the base added to the enzyme first followed by ribose-1-phosphate. It was not clearly indicated whether the same enzyme sources were used.

2. Present Approach

Initial velocity and product inhibition studies were used in the present investigation since they constitute one of the common approaches at this time for the determination of the binding order of enzymes. Although Alberty was the first one to show that product inhibition studies could be used to distinguish between certain mechanisms (158), for example, Theorell-Chance, ordered ternary complex and rapid equilibrium random mechanisms (discussed later), it was not until Cleland published a general approach to the subject in 1963 (159) that product inhibition studies became an extensively used technique (4, 86, 158-167). A review by Cleland on 'Enzyme Kinetics' gives the present day approach to its study (168).

3. Kinetic Notation

The Cleland method of kinetic notation is used in the present work (159). Other common notations are those of Alberty, Dalziel, and Bloomfield et al (168). The Cleland method has the advantage,

> "...that all constants have the dimensions of concentrations. It can be used to express distribution equations, rate equations for initial velocity in the presence and absence of products and equations for rates of isotopic exchange. As such it provides a universal method of kinetic notation" (168, p. 81).

4. Theory

a) Interpretation of Michaelis constants

For clarity, it would be appropriate to comment briefly on the meaning of a Michaelis constant. One of the most explicit statements on the interpretation of K_m was given by Boyer:

"Perhaps this review is a pertinent place to make a plea that the time-honoured practice of equating Michaelis constants with dissociation constants be discontinued...most enzymologists use an operational concept of the Michaelis constant as an experimentally measurable parameter for a system following 'Michaelis-Menten' kinetics" (156).

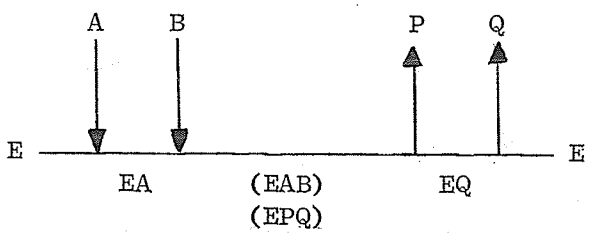
In two cases in the present study the calculated constants are equal to dissociation constants (K_{ia} and K_{iq}), but the physical meaning of the other constants is unknown. Nevertheless, the Michaelis constants are useful in specifying the quantitative dependence of the rate of the enzyme catalyzed reaction on the substrate concentration (64, Vol I, p. 21). Webb explored the meaning of K_m in more detail and he described three general situations that could exist for this constant (64, Vol I, p. 20). This may be illustrated with the following reaction:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

where E, enzyme; S, substrate; P, product; k, rate constant.
Thus one may obtain a general Michaelis constant $((k_{-1} + k_2)/k_1)$; a dissociation constant (k_{-1}/k_1); and a kinetic constant (k_2/k_1).

b) Initial velocity and product inhibition equations

It will be assumed here, but justified later, that the reaction mechanism may be of the ordered Bi Bi type (159) as represented below:



A,B,P,Q,E, represent inorganic phosphate, uridine, uracil, ribose-1-phosphate, and enzyme, respectively. The complete rate equation for this reaction mechanism in terms of the constants used by Cleland (169) is:

$$v = \frac{V_1 (AB - PQ/K_{eq})}{K_{ia}K_b + K_b A + K_a B + AB + K_{ia}K_b Q/K_i Q + K_{ia}K_b K_q P/K_{iq}K_p + K_{ia}K_b PQ/K_p K_{iq} + \frac{K_b K_q AP/K_{iq} Kp + K_a BQ/K_{iq} + ABP/K_{iq} + K_{ia}K_b BPQ/K_p K_{iq} K_{ib}}{}} \quad [1]$$

This equation may be modified to give all the rate equations for initial velocity in the absence or presence of a product (by setting the appropriate reactant equal to zero).

(1) in the absence of products

$$v = \frac{V_1}{K_{ia}K_b/AB + K_b/B + K_a/A + 1} \quad [2]$$

In the reciprocal form with A as the variable substrate:

$$1/v = K_a/V_1 \ (1+K_{ia}K_b/K_a B) \ 1/A + 1/V_1 \ (1+K_b/B) \quad [3]$$

or B as the variable substrate:

$$1/v = K_b/V_1 \ (1+K_{ia}/A) \ 1/B + 1/V_1 \ (1+K_a/A) \quad [4]$$

(2) in the presence of products

P as inhibitor and A varied:

$$1/v = K_a/V_1\ (1+K_{ia}K_b/K_aB)\ \left(1+\frac{P}{K_pK_{iq}/K_q\ (1+K_aB/K_{ia}K_b)}\right)\ 1/A + 1/V_1\ (1+K_b/B)\ \left(1+\frac{P}{1+K_b/B}\right) \Big/ 1/K_{ip}+K_qK_b/K_pK_{iq}B) \quad [5]$$

P as inhibitor and B varied:

$$1/v = K_b/V_1\ (1+K_{ia}/A)\ \left(1+\frac{P}{K_pK_{iq}/K_q}\right)\ 1/B + 1/V_1\ (1+K_a/A)\ \left(1+\frac{P}{K_{ip}\ (1+K_a/A)}\right) \quad [6]$$

Q as inhibitor and A varied:

$$1/v = K_a/V_1\ (1+K_{ia}K_b/K_aB)\ (1+Q/K_{iq})\ 1/A+1/V_1\ (1+K_b/B) \quad [7]$$

Q as inhibitor and B varied:

$$1/v = K_b/V_1\ (1+K_{ia}/A)\ \left(1+\frac{Q}{K_{iq}\ (1+A/K_{ia})}\right)\ 1/B + 1/V_1\ (1+K_a/A)\ \left(1+\frac{Q}{K_{iq}\ (1+A/K_a)}\right) \quad [8]$$

V_1 represents the maximum velocity in the forward direction. The maximum velocity for the reverse reaction was eliminated from equation (1). K_{ia} and K_{iq} represent dissociation constants for the reactions of the free enzyme with A and Q respectively. The constants K_a, K_b, K_p, K_q are Michaelis constants for A, B, P, and Q respectively: K_{ip} and K_{ib} are inhibition constants.

The theoretical predictions, based on equations 3 to 8, which an ordered Bi Bi mechanism must obey are given in section IV D 3c.

c) Inhibition types

Products which combine with only one enzyme form (with which it would react with as a substrate in the reverse direction), always give linear inhibitions which may be:

(1) competitive - the slopes of a family of reciprocal plots vary, and the lines intersect on the vertical axis.

[9] $1/v = K/V\ (1+I/K_i)\ (1/A) + 1/V$

(2) uncompetitive - the intercepts vary and the family of reciprocal plots form a series of parallel lines.

[10] $1/v = K/V\ (1/A) + 1/V\ (1+I/K_i)$

(3) noncompetitive - both the slopes and intercepts vary with the family of reciprocal plots crossing to the left of the vertical axis with the intersection point being above, below, or on the horizontal axis, depending on whether K_{is} (slope) is smaller, greater or equal to K_{ii} (intercept), respectively.

[11] $1/v = K/V\ (1+I/K_{is})\ (1/A) + 1/V\ (1+I/K_{ii})$

B. METHODS

1. Initial Velocity Studies

Enzyme activity was assayed in the direction of nucleoside cleavage for initial velocity and product inhibition studies, and in the direction of uridine synthesis for an initial velocity study. The general kinetic assay procedure was described in section II C 1b. Typical incubation media are given below:

Ingredients	Uridine cleavage (µl)	Deoxyuridine cleavage (µl)	Uridine synthesis (µl)
1 M G-G, pH 8.4	0	0	150
1 M G-G, pH 8.9	250	0	0
1 M Tris, pH 7.4	150	0	0
0.05 M P_i, pH 7.0	50	0	0
0.05 M P_i, pH 6.2	0	50	0
0.05 M Tris, pH 7.2	450	0	0
Water	500	1400	1140
0.15 M 2-SH	50	0	0
Enzyme (diluted with 0.05 M Tris, pH 7.2)	5	5	10
0.1 M Ur, pH 7.0	50	0	0
0.1 M Udr, pH 7.0	0	50	0
0.043 M R-1-P, pH 7.0	0	0	100
0.01 M uracil	0	0	100
Final pH 37°	8.1-8.2	6.5-6.6	8.1-8.2

In the case of phosphate as the variable substrate, 0.05 M P_i and 0.05 M Tris were varied together to give a final volume of 500 µl; with uridine as the variable substrate, water at pH 7.0 and uridine were usually varied together to give a final volume of 50 µl. In some studies, however, water at pH 7.0 was not used and instead water and uridine were varied together to give a final volume of 550 µl. In nucleoside synthesis, water, ribose-1-phosphate, and uracil were varied to give a final

volume of 1340 µl.

2. Product Inhibition Studies

The assay procedure was the same as in the above study. A typical incubation medium is illustrated below for the particular case of uracil (inhibitor) and phosphate (variable substrate).

Ingredients	Volume (µl)
1 M G-G, pH 8.9	250
1 M Tris, pH 7.4	150
0.05 M Tris, pH 7.2	480
0.05 M P_i, pH 7.0	20
Water	300
0.181 mM uracil, pH 5.7	200
0.15 M 2-SH	50
Enzyme	5
0.1 M uridine, pH 7.0	50
Final pH 37°	8.1-8.2

In the case of phosphate as the variable substrate, 0.05 M Tris and 0.05 M P_i were varied together to give a final volume of 500 µl. The product inhibitor (uracil or ribose-1-phosphate), uridine, and water were varied together to give a final volume of 550 µl. The product was preincubated with the enzyme in the incubation medium for about five minutes at 25° before the reaction was started by the addition of uridine.

3. Enzyme Fractions Used

The enzyme preparations have already been given in Table IV. The amount of protein added to the assay tube depended upon the concentrations of substrates and inhibitors.

Study	Protein used (μg)
Initial velocity	
uridine cleavage	7 to 57
deoxyuridine cleavage	13
uridine synthesis	2.3
Product inhibition	20 to 40

4. Preparation of Solutions

The preparation of substrate solutions was the same as described in section II C 4. The preparation of solutions of products for uridine cleavage is given below.

Uracil: 10.2 mg (0.181 mM) were dissolved in 10 ml of water with no pH adjustment. The final pH was about 5.7.

Ribose-1-phosphate: 75.2 mg (14.6 mM) were dissolved in 12 ml of water with no pH adjustment. The final pH was about 5.7.

5. Data Processing

Reciprocal velocities were plotted graphically against the reciprocals of substrate concentrations (170). Points which were far off this drawn line in relation to other points were not used for computer analysis (169). The data were fitted to the equation, $v = VS/(K + S)$, where v is initial velocity; V is maximum velocity; K, constant; S, substrate concentration. The least squares method of Wilkinson was used (171). All calculations were done on an Olivetti-Underwood Programma 101 computer. This provided values of K, V, and the standard error of their estimates. Slopes (K/V) and intercepts (1/V) were then plotted graphically against the reciprocal concentrations of the non-variable substrate for the initial velocity experiments, and against

inhibitor concentrations for product inhibition experiments. Constants were obtained from these replots:

(a) X-axis intercept of 1/V replot: K_a, K_b, K_p, K_q, K_i intercept.

(b) X-axis intercept of slope replot: K_{iq}, K_i, K_{ia} slope.

(c) Y-axis intercept of 1/V replot: V_M.

(d) Calculation from a complex intercept value: K_{ip}, K_{iq}.

If a constant was not calculated from a replot it will be called an apparent constant, that is, the true Michaelis constant is the value obtained after extrapolation to infinite concentration of the non-variable substrate (as done in the replots). The points on the initial velocity and product inhibition graphs are experimentally determined, but the lines drawn through these points were obtained from the K and V values provided by the computer.

C. RESULTS

1. Introduction

A summary of constants calculated from initial velocity and product inhibition studies is given in Tables XX and XXI, respectively, with literature values for K_m where available. Each velocity pattern shown was determined from one experiment. If only one point appears at a given concentration of substrate, it represents at least a mean of duplicate determinations, however, if two or more points appear, each point represents the result of a single determination.

2. Initial Velocity Patterns

Linear lines were obtained which intersected to the left of the vertical axis, and above the horizontal axis. A single intersection point was not evident for any of the patterns.

a) Nucleoside cleavage at pH 8.1

Figure 22 shows phosphate as the variable substrate and uridine as the nonvariable substrate (over a 20-fold concentration range).* Figure 23 shows uridine as the variable substrate and phosphate as the nonvariable substrate (over a 20-fold concentration range). In both cases the replots of intercepts and slopes against the reciprocal of the nonvariable substrate appeared to be linear (inserts). The Michaelis constants for phosphate (K_a) and uridine (K_b) were found to be 0.34 mM and 0.28 mM, respectively. The dissociation constant for phosphate (K_{ia}) was 3.3 mM as calculated from the X-axis intercept of the slope replot of Figure 23 (K_{ia} = A) and 7.5 mM as calculated from the X-axis

* Although the slope replot appears to pass through the origin, the calculated value of the vertical intercept is 1.3 (equation 3).

intercept of the slope replot from Figure 22 ($K_{ia} = K_a B/K_b$). If it is assumed at this time that phosphate is the first substrate to bind to the enzyme surface, then K_{ia} is equal to the numerical value of the substrate concentration at the point of intersection of the lines in Figure 22 (where 1/v at $[B_1]$ equals 1/v at $[B_2]$, etc.) (158). Although the lines did not intersect at one point, it still appears reasonable to estimate K_{ia} as being between 2-4 mM.

The complete initial velocity study at pH 8.1 with uridine as the nucleoside was done two other times, however, on these other occasions, the standard error of the constants was large, presumably because of low absorbance readings (preparation B, Table IV). Still, at saturating concentrations of phosphate (0.1 M), the apparent K_b (uridine) was 0.18 mM, and at saturating levels of uridine (3.3 mM) the apparent K_a for phosphate was 0.19 mM and 0.39 mM. The amount of protein used in these studies ranged from 9 to 13 μg/tube (a figure is not given).

b) Nucleoside cleavage at pH 6.5

Figure 24 shows phosphate as the variable substrate and deoxyuridine as the nonvariable substrate (over a 10-fold concentration range). The replots of the intercepts and slopes against phosphate concentration appeared to be linear. Figure 25 shows deoxyuridine as the variable substrate and phosphate as the nonvariable substrate (over a 140-fold concentration range). The replots against phosphate concentration were linear. The Michaelis constants for phosphate (K_a) and deoxyuridine (K_b) were calculated to be 0.64 mM and 0.36 mM, respectively. The dissociation constant for phosphate (K_{ia}) was calculated to be 1.8 mM from the X-axis intercept of the slope replot from Figure 25.

c) Nucleoside synthesis at pH 8.1

Figure 26 shows uracil as the variable substrate and ribose-1-phosphate as the nonvariable substrate (over a 20-fold concentration range). Figure 27 shows ribose-1-phosphate as the variable substrate and uracil as the nonvariable substrate (over a 7-fold concentration range). Above approximately 0.7 mM uracil, substrate inhibition by uracil would be evident (section II D 2g, Fig. 14). The replots of slopes and intercepts were linear (Fig. 28), although there is scatter around the drawn lines, especially for the slope replot from Figure 26.

The Michaelis constants for ribose-1-phosphate (K_q) and uracil (K_p) were found to be 0.07 mM and 0.31 mM, respectively. The dissociation constant for ribose-1-phosphate (K_{iq}) was found to be 0.070 mM from the x-intercept of the slope replot from Figure 27. If it is assumed that ribose-1-phosphate is the first substrate to bind in the direction of nucleoside synthesis then K_{iq} may be estimated to be 0.08-0.10 mM from the point at which the lines intersect in Figure 27.

3. Product Inhibition Patterns

All of the patterns showed linear lines that intersected either to the left or on the vertical axis, but above the horizontal axis. Slope and intercept replots were usually linear, as drawn by eye.

a) Inhibition by uracil

With phosphate as the variable substrate at a low concentration of uridine, uracil appeared to give noncompetitive inhibition (Fig. 29). The apparent V_M ($\pm$ standard error) of the control line was (0.18 ± 0.020) $\times 10^{-3}$ whereas that for 0.12 mM and 0.24 mM uracil were $0.11 \pm 0.017 \times 10^{-3}$

and $0.093 \pm 0.020 \times 10^{-3}$, respectively. The same type of inhibition may have been produced at a four-fold greater concentration of uridine but the lines appeared more parallel (Fig. 30). The change in slope was still apparent in the slope replot shown in Figure 37 II. The slope of the control was $0.94 \pm 0.19 \times 10^{-3}$ while the slope of the most inhibited line was $1.2 \pm 0.063 \times 10^{-3}$. Thus the slopes appear to be identical, and this would suggest that uracil inhibited uncompetitively.

With uridine as the variable substrate at a low concentration of phosphate, uracil appeared to give noncompetitive inhibition (Fig. 31); the apparent V_M value of the control was $0.15 \pm 0.0034 \times 10^{-3}$ whereas the value in the presence of 0.36 mM uracil was $0.091 \pm 0.0059 \times 10^{-3}$. The same type of inhibition was produced at a ten-fold greater concentration of phosphate (Fig. 32); the apparent V_M value of the control line was $0.11 \pm 0.0030 \times 10^{-3}$ whereas the value in the presence of 0.36 mM uracil was $0.085 \pm 0.0036 \times 10^{-3}$. The slope and intercept replots against uracil concentrations appeared to be linear (Fig. 37 I to IV) except for the replots of Figure 31 (Fig. 37 III). This might be expected from the irregular pattern obtained in Figure 31, and thus the replot need not imply nonlinear inhibition.

b) Inhibition by ribose-1-phosphate

With phosphate as the variable substrate at a low concentration of uridine, ribose-1-phosphate gave an inhibition pattern which could not easily be labeled (Fig. 33). Nevertheless, the apparent V_M value of the control was $0.17 \pm 0.013 \times 10^{-3}$ whereas the value in the presence of 0.83 mM inhibitor was $0.14 \pm 0.024 \times 10^{-3}$. Thus, the pattern might indicate competitive inhibition. With a twelve-fold greater concentration of uridine the inhibition was competitive (Fig. 34); the

apparent V_M value of the control line was $0.15 \pm 0.0061 \times 10^{-3}$ whereas the value in the presence of 0.73 mM inhibitor was $0.16 \pm 0.033 \times 10^{-3}$. The replot of the intercepts against ribose-1-phosphate concentrations from Figures 33 and 34 gave horizontal lines (Fig. 38 V and VI), as expected for competitive inhibitions. The errors involved in the slope values were too great to establish whether the lines were linear or nonlinear.

With uridine as the variable substrate at a low concentration of phosphate, ribose-1-phosphate inhibited noncompetitively (Fig. 35); the apparent V_M value of the control line was $0.17 \pm 0.028 \times 10^{-3}$ whereas the value in the presence of 0.73 mM inhibitor was $0.12 \pm 0.0075 \times 10^{-3}$. The replot of intercepts against ribose-1-phosphate appeared to be linear, but the errors involved in the slope replot were too great to make a similar conclusion (Fig. 38 VII). With a ten-fold greater concentration of phosphate (Fig. 36), ribose-1-phosphate appeared to inhibit competitively. A similar result was obtained with a 25-fold greater concentration of phosphate than used in Figure 35. Perhaps the pattern could be interpreted as indicating a change-over from noncompetitive inhibition to no inhibition when approaching saturating amounts of phosphate.

c) Constants calculated from the product inhibition study

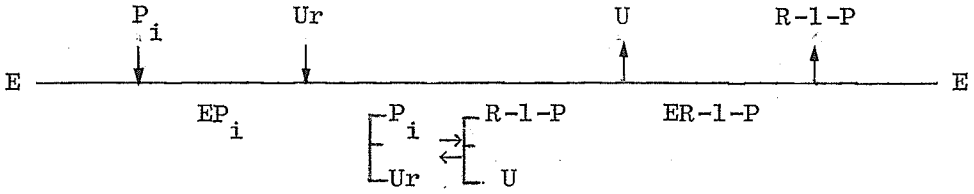
There are a number of ways of calculating the constants K_{ip} and K_{iq} as shown in Table XXI. In addition, K_{iq} may be compared to the same constant obtained from initial velocity studies (Table XX). The K_{iq} values calculated from ribose-1-phosphate inhibition studies were 8, 10, and 18-fold greater than the value calculated from initial velocity studies. Nevertheless, it should be noted that the two values calculated

with phosphate as the variable substrate are in harmony with each other, as are the two values with uridine as the variable substrate. The K_{iq} values calculated from uracil inhibition patterns were 0.4, 0.7, and 1.4 times the initial velocity value. Two out of the three K_{ip} values were in good agreement (1.8, 2.3 and 10.5 x 10^{-4} M). One possible explanation for the variation of K_{iq} values is given in section IV D 3g.

D. DISCUSSION

1. General Conclusions and Organization

The initial velocity and product inhibition data appear to be consistent with at least three sequential mechanisms: ordered Bi Bi, iso Theorell-Chance, and rapid equilibrium random (with four dead end complexes). Although an absolute choice is not possible from the present study the reasons for favouring the ordered Bi Bi mechanism will be discussed. The favoured mechanism is schematically shown below with the horizontal line indicating the point of contact between enzyme and reactant.



The complete rate equation corresponding to this mechanism has already been given in the 'Introduction' to section IV (equation 1).

The 'Discussion' from this point onwards will consist of three main parts: initial velocity studies, product inhibition studies, and comparison with mechanisms on phosphorylases from the literature.

2. Initial Velocity Studies

a) Sequential mechanism

The consistent intersecting initial velocity patterns for the forward and reverse reactions of uridine phosphorylase indicate a sequential mechanism, that is, both substrates must bind to the enzyme before a product is released (159). The lack of intersection of the

lines at a common point is not an unusual feature of kinetic studies in which sequential mechanisms are favoured (4, 164, 169), and this finding will not be further mentioned, except to suggest that experimental error is presumably the best explanation for this occurrence. The standard errors for apparent K_m and V_M values appeared to indicate this, in most cases at least.

Parallel double reciprocal initial velocity patterns are normally considered to indicate a ping pong mechanism in which the first product is released before the second substrate combines with the enzyme (159). Therefore, this type of mechanism need not be considered further. In a recent kinetic study on hypoxanthine-guanine phosphoribosyltransferase (from dialyzed lysates of human erythrocytes), however, it has been shown that a parallel pattern may still be associated with a sequential ordered Bi Bi mechanism, if the "true dissociation constants of both substrates with enzyme are less than one-tenth of the substrate concentrations which are used" (165).

From the slopes of rate equations (3) and (4), i.e., K_a/V_1 $(1 + K_{ia}K_b/K_aB)$ and K_b/V_1 $(1 + K_{ia}/A)$, respectively, it is apparent that the slopes change as a function of the nonvariable substrate; thus an intersecting initial velocity pattern is predicted for a sequential ordered mechanism. Nevertheless, if K_{ia}/B and K_{ia}/A are very small, that is, about 0.1, then as Henderson et al have suggested (165), the initial velocity lines may appear parallel. A more recent study on the above enzyme (purified 50-fold from lysates of human erythrocytes) indicated that the predominant mechanism is ping pong at high magnesium ion concentrations, and ordered Bi Bi at low magnesium ion concentrations (172).

The intersection point of the lines in the initial velocity study should be above the horizontal axis if the ratio K_{ia}/K_a is greater than one. This is the case for uridine cleavage where the ratio is at least eight (Fig. 22). With uridine synthesis, however, the ratio is one, and the lines do appear to be close to intersection on the horizontal axis, as would be predicted (Fig. 27). In this connection, it is interesting to note that in the kinetic study of pigeon liver malic enzyme (ordered Bi Ter) the ratio and the intersection pattern did not conform with the theoretical prediction (174).

b) Ordered versus random

Initial velocity studies do not always give a clear indication of whether there is an obligatory order of addition or whether binding occurs in a random order, especially if the latter is in a minor pathway (173). Until recently, initial velocity equations (3) and (4) were believed to be obeyed only by an ordered mechanism. Random mechanisms were thought to result in nonlinear double reciprocal plots (this would not be true for the rapid equilibrium condition (173)). Unfortunately,

> "...the unimolecular rate constants for release of substrates from the enzyme must be considerably smaller than the turnover number in order to see clearly the nonlinear (hyperbolic) reciprocal plots that have been thought to characterize random mechanisms" (173).

c) Constants

i) Maximum velocity

Although the maximum velocity for the forward and reverse reactions are included in Table XX, a comparison between them would not be reliable. The values were corrected for protein concentration and incubation time differences, but the enzyme concentration itself is not known, since

the enzyme is not exceptionally stable; moreover the relevant experiments were done at different times and with different preparations.

ii) Dissociation constants

Phosphate may have as much as a four-fold greater affinity for the enzyme at pH 6.5 than at pH 8.1, or the difference may be as little as two-fold (Table XX). Although different nucleosides were used for these two studies, one would not expect them to have an effect on the affinity of the enzyme for phosphate, if the ordered Bi Bi mechanism is the predominant one. The affinity of phosphate at pH 6.5 with uridine as the nucleoside, or at pH 8.1 with deoxyuridine as the nucleoside was not determined due to technical difficulties (large quantities of enzyme would have been necessary for such studies).

If future work also indicates the above increase in affinity, this might be explained by different affinities of the enzyme for the anionic forms of phosphate which one might expect to predominate at such pH values (110), i.e. pH 8.1 - dianion; pH 6.5 - monoanion. This may be due to different conformations at the active site, i.e. a relative change in ionic environment of the active site from negative at pH 8.1 to positive at pH 6.5 (153). These changes with pH may also have an effect on the binding of the nucleoside to the binary complex (EP_i) or on the rate of bond cleavage of the nucleosides. The low activity with uridine at pH 6.5 compared to pH 8.1 does not appear to be explained by a decreased affinity for uridine at the lower pH, because uridine competes strongly with deoxyuridine at pH 6.5 as shown by the sulfhydryl group modification study with the Ellman reagent (section III C 3). Actually the apparent K for uridine at pH 6.5 decreased by a factor of two over its K_m at pH 8.1; this is not necessarily related to an affinity

effect (section IV A 4 a). Although it is not apparent from Table XX, the app V_M of uridine at pH 6.5 was also about one-half that at pH 8.1, when one enzyme sample was used (Figure 11). This itself would explain the decrease in apparent K, and it would suggest that the affinity of the binary complex for uridine may not be substantially different at pH 6.5 and 8.1. In contrast to uridine, the affinity effect may explain to some extent the decreased activity with deoxyuridine at pH 8.1 compared to pH 6.5,for again in the Ellman reagent study, deoxyuridine was a poor inhibitor of uridine cleavage at pH 8.1.

To summarize the above speculative remarks, the low activity with uridine at pH 6.5 may be due to a low rate of cleavage of this nucleoside, whereas the low activity with deoxyuridine at pH 8.1 may be due to a low affinity of the enzyme for deoxyuridine.

In contrast to nucleoside cleavage, the dissociation constant (K_{iq}) and the Michaelis constant are virtually identical for uridine synthesis. This is still in harmony with theory, for dissociation constants may be greater or less than K_m (156). It may be of importance to note that the affinity of ribose-1-phosphate for uridine phosphorylase is at least 46 times greater than that of phosphate at pH 8.1. As explained in section I B 4, uridine phosphorylase is one of the enzymes necessary for the catabolism of nucleic acids, and it is also involved in what may be an important 'salvage pathway' for nucleic acid anabolism. These points, taken together with the fact that the equilibrium of the reaction is in favour of nucleoside synthesis (about 4:1, (1, 13)), suggest a possible means by which uridine phosphorylase may readily function in an anabolic pathway, even though ribose-1-phosphate is presumably not present in as great a concentration as the ubiquous inorganic phosphate.

iii) Comparison with Michaelis constants from the literature

There is as much as a ten-fold difference in the constants of the present study from the corresponding constants available from the literature. One must consider that the literature values give apparent K values only, and for these studies, preparations from different sources and at different stages of purity (homogenate to 250-fold pure) were used. Even the literature values show considerable variation, for example, the apparent K for uridine at pH 7.4 varies from 0.76 mM (13) to 0.13 mM (14) in highly purified Ehrlich ascites cell preparations and reaches 2.5 mM at pH 8.0 in a crude preparation (58). The only study in which constants were determined for phosphate showed that the apparent K values were the same for the phosphorolysis of uridine (pH 8.0) or deoxyuridine (pH 6.5) (13). This is in fair agreement with the present study.

3. Product Inhibition Studies

a) General conclusions

Product inhibition studies are able to distinguish between certain sequential mechanisms. The theoretical predictions of the eight possible inhibition patterns from each of seven different sequential mechanisms, as listed by Cleland, are given in Table XXII along with a list of the inhibitions observed in the present study. If the assignment of the type of inhibition obtained for uridine phosphorylase is taken as being correct, it is apparent that the results are consistent with an ordered Bi Bi reaction, with phosphate binding first and ribose-1-phosphate being released last, as well as with the two other mechanisms

mentioned previously (also to be discussed here). Even though each product inhibition pattern is based on one determination, it would be fortuitious indeed to find that the patterns happened by chance to obey the theoretically predicted inhibitions.

b) Comparison of the present study with similar studies of other enzymes

To place the present kinetic study in the proper perspective in relation to similar studies in the current literature, a number of important features concerning initial velocity and product inhibition studies are listed below. The present study on uridine phosphorylase is briefly contrasted to these general findings.

(1) Number of product inhibition patterns. The experimental determination of all possible inhibition patterns is not generally done (4, 6, 160, 162, 164, 165, 166, 169, 172-175); four and often fewer patterns are shown. In only one paper are five patterns given for an ordered Bi Bi mechanism (165).

(2) Number of lines per pattern. It appears to be a common practice to show three or four lines per pattern, although only three lines are frequently shown. Occasionally more lines are given (162, 164, 176, 177).

(3) Data processing. Computer analysis is usually stated as having been used for assessing kinetic data, but a number of papers do not state whether the data has been so analyzed (46, 160, 165, 172):

"Since the conclusions reached from kinetic studies depend on telling one pattern from another...it is imperative that the kineticist of today have an objective evaluation of the significance of his data" (168, p. 96).

(4) Reproducibility of the pattern. Few studies actually state whether an experiment has been repeated two or more times to establish

a consistent pattern (167, 174, 176).

(5) Purity of the enzyme. A high degree of purity does not appear to be an essential prerequisite in performing detailed kinetic studies (4, 6, 35, 165, 178, 179), but in one of these cases the possibility of interfering enzymes was eliminated (178). In some studies, commercial crystalline enzymes were used without a check on their purity (6, 64, 169).

In the present study, a highly purified uridine phosphorylase preparation was used; the maximum number of patterns were determined; four lines were indicated with all patterns except one; each pattern was usually based on one experiment; and the data was analyzed with a computer. Thus, overall, the present kinetic study compares favourably in its experimental approach with similar studies of other enzymes from the current literature.

c) Elimination of some possible mechanisms

Product inhibition patterns may be theoretically predicted from rate equations derived for the postulated mechanism, i.e. equations 5 to 8 from equation 1 (159), or by following three rules set up by Cleland (159, p. 188) for scanning a schematic representation of the mechanism (Fig. 39). Neither of these approaches will be explained in detail here, but both will be used in this discussion.

Ideally the maximum number of inhibition patterns should be shown to occur experimentally before one of the simple sequential mechanisms is assigned to the enzyme catalyzed reaction. But three of these patterns will suffice as a first approach to eliminate the iso ordered Bi Bi, Theorell-Chance (T-C), rapid equilibrium (RER), and random mechanisms

(Figure 39) - four of the most frequently suggested sequential mechanisms (159) - as the predominant mechanism. This approach has been taken with other studies in discussing product inhibition results (169, 173). The term 'simple' is applied to these mechanisms for other enzyme forms (or low levels of certain forms) may occur, but the above names will still be used to describe the overall mechanism. Other enzyme forms may be detectable on the initial velocity or product inhibition plot itself (165-167), on replots of slopes and intercepts (164, 167, 174), or on other studies, for example, isotope exchange (162, 173). The RER mechanism is similar to the ordinary random mechanism except that the interconversion of the ternary complexes constitutes the rate determining step. A T-C mechanism is similar to an ordered Bi Bi mechanism except for the absence of ternary complexes, with the iso T-C involving, in addition, a different stable enzyme form (Figure 39).

Pattern 1. With A as the variable substrate at high or low concentrations of B, competitive inhibition by Q is predicted for mechanisms 5 and 6, because both reactants compete for the enzyme form E (Figures 33, and 34). Pattern 2. With B as the variable substrate at low concentrations of A, noncompetitive inhibition by Q (Fig. 35) is predicted for mechanisms 5 and 6. These two patterns eliminate mechanism 7, because competitive inhibition should be observed with either substrate. The random and iso ordered Bi Bi mechanisms are also eliminated for noncompetitive inhibition should be observed with either substrate. The difference in inhibition patterns between mechanisms is a direct consequence of the distribution of the enzyme as indicated by the denominator of the complete rate equation (equation 1). For example, the RER mechanism does not have the forms EABP, EBPQ, EAP, and EBQ. Pattern 3. With P

as the inhibitor and B as the variable substrate at high or low concentrations of A, noncompetitive inhibition is predicted for mechanism 5, and competitive inhibition for mechanism6(Figs. 31 and 32). The Theorell-Chance mechanism does not have the enzyme forms EABP and EBPQ. The iso Theorell-Chance mechanism, however, is not eliminated since noncompetitive inhibition is also predicted. Binding studies (159) and an analysis of dead end inhibition patterns (180) are the present methods available for distinguishing between ordered Bi Bi and iso Theorell-Chance mechanisms. Few papers have shown experiments to distinguish these mechanisms. Ordered Bi Bi may be the more likely mechanism for this type is frequently met in the literature (168), but to the best of the author's knowledge only one case of an iso Theorell-Chance mechanism has been postulated (181). In addition, this mechanism is not favoured because of the "...high improbability of (sequential) mechanisms without central complexes" (169).

The third mechanism which is consistent with the data is a RER mechanism with dead end EAP, EABP, EBQ, and EBPQ complexes. This one cannot be definitely ruled out on the basis of the results from the present study (unfortunately, the present data is not good enough to rule out nonlinearity for at least two of the slope replots (Fig. 38 VI, VII)). Equilibrium exchange experiments should be able to eliminate the possibility of a predominant RER mechanism (as well as the random mechanism) (168, 182).

What purpose could be served by phosphate binding to the enzyme first? Although one could make the general statement concerning the importance of active site conformational changes that must proceed the binding of nucleoside, a recent review (183) on 'sulfhydryl group and

S-S bridges in the structure of enzymes', advanced an hypothesis that is very appealing in light of the results presented in this thesis. Actually this hypothesis carries a little further the speculation by Sugino (109) already discussed in section II E 4b.

"S-S bonds in enzymes may be in a dynamic state in vivo, the S-S bond being opened and reformed all the time. Instead of a fit induced by the substrate there is a fluctuating enzyme molecule, one particular form of which is able to bind the substrate. A variation in the environment would cause a strain in the rigid structure of a fluctuating molecule and result in a shift in the equilibrium to a structure which is stable in the changed environment and yet able to take up the conformation needed for its enzyme activity".

Thus, phosphate, in binding to the reduced form of the free enzyme, might cause a shift to the enzyme form in which the two sulfhydryl groups proposed for nucleoside binding are placed in a receptive conformation. The above two hypotheses (109, 183) were incorporated into a model for uridine cleavage (section VI B).

d) Degree of randomness

The product inhibition patterns and the fact that not all the replots appeared to be nonlinear, suggest that a predominant random Bi Bi mechanism does not occur for uridine phosphorylase. After product inhibition studies on aspartate transcarbamylase, the authors stated that,

"competitive inhibition of carbamylphosphate(A) by phosphate (Q) indicates that any contribution...of an alternate pathway in which B [aspartate] binds first is very small compared to the major pathway" (167).

Since the inhibition between the proposed A and Q components of the present study was also competitive, one might conclude that at the most 1-2% of the phosphorolytic mechanism goes by an alternate order of addition (169). It is not possible to eliminate completely a minor

random pathway solely from initial velocity and product inhibition studies for,

> "...it seems quite probable that a minor random component is associated with some if not all 'compulsory' pathways...it seems quite probable that many compulsory pathways will eventually have to be re-classified as 'kinetically ordered' implying that an alternative order of addition exists, but that is kinetically unimportant"(184).

Sensitive techniques such as isotope exchange studies (162, 169) and difference spectroscopy (167) could be used to estimate the degree of randomness.

e) Substrate protection and order of binding

Perhaps the finding that both uridine (or deoxyuridine) and phosphate protect uridine phosphorylase against inhibition by most of the sulfhydryl reagents used in this work, might indicate 'kinetically unimportant randomness' in its mechanism. Indeed, since the substrates protect equally as well in most cases, one might at first suspect a significant degree of randomness. Nevertheless, from the list of enzymes in Table XXIII there does not appear to be a clear relationship between protection and order of binding, that is, an enzyme catalyzing an ordered mechanism may be protected by both substrates, whereas in a random mechanism it may be protected by one or two substrates. Some explanation validating protection findings as an indication of binding order would be helpful, yet none has been found in the literature.

Only in the case of yeast adenylosuccinate lyase was the type of protection used as supporting evidence for the predominating Uni Bi mechanism (162). Still, one might have expected some protection at high concentrations of B, since an alternate mechanism of B binding first, albeit small, occurred at high concentrations of B. But this

was not observed with concentrations of B as high as twelve times its K_m value. Thus, a small degree of randomness, or complete randomness does not automatically yield to protection by both substrates. One might speculate that protection by substrates depends on the relationship between the substrate and enzymic sulfhydryl groups. Even if B combines with the enzyme first, it may not be in a position to protect a sulfhydryl group. This suggestion, however, would not explain the protection findings for a postulated ordered mechanism. For this case, the following explanations are put forward:

(1) The degree of randomness that exists in a mechanism that is predominantly ordered Bi Bi is sufficient to account for protection by both substrates (at least under saturating concentrations of substrate).

(2) The binding order as determined from kinetic studies gives the order of addition of substrates which results in the active intermediates involved in enzyme catalysis (Fig. 39). Unreactive intermediates may or may not be detected by kinetic studies, depending on the relationship between the rate constants, although they could be present in appreciable quantities under the proper conditions; that is, EB may be present to a significant extent at high concentrations of B with A being absent. The presence of an EB complex has been observed for aspartate transcarbamylase using the technique of difference spectroscopy (167). If the above proposal is accepted, at this time, for uridine phosphorylase, at least two predictions seem possible:

(1) Uridine and phosphate may protect different sulfhydryl groups or the same sulfhydryl groups but in a different way (as has been discussed in section III C 8e), that is, by conformation changes

and/or by substrate binding. Titration studies may be able to detect differences in protection, but the inhibition study with o-iodosobenzoate has already suggested differences in protection by uridine and phosphate.

(2) If uridine has a lower affinity for the enzyme than phosphate has, a substrate concentration study might show that uridine does not protect the enzyme as well as phosphate does, at low concentrations of each substrate. This would then explain why saturating levels of uridine or phosphate gave equal protection in most cases.

In summary, the significance of protection by both substrates with respect to the order of binding is a moot point. The small contribution from a random pathway would be "of great theoretical interest in connection with the arrangement of reactants at the active site" (169), but its possible presence should not blur the significance of the major pathway. Although there may be a mechanism which has not been considered, and which may still be consistent with the experimental data, the common mechanisms have been discussed. The paper on a kinetic study of inosine 5'-phosphate dehydrogenase illustrates a case where this was not done (160). The authors concluded that contrary to other dehydrogenases their enzyme catalyzed an ordered Bi Bi mechanism with the coenzyme adding second. Data processing was not used; reproducibility was not indicated; four patterns were shown, with three lines on two of the patterns; the enzyme preparation had two major active bands and, in addition, 20% inactive protein. As with other ordered Bi Bi mechanisms, product inhibition data cannot exclude the possibility of an iso Theorell-Chance mechanism (Table XX) with the coenzyme adding first. Isomerization of the free enzyme has been postulated in the

past (6, 166), and difficulty in the detection of a ternary complex by product inhibition studies has also been reported (167, 169).

f) Anomalies in the product inhibition study

i) Irregular intersecting patterns

The two irregular intersecting patterns (Figures 31, 33) were obtained at low concentrations of the nonvariable substrate (uridine and phosphate concentrations were approximately equal to their K_m values). These patterns were classified with respect to inhibition after a consideration of the errors involved in the vertical intercept, and they agreed with the prediction for an ordered Bi Bi mechanism (as well as with the other two possibilities). Nevertheless, it is important to note that even if any assignment for these two patterns based on the present data is considered dubious, it would not weaken the conclusion that uridine phosphorylase can catalyze an ordered Bi Bi reaction, if the study is considered as a whole, and in the light of kinetic studies from the literature.

ii) Inhibition under 'saturating' conditions

Two product inhibition patterns only appear to be approaching the predicted patterns (Figures 30 and 36). Nevertheless, the results may simply reflect the lack of high enough concentrations of the non-variable substrate. Still, the fact that the predicted inhibitions were being approached should be considered as a good indication for an ordered Bi Bi mechanism (as well as for the other two possibilities).

iii) Nonlinear slope replots

The irregular slope replot shown in Figure 37 III is presumably due to the irregular product inhibition pattern obtained for Figure 31.

In the case of inhibition with ribose-1-phosphate, there are two non-linear, possibly parabolic, slope replots (Figs. 38 VI and VII). To begin with, if one considers the errors of the slopes themselves (indicated with each point), it is apparent that they are large enough to make the deviation from linearity of doubtful significance. If these replots are found to be reproducible in future work, however, they might be explained adequately by postulating a EQ_2 complex. With aspartate transcarbamylase this type of complex was suggested as being "....the only species which can provide parabolic inhibition...without disrupting the competitive pattern" (167), between A and Q. This proposal appears even more reasonable when one considers that both ribose-1-phosphate and phosphate (in the present study) are anions.

Nonlinearity would probably have been observed with uracil (P) as the product, if sufficiently high concentrations were used (0.24 mM was the greatest amount used, whereas about 0.70 mM is necessary for substrate inhibition). If nonlinearity were observed, one could postulate a dead end combination of P with the free enzyme as has been proposed for the ordered Bi Ter mechanism of malic enzyme (174).

g) Comparison of constants from initial velocity and product inhibition studies

It is only possible to compare the K_{iq} values. Two out of the three values, calculated with uracil as the inhibitor are not too different from the initial velocity value; however, the four values calculated from the inhibition study with ribose-1-phosphate are not as close. Perhaps the following two points should be considered in any explanation for the variation in the ribose-1-phosphate K_{iq} values with

respect to the uracil values:

(1) Uridine and phosphate were present in the product inhibition studies along with one product, whereas only ribose-1-phosphate and uracil were present in the initial velocity study in the direction of nucleoside synthesis.

(2) Possible nonlinear slope replots were only evident with ribose-1-phosphate as the inhibitor.

Since the explanation for the nonlinear slope replot of ribose -1-phosphate entailed competition between ribose-1-phosphate for the phosphate site, thus forming an EQ_2complex, one can further say that by inhibiting the combination of ribose-1-phosphate with the free enzyme, phosphate increased the dissociation constant of ribose-1-phosphate calculated from product inhibition studies.

With uridine, presumably, there is no direct competition with ribose-1-phosphate for a similar site on the free enzyme, but there might be competition for the free enzyme itself, since uridine may be able to form a EB complex. Thus, the formation of a complex between ribose-1-phosphate and the free enzyme is again inhibited and its dissociation constant will appear to increase. The variation in K_{ip} values cannot be explained in the above manner for K_{ip} does not have an obvious physical significance.

h) Other mechanisms for phosphorolysis (from the literature)

In the two other studies on uridine phosphorylase, the authors postulated ordered mechanisms with nucleoside binding first (25, 35). These studies were discussed in detail in section IV A 1b. The discrepancies with the present mechanism may have been due to different methods of study, or perhaps to the different enzyme sources (35). For

purine nucleoside phosphorylase, three different types of ordered mechanisms have been postulated (4, 6, 157), but in each study a different enzyme source was used as well as a different experimental approach (section IV A 1c). A recent paper summarized a number of kinetic differences between purine nucleoside phosphorylases from bovine spleen and human erythrocytes (5). Perhaps, at first, one might think that as a class nucleoside phosphorylases should have a similar order of binding. This is not necessarily true, since kinases may have RER or ping pong mechanisms (168), while dehydrogenases may have ordered (169, 176), ordered with enzyme isomerization (166), iso ping pong Bi Bi (188), RER (185), or random (179) mechanisms.

Since initial velocity and product inhibition studies were the sole reason for concluding that for one purine nucleoside phosphorylase preparation the nucleoside binds first to the enzyme, it would be interesting, in light of the present study on uridine phosphorylase, to examine the data in some detail (4). The enzyme was highly purified; data processing was not used; four patterns were shown with three lines in each one; replots of slopes and intercepts were always linear. For clarity the inhibition patterns are tabulated below. The alternate product, guanine, was used instead of hypoxanthine.

Variable substrate	Guanine (Q)	Ribose-1-phosphate (P)
Inosine (A)	competitive	noncompetitive
Phosphate (B)	noncompetitive	noncompetitive

nonvariable substrate: inosine - 9 times its K_m value; phosphate - 150 times its K_m value.

If one concludes that the inhibition patterns were obtained at sufficiently low concentrations of the nonvariable substrate, then the patterns are consistent with the mechanism proposed by the authors. The above concentrations, however, appear to be high enough so that at least an approach to the patterns predicted for saturating conditions might be expected. This was not the case, and for only two of the above patterns will the inhibition be the same under low and high concentrations of the nonvariable substrate (Table XXII). The random Bi Bi mechanism appears to be most consistent with the primary plots. Although the replots in this study were linear, only three points were given for each replot.

In considering other phosphorylases, it is interesting to note that aspartate transcarbamylase (1, 167) has an ordered Bi Bi mechanism with phosphate binding first; glyceraldehyde 3-phosphate dehydrogenase (1, 185) and maltodextrin phosphorylase have RER mechanisms (189). With the latter enzyme there is no inversion of configuration of the glycosyl moiety upon cleavage, whereas there is such an inversion with uridine phosphorylase (1).

i) Possible future work

Further kinetic studies are necessary to verify and to investigate the ordered Bi Bi mechanism proposed for uridine phosphorylase. The present controversy in the mechanism of yeast hexokinase should be noted even though many detailed studies have been done (190).

(1) Product inhibition studies. In addition to a study of nucleoside synthesis, further work in the direction of nucleoside cleavage would also be useful: (a) to confirm some of the patterns already done; (b) to study the effect of pH on some patterns; (c) to obtain

other patterns, such as one at different levels of B with Q as inhibitor, and A as the variable substrate (159). An ordered Bi Bi mechanism will have a constant K_{is} whereas a RER mechanism will have a variable K_{is}.

(2) Alternate substrate studies. These studies may not just clarify whether uridine and deoxyuridine occupy a common binding site (4, 165) or interacting sites, but they may also allow a choice to be made between random and ordered pathways (190).

(3) Analysis of dead end inhibition patterns. If the nonlinear replots are confirmed, it may be possible to exploit their presence in order to differentiate between an ordered Bi Bi and an iso Theorell-Chance mechanism (180).

(4) Isotope exchange studies. This technique supplements the information obtained from initial velocity and product inhibition studies (168). It can indicate the degree of randomness in a predominantly ordered mechanism (162, 169).

SECTION V

SUMMARY AND CONCLUSIONS

Welcome, stranger, to this place,
Where joy doth sit on every bough,
Paleness flies from every face;
We reap not what we do not sow.

William Blake (poems written in a copy of Poetical Sketches)

A. PURIFICATION AND PROPERTIES

1. Five purified preparations of uridine phosphorylase were obtained with a 300 to 1900-fold increase in specific activity relative to the homogenate. There was no indication of a separation of uridine and deoxyuridine cleaving activities during purification. About 75% of both activities were generally recovered in the soluble fraction of the cytoplasm. Disc gel electrophoresis always showed two major bands in the purified preparations (sections II D 1, II D 2c).

2. The best purification scheme at this time utilized heat treatment; and DEAE-Sephadex (pH 8.0), DEAE-Sephadex (pH 7.2), Sephadex G-200 (pH 8.0), and hydroxylapatite (pH 7.2) chromatography (Tables V, VI, VII).

3. Uridine phosphorylase has a pH optimum of 8.1 for uridine cleavage, 8.5 for uridine synthesis, and 6.5 for deoxyuridine cleavage. The enzyme catalyzed the cleavage of Ur > Udr $\gg$ Tdr at pH 7.4, but Udr > Ur (> Tdr) at pH 6.5. At about pH 7.2 the cleavage of uridine and deoxyuridine occurred at the same rate (section II D 2d).

4. Most purified uridine phosphorylase preparations were fairly stable, with aliquots frozen at -40° retaining about 80% of their activity after 60 days (section II D 2b).

5. Purified preparations, inactivated at room temperature for four days, could not be reactivated under the conditions used; purified preparations aged at -40°, however, could be substantially reactivated (section II D 2 biii).

6. Uridine phosphorylase and thymidine phosphorylase both have molecular weights of about 110,000 by a gel filtration estimation (section II D 2e).

7. Uridine phosphorylase did not catalyze a direct transfer of ribose between uracil and uridine under the present conditions, but it had indirect transfer activity. Uridine cleavage (pH 7.3 and 8.1), deoxyuridine cleavage (pH 6.5 and 7.3), and uridine synthesis (pH 8.1), were inhibited by deoxyglucosylthymine. The I_{50} values for deoxyuridine cleavage were about one-fifth that of the other two reactions. The phosphorolysis of deoxyuridine and thymidine by thymidine phosphorylase was not inhibited by deoxyglucosylthymine (section II D 2f).

8. Uridine and deoxyuridine synthesis by uridine phosphorylase were inhibited by uracil with 50% inhibition at about 1 mM (section II D 2g).

B. SULFHYDRYL GROUP MODIFICATION STUDIES

1. Time, concentration, pH, substrate protection, and reversal experiments with 2-mercaptoethanol were done with seven sulfhydryl reagents. Based on the results, only a generalization is appropriate at this time: uridine phosphorylase may contain sulfhydryl group(s) which may be essential for binding of substrates and/or for the catalytic step itself and/or for the maintenance of the conformation of the active site (section III D).

2. Uridine protected uridine phosphorylase to a greater degree than phosphate did against inhibition with o-iodosobenzoate (section III C 4). Differential protection was not evident with the other inhibitors.

C. INITIAL VELOCITY AND PRODUCT INHIBITION STUDIES

1. Initial velocity patterns of uridine cleavage, deoxyuridine cleavage, and uridine synthesis, and eight product inhibition patterns in the direction of uridine cleavage - with slope and intercept replots - were shown.

2. Initial velocity and product inhibition results were consistent with ordered Bi Bi, iso Theorell-Chance and rapid equilibrium random mechanisms (with four dead end complexes) (section IV D 3). The reasons for favouring an ordered Bi Bi mechanism were discussed (section IV D 3c).

SECTION VI

SPECULATIONS

Things not yet known are coveted by men,
   Our desires give them fashion, and so
As they wax lesser, fall, as they size, grow.

John Donne (Farewell to Love).

A. SUMMARY OF SPECULATIONS MADE IN THIS THESIS

1. Uridine phosphorylase may not be a soluble or a cytoplasmic enzyme (section II E 1).

2. The different pH optima for uridine and deoxyuridine may be explained by different reactivities of sulfhydryl groups and the existence of overlapping sites for uridine and deoxyuridine; a positively charged group may be near an essential sulfhydryl group in the deoxyuridine site (sections II D 2g, III D 5b, III D 8c).

3. Some type of modification of sulfhydryl groups produced on aging of purified preparations may cause differential loss of uridine cleaving activity (section II D 2h). Uridine, if stored with the purified preparation, may prevent rapid aging of uridine phosphorylase (section II E 4a).

4. An equilibrium may exist between reduced sulfhydryl groups and oxidized sulfhydryl groups as postulated by Sugino et al (109) and Bruno (183) (section II E 4b).

5. Uridine phosphorylase may have three essential sulfhydryl groups in the active site, along with many more in the molecule as a whole (sections III A 1, III D 6a).

6. Uridine phosphorylase may have direct transferase activity under special conditions (section II E 6).

7. Uracil may be able to produce an essentially unidirectional reaction in the direction of nucleoside synthesis (sections I B 4, II E 7c).

8. The low dissociation constant for ribose-1-phosphate may be of importance for the role of uridine phosphorylase in the salvage pathway (section IV D 2cii).

9. Dead end complexes EQ_2 and EB may be present in the simple ordered Bi Bi mechanism (sections IV D 3 fiii, IV D 3e).

B. MODEL FOR THE PHOSPHOROLYSIS OF URIDINE

At the present time the most detailed model on the mechanism of action of uridine phosphorylase revolves about an explanation for the inversion of configuration of C'-1 after cleavage of the C'-N linkage (191):

> "The stereochemical logic, moreover leads to the conclusion that enzymes that catalyze reactions leading to inversion must involve an odd number of displacements. Since the smallest odd number is 1, it was postulated that those enzymes acting to give inversion proceeded by one direct displacement of the acceptor Y, on the original substrate BX."

With uridine phosphorylase, Y is phosphate, and BX is the nucleoside.

A model allowing for a full explanation of both uridine and deoxyuridine cleavage would require an intimate knowledge of the conformational properties of ribo- and deoxyribonucleosides in solution (assuming one enzyme is involved for both nucleosides). Jardetzky felt that the furanose conformation might be implicated in the specificity of enzymes (192). In a more recent article, the authors suggested that "...knowledge of the conformation of nucleosides and nucleotides about the glycosidic bond may be important in the consideration of reactions of the species" (193). It may be that purine nucleosides (192), pyrimidine ribo- and deoxyribonucleosides (194, 195) have different conformations

in solution. In this regard it is interesting to speculate on whether uridine phosphorylase induces different changes in the conformation of uridine and deoxyuridine in carrying out its catalytic function. Conformational analysis, however, is beyond the scope of this thesis (as well as the knowledge of the author).

More fundamental modification studies are essential if an unnecessarily speculative model is not to be proposed. But one mitigating factor in favour of presenting some kind of model here, is that no detailed model has as yet been found in the literature for the action of uridine phosphorylase. Thus, it might be useful, even at this early stage, for the benefit of future investigators, to bring the findings and speculations of this thesis together in a simple diagrammatic presentation, for at least uridine cleavage. In some respects this presentation could also serve for deoxyuridine cleavage, that is, for the binding of substrates and for the catalytic step; but it does not offer an explanation for the different pH optima of the nucleosides (see speculation 2).

This model incorporates the sulfhydryl equilibrium features of the models of Sugino et al (109) and Bruno (183) (Figure 40). Inorganic phosphate is postulated to bind only to the reduced form of the free enzyme (form B). This may produce a shift of the equilibrium towards the reduced form (as well as conformational changes in the position of the reduced sulfhydryl groups). Although uridine may also bind to this reduced form first, it would produce an inactive enzyme conformation, as discussed in section IV D 3e. In addition, uridine may have a lower affinity for this form than phosphate has, and if the binding with uridine were reversible, the formation of the complex could explain

the protection given by uridine against inhibition by sulfhydryl reagents.

This model also emphasizes the importance of facilitated proton transfer in enzyme catalysis as postulated by Wang (196):

"...facilitated proton transfer along rigidly and accurately held hydrogen bonds in enzyme-substrate complexes...may play a crucial role in enzyme catalysis."

Thus, in the catalytic step, the present model shows that hydrogen bonding occurs between P = O and an enzyme sulfhydryl group as well as between P - OH and N - 3. The bond breaking step (that is, C'-1/N-3) involves the formation of 'new' P = O and 'new' P-OH groups during the transfer of electrons in the transition state, and the transfer of a hydrogen ion to N-3 (form D). The transition state involves a six-membered ring which could serve to stabilize the complex.

The HPO_4^- species is shown, since it should predominate at pH 8 (110). One immediate problem with this proposal may be the reluctance of this species to give up a hydrogen ion, for the pK of this third ionizable hydrogen is normally about 12 (110). Perhaps the rigid binding of the hydrogen reduces the pK, but also it should be noted that another P - OH group is formed in the transition state, possibly simultaneous with the transfer of the hydrogen ion from HPO_4^- to N-3 (form D). The action of the phosphate anion implicates the P - OH group as an acid, and the P - O^- group as a base, although one usually would think of groups in the enzyme acting as an acid-base unit. In any case, an acid-base unit involving rigidly held hydrogen bonds"...capable of such synchronous action may well be the reason for the efficiency of enzyme catalysis" (197). The uncatalyzed hydrolysis of pyrimidine nucleosides is slow, and it occurs best at 75-95^{o}, in acid medium, after an incubation period of several hours (198).

The reactions that are shown for binding of substrates are common reactions for sulfhydryl groups (1), that is, the attack on a double bond and hydrogen bonding. The binding of the sulfhydryl groups to C-2 and the 'O' of C-4 of the uracil moiety would allow the electron withdrawing sulfhydryl groups to be ortho to N-3. If in the transition state N-3 acquires a partial negative charge, i.e. the formation of a carbanion, an ortho effect (or para) by an electron-withdrawing group would stabilize this incipient charge best (199). In passing, it is interesting to note that a model for the hydrolysis of pyrimidine nucleosides, which is not catalyzed by an enzyme, has been proposed (198). It is a general acid catalyzed reaction, possibly S_N1 (substitution nucleophilic unimolecular).

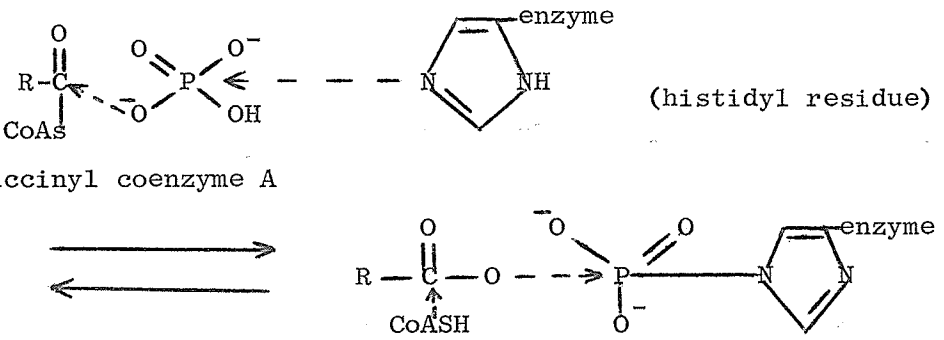
It should be made clear that the model for the phosphorolysis of uridine by uridine phosphorylase offers suggestions only on possible ways by which sulfhydryl groups could participate in the mechanism. This model is not intended to imply that other groups in the enzyme do not participate (5, 8). For example, the negative groupings in the inorganic phosphate molecule might be bound to positive centers in the enzyme as postulated for aspartate transcarbamylase (167).

C. VARIATIONS

1. Phosphate may form a covalent linkage with the sulfhydryl group, i.e. $S\text{-}PO_3^-$. This intermediate has been proposed to be part of the mechanism for glutamine synthetase (200). If this is the case for uridine phosphorylase, another source for the hydrogen which is acquired by N-3 must be proposed; perhaps one could come from a water molecule or it could come in the manner described in point 2.

2. An enzymic sulfhydryl group may take part directly in the catalytic step by a frontal attack on the glycosidic bond. It may be that in the process the hydrogen of the sulfhydryl group is transferred to N-3. Subsequently the $R\text{-}S^-$ group would be replaced by a backside attack by the phosphate anion in order for ribose-1-phosphate to show complete inversion of configuration about C'-1. These methods of attack are illustrated by Koshland (201) and in a review by Cohen (1).

3. A concerted mechanism for the reversible formation of EP_i from phosphate and succinyl coenzyme A by succinyl CoA synthetase (E. coli) has a striking similarity to variation 1 and to parts of the model in Figure 40 (202). It is reproduced below.



Thus, histidine is implicated for binding phosphate instead of a sulfhydryl group as in variation 1. The attack on the carbonyl group by phosphate is similar to the attack shown on C'-1 in Figure 40. Although a phosphate dianion is shown by the authors, one would expect a substantial concentration of the monoanion at pH 7.4 (110).

4. The attack by a sulfhydryl group as described in point 2 may involve the opening and closing of the furanose ring as has been proposed for the uncatalyzed hydrolysis of pyrimidine nucleosides (198). Although ring opening is not involved, the formation of an enzyme-S-C=O

intermediate has been proposed as part of the mechanism of glyceraldehyde 3-phosphate dehydrogenase. In addition a neighbouring histidyl residue was implicated in the mechanism (120). The interaction of the above complex with the phosphate anion was not explicitly shown. It is interesting to note that the interconversion of the diol to the free aldehyde of glyceraldehyde-3-phosphate was suggested to be the rate-limiting step in the enzyme catalyzed reaction under certain concentration ranges of substrate and enzyme (203). For aldolase, it was suggested that the "...comparatively low rate of aldol cleavage of fructose-1-phosphate may be due to the rate-limiting conversion of the pyranose or furanose, to the open-chain form" (204).

It is possible that ring opening could involve a polarization of charge about C'-1 and the ring oxygen, and, in effect, produce a carbonium ion on C'-1 (in addition to the carbanion on N-3). In nucleophilic reactions, one generally associates a carbonium ion with at least partial racemization of the product (199, p. 378). This point was briefly discussed by Bender and Breslow (205):

> "It is entirely possible that a carbonium ion process on an enzyme surface could result in complete inversion or retention of configuration if stereospecific solvation (ion pair formation) could be performed by a nucleophilic group of the enzyme or a water molecule suitably placed on the enzyme surface."

REFERENCES

SECTIONS I AND II. INTRODUCTION; PURIFICATION AND SOME PROPERTIES OF URIDINE PHOSPHORYLASE

1. Cohen, M. in The Enzymes, P.D. Boyer, H. Lardy, and K. Myrbach (Editors); Academic Press, New York, Vol. 5, p. 179 (1961).

2. Friedkin, M. and Kalckar, H. ibid., p. 237.

3. Kim, B.K., Cha, S., and Parks Jr., R.E., J. Biol. Chem., 243, 1763 (1968).

4. Kim, B.K., Cha, S., and Parks Jr., R.E., J. Biol. Chem., 243, 1771, (1968).

5. Agarwal, R.P. and Parks Jr., R.E., J. Biol. Chem., 244, 644 (1969).

6. Krenitsky, T.A., Mol. Pharm., 3, 526 (1967).

7. Tarr, H.L.A. and Roy, J.E., Can. J. Biochem., 45, 409 (1967).

8. Zimmerman, M., J. Biol. Chem., 239, 2622 (1964).

9. Lyon Jr., G.M., Biochem. Biophys. Acta, 159, 38 (1968).

10. Gallo, R.C., Perry, S., and Breitman, T.R., J. Biol. Chem., 242, 5059 (1967).

11. Gallo, R.C. and Breitman, T.R., J. Biol. Chem., 243, 4936 (1968).

12. Tarr, H.L.A., Roy, J.E. and Yamamoto, M., Can. J. Biochem., 46, 407 (1968).

13. Pontis, H., Degerstedt, G., and Reichard, P., Biochem. Biophys. Acta, 51, 138 (1961).

14. Krenitsky, T.A., Barclay, M., and Jacquez, J.A., J. Biol. Chem., 239, 805 (1964).

15. Krenitsky, T.A., Mellors, J.W., and Barclay, R.K., J. Biol. Chem., 240, 1281 (1965).

16. Yamada, E.W., Can. J. Biochem., 42, 317 (1964).

17. Yamada, E.W., Can. J. Biochem., 43, 41 (1965).

18. Yamada, E.W., Biochem. Biophys. Acta, 149, 299 (1967).

19. Yamada, E.W., J. Biol. Chem., 243, 1649 (1968).

20. Etzold, G., Preussel, B., and Langen, P., Mol. Pharm., 4, 20 (1968).

21. Lindsay, R.H., Romine, C.J., and Wong, M.Y., Arch. Biochem. Biophys., 126, 812 (1968).

22. Skold, O., Biochem. Biophys. Acta, 44, 1 (1960).

23. Canellakis, E.S., J. Biol. Chem., 227, 701 (1957).

24. Canellakis, E.S., Jaffe, J.J., Mantsavinos, R., and Krakow, J.S., J. Biol. Chem., 234, 2096 (1959).

25. De Verdier, C.H., and Potter, V.R., J. Nat. Cancer Inst., 24, 13 (1960).

26. Canellakis, E.S., J. Biol. Chem., 227, 329 (1957).

27. Paege, L.M. and Schlenk, F., Arch. Biochem. Biophys., 40, 42 (1952).

28. Imada, A., and Igarasi, S., J. of Bact., 94, 1551 (1967).

29. Akamatsu, N., Lindsay, R.H., and Cohen P.P., J. Biol. Chem., 239, 2246 (1964).

30. Zimmerman, M., and Seidenberg, J., J. Biol. Chem., 239, 2618 (1964).

31. Krenitsky, T.A., Elion, G.B., Strelitz, R.A., and Hitchings, G.H., J. Biol. Chem., 242, 2675 (1967).

32. Zimmerman, M., J. Biol. Chem., 241, 4914 (1966).

33. Langen, P., and Etzold, G., Biochem. Z., 339, 190 (1963).

34. Zimmerman, M., Biochem. Biophys. Res. Commun., 16, 600 (1964).

35. Krenitsky, T.A., J. Biol. Chem., 243, 2871 (1968).

36. Kornberg, A., in Advances in Enzymology, F.F. Nord (Editor), Interscience Publishers Inc., New York. 18, 191 (1957).

37. Kalckar, H.M. J. Biol. Chem., 167, 477 (1947).

38. Friedkin, M. and Roberts, D., J. Biol. Chem., 207, 245 (1954).

39. Hatfield, D. and Wyngaarden, J.B., J. Biol. Chem., 239, 2580 (1964).

40. Razzell, W.E. and Khorana, H.G., Biochem. Biophys. Acta, 28, 562 (1958).

41. Strominger, D.B. and Friedkin, M., J. Biol. Chem., 208, 663 (1954).

42. Gallo, R.C. and Breitman, T.R., J. Biol. Chem., 243, 4943 (1968).

43. Tono, H. and Cohen, S.S., J. Biol. Chem., 237, 1271 (1962).

44. Abrams, R., Edmonds, M., and Libenson, L., Biochem. Biophys. Res. Commun., 20, 310 (1965).

45. Pinto, B. and Touster, O., J. Biol. Chem., 241, 772 (1966).

46. Koch, A.L., J. Biol. Chem., 223, 535 (1956).

47. Roush, A.H. and Betz, R.F., J. Biol. Chem., 233, 261 (1958).

48. Wang, T.P. and Lampen, J.O., J. Biol. Chem., 192, 339 (1951).

49. Carter, C.E., J. Am. Chem. Soc., 73, 1508 (1951).

50. Achar, B.S. and Vaidyanathan, C.S., Arch. Biochem. Biophys., 119, 356 (1967).

51. Tarr, H.L.A., Biochem. J., 59, 386 (1955).

52. Reichard, P., in Advances in Enzymology, 21, 263 (1959).

53. Sugino, Y., Annual Report of the Institute for Virus Research, Kyoto Univ., 9, 1 (1966).

54. Reyes, P., Biochem., 8, 2057 (1969).

55. Reichard, P. and Skold, O., Biochem. Biophys. Acta, 28, 376 (1958).

56. Fritzson, P., Eur. J. Biochem., 1, 12 (1967).

57. Rutman, R.J., Cantarow, A., and Paschkis, K.E., J. Biol. Chem., 210, 321 (1954).

58. Rainnie, D.J. and Blair, D.G.R., Can. J. Biochem., 47, 429 (1969).

59. Skold, O., J. Biol. Chem., 235, 3273 (1960).

60. Sakai, H. and Kehara, H.R., Biochem. Biophys. Acta, 157, 630 (1968).

61. Brown, E.G. and Mangat, B.S., Biochem. Biophys. Acta, 177, 427 (1969).

62. Gallo, R.C., Perry, S., and Breitman, T.R., Biochem. Pharm., 17, 2185 (1968).

63. Yamada, E.W., J. Biol. Chem., 236, 3043 (1961).

64. Webb, J.L., Enzyme and Metabolic Inhibitors, Vol. I-III, Academic Press, New York, 1965-1966.

65. Reichard, P. and Skold, O., in Methods in Enzymology, S.P. Colowick and N.O. Kaplan (Editors); Academic Press, New York, Vol. VI, p. 189 (1963).

66. Schwimmer, S. and Pardee, A.B., in Advances in Enzymology, 14, 375 (1953).

67. Dixon, M. and Webb, E.C., Enzymes, Longmans, Green and Co. Ltd., London, 2nd ed., 1965.

68. Sober, H.A., Hartley Jr., R.W., Carroll, W.R., and Peterson, E.A., in The Proteins, H. Neurath, (Editor), Academic Press, New York, Vol. III, 2 (1965).

69. Tsuboi, K.K. and Hudson, P.B., J. Biol. Chem., 224, 879-897 (1957).

70. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., J. Biol. Chem., 193, 265 (1951).

71. Warburg, O. and Christian, W., Biochem. Z., 310, 384 (1942).

72. Setlow, B. and Lowenstein, J.M., J. Biol. Chem., 242, 607 (1967).

73. Kohn, J., Nature, 183, 1055 (1959).

74. Levin, O., in Methods in Enzymology, 5, 27 (1962).

75. Andrews, P., Biochem. J., 91, 222 (1964).

76. Davis, B.J., Ann. New York Academy of Science, 121, 404 (1964).

77. Williams, D.E. and Reisfeld, R.A., ibid. p. 373.

78. Chrambach, A., Reisfeld, R.A., Wyckoff, M., and Zaccari, J., Anal. Biochem., 20, 150 (1967).

79. Canalco Product

80. Hooton, B.T., Biochem., 7, 2063 (1968).

81. Younathan, E.S., Paetkau, V., and Lardy, H.A., J. Biol. Chem., 243, 1603 (1968).

82. Sugino, Y., Teraoka, H., Shimono, H., J. Biol. Chem., 241, 961 (1966).

83. Goffeau, A., Pedersen, P.L., and Lehninger, A.L., J. Biol. Chem., 243, 1685 (1968).

84. Hsu, R.Y. and Lardy, H.A., J. Biol. Chem., 242, 520 (1967).

85. Froede, H.C., Geraci, G., and Mansour, T.E., J. Biol. Chem., 243, 6021 (1968).

86. Plowman, K.M. and Cleland, W.W., J. Biol. Chem., 242, 4239 (1967).

87. Kobes, R.D. and Dekker, E.E., J. Biol. Chem., 244, 1919 (1969).

88. Rechler, M.M., J. Biol. Chem., 244, 551 (1969).

89. Silverstein, R., Voet, J., Reed, D., and Abeles, R.H., J. Biol. Chem., 242, 1338 (1967).

90. Taylor, R.T. and Jenkins, W.T., J. Biol. Chem., 241, 4396 (1966).

91. Rosso, R.G. and Adams, E., J. Biol. Chem., 242, 5524 (1967).

92. Parsons, S.J. and Burns, R.O., J. Biol. Chem., 244, 996 (1969).

93. Hayashi, S-I., Granner, D.K., and Tomkins, G.M., J. Biol. Chem., 242, 3998 (1967).

94. Geraci, G., Rossi, M., and Scarano, E., Biochem., 6, 183 (1967).

95. Ide, S., Hayakawa, T., Okabe, K., and Koike, M., J. Biol. Chem., 242, 54 (1967).

96. Scher, W. and Jacoby, W.B., J. Biol. Chem., 244, 1878 (1969).

97. Allen, R.H. and Jacoby, W.B., J. Biol. Chem., 244, 2078 (1969).

98. Voll, M.J., Appella, E., and Martin, R.G., J. Biol. Chem., 242, 1760 (1967).

99. Saunders, P.P., Wilson, B.A., and Saunders, G.F., J. Biol. Chem., 244, 3691 (1969).

100. Siebert, G. and Humphrey, G.B., in Advances in Enzymology, 27, 239 (1965).

101. Rose, I.A. and Warms, J.V.B., J. Biol. Chem., 242, 1635 (1967).

102. Fritzson, P., Biochem. Biophys. Acta, 178, 534 (1969).

103. Green, D.E., Murer, E., Hultin, H.O., Richardson, S.H., Salmon, B., Brierley, G.P., and Baum, H., Arch. Biochem. Biophys., 112, 635 (1965).

104. Baltus, E., Biochem. Biophys. Acta, 15, 263 (1954).

105. Determann, H. and Walter, I., Nature, 219, 604 (1968).

106. Monod, J., Wyman, J., and Changeux, J-P., J. Mol. Biol., 12, 88 (1965).

107. Linderstrom-Lang, K.U. and Schellman, J.A., in The Enzymes, Vol. 1. 443 (1959).

108. Pontremoli, S., Traniello, S., Enser, M., Shapiro, S., Horecker, B.L., Proc. Natl. Acad. Sci. U.S., 58, 286 (1967).

109. Sugino, Y., Kobayashi, S., Nakamura, H., Tzukamoto, K., Shimono, H., Shimada, K., Sonoda, S., and Kozai, Y., Annual Report of the Institute for Virus Research, Kyoto Univ., 9, 148 (1966).

110. Cooke Jr., S.L., J. Chem. Educ., 42, 620 (1965).

111. Weimberg, R. and Doudoroff, M., J. of Bact., 68, 381 (1954).

SECTION III. SULFHYDRYL GROUP MODIFICATION STUDIES

112. Parker, D.J. and Allison, W.S., J. Biol. Chem., 244, 180 (1969).

113. Battell, M.L., Smillie, L.B., and Madsen, N.B., Can. J. Biochem., 46, 609 (1968).

114. Huennekens, F.M., Nurk, E., and Gabrio, B.W., J. Biol. Chem., 221, 971 (1956).

115. Boyer, P.D., in The Enzymes, Vol. 1, p. 511, 1959.

116. Taylor, J.E., Yan, J.F., and Wang, J-L., J. Am. Chem. Soc., 88, 1663 (1966).

117. Cavallini, D., De Marco, C., Dupre, S., and Rotilio, G., Arch. Biochem. Biophys., 130, 354 (1969).

118. Boyer, P.D., J. Am. Chem. Soc., 76, 4331 (1954).

119. Koeppe, O.J., Boyer, P.D., and Stulberg, M.P., J. Biol. Chem., 219, 569 (1956).

120. Mathew, W., Meriwether, B.P., and Park, J.H., J. Biol. Chem., 242, 5024 (1967).

121. Folk, J.E. and Cole, P.W., Biochem. Biophys. Acta, 122, 244 (1966).

122. Lynen, F., Fed. Proc., 20, 941 (1961).

123. Barns, R.J. and Keech, D.B., Biochem. Biophys. Acta, 159, 514 (1968).

124. Smith, E., J. Biol. Chem., 233, 1392 (1958).

125. Coleman, R.F., Biochem., 8, 888 (1969).

126. Rabin, B.R., Whitehead, E.P., Nature, 196, 658 (1962).

127. Grazi, E. and Rossi, N., J. Biol. Chem., 243, 538 (1968).

128. Edwards, J.B. and Keech, D.B., Biochem. Biophys. Acta, 146, 576 (1967).

129. Hellerman, L., Coffey, D.S., and Niems, A.H., J. Biol. Chem., 240, 290 (1965).

130. Turano, C., Giartosio, A., and Fasella, P. Arch. Biochem. Biophys., 104, 524 (1964).

131. Holbrook, J.J., Biochem. Z., 344, 141 (1966).

132. Kress, L.F., Bono Jr., V.H., and Noda, L., J. Biol. Chem., 241, 2293 (1966).

133. Liu, T-Y., Neumann, N.P., Elliott, S.D., Moore, S., and Stein, W.H., J. Biol. Chem., 238, 251 (1963).

134. Thoma, J.A., Koshland Jr., D.E., Shinke, R., and Ruscica, J., Biochem., 4, 714 (1965).

135. Parry, M.J., and Walker, D.G., Biochem. J., 105, 473 (1967).

136. Cottam, G.L. and Srere, P.A., Arch. Biochem. Biophys., 130, 304 (1969).

137. Bocchini, V., Alioto, M.R., and Najjar, V.A., Biochem., 6, 313 (1967).

138. Chatterjee, G.C. and Noltmann, E.A., J. Biol. Chem., 242, 3440 (1967).

139. Robinson, G.W., Bradshaw, R.A., Kanarek, L., Hill, R.L., J. Biol. Chem., 242, 2709 (1967).

140. Shapiro, B.M. and Stadtman, E.R., J. Biol. Chem., 242, 5069 (1967).

141. Matsuzawa, T. and Segal, H.L., J. Biol. Chem., 243, 5929 (1968).

142. Guha, A., Englard, S., and Listowsky, I., J. Biol. Chem., 243, 609 (1968).

143. Szabolcsi, G. and Biszku, E., Biochem. Biophys. Acta, 48, 335 (1961).

144. Cremona, T. and Kearney, E.B., J. Biol. Chem., 240, 3645 (1965).

145. Ellman, G.L., Arch. Biochem. Biophys., 82, 70 (1959).

146. Holbrook, J.J. and Jeckel, R., Arch. Biochem. Biophys., 122, 519 (1967).

147. Kemp, R.G. and Forest, P.B., Biochem., 7, 2596 (1968).

148. Wassarman, P.M. and Major, J.P., Biochem., 8, 1076 (1969).

149. Connellan, J.M. and Folk, J.E., J. Biol.Chem., 244, 3173 (1969).

150. Little, C. and O'Brien, P.J., Arch. Biochem. Biophys., 122, 406 (1967).

151. Rabin, B.R. and Watts, D.C., Nature, 188, 1163 (1960).

152. Brewer, C.F. and Riehm, J.P., Anal. Biochem., 18, 248 (1967).

153. Gerwin, B.I., J. Biol. Chem., 242, 451 (1967).

154. Bradshaw, R.A., Robinson, G.W., Hass, G.M., Hill, R.L., J. Biol. Chem., 244, 1755 (1969).

155. Barron, E.S.G. and Levine, S., Arch. Biochem. Biophys., 41, 175 (1952).

SECTION IV. INITIAL VELOCITY AND PRODUCT INHIBITION STUDIES

156. Boyer, P.D., in Ann. Review Biochem., J-M. Luck, R.W. Allen and G. MacKinney (Editors). 29, 15 (1960).

157. Fiers, W. and de Bersaques, J., Enzymologia, 24, 197 (1962).

158. Alberty, R.A., J. Am. Chem. Soc., 80, 1777 (1958).

159. Cleland, W.W., Biochem. Biophys. Acta, 67, 104, 173, 188 (1963).

160. Brox, L.W. and Hampton, A., Biochem., 7, 2589 (1968).

161. Noat, G., Ricard, J., Borel, M., and Got, C., Eur. J. Biochem., 5, 55 (1968).

162. Bridger, W.A. and Cohen, L.H., J. Biol. Chem., 243, 644 (1968).

163. Joyce, B.K. and Himes, R.H., J. Biol. Chem., 241, 5725 (1966).

164. Black, W.J., Can. J. Biochem., 44, 1301 (1966).

165. Henderson, J.F., Brox, L.W., Kelley, W.N., Rosenbloom, F.M., and Seegmiller, J.E., J. Biol. Chem., 243, 2514 (1968).

166. Sanwal, B.D. and Smando, R., J. Biol. Chem., 244, 1824 (1969).

167. Porter, R.W., Modebe, M.O., Stark, G.R., J. Biol. Chem., 244, 1846 (1969).

168. Cleland, W.W., in Ann.Review Biochem., P.D. Boyer, A. Meister, R.L. Sinsheimer, and E.E. Snell (Editors), Palo Alto, Calif.; Vol. 36, 77 (1967).

169. Wratten, C.C. and Cleland, W.W., Biochem., 2, 935 (1963).

170. Lineweaver, H. and Burk, D.J., J. Am. Chem. Soc., 56, 658 (1934).

171. Wilkinson, G.N., Biochem. J., 80, 324 (1961).

172. Krenitsky, T.A. and Papaioannou, R., J. Biol. Chem., 244, 1271 (1969).

173. Gulbinsky, J.S. and Cleland, W.W., Biochem., 7, 566 (1968).

174. Hsu, R.Y., Lardy, H.A., Cleland, W.W., J. Biol. Chem., 242, 5315 (1967).

175. Nordlie, R.C. and Fromm, H.J., J. Biol. Chem., 234, 2523 (1959).

176. Heyde, E. and Ainsworth, S., J. Biol. Chem., 243, 2413 (1968).

177. Raval, D.N. and Wolfe, R.G., Biochem., 1, 1112 (1962).

178. Sanwal, B.D., J. Biol. Chem., 244, 1831 (1969).

179. Cleland, W.W. and Thompson, V.W., Fed. Proc., 24, 228 (1965).

180. Hurst, R.O., Can. J. Biochem., 47, 111 (1969).

181. Zewe, V. and Fromm, H.J., Biochem., 4, 782 (1965).

182. Joyce, B.K. and Himes, R.H., J. Biol. Chem., 241, 5716 (1966).

183. Bruno, S.F., Proc. Plenary Sess., Int. Congr. Biochem., 7th, 1967 (Publ. 1968), in Chem. Abstracts, Vol. 170, 25 (1969).

184. Ray Jr., W.J. and Roscelli, G.A., J. Biol. Chem., 241, 3499 (1966).

185. Furfine, C.S. and Velick, S.F., J. Biol. Chem., 240, 844 (1965).

186. Goffeau, A., Pedersen, P.L., and Lehninger, A.L., J. Biol. Chem., 242, 1845 (1967).

187. Segal, H.L. and Boyer, P.D., J. Biol. Chem., 204, 265 (1953).

188. Toews, C.J., Biochem. J., 105, 1067 (1967).

189. Chao, J., Johnson, G.F., and Graves, D.J., Biochem., 8, 1459 (1969).

190. Fromm, H.J., Eur. J. Biochem., 7, 385 (1969).

SECTION VB. MODEL FOR THE PHOSPHOROLYSIS OF URIDINE

191. Koshland Jr., D.E., in The Enzymes, Vol. 1, 305 (1959).

192. Jardetzky, C.D., J. Am. Chem. Soc., 82, 229 (1960).

193. Hart, P.A. and Davis, J.P., Biochem. Biophys. Res. Commun., 34, 733 (1969).

194. Jardetzky, C.D., J. Am. Chem. Soc., 83, 2919 (1961).

195. Haschemeyer, A.E.V. and Rich, A., J. Mol. Biol., 27, 369 (1967).

196. Wang, J.H., Science, 161, 328 (1968).

197. Herr Jr., E.B. and Koshland Jr., D.E., Biochem. Biophys. Acta, 25, 219 (1957).

198. Shapiro, R. and Kang, S., Biochem., 8, 1806 (1969).

199. Morrison, R.T. and Boyd, R.N., Organic Chemistry, Allan and Bacan Inc., Boston, 1960, p. 404.

200. Wieland, T., Pfleiderer, G., and Sandmann, B., Biochem. Z., 330, 198 (1958).

201. Koshland Jr., D.E., in The Mechanism of Enzyme Action, W.D. McElroy and B. Glass (Editors); Johns Hopkins Press, Baltimore, p. 608 (1954).

202. Robinson, J.L., Benson, R.W., and Boyer, P.D., Biochem., 8, 2503 (1969).

203. Trentham, D.R., McMurray, C.H., and Pogson, C.I., Biochem. J., 114, 19 (1969).

204. Rutter, W.J., in The Enzymes, Vol. 5, p. 341 (1961).

205. Bender, M.L. and Breslow, R., in Comprehensive Biochemistry, M. Florkin and E.H. Stotz (Editors), Elsevier Publ. Co., Vol. 2, p. 38 (1962).

APPENDIX A

TABLE I

TISSUE DISTRIBUTION OF URIDINE PHOSPHORYLASES

Source	Author
Rat (liver, kidney, spleen, heart, brain, thymus)	De Verdier and Potter, 1960 (25).
Mouse (heart, brain, liver, kidney, spleen, intestine, Ehrlich ascites cells) Rat (bone marrow, liver) Rabbit (bone marrow)	Skold, 1960 (22).
Rabbit (bone marrow, liver, intestine)	Yamada, 1961 (63).
Rat (liver)	Yamada, 1964-8 (16-19).
A. Mouse (intestinal epithelium, liver, ascites cells) Rat (intestinal epithelium, liver) Dog (intestinal epithelium, liver, kidney)	Krenitsky, 1965 (15)
B. Chick, guinea pig, frog (intestinal epithelium) Human (liver, fibrosarcoma) Escherichia coli	
Calf (thymus) Rat (thyroid, liver)	Lindsay (21), 1968.
Fish (muscle)	Tarr (7), 1967

TABLE II

SPECIFIC ACTIVITY VALUES OF SOME URIDINE PHOSPHORYLASES

Animal	Tissue	Fraction used	Specific activity	Definition of specific activity
Rat (22)*	Liver	Extracts of acetone powder	0.50**	mμmoles uracil/mg acetone powder/15 min.
	Bone marrow		0.22	
Rat (25)	Liver	114,000 g supernatant	4 to 10	μmoles thymine-2-^{14}C incorporated into thymine riboside/gram fresh weight/hour
	Kidney		7.3	
	Brain		4.8	
	Heart		4.5	
	Spleen		3.5	
	Thymus		2.4	
	Dunning hepatoma		47.0	
	Novikoff hepatoma		9.0	
Rat (21)	Thyroid	Partially purified	0.83	μmoles uracil/100mg wet weight of tissue per hour
	Liver		0.67	
Rat (19)	Regenerating Liver	151,000 g supernatant	0.23	μmoles uracil/hour/ mg protein
Mouse (22)	Intestine	Extracts of acetone powder	34.6	mμmoles uracil/mg acetone powder/15 min.
	Kidney		17.1	
	Liver		12.8	
	Heart muscle		10.3	
	Spleen		5.9	
	Brain		5.0	
Rabbit (63)	Liver	105,000 g supernatant	0.08	μmoles uracil/hour/ mg protein
	Intestinal mucosa		1.3	
	Bone marrow		1.4	

* Reference.

** Enzyme activity was assayed in the direction of nucleoside cleavage with uridine as the nucleoside except in reference 25 where a transferase assay was used.

TABLE III

PURIFICATION OF NUCLEOSIDE CLEAVING ENZYMES

Enzyme	Source	pH Optimum Ur	pH Optimum Udr	pH Optimum Tdr	Purification	Final specific activity	% Yield
Tdrpase[a] (8)[b]	Human spleen	6		6.0	3700	1100	21
Tdrpase[a] (11)	Human leukocytes	-		-	140	90	3
Tdrpase[a] (9)	Rabbit liver	-		5.5	490	5	35
PNP (3,4,5)	Human erythrocytes	-		-	5560	3660[d]	35
PNP (69)	Human erythrocytes	-		-	510	1260[d]	30
PNP (63)	Rabbit bone marrow	-		-	270	292	3
Urpase (26)	Rat liver	8.0		-	10	4[d]	81
Urpase (13)	Ehrlich ascites cells	8.2	6.5	-	300	60[d]	41
Urpase (14)	Ehrlich ascites cells	8.2	6.4	6.8	260	106	3
Urpase (28)	E. coli	7.6		-	15	342[d]	36
Urpase (27)	E. coli	7.2		-	several hundredfold	130-240	5
Uridine hydrolase (50)	Mung bean	7.4		-	132	0.7[d]	24
Urpase (present work)	Rat liver	8.1	6.5	-	300-1900	19-144	6-20

[d] Converted to μmole/hour/mg protein from authors' units.

[b] Reference.

[c] Not given.

[a] Assay in the direction of nucleoside synthesis.

TABLE IV

STUDIES DONE ON URIDINE PHOSPHORYLASE

PURIFIED IN THE PRESENT WORK

Preparation	Purity (-fold)	Total units	Major studies
A	300	88[a]	Time, pH[b], enzyme concentration, p-mercuribenzoate[b].
B	500	265	Initial velocity[b] (uridine → uracil, pH 8.1)
C	900	1409	Product inhibition (uridine → uracil, pH 8.1)
D	1900	578	Disc gel electrophoresis (elution), initial velocity[c] (uracil → uridine, pH 8.0)
E_a	200[d]	390	Reactivation, deactivation, pH[c] (Ur → uracil, Udr → uracil, uracil → Ur), time study (uracil → Ur), DGT study[c] (uracil → Ur)
E_b	1100[d]	1531	Initial velocity (Ur → uracil, pH 8.1; Udr → uracil[c], pH 6.5; Ur → uracil[c], pH 6.5), reactivation Sulfhydryl group modification studies (Ur → uracil); DGT study (Ur → uracil; Udr → uracil)

[a] Uridine cleaving activity.

[b] These studies, for the most part, are not reported in this thesis.

[c] These studies were done using reactivated enzyme preparations.

[d] Uridine phosphorylase activity in the final step of the purification procedure was collected in two fractions.

TABLE V

PURIFICATION SCHEME FOR PREPARATION C

Step	Fraction	Volume (ml)	Protein (mg)	Uridine			Deoxyuridine			Thymidine			Purity (-fold (Ur))
				Total units	Sp. act.[a]	% Recov.	Total units	Sp. act.	% Recov.	Total units	Sp. act.	% Recov.	
1	Homogenate	3544	110245	7432[b]	0.067[c]	(100)	19027	0.17[c]	(100)	11229	0.10[c]	(100)	1
2A	Nuclear fraction	1359	43633	832	0.019	11	1448	0.033	8	727	0.017	7	0.30
2B	Supernatant 1	3805	60327	6516	0.11	95	16953	0.28	89	8887	0.14	79	1.6
3	Supernatant 2	3240	27736	5816	0.21	78	13263	0.48	75	7832	0.28	75	3.2
4A	AS_1, 0-30%	93	5094	129	0.025	2	1990	0.39	11	1244	0.24	11	0.40
4B	AS_2, 30-50%	126	13037	4705 (4047)[d]	-	64	12637 (11067)	-	66	6513 (5565)	-	58	-
4C	AS_{2c}	134	12281	3391[d]	0.28	-	12442	1.0	-	6766	0.55	-	5.4
5	AS_{2cd}	216	12571	3865[d]	0.29	49	12549	1.0	65	6704	0.53	60	4.3
6	DEAE-Sephadex, pH 8	140	4984	3384	0.68	45	6195	1.2	32	2651	0.53	24	10
7A	Fraction 1	50	1643	3397	2.1	45	2501	1.5	13	318	0.19	3	31
7B	Fraction 2	45	1215	41	0.030	1	4023	3.3	21	2687	2.2	24	0.50
8	DEAE-Sephadex, pH 7	6	359	2496	7.0	33	1561	4.4	8	213	0.60	2	101
9	Sephadex G-200, pH 7	15	139	1898	14	25	1382	9.9	7	200	1.4	2	200
10	Hydroxylapatite	6	25	1409	56	19	1069	42	6	133	5.2	1	820

[a] Specific activity; [b] Enzyme activity was assayed by procedure (ai); [c] Rounded off to two significant digits;

[d] See section II D 1c.

TABLE VI

BRIEF SUMMARY OF THE PURIFICATION SCHEME FOR PREPARATION A

Step[a]	Fraction	Volume (ml)	Protein (mg)	Uridine					Purity (-fold (Ur))
				Total units	Spec. act.	% Recov.	$\frac{Ur}{Udr}$[b]	$\frac{Ur}{Tdr}$	
1[c]	Homogenate	752	24910	1538	0.062[d]	(100)	0.43[d]	0.75[d]	1
4B	AS_2 (30-50%)	52	3625	1113	0.32	75	0.45	0.94	5
5	AS_{2cd}	60	2781	1089	0.39	70	0.68	1.2	6
7A	DEAE-Sephadex Fraction #1	10	390	542	1.4	35	1.1	7.3	22
7B	Fraction #2	8	145	14	0.099	1	0.023	0.039	2
9	Sephadex G-200, pH 7	11	207	436	2.6	28	1.5	9.1	42
8	DEAE-Sephadex, pH 7	11	72	271	3.1	18	1.4	10	51
10	Hydroxylapatite	5	5	88	19	6	1.6	11	300

[a] For the sake of clarity, steps 2, 3, and parts of 4 are not included.

[b] Ratios of specific activity values.

[c] The numbers refer to the order relative to preparation C. The order of the fractions represents the order of the steps in this purification procedure.

[d] Rounded off to two significant digits.

TABLE VII

BRIEF SUMMARY OF THE PURIFICATION SCHEME FOR PREPARATION D

Step[a]	Fraction	Volume (ml)	Protein (mg)	Uridine			$\frac{Ur}{Udr}$[b]	$\frac{Ur}{Tdr}$	Purity (-fold (Ur))
				Total units	Sp. act.	% Recov.			
1	Homogenate	3673	94197	7139	0.076	(100)	0.40[c]	0.47	1
4C	AS_{2c}	172	11988	4472	0.37	63	0.39	0.76	5
extra step	Heat treatment	170	8262	3650	0.44	51	0.43	0.69	6
6	DEAE-Sephadex	49	1568	3149	2.0	44	1.5	6.4	28
7A	DEAE-Sephadex Fraction #1	23	488	2018	4.1	28	1.3	11	57
8	DEAE-Sephadex, pH 7	6	193	1226	6.3	17	1.4	11	87
9	Sephadex G-200, pH 8	3	26	832	32	11	1.4	10	440
10	Hydroxylapatite	4	4	578	144	8	1.4	10	1900

[a] For the sake of clarity, steps 2, 3, parts of 4 and 5 are not included.

[b] Ratio of specific activity values.

[c] Rounded off to two significant digits.

TABLE VIII

SUMMARY OF CERTAIN PARAMETERS FOR FIVE PURIFIED PREPARATIONS (A-E)

Date of preparation	Specific act. range (μmole/hr /mg)	Rat			Purity (-fold (Ur))	% yield	Total units (Ur cleavage)
		number	body wt (gm)	liver wt (gm)			
Prep. A: 8.3.67-18.4.67	0.062-19	13	302*	14.2*	300	6	88
Prep. B: 11.9.67-22.11.67	0.070-35	49	246	13.4	500	samples spilled	265
Prep. C: 11.12.67-22.3.68	0.063-56	57	327	15.3	900	19	1409
Prep. D: 20.2.68-25.5.68	0.076-144	52	304	15.7	1900	8	578
Prep. E:	-	(1) 64	291	11.5	-	-	-
27.5.68-19.8.68	-	(2) 16	342	14.7	-	-	-
Fraction (a)**	0.087-20	-	-	-	200	3	390
Fraction (b)**	0.087-97	-	-	-	1100	13	1531

* Average weight.

** Two fractions were collected from step 10 of preparation E.

TABLE IX

SPECIFIC ACTIVITY RATIOS FOR PREPARATION C

Step	Fraction	Ur/Udr	Ur/Tdr	Udr/Tdr
1*	Homogenate	0.39**	0.67	1.7
2A	Nuclear fraction	0.58	1.2	1.9
2B	Supernatant 1	0.41	0.74	1.8
3	Supernatant 2	0.44	0.75	1.7
4A	AS_1	0.064	0.11	1.6
4B	AS_2	0.37	0.72	1.9
4C	AS_{2c}	0.28	0.51	1.8
5	AS_{2cd}	0.29	0.55	1.9
6	A-50	0.57	1.3	2.3
7A	A-50, #1	1.4	11	7.8
7B	A-50, #2	0.010	0.014	1.5
8	A-50	1.6	12	7.5
9	G-200	1.4	10	7.1
10	Hydroxyl-apatite	1.3	11	8.1

* A purification scheme for preparation C is given in Table V.

** The specific activity ratios are rounded off to two significant digits.

TABLE X

SPECIFIC ACTIVITY RATIOS FROM COLUMN STEPS OF PREPARATIONS A-E

Prep.	Step 6			Step 7A			Step 8			Step 9			Step 10		
	Ur/Udr	Ur/Tdr	Udr/Tdr	Ur/Udr	Ur/Tdr	Udr/Tdr	Ur/Udr	Ur/Tdr	Udr/Tdr	Ur/Udr	Ur/Tdr	Udr/Tdr	Ur/Udr	Ur/Tdr	Udr/Tdr
A				1.3 (1.4)	7.3 (10)	6.2 * (7.4)**	1.7	12	7.0	1.4 (1.5)	10 (9.1)	7.7 (6.2)	1.6	11	7.0
B	0.69	1.2	1.7	1.6	10	6.3	1.7	11	6.3	1.4	-	-	1.9	9.5	5.1
C	0.57	1.3	2.3	1.4	11	7.8	1.6	12	7.5	1.4	10	7.1	1.3	11	8.1
D	1.5	6.4	4.4	1.3	11	8.2	1.4	11	8.0	1.4	10	7.5	1.4	10	7.5
E	1.2	6.2	5.1	1.4 (1.6)	9.7 (11)	6.8 (7.4)	1.5 (1.2)	16 (12)	11 (9.8)	1.5 (1.4)	11 (12)	7.3 (8.8)			
a)													1.4	11	7.7
b)													1.4	11	7.7

* The specific activity ratios are rounded off to two significant digits.

** Values in parenthesis are those obtained after dialysis of the fraction.
— Assay for thymidine cleaving activity was not done.

TABLE XI

THIOL GROUP PROTECTORS AND EDTA IN STUDIES ON NUCLEOSIDE CLEAVING ENZYMES

Enzyme source	SH protector in purification	SH protector in storage	SH protector in assay	EDTA	Other conditions	Stability
E. coli; Urpase (27)*	——	——	——	——	——	——
Ehrlich ascites cells; Urpase (13)	——	——	——	——	1% albumin (S)	active for two weeks
Ehrlich ascites cells; Urpase (14)	10 mM	10 mM	——	——	sat. AS (S)	labile
Human spleen; Tdrpase (8)	2-SH	2-SH	——	3 mM (H)	2% mannitol (P,S)	stable for more than 1 yr.
Rabbit liver; Tdrpase (9)	——	——	——	1 mM (P,S)	50 mM (P,S) imidazole 60 mM sucrose (P,S)	——
Human leukocytes; Tdrpase (11)	5 mM DTT	5 mM DTT	——	——	——	——
Human erythrocytes; PNP (3)	——	——	——	(B)	80% AS (S)	moderately stable for several months
Mung bean; Uridine hydrolase (50)	——	——	——	——	——	——
Rabbit bone marrow PNP (63)	5 mM 2-SH	5 mM 2-SH	5 mM 2-SH	——	——	no loss in activity after one month
Present work	5-10 mM 2-SH	5 mM 2-SH	5 mM 2-SH	1 mM (P,B)	——	moderately stable

* Reference; P, purification; S, storage; H, homogenate; B, dialyzing bag.
\- SH protector, EDTA or other conditions were not reported.

TABLE XII

REACTIVATION OF AGED PURIFIED PREPARATIONS WITH DITHIOTHREITOL

Preparation	Storage time	Uridine % activity		Deoxyuridine % activity	
		before activation	after activation	before activation	after activation
C	9 months[a]	0	25[b]	0	20
D	7 months	0	46	0	31
E_a	3 months[c]	74	100	75	108
E_b	3 months[c]	30	62	–	–

[a] The storage time begins from the preparation of the fraction from Step 10 of the purification scheme. The nucleoside cleaving activity present at this time was taken as 100%. The time involved from Step 1 may be arrived at from information given in Table VIII.

[b] Time of preincubation with dithiothreitol was 180 minutes at room temperature. Enzyme activity was assayed by procedure (bi) at pH 7.4. Concentration of substrates: nucleosides (3.3 mM); phosphate (100 mM for preparations C and D, and 10 mM for fractions E_a and E_b).

[c] Figure 9 gives more information on reactivation of fractions E_a and E_b.

– Control value was not available.

TABLE XIII

ANOMALOUS RESULTS OF AGED REACTIVATED FRACTION E_b

	Protein conc. (mg/ml)	Absorbance reading (units)	Time of incubation (minutes)	Substrates	pH of incubation medium
(1)[a]	8.4	0.297[b]	10	Udr + P_i[c]	6.5
(2)	8.4	0.266[b]	30	Ur + P_i	6.5
(3)	1.3	0.056	65	Ur + P_i	7.3
(4)	↓	0.072	↓	↓	7.7
(5)	↓	0.236	↓	↓	7.2 (G-G)[d]
(6)	↓	0.309	↓	↓	8.1 (G-G)
(7)	↓	0.241	↓	↓	8.8 (G-G)
(8)	↓	0.025	↓	Udr + P_i	7.3
(9)	↓	0.284	↓	↓	6.4
(10)	↓	0.307	↓	↓	5.8
(11)	↓	0.306	↓	↓	6.0
(12)	1.4	0.161	↓	Uracil + dR-1-P	6.5
(13)	1.4	0.391	↓	Uracil + R-1-P	8.2 (G-G)

[a] Experiment number.

[b] These assays were done 27 days before the others and they are intended to serve as controls.

[c] Nucleoside cleavage was assayed by procedure (bi) and nucleoside synthesis was assayed by procedure (bii). Concentration of substrates: nucleoside (3.3 mM), phosphate (100 mM), uracil (0.6 mM), pentose-1-phosphate (about 2 mM). To obtain the final pH values the following buffers were used, (the numbers correspond to the experiment number): (1) 100 mM P_i, 6.0; (3) 100 mM P_i, 7.1; (4) 100 mM P_i, 7.8; (5) 100 mM P_i, 6.0 and 100 mM G-G, 8.4; (6) 100 mM P_i, 7.7 and 100 mM G-G, 8.4; (7) 100 mM P_i, 7.7 and 100 mM G-G, pH 9.3; (10) 100 mM P_i, 5.5; (11) 100 mM P_i, 6.0 and 100 mM Ac, 5.5; (12) 100 mM Tris, 6.5; (13) 100 mM G-G, 8.4.

[d] G-G: glycylglycine was the buffer or part of the buffer system.

TABLE XIV

INHIBITION STUDIES ON NUCLEOSIDE CLEAVING ENZYMES

Enzyme	Inhibitor	Concentration (mM)	Time of preincub. (min)	% Inhibition	Reversal
PNP (3)[a]	DTNB	2.0	30	92	partial[b]
	pMB	2.0	30	83	-
Tdrpase (8)	pMB	0.001	15	20	
	pMB	0.01	15	58	
	pMB	0.1	15	78	
	IA	10.0	-	0	
PNP (69)	pMB	0.0001	10	100	partial
	IOB	0.1	-	90-100	-
	IA	0.1		0	
	INH_2	0.1		0	
	Hg^{++}	0.01		100	
	Hg^{++}	0.001		85	
PNP (63)	pMB	0.2		100	
PNP (114)	pMB	0.1		100	
	IA	1.0		100	
Uridine hydrolase (50)	pMB	0.1	5	50	100%[c]
	Hg^{++}	0.1	5	100	-
	NEM	10.0	5	100	
	NEM	0.1	5	80	

[a] Reference.

[b] Reversal time of 120 minutes with 7 mM dithiothreitol.

[c] Reversal with 10 mM 2-mercaptoethanol.

- Information was not given.

TABLE XV

CLASSIFICATION OF SOME SULFHYDRYL ENZYMES

	SH groups in the catalytic process	SH groups in binding of substrates	SH groups in maintaining protein structure a) catalytic site	b) overall conformation
1.	Glyceraldehyde 3-phosphate dehydrogenase* (119, 120)**	Alcohol dehydrogenase (126)	Lactate dehydrogenase (131)	Phosphoglucomutase (137)
2.	Transglutaminase (121)	Transamidinase (127)*	Myokinase (132)	Phosphoglucoisomerase (138)
3.	Fatty acid synthetase (122)	Isopropyl CoA carboxylase (128)	Creatine kinase (80)	Fumarase (139)
4.	Phosphoenolpyruvate carboxykinase (123)	D-amino acid oxidase (129)	Streptococcal proteinase (133)	Glutamine synthetase (140)
5.	Papain (124)	Glutamate-oxaloacetate transaminase (130)	β-amylase (134)	Alanine aminotransferase (141)
6.	Isocitrate dehydrogenase (125)	——	Glucokinase (135)	Malic dehydrogenase (142)
7.	——	——	Nucleoside diphosphokinase (83)	Aldolase (143)
			Citrate cleaving enzyme (136)	Phosphofructokinase (81)
				DPNH dehydrogenase (144)

* Strong evidence for some enzyme intermediate in binding or in catalysis.

** Reference.

TABLE XVI

SUMMARY OF RESULTS OF SULFHYDRYL GROUP MODIFICATION STUDIES ON URIDINE PHOSPHORYLASE

Inhibitor[a]	I_{50} (mM)		pH	Time[b] (min)	Protection			Reversal (decrease with 2-SH in %)[c]
	uridine	deoxyuridine			Ur	Udr	P_i	
					change in inhibition (%)			
pMB	0.00062	-	8.2	5	N.P.	-	N.P.	0 (5 mM)
	0.00085	0.00037	7.4	5	-	-	-	
	-	0.00055	6.7	5	-	N.P.	N.P.	0 (5 mM)
pMPS	0.00040	-	8.2	5 (3)[E]	N.P.	-	N.P.	60-8 (5 mM)
DTNB	0.0018	-	8.2	30[E]	30-15	-	22-10	40-30 (5 mM)
	-	0.017	6.7	5	-	44-20	42-17	-
IOB	0.0035	-	8.2	30[E]	42-4	-	35-28	40-30 (5 mM)
	0.0035	-	7.4	30	41-8	-	35-17	-
	-	0.0036	6.7	30	-	63-36	0	-
NEM	0.16	-	8.2	5 (30)[E]	40-10	-	35-14	-
	0.50	0.23	7.4	5	-	-	-	-
	-	1.7	6.5	5	-	44-12	42-7	-
IA	5.6	-	8.2	120 (>120)[E]	74-17	-	74-27	74-53 (50 mM)
	6.3	-	7.4	120	-	-	-	-
	-	10.0	6.7	120	-	-	-	-
INH_2	1.8	-	8.1	60 (>60)[E]	60-1	-	50-6	0 (5 mM)
	5.8	-	7.3	60	-	-	-	-

[a] Experimental procedure is described in section III B; [b] Preincubation time; [c] Reversal times are given in section III B 4; E: equilibrium time; N.P.: no protection.

TABLE XVII

NUCLEOSIDE PROTECTION AGAINST INHIBITION WITH ELLMAN'S REAGENT (DTNB)

	Protecting substrate	pH	Inhibitor conc (mM)	Substrates added after 30 min inhibition	Absorbance reading (290 mμ)
1)[a]	None	8.1	0	Ur + P_i	0.215
2)	-		0	Udr + Ur + P_i[b]	0.196
3)	-		0	Udr + P_i	0.022
4)	-		0.001	Ur + Udr + P_i	0.066
5)	Udr		0.001	Ur + P_i	0.134
6)	Ur		0.001	Udr + P_i	0.159
7)	Ur		0.001	P_i	0.187
8)	None	6.3	0	Udr + P_i	0.100
9)				Udr (5 min preincub.) + Ur + P_i	0.038
10)		6.3	0	Ur (5 min preincub.) + Udr + P_i	0.015
11)		6.5	0	Ur + P_i	0.051
12)			0.017	Udr + P_i	0.025
13)				Ur + P_i	0.004
14)	Udr			P_i	0.064
15)	Udr			Ur + P_i	0.029
16)	Ur			P_i	0.051
17)	Ur			Udr + P_i	0.045

[a] Experiment number; experimental procedure with fraction E_b is described in section III B. Nucleoside concentration was 3.3 mM and phosphate concentration was 10 mM.

[b] A preincubation period of 5 minutes of either nucleoside with enzyme before the addition of the other nucleoside did not affect the absorbance reading.

TABLE XVIII

INHIBITION OF ACTIVITY BY IODOACETAMIDE

	Time (min)[a]	INH_2 (mM)	Absorbance	Inhibition (%)	pH
			Uridine		
1)[b]	5	0	0.189	0	8.1
2)	20	6.5	0.090	53	
3)	20	13.0	0.018	92	
4)	20	0	0.142	0	7.3
5)	20	6.5	0.131	8	
6)	60	6.5	0.057	60	
7)	20	13.0	0.111	22	
8)	60	13.0	0.048	66	
9)	20	0	0.059	0	6.6
10)	20	6.5	0.068	- 15[c]	
11)	60	6.5	0.060	- 1	
			Deoxyuridine		
12)	20	0	0.085	0	7.3
13)	20	6.5	0.084	1	
14)	60	6.5	0.057	33	
15)	20	13.0	0.079	7	
16)	60	13.0	0.060	29	
17)	20	0	0.115	0	6.6
18)	60	0	0.114	0	
19)	20	6.5	0.107	7	
20)	60	6.5	0.101	11	
21)	20	13.0	0.083	28	
22)	60	13.0	0.084	26	

[a] Preincubation period of inhibitor and enzyme.

[b] Experiment number; experimental procedure is described in section III B.

[c] Possible stimulation.

TABLE XIX

SENSITIVITY OF ENZYMES TO SULFHYDRYL REAGENTS

Inhibitor	Conc. (mM)	% Inhibition	Enzyme*	Source
pMB	0.000025	50	Isocitrate dehydrogenase (0.99 µg protein)	Pig heart
	0.000015	50	Myokinase (0.5 µg)	Rabbit muscle
	0.00004	50	Oxalosuccinate dehydrogenase (0.99 µg)	Pig heart
	0.0001	56	Ornithine carbamyl transferase	Rat liver
	0.0001	29	Creatine kinase (5 µg nitrogen)	Sheep muscle
	0.0002	50	Glyceraldehyde 3-phosphate dehydrogenase	Rabbit muscle
	0.0003	69	UDP-glucose dehydrogenase (25 µg)	Calf liver
	0.0005	41	Succinate dehydrogenase (90 µg)	Beef heart
	0.0005	50	Aminopeptidase (5-10 µg)	Beef liver
	0.0006	50	Arylesterase	Human serum
	0.0006	50	Uridine phosphorylase (7 µg)	Rat liver
IOB	0.00013	100	Xanthine oxidase	Milk
	0.0025	100	Creatine kinase (5 µg nitrogen)	Sheep muscle
	0.0035	50	Uridine phosphorylase	Rat liver

TABLE XIX
(continued)

Inhibitor	Conc. (mM)	% Inhibition	Enzyme*	Source
NEM	0.001	38	Creatine kinase	Sheep muscle
	0.010	86	α-glycerophosphate dehydrogenase	Rabbit muscle
	0.010	50	Succinate dehydrogenase	Monkey brain
	0.036	50	Fructokinase	Beef liver
	0.033	48	NAD (H) diaphorase	Beef brain
	0.130	50	Glyceraldehyde 3-phosphate dehydrogenase	Rabbit muscle
	about 0.04	50	Uridine phosphorylase	Rat liver
IA	0.0012	50	Cathepsin	Liver
	0.10	60	Glyceraldehyde 3-phosphate dehydrogenase	Rabbit muscle
	0.10	100	Creatine kinase	Sheep muscle
	0.10	45	Proteinase	Rat liver
	0.23		Alcohol dehydrogenase	Rat liver
	1.0	52	Succinate dehydrogenase	Rat liver
	1.5	3	Xanthine oxidase	Milk
	5.6	50	Uridine phosphorylase	Rat liver

* The enzymes shown are the most sensitive ones found for pMB, IOB, and NEM in mammalian systems as listed by Webb (64). For IA, the most sensitive ones are listed here, but only a few of the enzymes inhibited in the 1 mM range are included.

TABLE XX

CONSTANTS FOR URIDINE PHOSPHORYLASE FROM INITIAL VELOCITY STUDIES

Substrate	pH	Constant	Value (mM)	Reference
Uridine	8.1	Michaelis (K_b)	0.28	Figure 22
Uridine	6.5	Apparent K_b	0.12	Section IV A 2cii
Udr	6.5	K_b	0.36	Figure 26
P_i	8.1	K_a	0.34	Figure 23
P_i	6.5	K_a	0.64	Figure 25
Uracil	8.1	K_p	0.31	Figure 28
R-1-P	8.1	K_q	0.070	Figure 28
P_i	6.5	K_{ia}	1.8	Figure 25
R-1-P	8.1	K_{iq}	0.071	Figure 26
	"	K_{iq}	0.080-0.10	Figure 27
P_i	8.1	K_{ia}	3.3	Figure 23
	"	K_{ia}	7.5	Figure 22
Uridine	8.1	V_M^a	0.21	Figures 22, 23
Udr	6.5	V_M	0.10	Figures 24, 25
Uracil	8.1	V_M	0.13	Figure 28

TABLE XX (continued)

Substrate	pH	Constant	Value (mM)	Reference
Uridine	8.0	Apparent K	0.12	(21)[b]
Uracil	8.0	Apparent K	0.26	(21)
Uridine	7.4	Apparent K	0.76	(13)
P_i	7.4	Apparent K	3.9	(13)
P_i	6.5	Apparent K	3.9	(13)
Udr	6.5	Apparent K	0.71	(13)
Uridine	8.1	Apparent K	0.069	(14)
Uridine	7.4	Apparent K	0.13	(14)
Uridine	6.7	Apparent K	0.11	(14)
Udr	8.1	Apparent K	0.40	(14)
Udr	7.4	Apparent K	0.59	(14)
Udr	6.7	Apparent K	0.60	(14)
Uridine	7.4	Apparent K	1.0	(16)
Udr	7.4	Apparent K	0.30	(16)
Uridine	8.0	Apparent K	2.5	(58)

[a] All V_M values were normalized to the same protein concentration and incubation times.

[b] Enzyme sources were given in Tables I and III. Ehrlich ascites cells were used in references 13, 14, and 58.

TABLE XX

CONSTANTS FROM PRODUCT I

Inhibitor	Variable substrate	Nonvariable substrate (mM)	Apparent K_{is} X 10^{-4}M	Definition of apparent K_{is}	Calculated Constant X 10^{-4}M	Appa K_{ii} X
Uracil	Phosphate	(0.133)	3.3	$\frac{K_p K_{iq}}{K_q}\left(1 + \frac{K_a B}{K_{ia} K_b}\right)$	K_{iq} = 0.52	2
Uracil	Phosphate	(0.533)	7.8	$\frac{K_p K_{iq}}{K_q}\left(1 + \frac{K_a B}{K_{ia} K_b}\right)$	K_{iq} = 1.1	2
Uracil	Uridine	(0.667)	-	$\frac{K_p K_{iq}}{K_q}$	-	-
Uracil	Uridine	(6.67)	1.6	$\frac{K_p K_{iq}}{K_q}$	K_{iq} = 0.26	10.
R-1-P	Phosphate	(0.266)	13.0	K_{iq}	-	Compet inhibi
R-1-P	Phosphate	(3.33)	13.0	K_{iq}	-	Compet inhibi
R-1-P	Uridine	(0.667)	8.0	$K_{iq}\left(1 + \frac{A}{K_{ia}}\right)$	K_{iq} = 7.0	14
R-1-P	Uridine	(6.67)	-	Approaching no inhibition	-	-

TABLE XXI

PRODUCT INHIBITION STUDIES

Apparent K_{ii} X 10^{-4}M	Definition of apparent K_{ii}	Calculated Constant X 10^{-4}M	Comments
2.5	$\frac{B + K_b}{\frac{B}{K_{ip}} + \frac{K_q K_b}{K_{iq} K_p}}$	K_{ip} = 1.8	Figure 37 I; equation 5. Values of K_b, K_q, K_p, and K_{iq} are from the initial velocity study.
2.5	$\frac{B + K_b}{\frac{B}{K_{ip}} + \frac{K_q K_b}{K_{iq} K_p}}$	K_{ip} = 2.3	Figure 37 II; equation 5.
-	$K_{ip}\left(1 + \frac{K_a}{A}\right)$	-	Figure 37 III; equation 6. Poor product inhibition pattern.
10.5	K_{ip}	K_{ip} = 10.5	Figure 37 IV; equation 6.
Competitive inhibition	-	-	Figure 38 V; equation 7. Poor product inhibition pattern.
Competitive inhibition	-	-	Figure 38 VI; equation 7. Nonlinear slope.
14.8	$K_{iq}\left(1 + \frac{A}{K_a}\right)$	K_{iq} = 5.7	Figure 38 VII; equation 8. Nonlinear slope.
-	Approaching no inhibition	-	Figure 36; equation 8.

TABLE XXII

PRODUCT INHIBITION PATTERNS FOR SEQUENTIAL Bi Bi MECHANISMS

Mechanism[1]	Product	Variable Substrate: A, B Not sat.	Variable Substrate: A, B Sat.	Variable Substrate: B, A Not sat.	Variable Substrate: B, A Sat.
Ordered Bi Bi[2]	P	NC[3]	UC[4]	NC	NC
	Q	Comp[5]	Comp	NC	—[6]
Theorell-Chance	P	NC	—	Comp	Comp
	Q	Comp	Comp	NC	—
Iso ordered Bi Bi	P	NC	UC	NC	NC
	Q	NC	NC	NC	UC
Iso Theorell-Chance[2]	P	NC	—	Comp	Comp
	Q	NC	NC	NC	UC
Rapid equilibrium random Bi Bi	P	Comp	—	Comp	—
	Q	Comp	—	Comp	—
Rapid equilibrium random Bi Bi (dead end Ebq complex)	P	Comp	—	Comp	—
	Q	Comp	Comp	NC	—
Random Bi Bi	P	NC[7]	NC[7]	NC[7]	NC[7]
	Q	NC	NC	NC	NC
Uridine phosphorylase	P	NC	UC(?)[8]	NC	NC
	Q	Comp	Comp	NC	—(?)

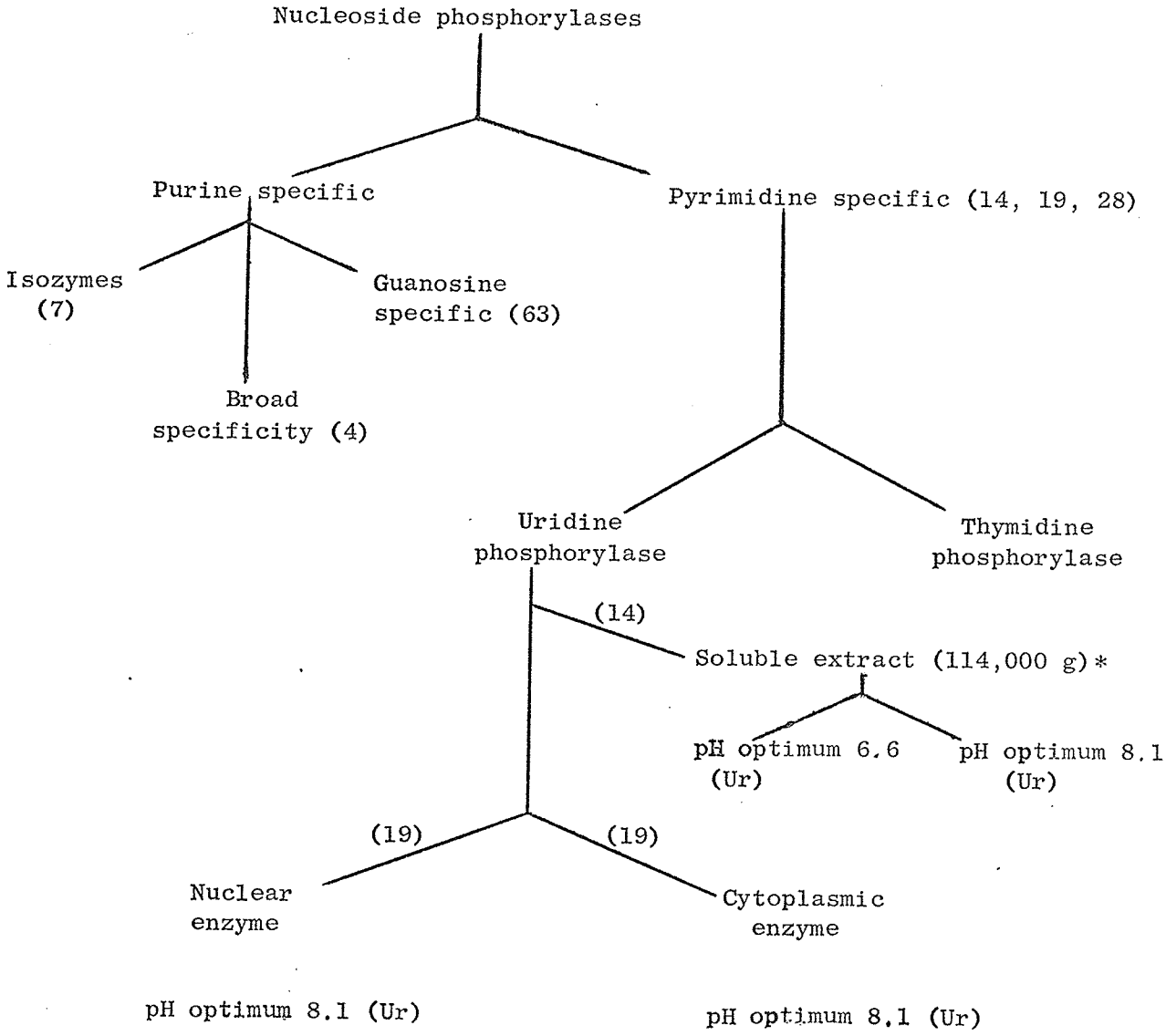
[1] The table is based on one from reference 159. [2] The mechanisms must be differentiated by binding studies. [3] NC: noncompetitive. [4] UC: uncompetitive. [5] Comp: competitive. [6] —: no inhibition. [7] :Reciprocal plots are nonlinear. [8] :Probable type.

TABLE XXIII

SUBSTRATE PROTECTION AGAINST INHIBITION BY SULFHYDRYL REAGENTS VIEWED IN CONNECTION WITH ORDER OF BINDING AS DETERMINED BY KINETICS

Enzyme	Mechanism	Protection
Alcohol dehydrogenase (yeast)	Ordered Bi Bi with some randomness (169)	Both substrates (155)
Adenylosuccinate lyase (yeast) (162)	Ordered uni bi, with some randomness	AMP or adenylosuccinate but not fumarate
Hypoxanthine-guanine phosphoribosyltransferase (human erythrocytes)	Ordered Bi Bi (165), with alternate ping pong pathway (172)	Only phosphoribosylpyrophosphate (172) (substrate A)
Isocitrate TPN dehydrogenase (pig heart)	Random (179)	(Isocitrate + $MnSO_4$ or TPNH + $MnSO_4$) (125)
Citrate cleavage enzyme	RER; alternate ping pong pathway (rat liver) (86)	Only Mg-citrate (chicken liver) (136)
Glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle)	RER (185)	Glyceraldehyde-3-P and glycerald. (187)
Glucokinase (135), rat liver	RER	Glucose (ATP not given)
Mitochondrial nucleoside diphosphokinase (bovine liver)	Ping pong with MgATP binding first; MgADP dead end inhibitor (186)	MgATP - slight MgADP - complete (83)
Phosphofructokinase	Not found in the literature, but other kinases as a rule are RER or Ping Pong	MgATP or fructose 6-phosphate (81)

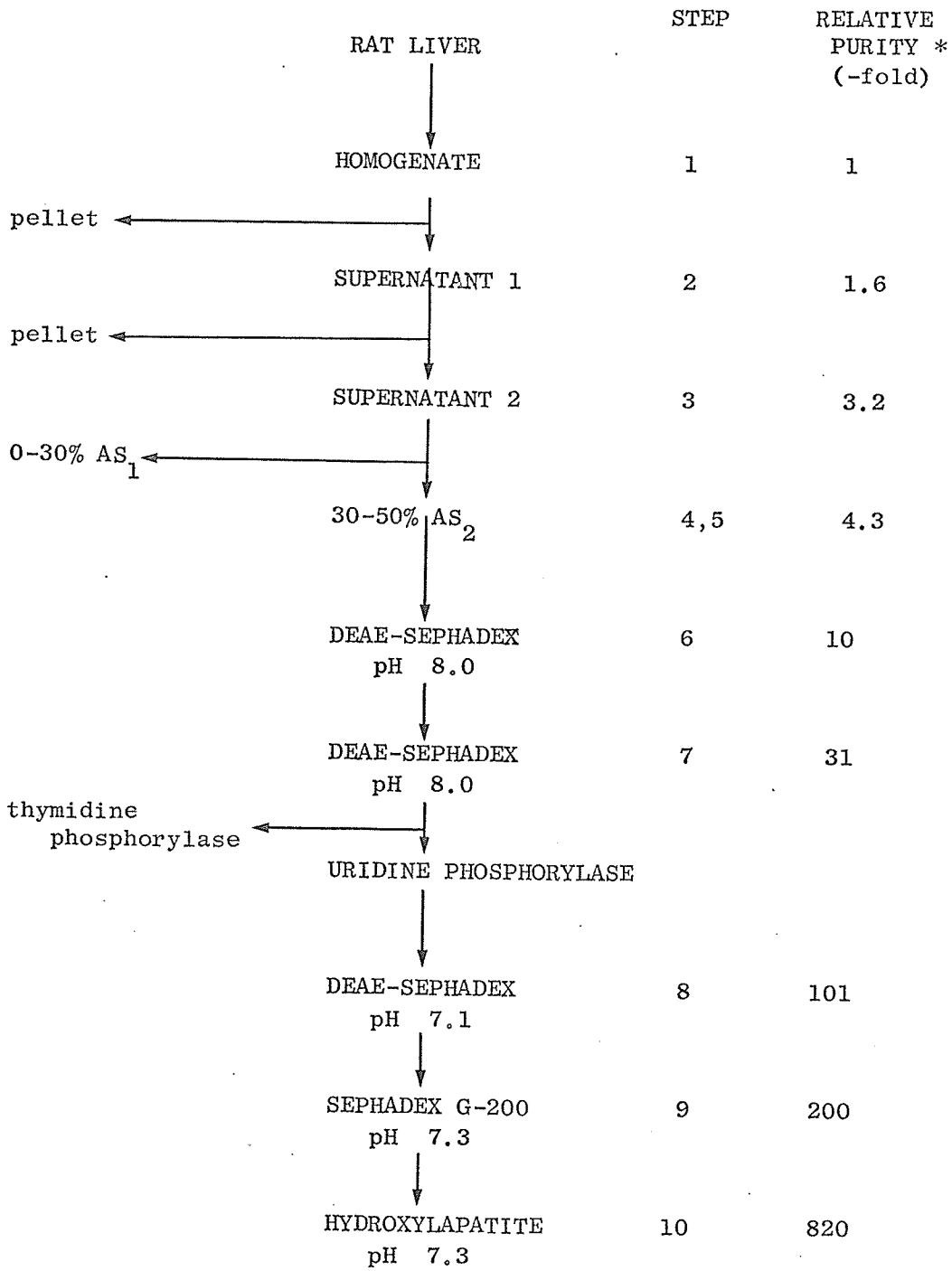
A P P E N D I X B



* The soluble extract contains uridine phosphorylase activity with one of the listed pH optima.

FIGURE 1A

A schematic classification of nucleoside phosphorylases.



* with respect to uridine cleavage relative to the homogenate.

FIGURE 1B

A schematic outline of the purification scheme for preparation C.

Figure 2. DEAE-Sephadex chromatography at pH 8.0 of fraction 5 from preparation A.

The relative position of this fraction in the purification procedure, as well as the fractions in the following four figures, are shown in Figure 1B.

Column parameters:

column size	:	2.5 cm by 60 cm
bed volume	:	255 ml
flow rate	:	30 ml/hour
aliquot	:	6 ml
volume of sample	:	60 ml

The sample contained 2781 mg of protein (80% recovered on elution), and 1595 units of deoxyuridine cleaving activity (93% was eluted from the column and 64% was recovered after concentration of the pooled fractions). The vertical arrow indicates the point at which elution was started with a linear gradient of 0-0.4 M KCl (see section II C 5). Column fractions (tubes 104-116 for Urpase, and tubes 123-146 for Tdrpase) were concentrated with ammonium sulfate at 80% saturation. Deoxyuridine cleaving activity of uridine phosphorylase (■——■), and deoxyuridine cleaving activity of thymidine phosphorylase (●——●) were assayed by procedure (aii); the region of overlap between Urpase and Tdrpase activity profiles is indicated by the dotted lines. Protein (O——O) was estimated spectrophotometrically.

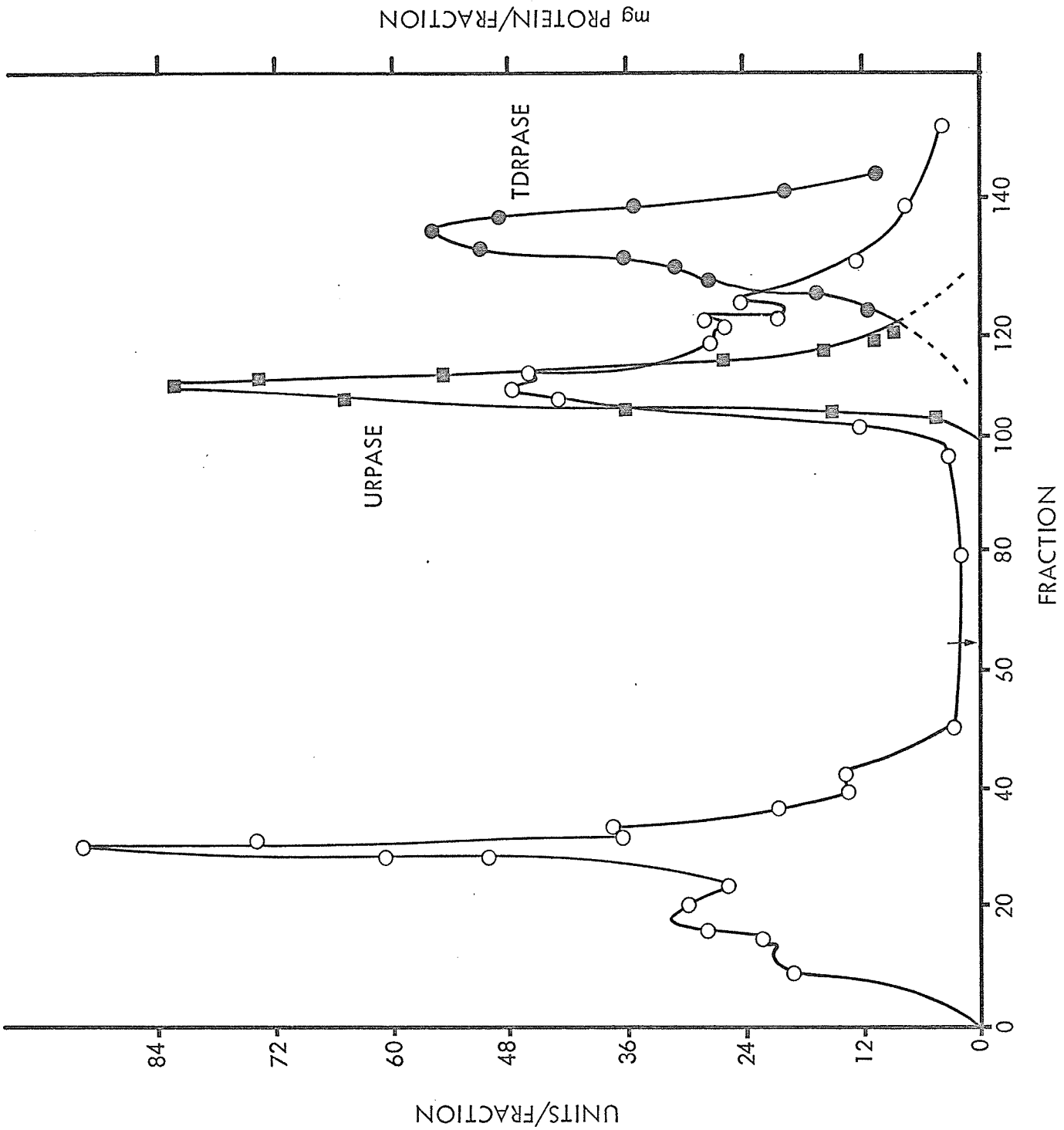

mg PROTEIN/FRACTION
TDRPASE
URPASE
UNITS/FRACTION
FRACTION
0
12
24
36
48
60
72
84
0
20
40
60
80
100
120
140

Figure 3. DEAE-Sephadex chromatography at pH 7.1 of fraction 7 from preparation C.

Column parameters are described in section II C 5. The sample contained 1643 mg of protein of which 95% was recovered, and 2501 units of deoxyuridine cleaving activity. The recoveries of uridine and deoxyuridine cleaving activities in fractions 39-42 were 80 and 70%, respectively, and the recoveries of uridine and deoxyuridine cleaving activities in fractions 47-53 were 10 and 9%, respectively. The values are based on the activity present in the pooled fractions after concentration with ammonium sulfate. The vertical arrow indicates the point at which elution with a linear gradient of 0-0.2 M KCl was started, and the horizontal arrows indicate the fractions that were combined for the subsequent concentration step. Deoxyuridine cleaving activity (●——●) was assayed by procedure (ai). Protein (O——O) was estimated spectrophotometrically.

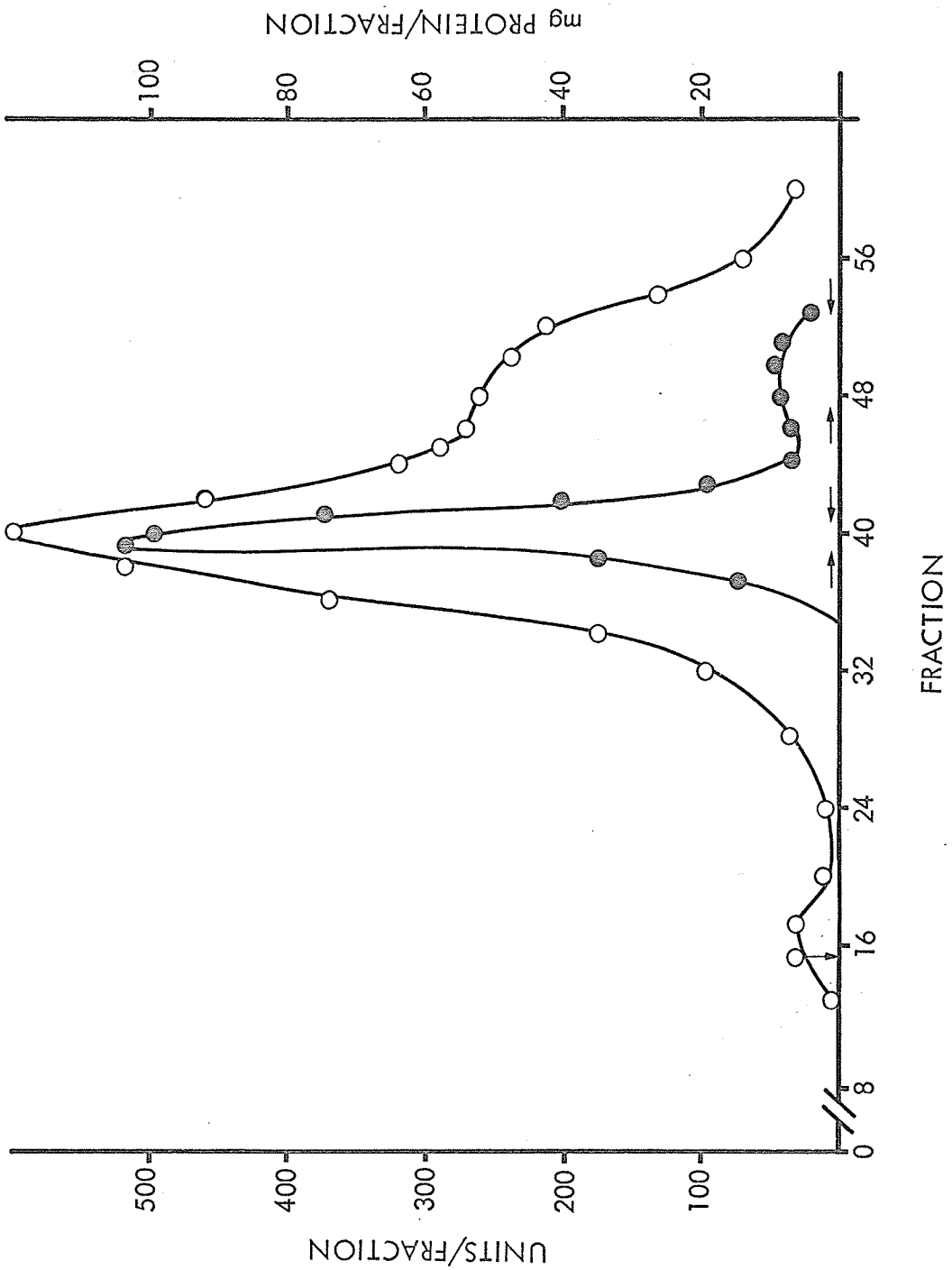

mg PROTEIN/FRACTION
100
80
60
40
20
UNITS/FRACTION
500
400
300
200
100
0
8
16
24
32
40
48
56
FRACTION

Figure 4. The effect of different linear gradients of potassium chloride on DEAE-Sephadex chromatography.

The initial buffer in the mixing chamber for each column was the same (0.02 M P_i -1 mM EDTA-10 mM 2-SH, pH 7.1). The ratio of protein applied (mg)/bed volume was, 2, 7, 9, 4, 16 for preparations A-E, respectively. For preparation A (fraction 9, see Table VI) a linear gradient of 0-0.40 M KCl was used (▩——▩); for preparation B (fraction 9), 0-0.40 M KCl (O——O); for preparation D (fraction 8, see Table VII), 0-0.15 M KCl (□——□); for preparation E (fraction 8), 0-0.25 M KCl (●——●). Chromatography of preparation C is shown in Figure 3. Deoxyuridine cleaving activity was assayed by procedure (ai).

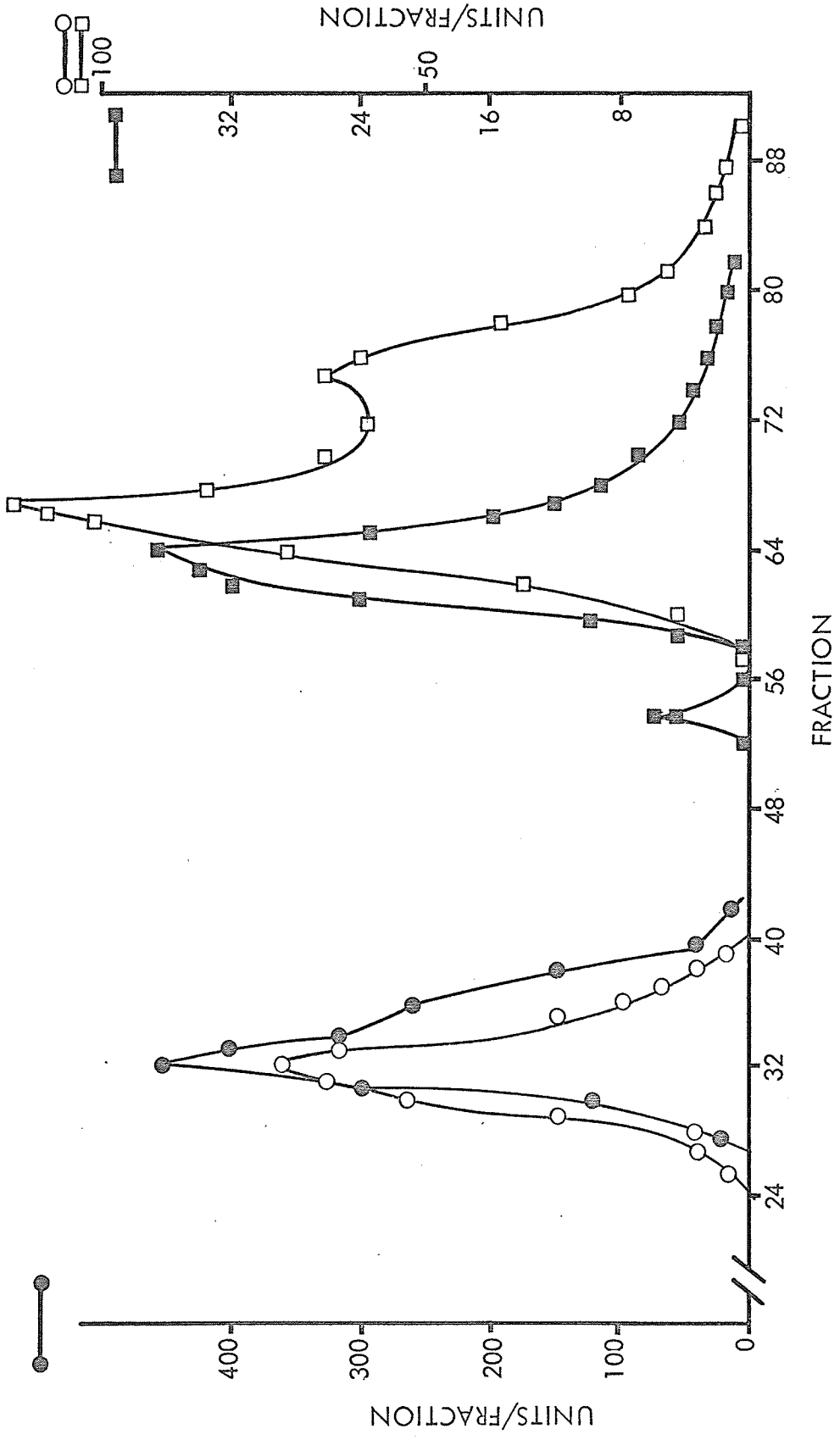

UNITS/FRACTION
100
50
32
24
16
8
88
80
72
64
56
48
40
32
24
FRACTION
400
300
200
100
0
UNITS/FRACTION

Figure 5A. Sephadex G-200 chromatography at pH 8.0 of fraction 8 from preparation D.

Column parameters:

column size	:	2.5 cm by 90 cm
bed volume	:	421 ml
flow rate	:	32 ml/hour
aliquot	:	8 ml
volume of sample	:	4.8 ml

The sample contained 193 mg of protein and 935 units of deoxyuridine activity of which 66% and 91%, respectively, were removed. Uridine cleaving activity (O——O) and deoxyuridine cleaving activity (■——■) were assayed by procedure (aii). The specific activity values (□——□) with deoxyuridine as the nucleoside are shown. Fractions 31-35 were combined (→←) for subsequent concentration in Carbowax for 6 hours. Protein (●——●) was estimated spectrophotometrically.

Figure 5B. Sephadex G-200 chromatography at pH 7.4 of Blue dextran 2000.

Blue dextran (0.2% solution in 0.02 M P_i-1 mM EDTA) was applied to a column similar to the one described in Figure 5A. The effluents were monitored spectrophotometrically at 290 mμ.

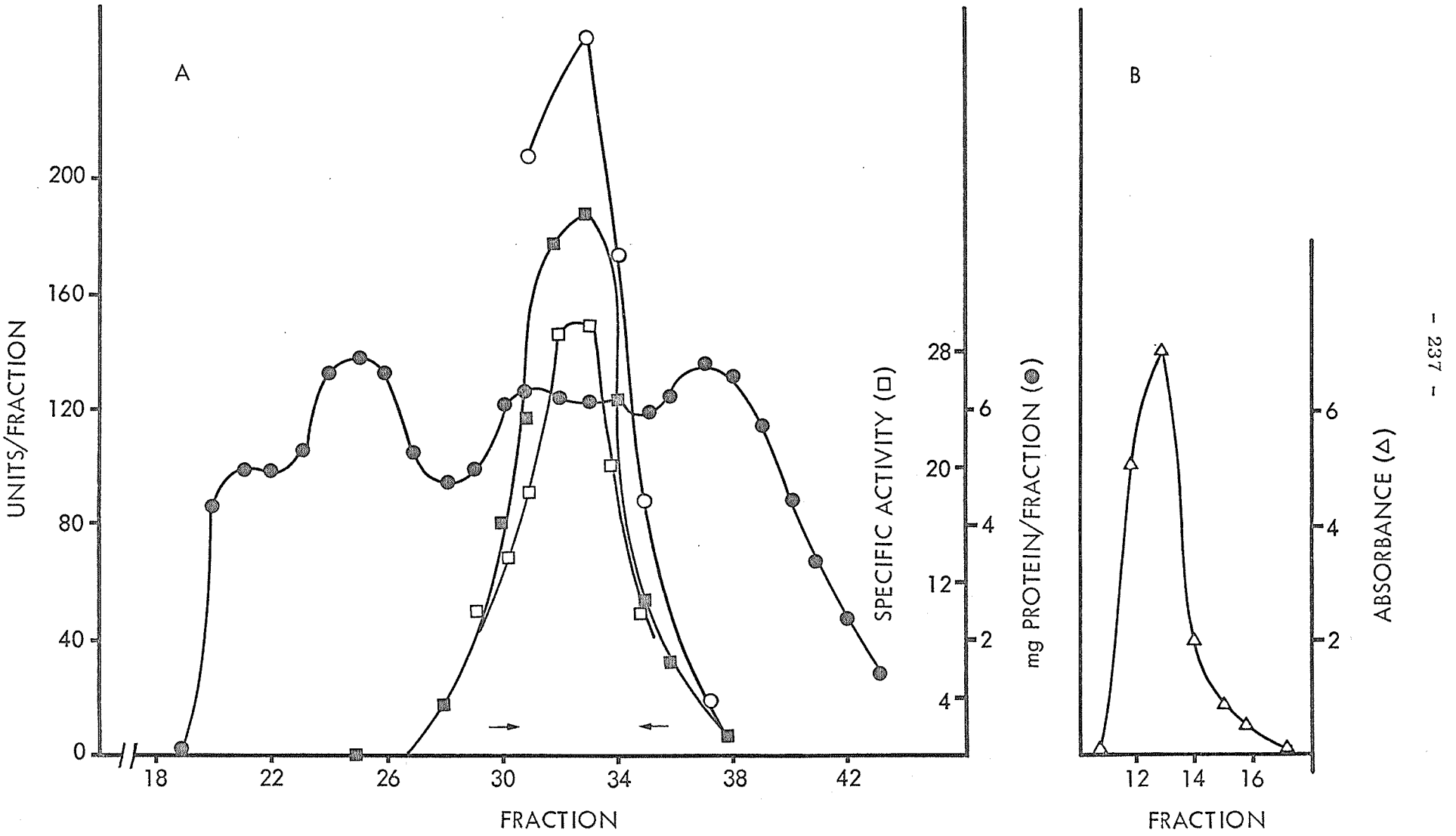

A
B
UNITS/FRACTION
SPECIFIC ACTIVITY (□)
mg PROTEIN/FRACTION (●)
ABSORBANCE (Δ)
FRACTION
FRACTION
0
40
80
120
160
200
4
12
20
28
2
4
6
18
22
26
30
34
38
42
12
14
16

Figure 6. Hydroxylapatite chromatography at pH 7.2 of fraction 9 from preparation D.

Column parameters:

column size	:	0.9 cm by 60 cm
bed volume	:	20 ml
flow rate	:	4 ml/hour
aliquot	:	2.5 ml
volume of sample	:	2.5 ml

The sample contained 26 mg of protein, 832 units of uridine cleaving activity, and 571 units of deoxyuridine cleaving activity of which 58%, 92%, and 92%, respectively, were recovered. The vertical arrow indicates the point at which elution was started with a linear gradient of 0.02-0.25 M phosphate. Uridine cleaving activity (■——■) and deoxyuridine cleaving activity (O——O) were assayed by procedure (ai). The specific activity values (□) with uridine as the nucleoside are shown. Fractions 34-42 (—► ◄—) were combined and concentrated with Carbowax for 4 hours. Protein (●——●) was estimated spectrophotometrically.

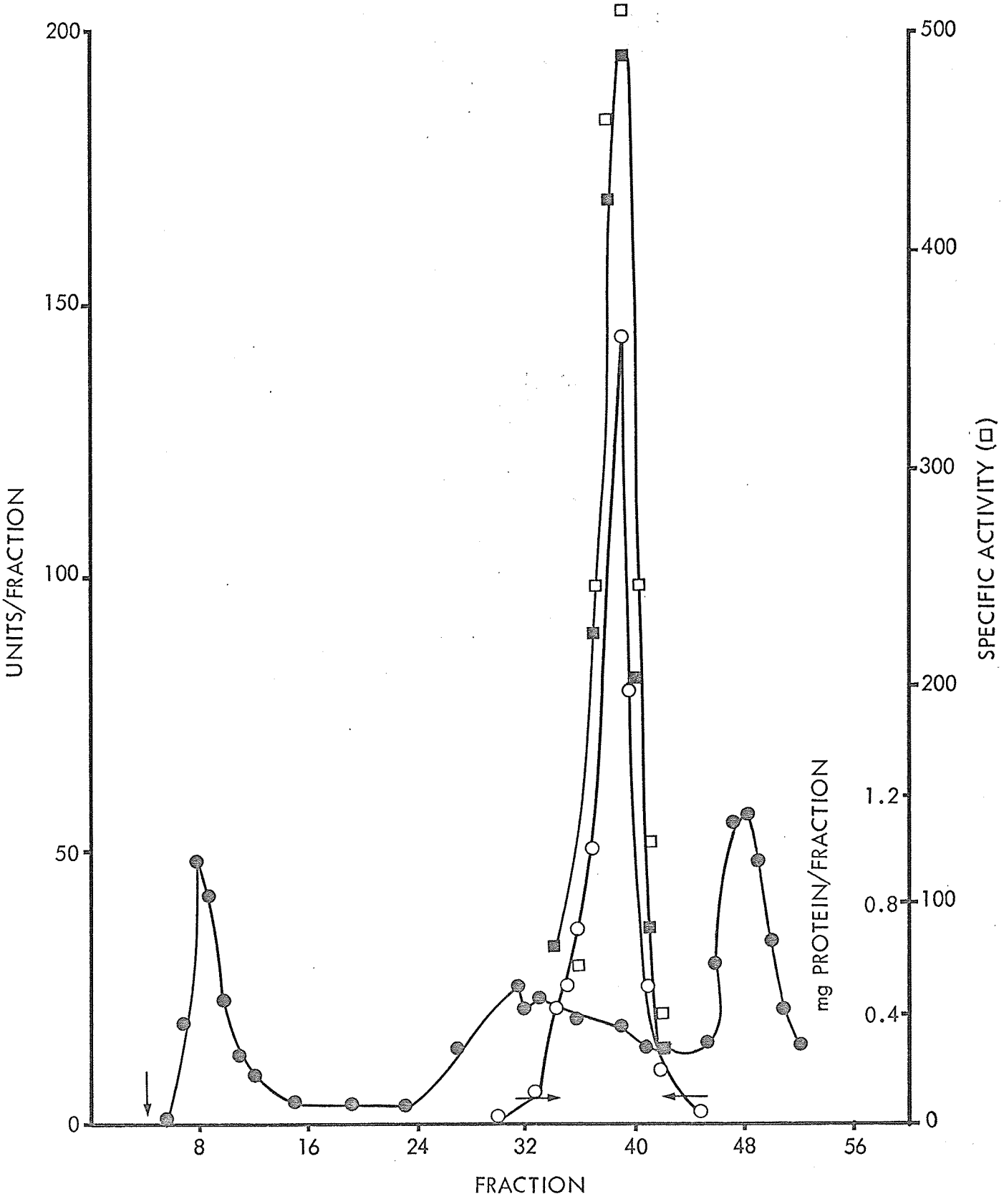

200
150
100
50
0
UNITS/FRACTION
500
400
300
200
100
0
SPECIFIC ACTIVITY (□)
1.2
0.8
0.4
mg PROTEIN/FRACTION
8
16
24
32
40
48
56
FRACTION

Figure 7. Time course of uridine cleavage and uridine synthesis by uridine phosphorylase.

For uridine cleavage (○——○) at pH 8.2, the incubation medium contained 0.1 M P_i, pH 7.4; 0.1 M glycylglycine, pH 8.7; 5 mM 2-mercaptoethanol; 3.3 mM uridine, pH 7.0; 9.8 μg of preparation A_f diluted to 0.5 ml with 0.05 M P_i, pH 7.0. Activity was assayed by procedure (ai). For uridine synthesis (●——●) at pH 8.14, the incubation medium contained 0.1 M glycylglycine, pH 8.4; 0.67 mM uracil; 2.9 mM ribose-1-phosphate, pH 7.0; 6 μg of fraction E_a. Activity was assayed by procedure (bii). Enzyme activity is given as the change in absorbance at 290 mμ. If only one experimental point is shown, it represents an average of at least two determinations; if, however, the duplicates did not agree, both readings are given.

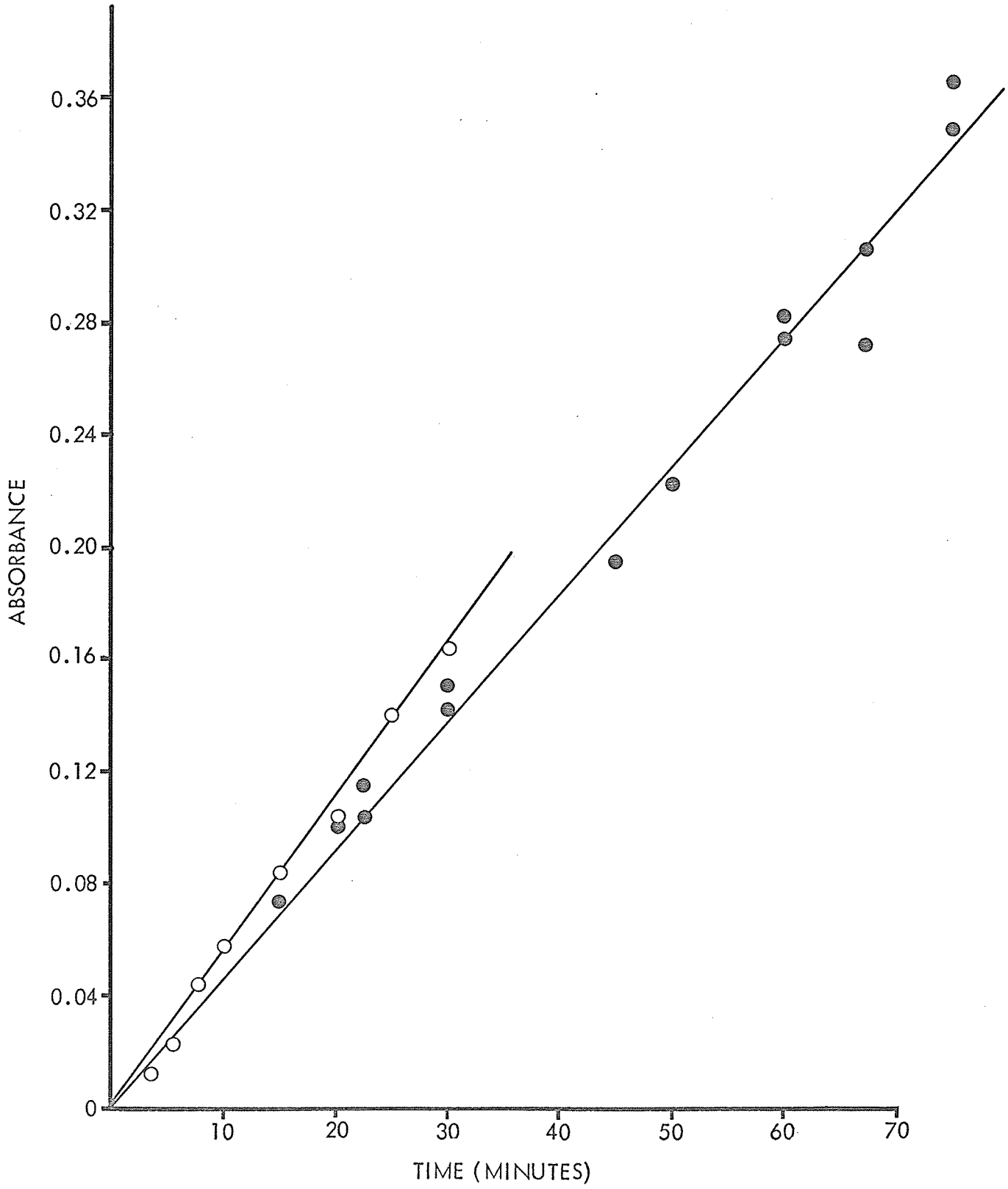

ABSORBANCE
0.36
0.32
0.28
0.24
0.20
0.16
0.12
0.08
0.04
0
10
20
30
40
50
60
70
TIME (MINUTES)

Figure 8. The relationship between protein concentration and enzyme activity.

The experimental conditions for uridine cleavage (O——O) were the same as those of Figure 7. Enzyme activity is shown as an increase in absorbance at 290 mμ. A microlitre of enzyme sample (preparation A_f) contained 0.98 μg of protein.

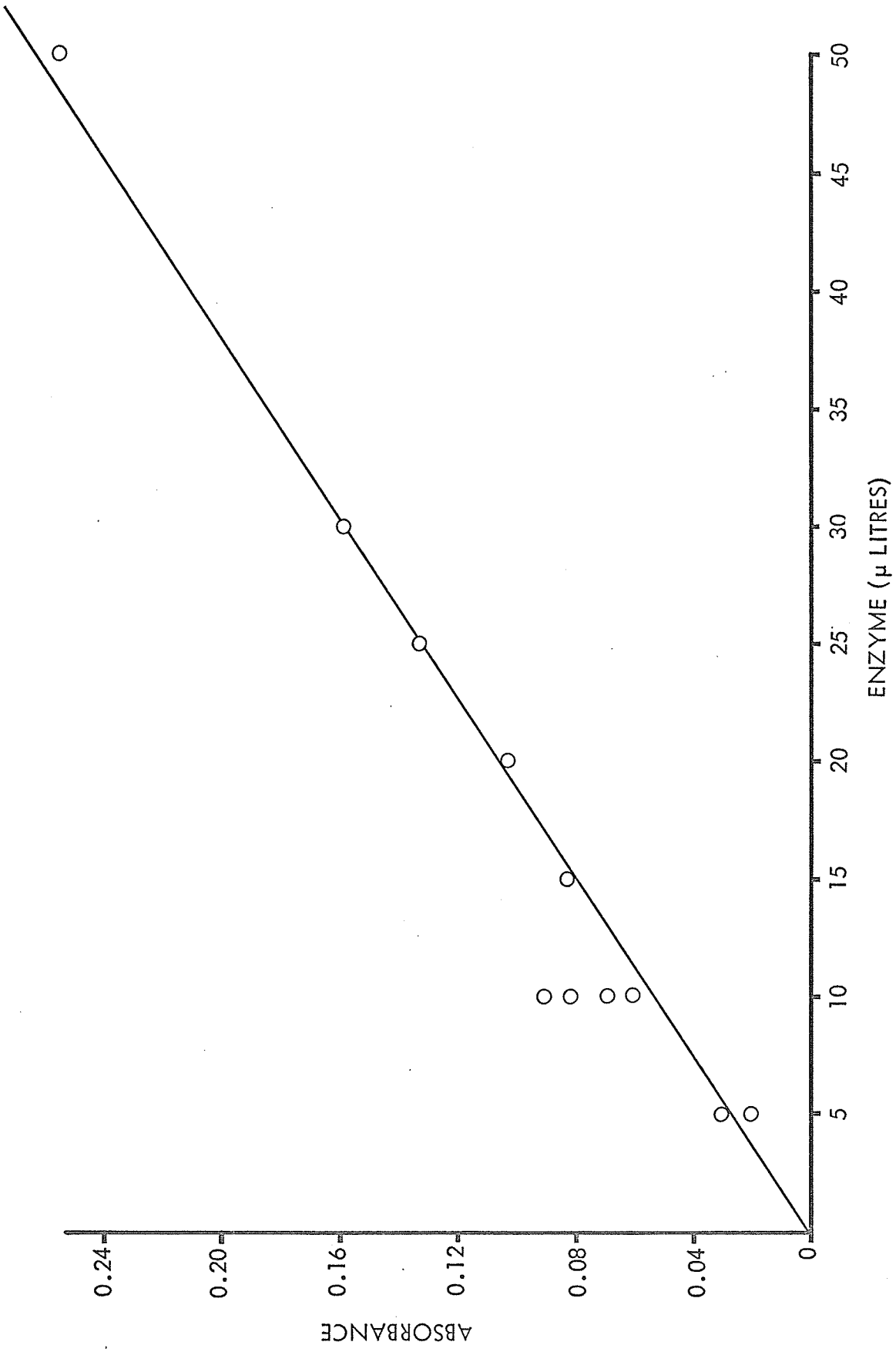

ABSORBANCE
0.24
0.20
0.16
0.12
0.08
0.04
0
5
10
15
20
25
30
35
40
45
50
ENZYME (μ LITRES)

Figure 9. Reactivation of uridine cleaving activity by 2-mercaptoethanol and dithiothreitol.

A-1. Effect of the time of preincubation on reactivation of uridine phosphorylase with 2-mercaptoethanol

Fraction E_a (aged for three months at -40°) was preincubated at room temperature with 50 mM 2-mercaptoethanol, for the times indicated, before either substrate was added to the reaction tube. Percent reactivation of uridine cleaving activity (●——●) was calculated from control readings in which 2-mercaptoethanol was omitted. The complete incubation medium contained 0.1 M Tris, pH 7.4; 0.16 mM glycylglycine, pH 8.9; 10 mM P_i, pH 7.0; 3.3 mM uridine, pH 7.0; 50 mM 2-mercaptoethanol; 35 μg of protein were diluted to 0.5 ml with 0.05 M Tris, pH 7.1. The final pH of the incubation mixture was 8.2. Activity was assayed by procedure (bi).

A-2. Effect of 2-mercaptoethanol concentration on reactivation of aged fraction E_a.

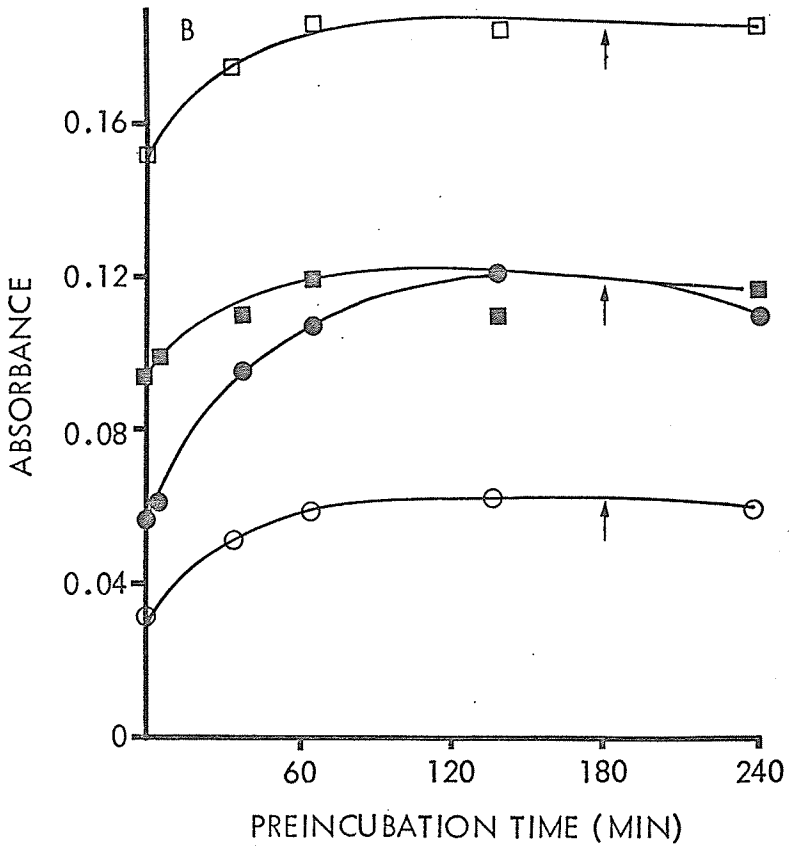
The enzyme fraction and 2-mercaptoethanol were preincubated for 120 minutes. Other conditions were the same as described in A-1.

B. Effect of dithiothreitol on uridine and deoxyuridine cleaving activities of fractions E_a and E_b

Activity is shown as an increase in absorbance at 290 mμ. Fraction E_b (11 μg of protein) was preincubated at the times indicated with 10 mM dithiothreitol and then assayed for uridine cleaving activity (●——●) or deoxyuridine cleaving activity (○——○). Fraction E_a (35 μg of protein) was preincubated with 10 mM dithiothreitol and then assayed for uridine cleaving activity (□——□) or decxyuridine cleaving activity (■——■). After 180 minutes, dithiothretiol was added (↑) to give a final concentration of about 20 mM. These fractions were aged for three months

Figure 9. (continued)

at -40° with intermittent freezing and thawing, and then dialyzed against 0.05 M Tris, pH 7.2-1 mM EDTA, pH 7.0 for 90 minutes. The incubation medium contained 0.1 M Tris, pH 7.4; 10 mM P_i, pH 7.0; 3.3 mM uridine or deoxyuridine, pH 7.0; enzyme diluted as in Figure 9 A-1. The final pH of the incubation mixture was 7.35. Activity was assayed by procedure (bi).


B
0.16
0.12
0.08
0.04
0
ABSORBANCE
60
120
180
240
PREINCUBATION TIME (MIN)

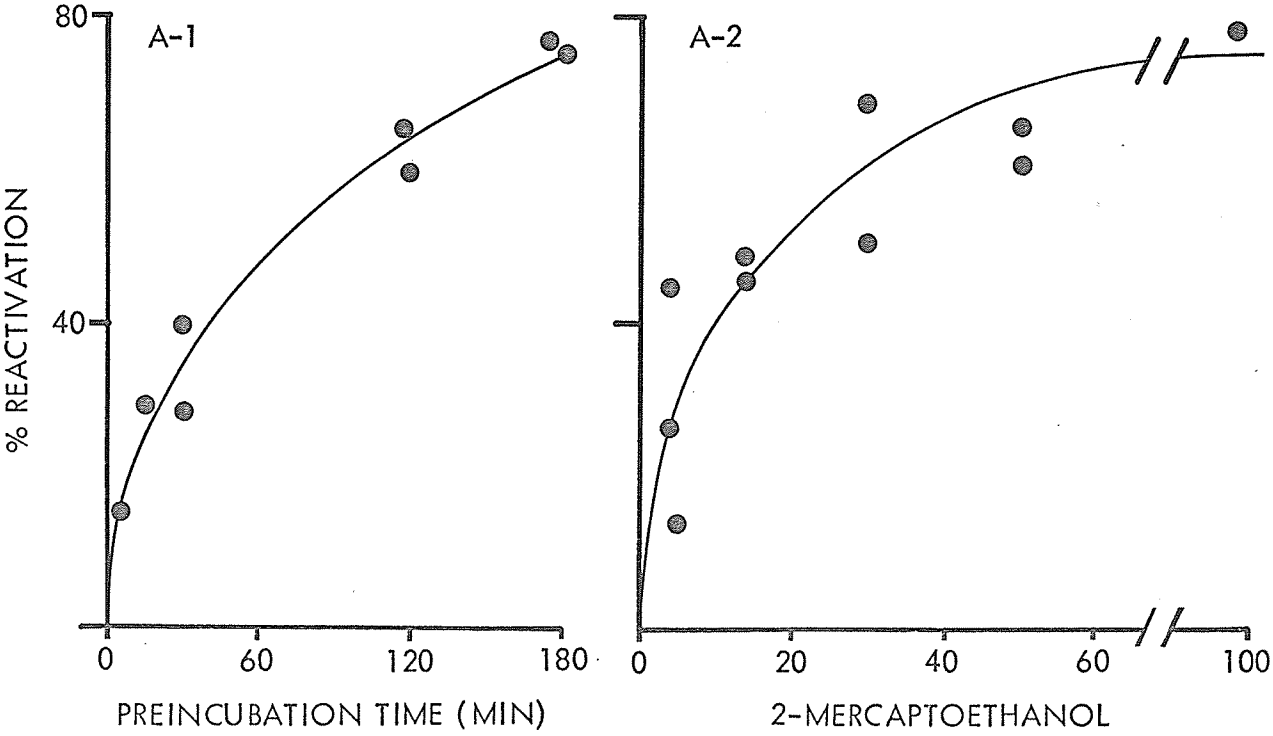

80
40
% REACTIVATION
A-1
0
60
120
180
PREINCUBATION TIME (MIN)
A-2
0
20
40
60
100
2-MERCAPTOETHANOL

Figure 10. Polyacrylamide gel electrophoresis of purified preparations of uridine phosphorylase.

Electrophoresis was carried out as described in section II C 7, with 7% acrylamide, glycine-phosphate buffer, pH 8.9, for 90 minutes, at 2 mA/tube. Preparation C_f was used for tubes 3-6, and preparation D_f was used for tubes 1 and 2. A major (A) and a minor (B) band were detected in each tube: ↓ , shows the direction of migration of the proteins; → , origin.

Tube	Stain	Protein applied (µg)	Age of enzyme preparation (weeks)
1	Amido black	50	1
2	Coomassie blue	50	1
3	Amido black	200	5
4	Amido black	50	5
5	Amido black	50	10
6	Coomassie blue	50	10

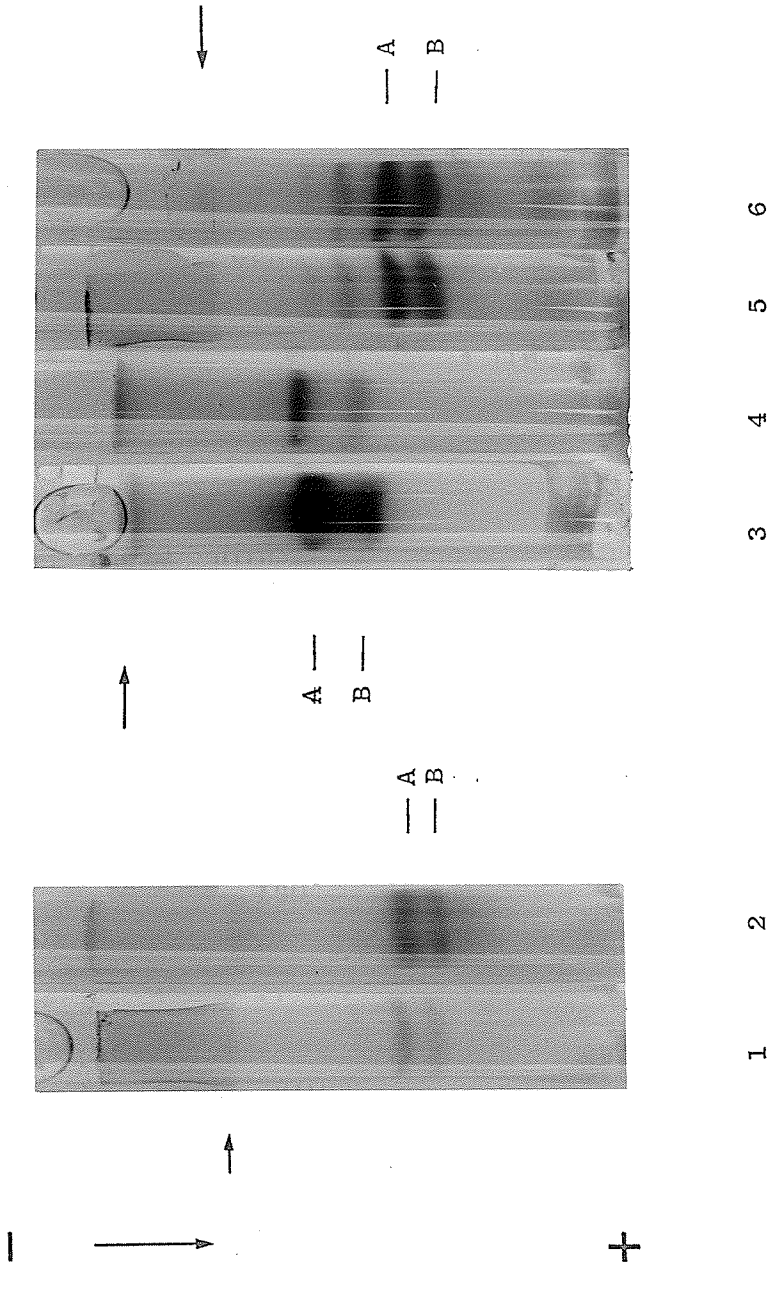

A
B
6
5
4
3
A
B
A
B
2
1
-
+

Figure 11. pH-Activity curve for uridine cleavage, deoxyuridine cleavage, and uridine synthesis.

Different pH values were obtained by using 100 mM Tris, 100 mM acetate, or 160 mM glycylglycine. Saturating concentrations of substrates were used: 3.3 mM uridine or deoxyuridine, 0.67 mM uracil, 0.11 M phosphate, 2.9 mM ribose-1-phosphate. These experiments were done after reactivation of fraction E_a (Table IV). For uridine and deoxyuridine cleavage, 35 μg of protein from fraction E_a were used, and for uridine synthesis 6 μg of protein from fraction E_a were used. Assay procedure (bi) was used for measuring uridine or deoxyuridine cleavage, and assay procedure (bii) was used for measuring uridine synthesis. The incubation period for uridine synthesis was 30 minutes. Other experimental conditions are described in section II C 1. —— , phosphorolysis of uridine; – – – , phosphorolysis of deoxyuridine; O—O , uridine synthesis; ●—● uridine cleavage (no phosphate added); X , deoxyuridine cleavage (no phosphate added). Buffers: □ , ■ , P_i-Ac; △ , ▲ , P_i; ● , P_i-Tris; O , G-G. For uridine synthesis the buffer was Tris (100 mM) below pH 8, and G-G (100 mM) above pH 8. For nucleoside cleavage the buffers are given in section II C 2d. ⊖ , enzyme preincubated in the absence of either substrate at pH 5.4 for 10 minutes, but assayed at pH 7.9 for uridine cleaving activity; ⊟ , enzyme preincubated in the absence of either substrate at pH 9.4 for 10 minutes, but assayed at pH 7.6 for uridine cleaving activity.

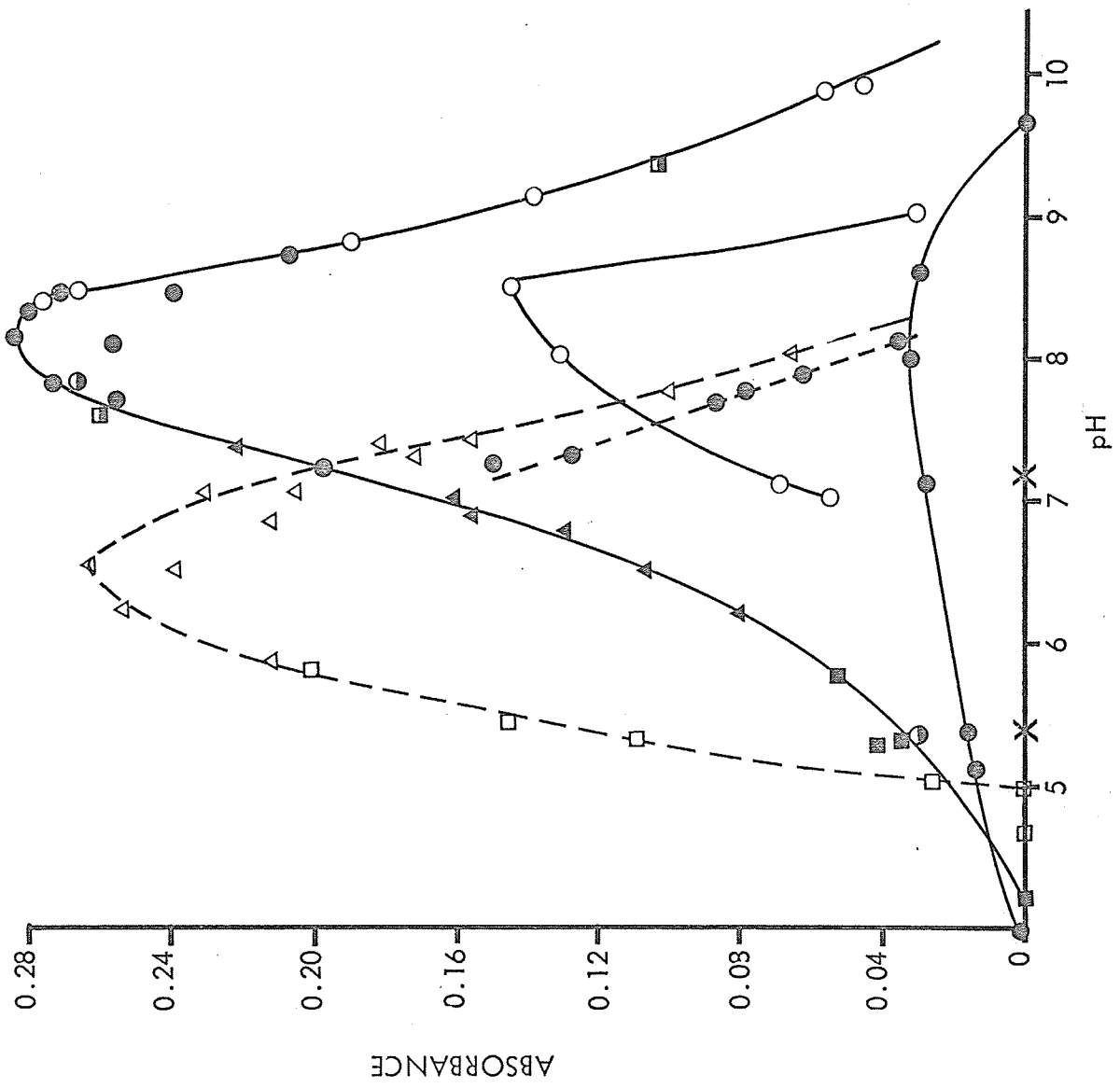

ABSORBANCE
0.28
0.24
0.20
0.16
0.12
0.08
0.04
0
5
6
7
8
9
10
pH

Figure 12. Molecular weight estimation of uridine phosphorylase and thymidine phosphorylase by Sephadex G-200.

Uridine and thymidine phosphorylase fractions from DEAE-Sephadex chromatography (Figure 1B) were used. Experimental conditions are described in section II C 6. Marker proteins were apoferritin (○), γ-globulin (▲), albumin (□), ovalbumin (△) and chymotrypsinogen (▣); X, uridine or thymidine phosphorylase. The profile of Blue dextran (●——●) was determined by monitoring the effluents spectrophotometrically at 290 mμ. With reference to the standard line, the horizontal axis indicates the fraction number in which maximum absorbance of protein occurred.

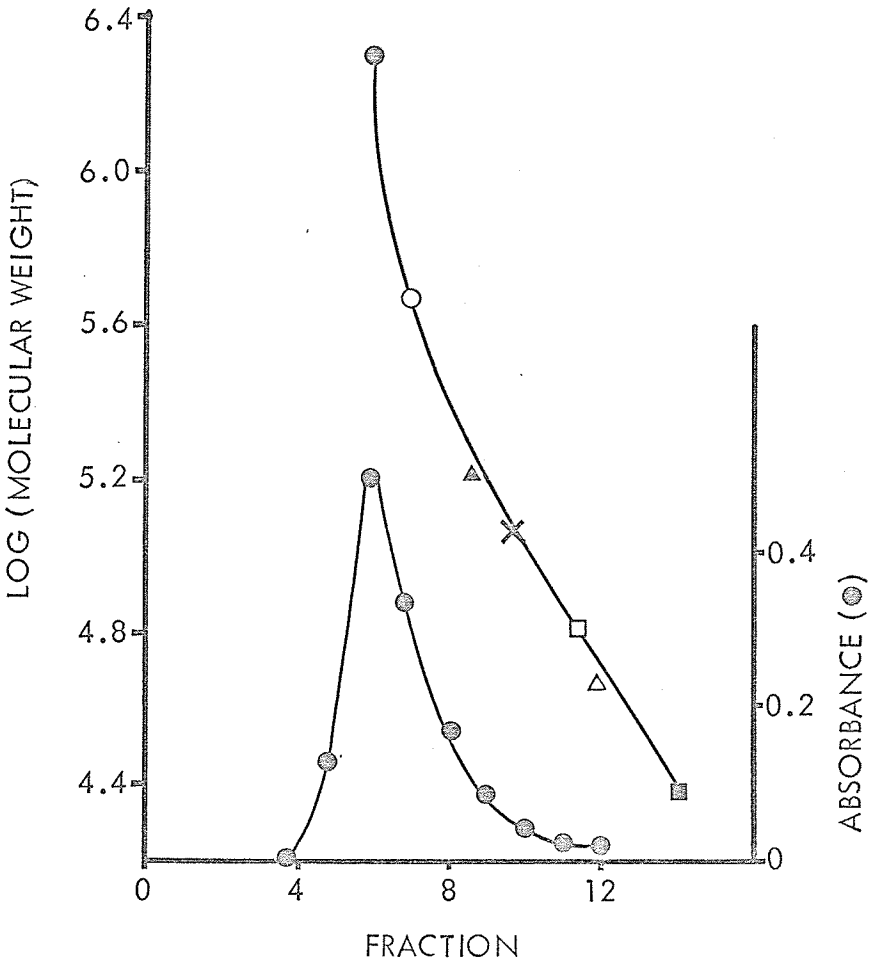

6.4
6.0
5.6
5.2
4.8
4.4
LOG (MOLECULAR WEIGHT)
0.4
0.2
0
ABSORBANCE (●)
0
4
8
12
FRACTION

Figure 13. Inhibition with deoxyglucosylthymine.

A. Inhibition of uridine cleavage (pH 8.1), deoxyuridine cleavage (pH 6.6), and uridine synthesis (pH 7.7) by deoxyglucosylthymine.

The incubation medium for uridine cleavage (O——O), contained 0.1 M Tris, pH 7.4; 0.16 mM glycylglycine, pH 8.9; 10 mM P_i, pH 7.0; 3.3 mM uridine, pH 7.0; 7 µg of protein from fraction E_b diluted to 0.2 ml with 0.05 M Tris, pH 7.2. The incubation medium for deoxyuridine cleavage (△——△) contained 100 mM acetate, pH 5.5; 100 mM Tris, pH 7.4; 10 mM P_i, pH 7.0; 7 µg of protein from fraction E_b diluted as described above. Nucleoside cleavage was assayed by procedure (bi). The incubation medium for uridine synthesis (□——□) contained 100 mM Tris, pH 7.8; 0.67 mM uracil; 1.4 mM ribose-1-phosphate; 0.64 µg of protein of fraction E_a (after reactivation). The incubation period for uridine synthesis was 65 minutes. Other experimental conditions for assay procedure (bii) are given in section II C 1. Deoxyglucosylthymine was preincubated for 5 minutes with the enzyme fraction at 25° before the addition of either substrate. A stock solution of 9.8 mM deoxyglucosylthymine in water was prepared.

B. Inhibition of uridine and deoxyuridine cleavage at pH 7.3 by deoxyglucosylthymine.

The incubation medium contained 100 mM Tris, pH 7.4; 10 mM P_i, pH 7.0; 3.3 mM uridine or deoxyuridine, pH 7.0; 7 µg of protein from fraction E_b diluted as described in Figure 13A. Assay procedure (bi) was used to assay enzyme activity: O——O , uridine cleavage (28.11.68); ● , uridine cleavage (8.12.68); △——△ , deoxyuridine cleavage (28.11.68). The control value for (8.12.68) was 20% lower than for (28.11.68).

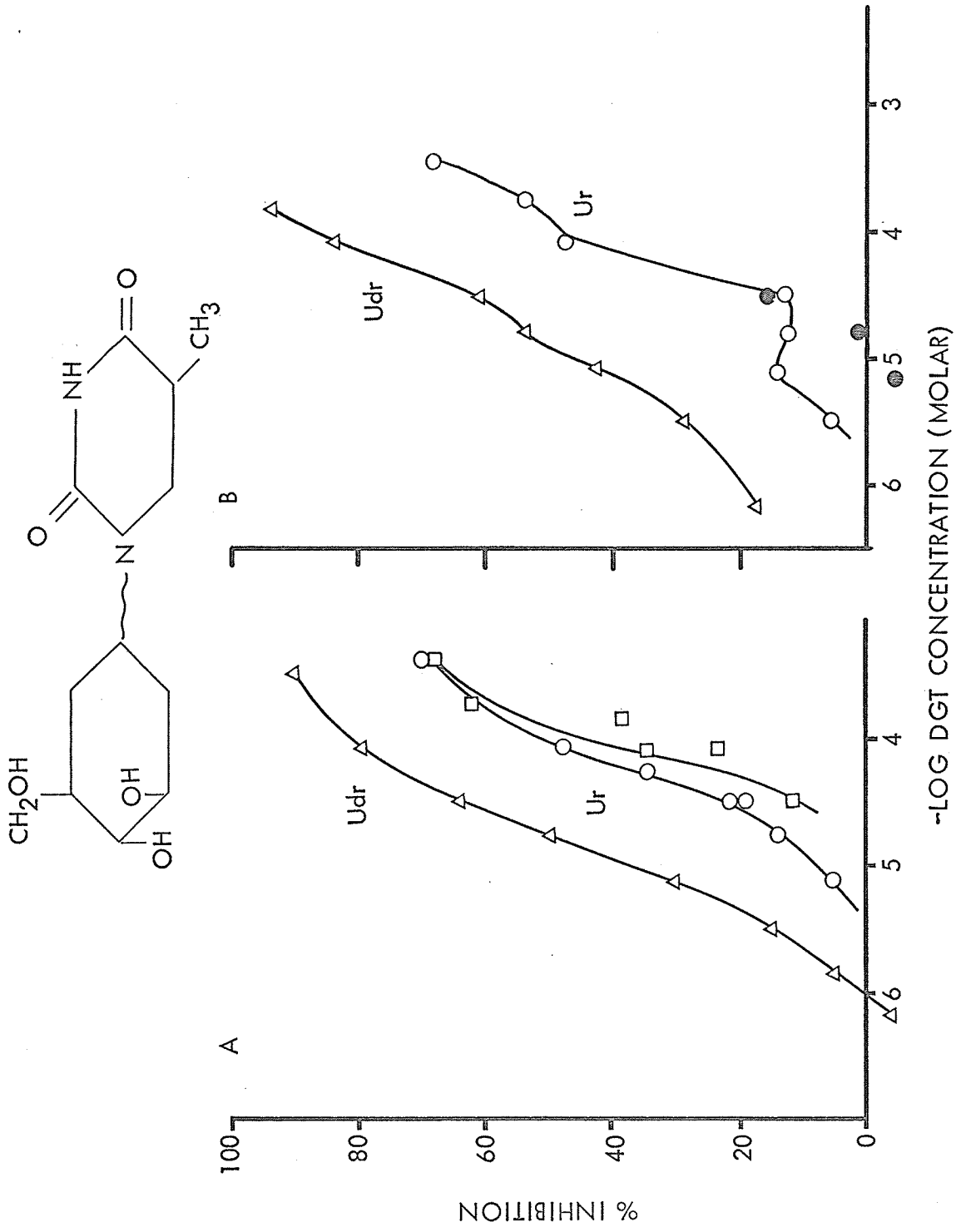

A
B
Udr
Ur
Udr
Ur
% INHIBITION
-LOG DGT CONCENTRATION (MOLAR)
100
80
60
40
20
0
3
4
5
6
NH
O
O
CH3
N
CH2OH
OH
OH

Figure 14. Uridine synthesis as a function of uracil concentration.

The incubation medium for uridine synthesis at pH 8.1 is similar to that of Figure 7. The reaction medium contained 2.2 μg of protein from preparation D_f (after reactivation). The incubation period was 65 minutes. Ribose-1-phosphate was held constant at concentrations of 1.43 mM (○——○) and 0.143 mM (●——●).

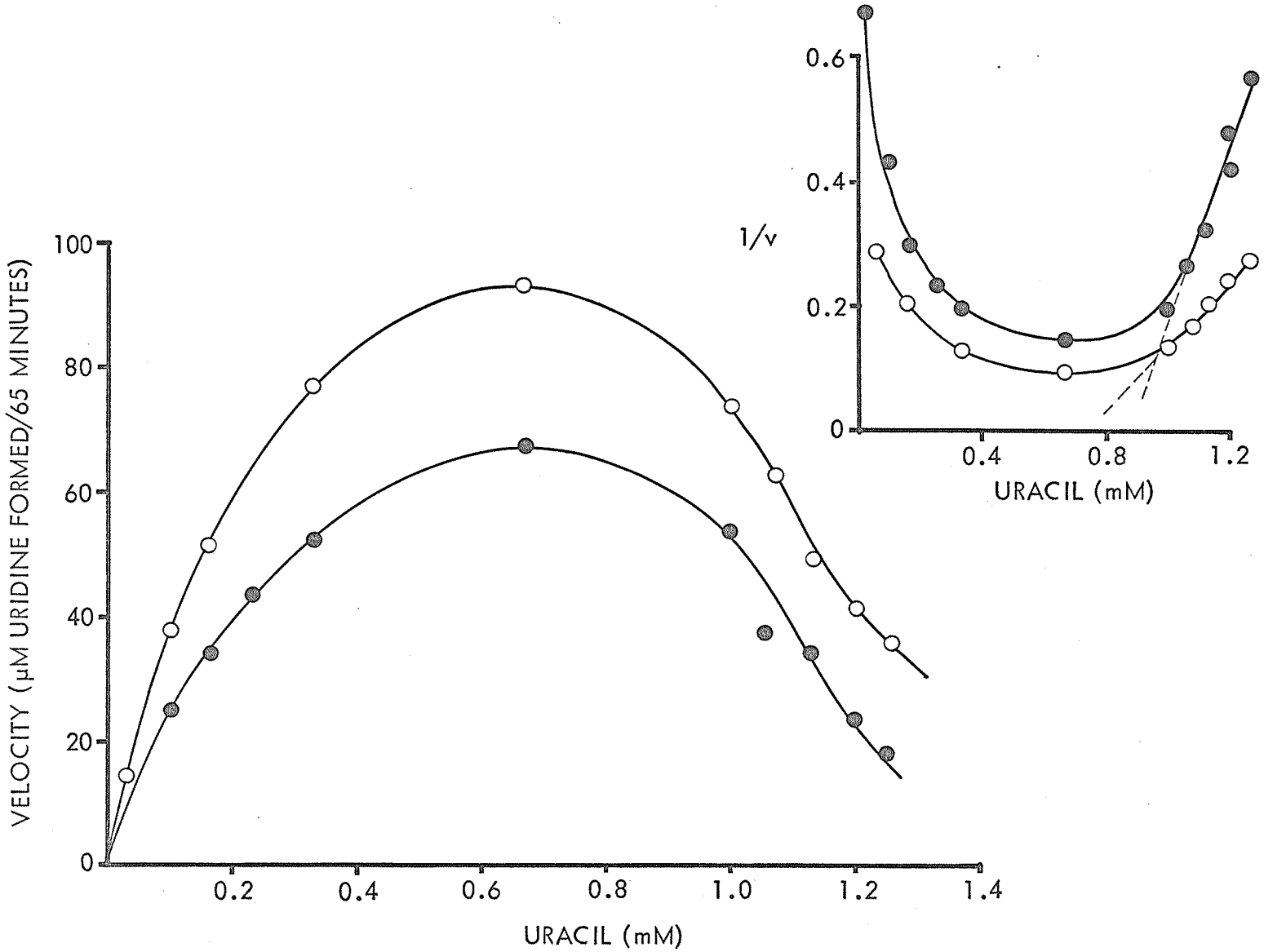

VELOCITY (μM URIDINE FORMED/65 MINUTES)
100
80
60
40
20
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
URACIL (mM)
1/v
0.6
0.4
0.2
0
0.4
0.8
1.2
URACIL (mM)

Figure 15. Inhibition of activity with p-mercuriphenylsulfonate.

The experimental procedure for these modification experiments is described in section III B. The reaction medium contained 7 μg of protein from fraction E_b. All assays were done in the direction of nucleoside cleavage in the presence of 3.3 mM uridine or deoxyuridine and 10 mM phosphate (assay procedure (bi)).

A. Time course of inhibition. An assay of uridine cleaving activity at pH 8.2 was performed after preincubation of the enzyme fraction with 0.65 μM pMPS in the absence of substrates for the times indicated.

B. Concentration study. The enzyme fraction and pMPS were preincubated in the reaction medium for 5 minutes before the addition of substrates. Uridine cleaving activity (O——O) was assayed at pH 8.2.

Protection experiments. After uridine phosphorylase and one substrate were preincubated for 5 minutes, 0.75 mμmoles pMPS were preincubated with the protected enzyme for 3 minutes: □ , uridine (0.63 μM pMPS); △ , phosphate (0.52 μM pMPS). The concentrations of inhibitor are corrected for different dilutions that occurred after the addition of phosphate or nucleoside.

C. Reversal of inhibition. After the enzyme fraction was preincubated with 0.65 μM pMPS for 1.5 minutes, 2-mercaptoethanol was added to the reaction medium. Assays of enzyme activity were done at the times indicated: ● , controls (no added 2-mercaptoethanol); ○ , 5 mM 2-mercaptoethanol.

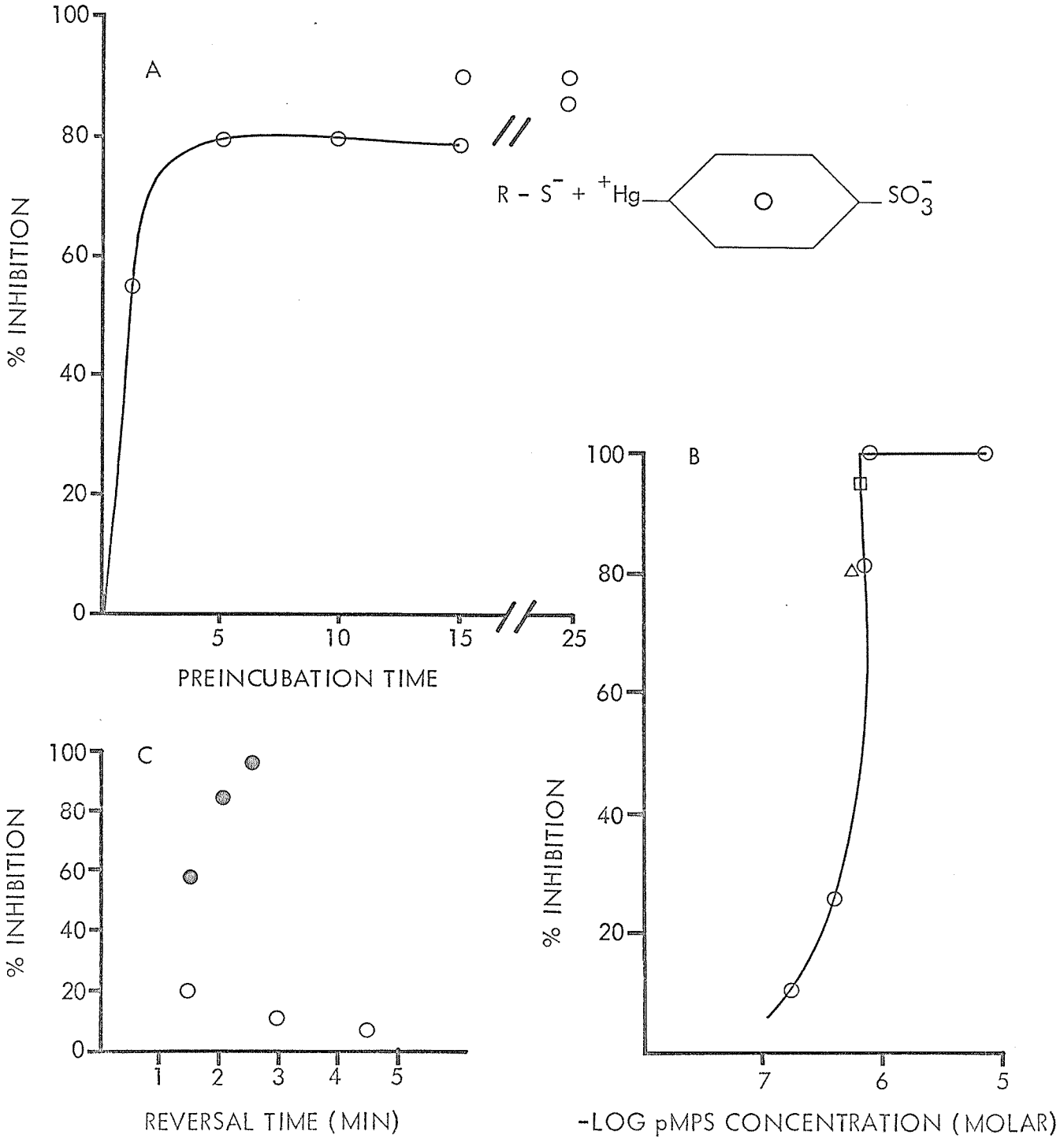

A
100
80
60
40
20
0
% INHIBITION
5
10
15
25
PREINCUBATION TIME
R – S⁻ + ⁺Hg
SO3⁻
B
100
80
60
40
20
% INHIBITION
7
6
5
-LOG pMPS CONCENTRATION (MOLAR)
C
100
80
60
40
20
0
% INHIBITION
1
2
3
4
5
REVERSAL TIME (MIN)

Figure 16. Inhibition of activity with p-mercuribenzoate.

A. The effect of concentration of pMB on uridine cleaving activity. Uridine phosphorylase and pMB were preincubated for 5 minutes before the addition of substrates at pH 7.4 (●—●), and at pH 8.2 (○—○). Other experimental conditions are described in Figure 15.

B. The effect of concentration of pMB on deoxyuridine cleaving activity. The experimental conditions are described in Figure 16. The reaction medium is described in section III B for pH 7.4 (▲—▲), and for pH 6.7 (△—△). Protection experiment. After uridine phosphorylase and one substrate were pre-incubated for 5 minutes at pH 6.7, 0.75 mμmoles pMB were preincubated with the protected enzyme for an additional 5 minutes: □ , phosphate (0.52 μM pMB); ■ , deoxyuridine (0.63 μM pMB).

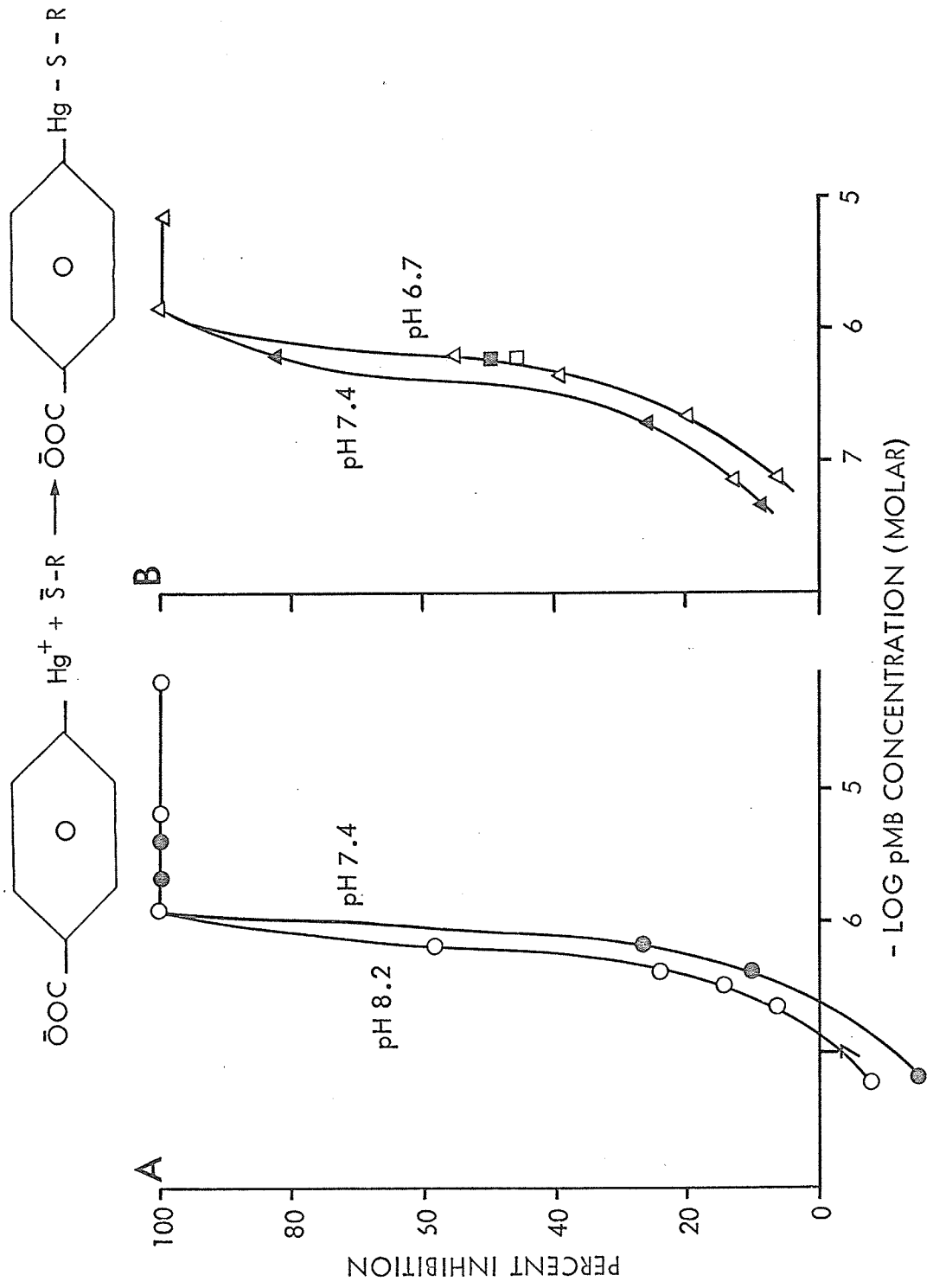

ŌOC
O
Hg+ + S̄-R
Hg - S - R
ŌOC
O
A
B
pH 8.2
pH 7.4
pH 7.4
pH 6.7
PERCENT INHIBITION
100
80
50
40
20
0
5
6
7
5
6
- LOG pMB CONCENTRATION (MOLAR)

Figure 17. Inhibition of activity with 5,5' dithiobis(2-nitrobenzoic acid).

A. Time course of inhibition. An assay for uridine cleaving activity at pH 8.2 was performed after preincubation of uridine phosphorylase with 6.52 μM DTNB (■—■), 4.35 μM DTNB (○—○), and 1.30 μM DTNB (●—●), in the absence of substrates. Other experimental conditions are described in section III B.

B. The effect of concentration of DTNB on uridine and deoxyuridine cleaving activities. After uridine phosphorylase and DTNB were preincubated in the absence of substrates for 5 minutes, an assay was performed for uridine cleaving activity at pH 8.2 (▲—▲) or deoxyuridine cleaving activity at pH 6.7 (△—△). After a 30 minute preincubation period an assay was done for uridine cleaving activity at pH 8.2 (○—○). Protection experiments. After uridine phosphorylase and uridine (□) or phosphate (●) were preincubated at pH 8.2, or after deoxyuridine (□) or phosphate (●) were preincubated at pH 6.7 for 5 minutes, 5.0 mμmoles DTNB were preincubated with the protected enzyme for an additional 5 minutes. Also, after uridine phosphorylase and uridine (□) or phosphate (●) were preincubated for 5 minutes at pH 8.2, 1.5 mμmoles DTNB were preincubated with the enzyme for an additional 30 minutes. Reversal of inhibition experiment. After uridine phosphorylase and 1.3 μM DTNB were preincubated for 30 minutes at pH 8.2, in the absence of substrates, 2-mercaptoethanol at a final concentration of 5 mM (⊖) was added to the reaction mixture for an additional 15 minute preincubation period (see section III B 4).

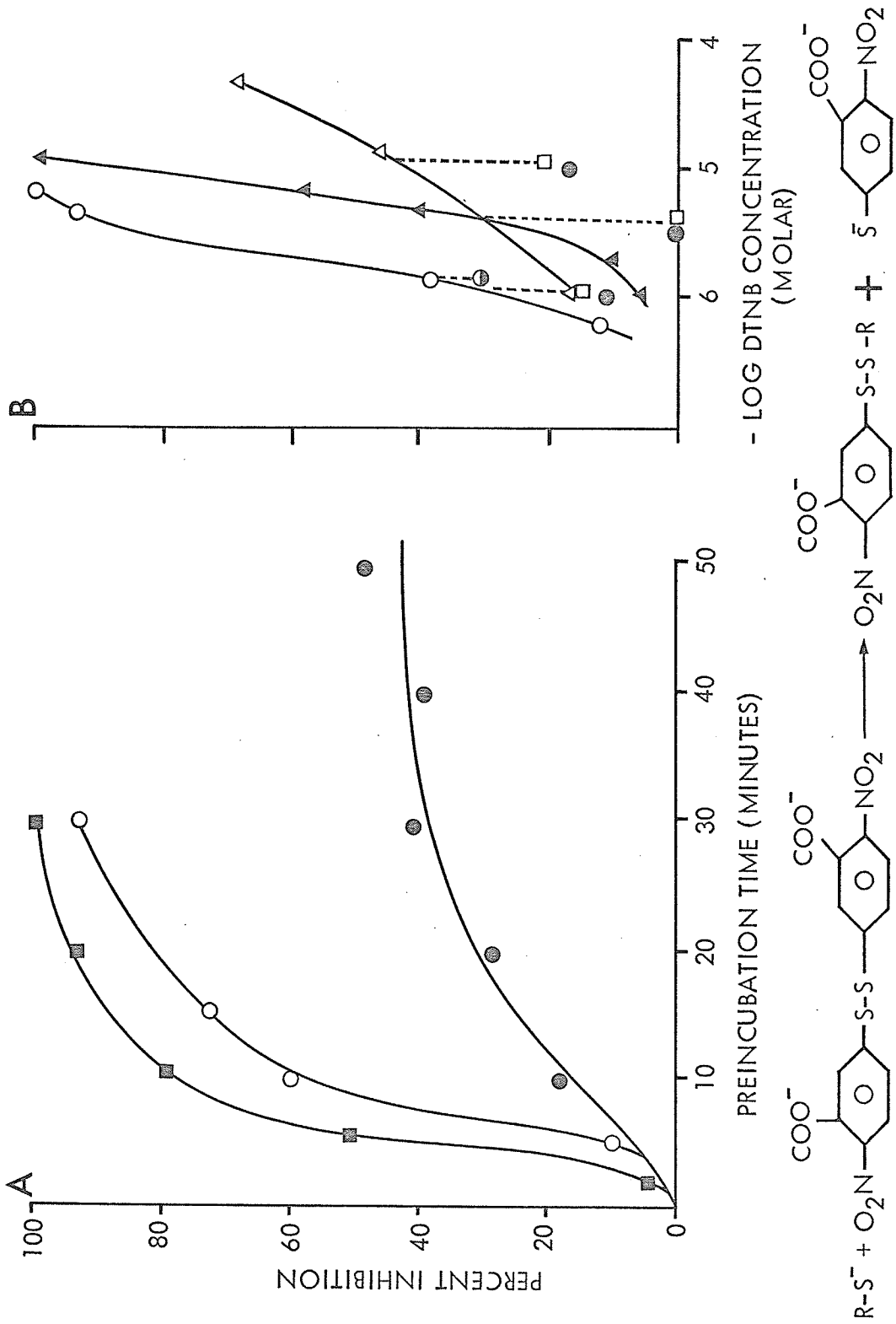

A
B
PERCENT INHIBITION
PREINCUBATION TIME (MINUTES)
- LOG DTNB CONCENTRATION (MOLAR)
R-S⁻ + O2N
COO⁻
S-S
NO2
S-S-R
S⁻

Figure 18. Inhibition of activity with o-iodosobenzoate.

A. Time course of inhibition. An assay for uridine cleaving activity at pH 8.2 was performed after preincubation, in the absence of substrates, of uridine phosphorylase and 3.25 μM IOB (●——●) or 2.16 μM IOB (○——○). Other experimental conditions are described in section III B.

B. The effect of concentration of IOB on uridine cleaving activity. Uridine phosphorylase and IOB were preincubated for 30 minutes in the absence of substrates before an assay was performed for uridine cleaving activity at pH 8.1 (○——○) and at pH 7.3 (▲——▲). Protection experiment. After uridine phosphorylase and a substrate were preincubated for 5 minutes, 3.8 mμmoles of IOB were preincubated with the protected enzyme for an additional 30 minutes. Protection is shown with phosphate at pH 8.1 (●), and at pH 7.3 (△); with uridine at pH 8.1 (■), and at pH 7.3 (□). Reversal experiment. After uridine phosphorylase and 3.3 μM IOB were preincubated for 15 minutes at pH 8.1, 2-mercaptoethanol at a final concentration of 5 mM (⊖) was preincubated with the inhibited enzyme for an additional 15 minutes. Other experimental conditions are described in section III B.

C. The effect of concentration of IOB on deoxyuridine cleaving activity. Uridine phosphorylase and IOB were preincubated for 30 minutes at pH 6.6 before the addition of substrates (△——△). Protection experiment. As described in Figure 18B but with deoxyuridine (■) or phosphate (●) as protectors.

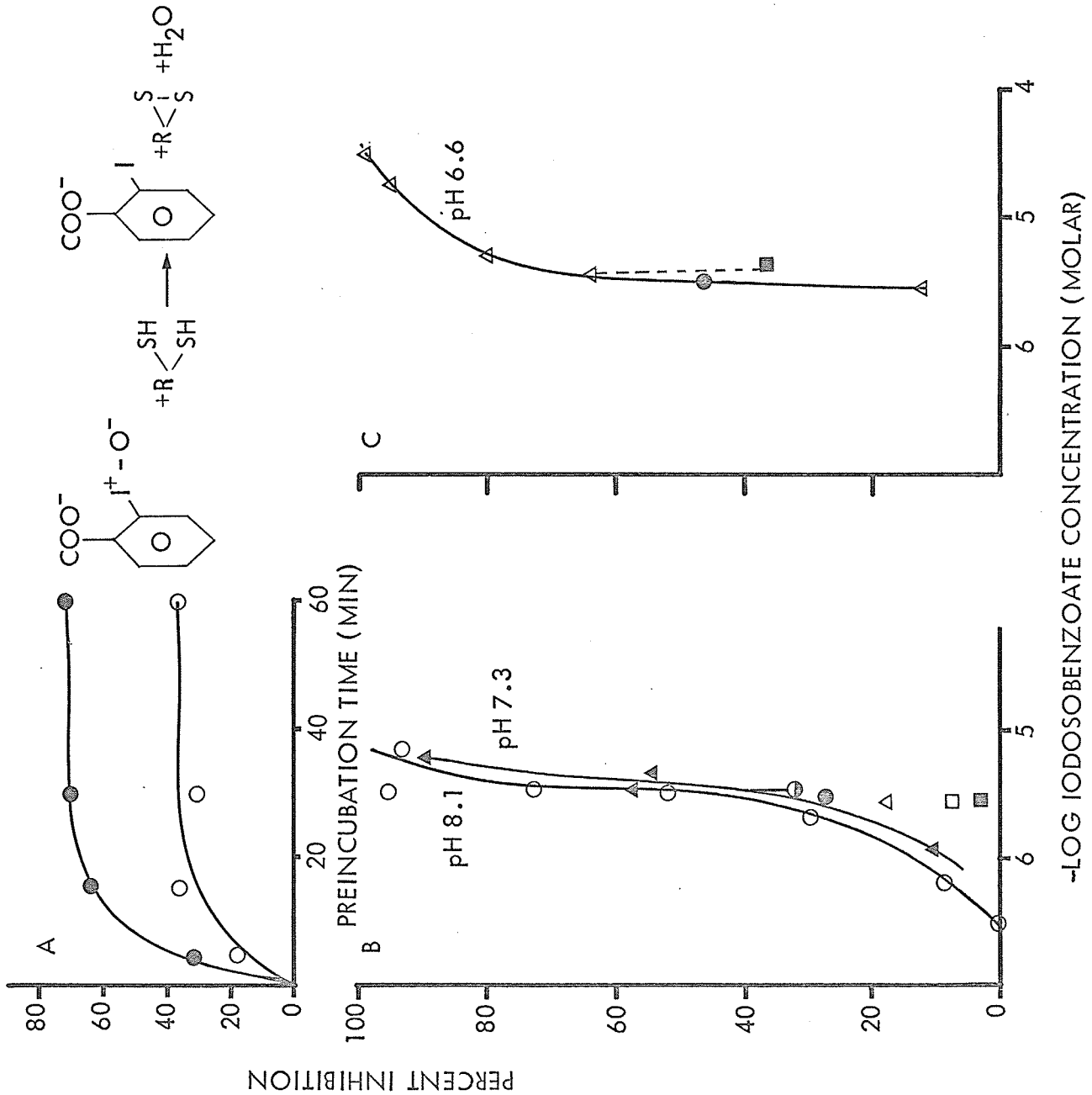

PERCENT INHIBITION
A
B
C
PREINCUBATION TIME (MIN)
-LOG IODOSOBENZOATE CONCENTRATION (MOLAR)
pH 8.1
pH 7.3
pH 6.6
COO⁻
I⁺-O⁻
+R SH SH
+H2O

Figure 19. Inhibition of activity with N-ethylmaleimide.

A. Time course of inhibition. An assay for uridine cleaving activity at pH 8.2 was performed after preincubation of uridine phosphorylase and 65.2 μM NEM, in the absence of substrates.

B. The effect of concentration of NEM on uridine cleaving activity. Uridine phosphorylase and NEM werepreincubated for 5 minutes, in the absence of substrates, before an assay was performed at pH 8.2 (○——○) and at pH 7.4 (▲——▲). Protection experiment. After uridine phosphorylase and a substrate were preincubated for 5 minutes, 0.15 μmoles of NEM were preincubated with the protected enzyme for an additional 5 minutes. Protection is shown with uridine (■) or with phosphate (●) at pH 8.2.

C. The effect of concentration of NEM on deoxyuridine cleaving activity. This study was performed as described above but at pH 6.5 (△——△), and pH 7.4 (▲——▲). Protection experiment. After uridine phosphorylase and a substrate were preincubated for 5 minutes, 1.5 μmoles NEM were preincubated with the protected enzyme for an additional 5 minutes. Protection is shown with deoxyuridine (■) or with phosphate (●) at pH 6.5.

Other experimental conditions are described in section III B.

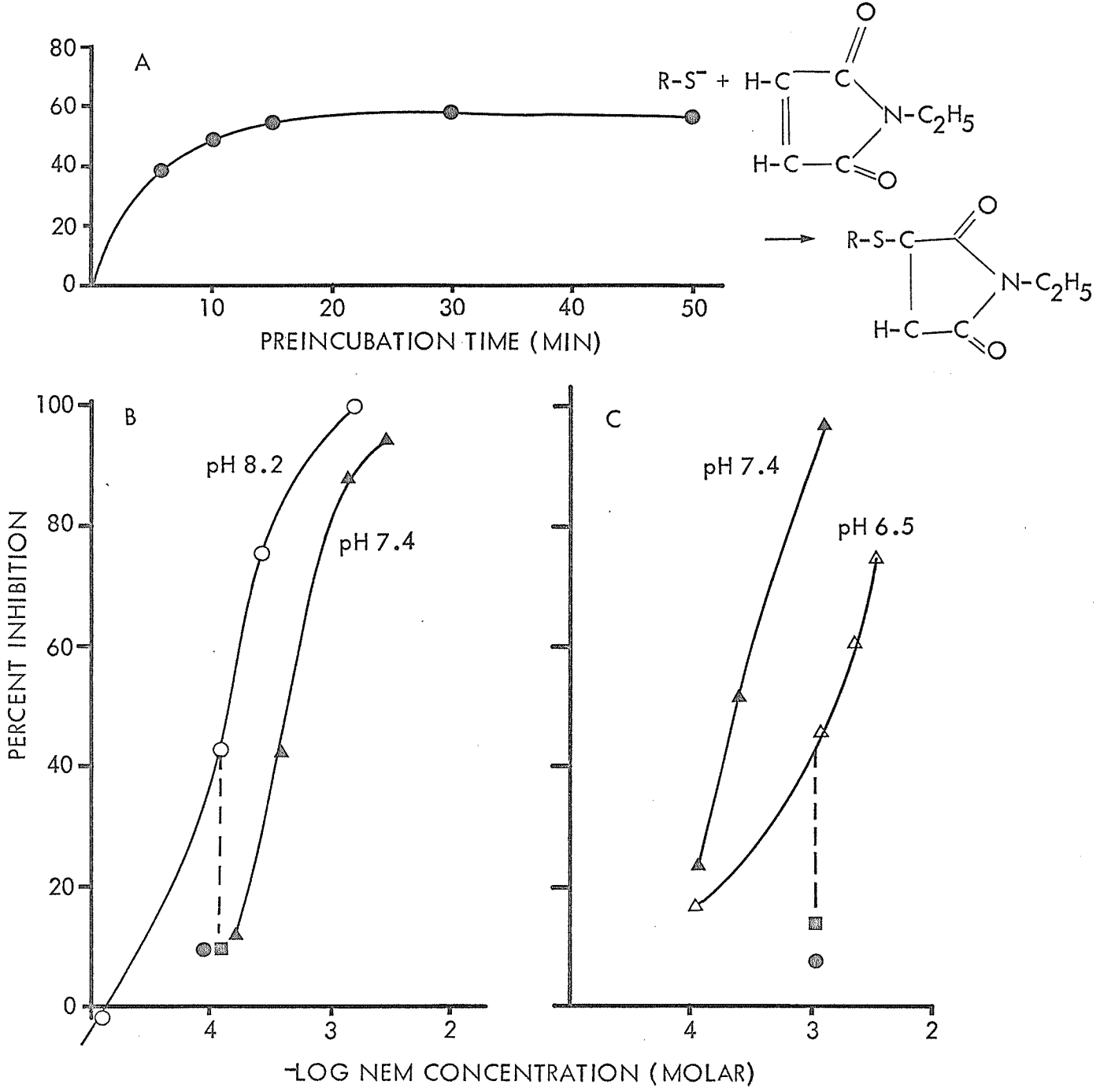

A
80
60
40
20
0
10
20
30
40
50
PREINCUBATION TIME (MIN)
R-S⁻ + H-C
C
O
N-C2H5
H-C
C
O
R-S-C
O
N-C2H5
H-C
C
O
B
100
80
60
40
20
0
pH 8.2
pH 7.4
PERCENT INHIBITION
4
3
2
C
pH 7.4
pH 6.5
4
3
2
-LOG NEM CONCENTRATION (MOLAR)

Figure 20. Inhibition of activity with iodoacetamide.

A. Time course of inhibition. An assay for uridine cleaving activity at pH 8.2 was performed after preincubation, in the absence of substrates, of uridine phosphorylase and 6.5 mM INH_2 (▲——▲) or 13.0 mM INH_2 (●——●). Reversal of inhibition experiment. After the enzyme fraction and 16.5 mM INH_2 were preincubated for 20 minutes, 2-mercaptoethanol (■), at a final concentration of 5 mM, was preincubated with the inhibited enzyme for an additional 20 minutes.

B. The effect of concentration of iodoacetamide on uridine cleaving activity. Uridine phosphorylase and iodoacetamide were preincubated for 60 minutes, in the absence of substrates, before performing an assay for uridine cleaving activity at pH 7.3 (●——●) or at pH 8.1 (○——○). Protection experiments. After the enzyme fraction and a substrate were preincubated for 5 minutes, 2.5 μmoles iodoacetamide were preincubated with the protected enzyme for an additional 60 minutes. Protection is shown with uridine (■) or with phosphate (▲) at pH 8.1.

Other experimental conditions are described in section III B.

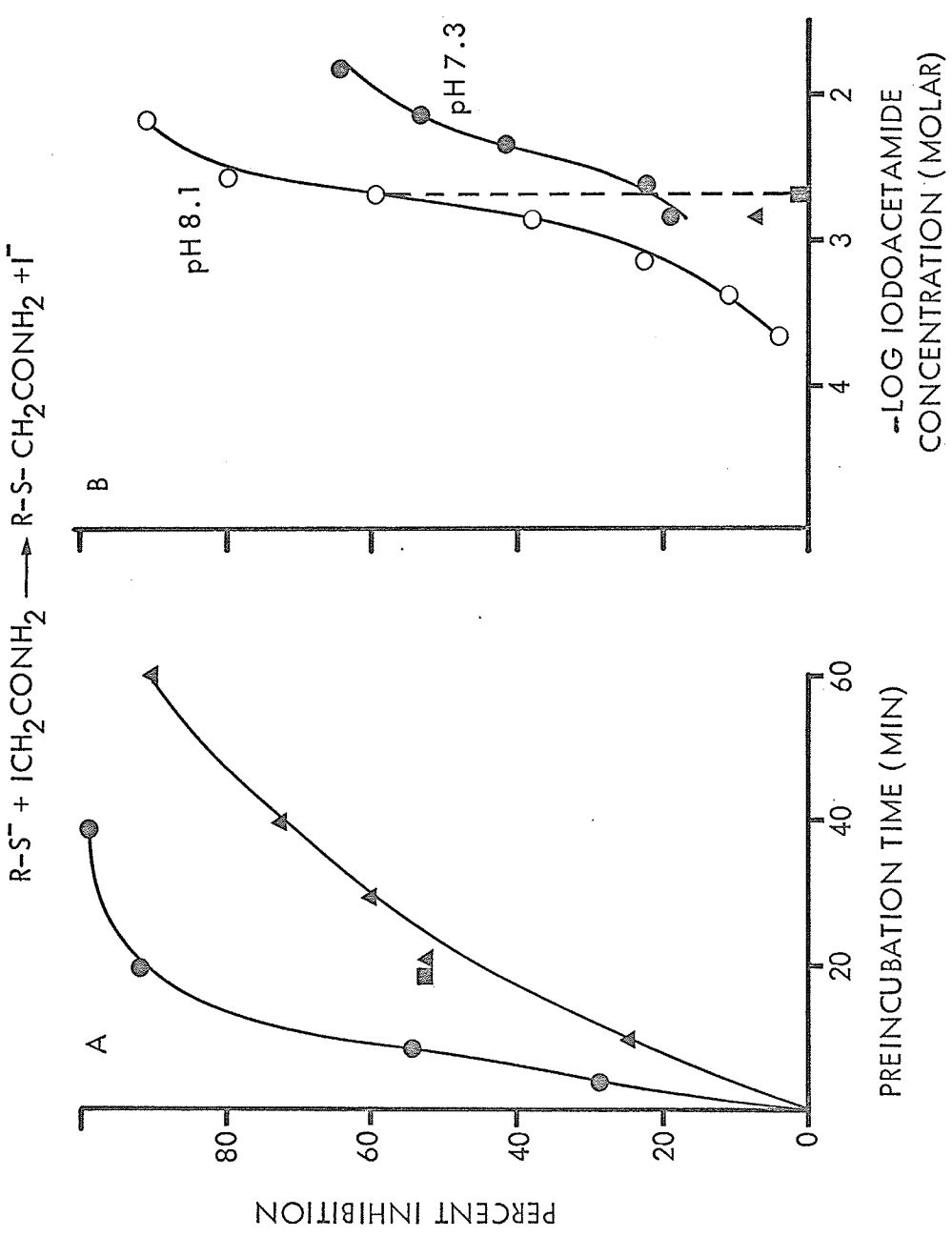

R-S⁻ + ICH2CONH2 ⟶ R-S- CH2CONH2 +I⁻
A
B
pH 8.1
pH 7.3
PERCENT INHIBITION
0
20
40
60
80
PREINCUBATION TIME (MIN)
-LOG IODOACETAMIDE CONCENTRATION (MOLAR)
2
3
4

Figure 21. Inhibition of activity with iodoacetate.

A. Time course of inhibition. An assay of uridine cleaving activity at pH 8.2 was performed after preincubation, in the absence of substrates, of uridine phosphorylase and 6.5 mM IA (●——●) and 13.0 mM IA (▲——▲). Protection experiment. After the enzyme fraction and a substrate were preincubated for 5 minutes at pH 8.2, 15 μmoles of IA were preincubated with the protected enzyme for an additional 60 minutes. Protection is shown with uridine (■) or phosphate (□). Reversal of inhibition experiment. After the enzyme fraction and 13 mM IA were preincubated for either 30 or 60 minutes, 2-mercaptoethanol was preincubated with the inhibited enzyme for an additional 30 minutes at a final concentration of 5 mM (△) or 50 mM (○), respectively.

B. The effect of concentration of iodoacetate on uridine and deoxyuridine cleaving activities. Uridine phosphorylase and iodoacetate were preincubated for 120 minutes in the absence of substrates before an assay was performed for uridine cleaving activity at pH 8.2 (○——○) or at pH 7.4 (●——●), and for deoxyuridine cleaving activity at pH 6.7 (△——△).

Other experimental conditions are described in section III B.

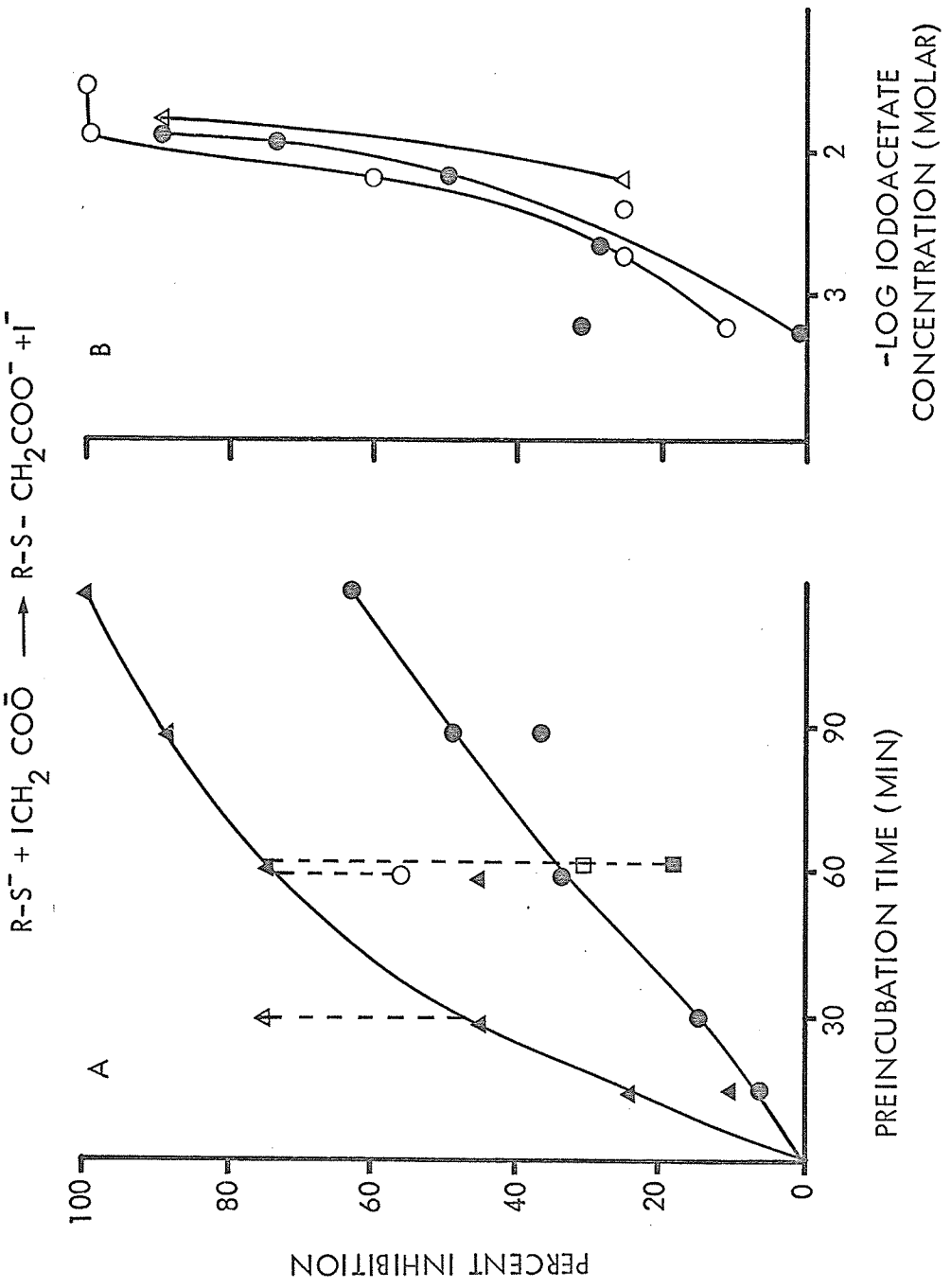
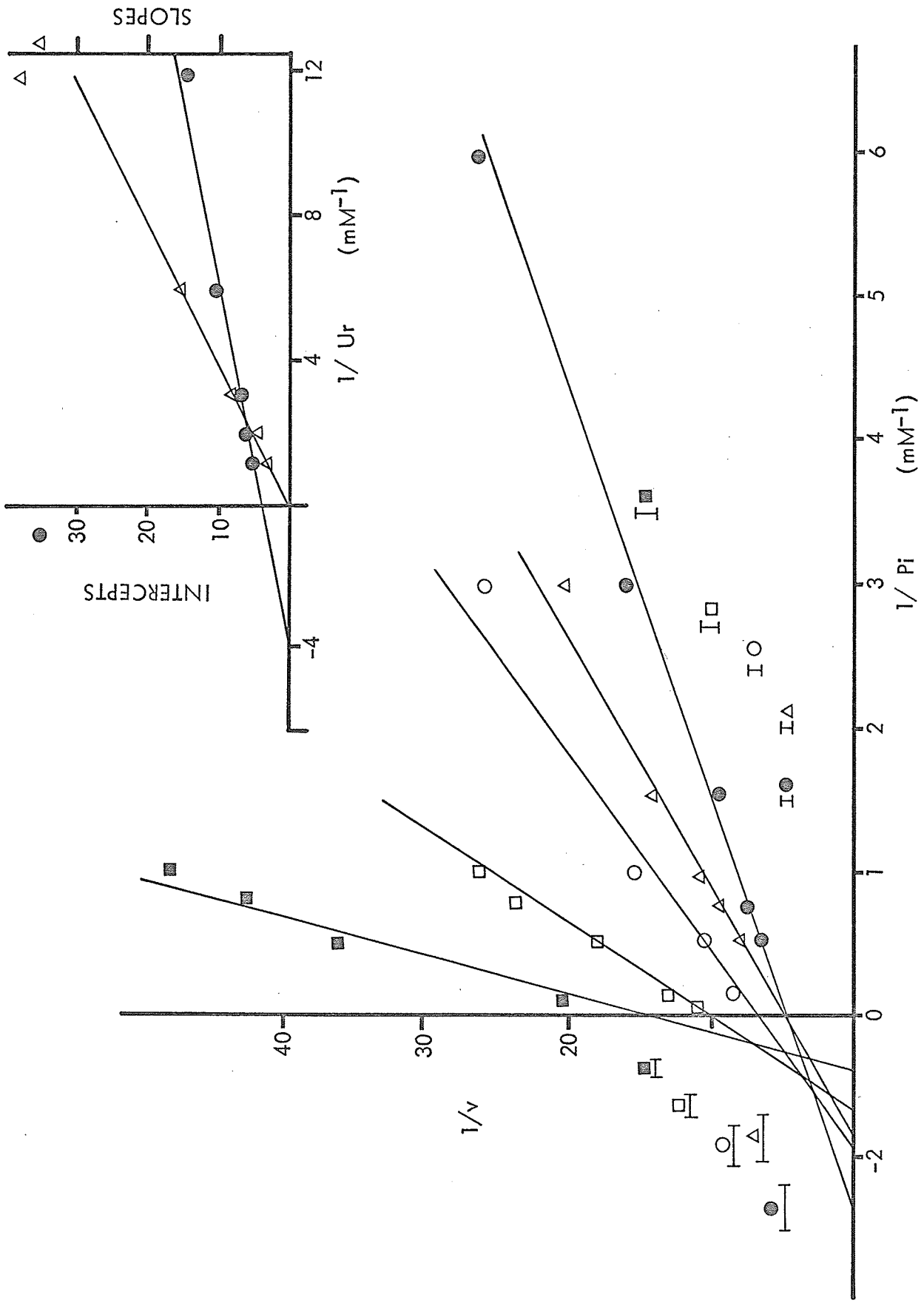

R-S⁻ + ICH2 COO⁻ ⟶ R-S- CH2COO⁻ +I⁻
A
B
PERCENT INHIBITION
100
80
60
40
20
0
30
60
90
PREINCUBATION TIME (MIN)
2
3
-LOG IODOACETATE
CONCENTRATION (MOLAR)

Figure 22. Double reciprocal plots of initial velocity against phosphate concentration.

The experimental procedure for the assay of uridine cleaving activity at pH 8.1 with fraction E_b (7 to 57 μg) is described in section IV B 1. Uridine was held constant at concentrations of 1.67 mM (●), 0.500 mM (△), 0.333 mM (○), 0.167 mM (□), and 0.0889 mM (■). The insert shows the replot of slopes (△ , K/V) and intercepts (● , 1/V) against the reciprocal of uridine concentration. Velocity (v) = uracil (mMolar) produced/ tube/10 minutes/14 μg protein. The errors in the estimation of apparent V_M values are given as plus or minus one standard error (I). Similarly, the errors in the estimation of apparent K_m are given as ± S.E. (⊢⊣).

Figure 23. Double reciprocal plots of initial velocity against uridine concentration.

Phosphate was held constant at concentrations of 6.67 mM (●), 1.99 mM (△), 0.999 mM (■), and 0.333 mM (○). The insert shows the replot of slopes (△) and intercepts (●) against the reciprocal of phosphate concentration. Other conditions are the same as in Figure 22. Some of the velocity values which were not included in data processing for Figures 22-36 are indicated by the double symbols.


SLOPES
INTERCEPTS
1/ Ur (mM⁻¹)
1/v
1/ Pi (mM⁻¹)

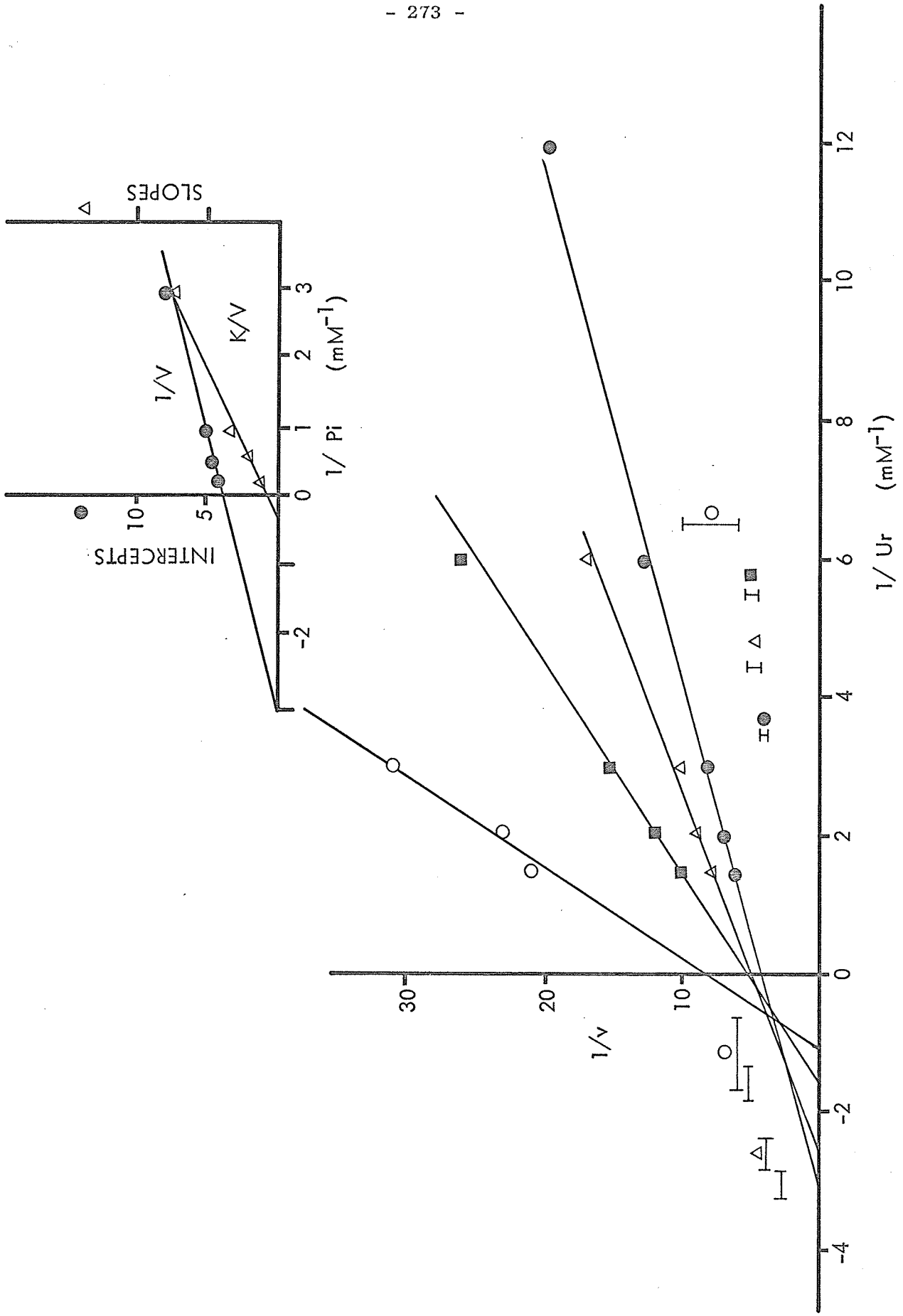
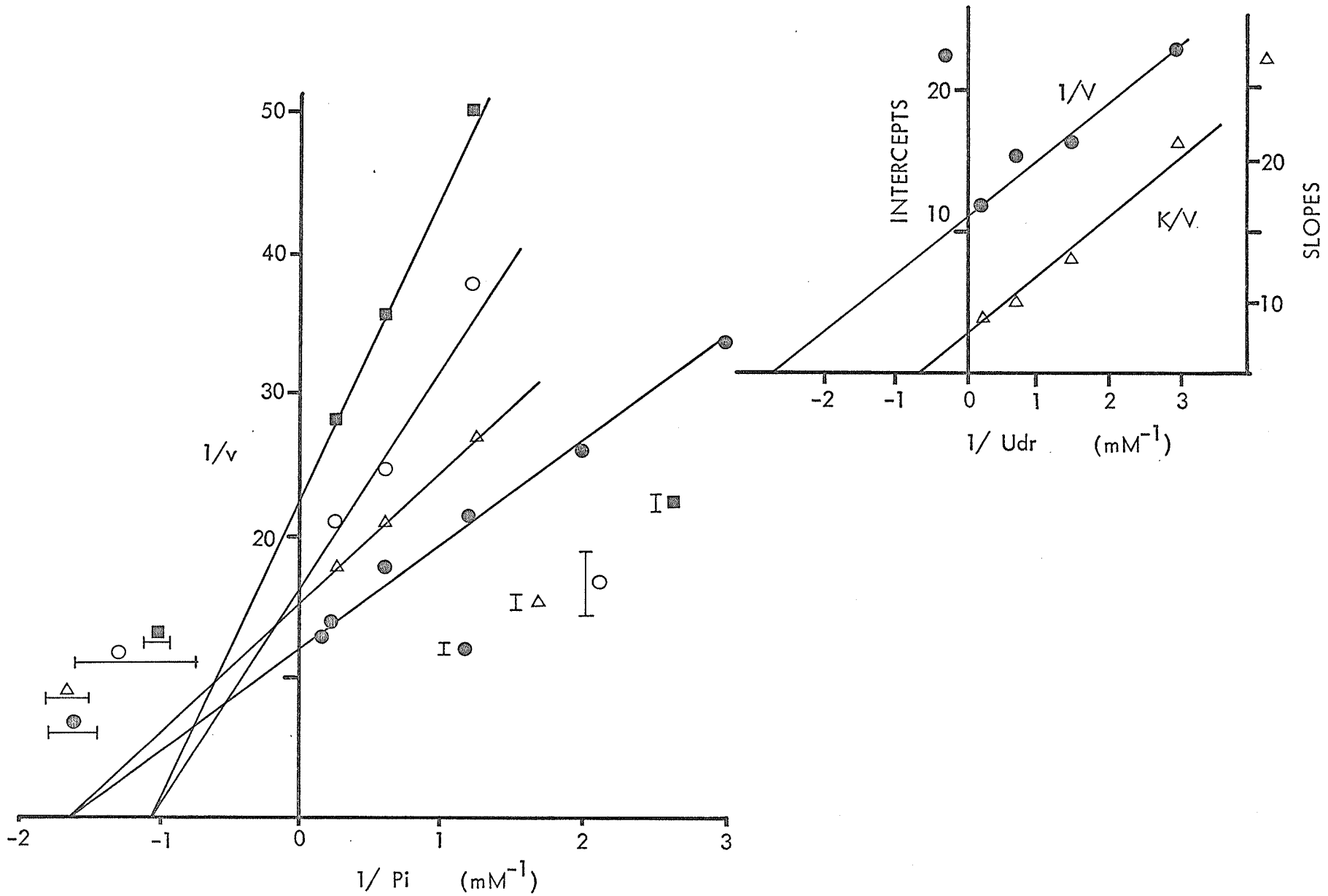

1/v
1/ Ur (mM^{-1})
1/ Pi (mM^{-1})
1/V
K/V
SLOPES
INTERCEPTS

Figure 24. Double reciprocal plots of initial velocity against phosphate concentration.

The experimental procedure for the assay of deoxyuridine cleaving activity at pH 6.5 with reactivated fraction E_b (13 μg) is described in section IV B 1. Deoxyuridine was held constant at concentrations of 3.33 mM (●), 1.33 mM (△), 0.667 mM (○), and 0.333 mM (■). The insert shows the replot of slopes (△ , K/V), and intercepts (● , 1/V) against the reciprocal of deoxyuridine concentration. Velocity (v) = uracil (mMolar) produced/tube/10 minutes/13 μg protein.

Figure 25. Double reciprocal plots of initial velocity against deoxyuridine concentration.

Phosphate was held constant at concentrations of 117 mM (●), 3.98 mM (■), 1.67 mM (△), and 0.835 (○). The insert shows the replot of slopes (△ , K/V) and intercepts (● , 1/V) against the reciprocal of phosphate concentration. Other conditions are the same as in Figure 24.


50
40
30
20
1/v
-2
-1
0
1
2
3
1/ Pi (mM^{-1})
20
10
INTERCEPTS
1/V
K/V
SLOPES
1/ Udr (mM^{-1})

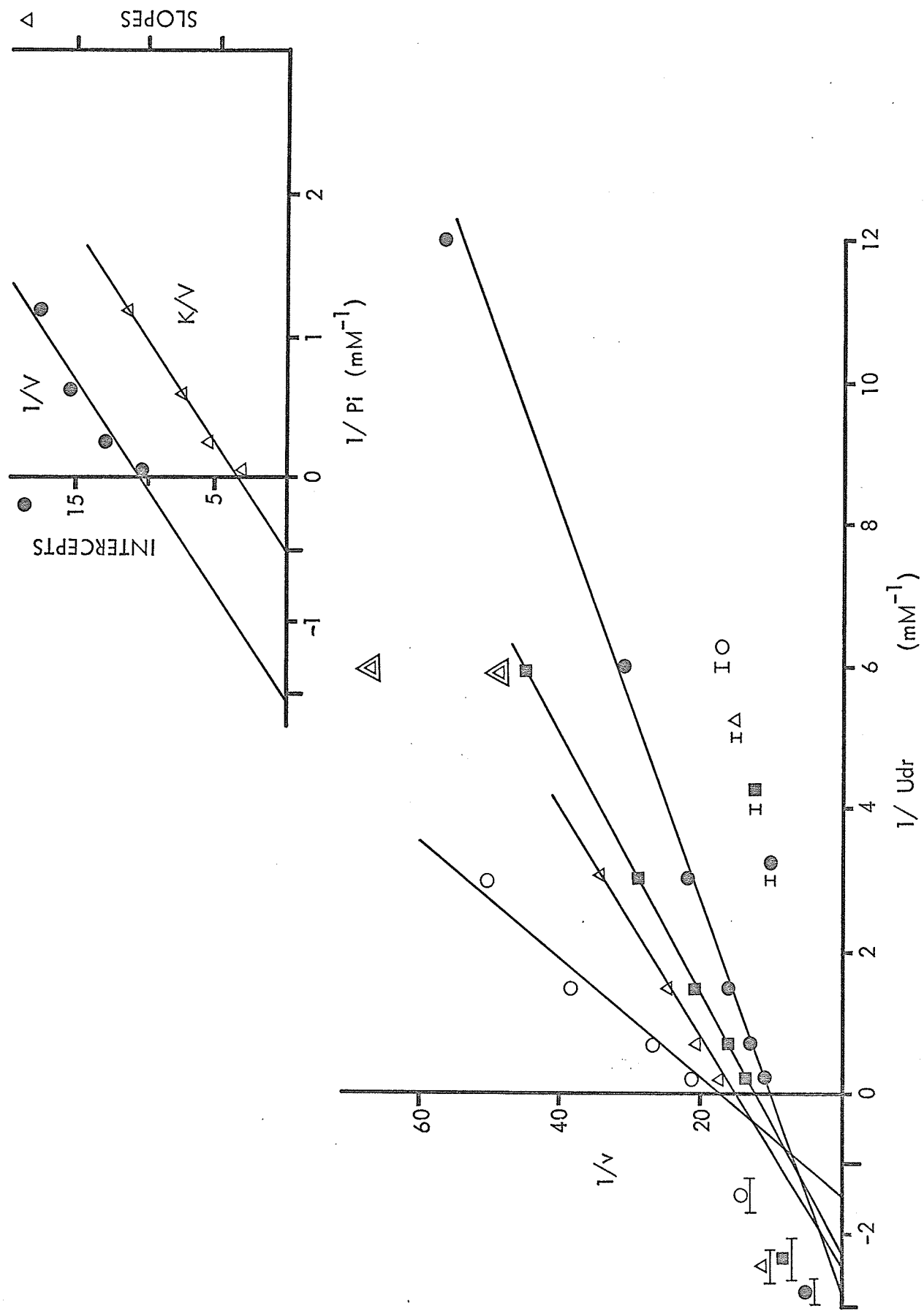

SLOPES
INTERCEPTS
1/V
K/V
15
5
0
1
2
-1
1/ Pi (mM^{-1})
1/v
60
40
20
0
2
4
6
8
10
12
-2
1/ Udr (mM^{-1})

Figure 26. Double reciprocal plots of initial velocity against uracil concentration.

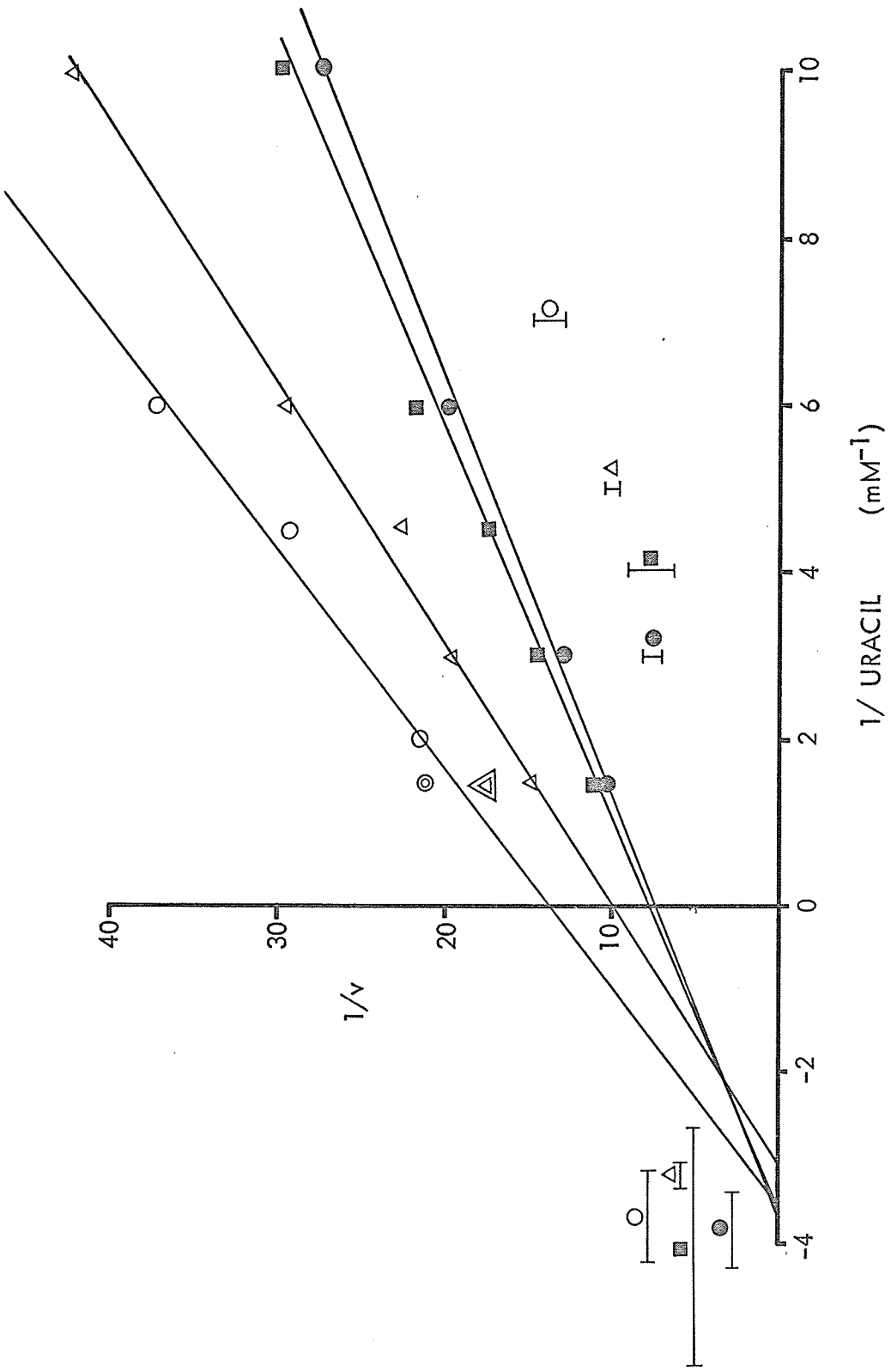
The experimental procedure for the assay of uridine synthesis at pH 8.1 with reactivated fraction D_f (2.3 μg) is described in section IV B 1. Ribose-1-phosphate was held constant at concentrations of 1.43 mM (●), 0.429 (□), 0.143 mM (△), and 0.0715 mM (○). Velocity (v)=uridine (mMolar) produced/tube/65 minutes/2.3 μg protein.

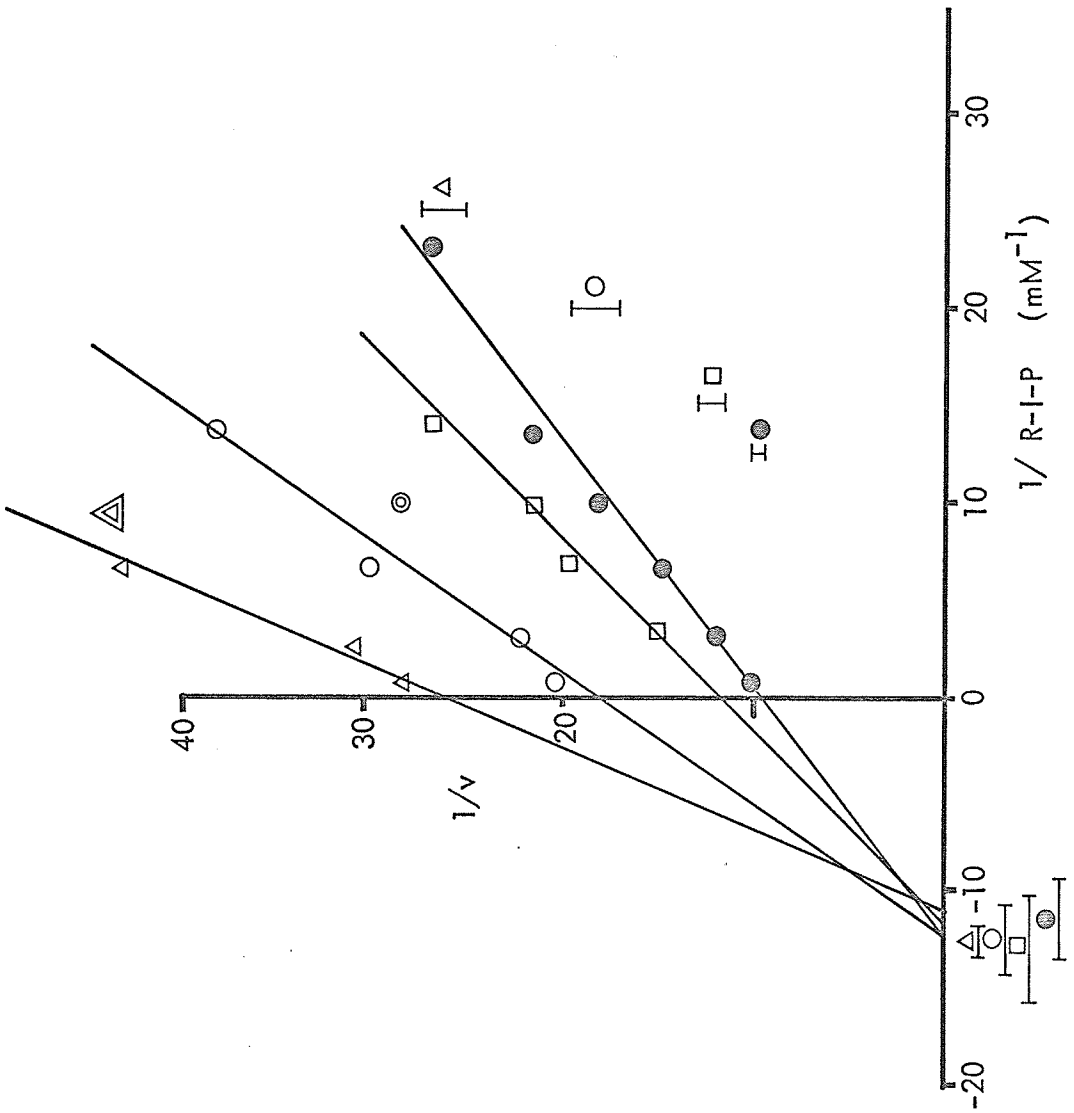
Figure 27. Double reciprocal plots of initial velocity against ribose-1-phosphate.

Uracil was held constant at concentrations of 0.667 mM (●), 0.333 mM (□), 0.167 mM (○), and 0.100 mM (△). Other conditions are the same as in Figure 26.

Figure 28. Replots of slopes and intercepts from Figures 26 and 27.

Replots are shown of intercepts (○) and slopes (□) from Figure 26 against the reciprocal of ribose-1-phosphate concentration, and intercepts (●) and slopes (■) from Figure 27 against the reciprocal of uracil concentration.


40
30
20
10
0
-2
-4
2
4
6
8
10
1/v
1/ URACIL (mM^{-1})


40
30
20
1/v
0
10
20
30
-10
-20
1/ R-I-P (mM^{-1})

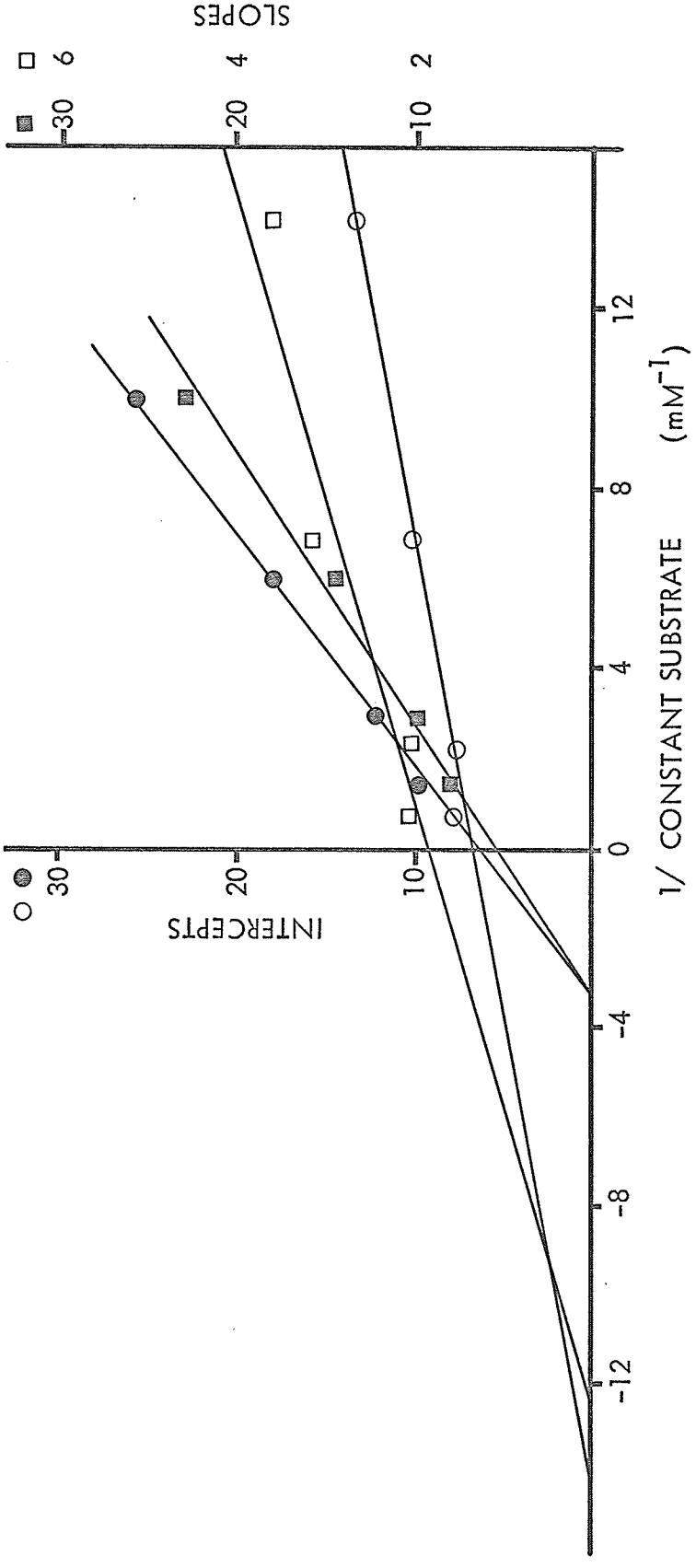

SLOPES
6
4
2
30
20
10
INTERCEPTS
30
20
10
0
-4
-8
-12
4
8
12
1/ CONSTANT SUBSTRATE (mM^{-1})

Figure 29. Product inhibition by uracil with phosphate as the variable substrate at low uridine concentration.

The experimental procedure for product inhibition of uridine cleavage at pH 8.1 with preparation C_f (20 to 40 μg of protein) is described in section IV B 2. The concentration of uridine was held constant at 0.133 mM. Uracil concentrations: 0 (●), 0.0605 mM (△), 0.121 mM (○), 0.181 mM (□), and 0.242 mM (▲). Velocity (v) = Uracil (mMolar) produced/tube/10 minutes/20 μg protein.

Figure 30. Product inhibition by uracil with phosphate as the variable substrate at high uridine concentration.

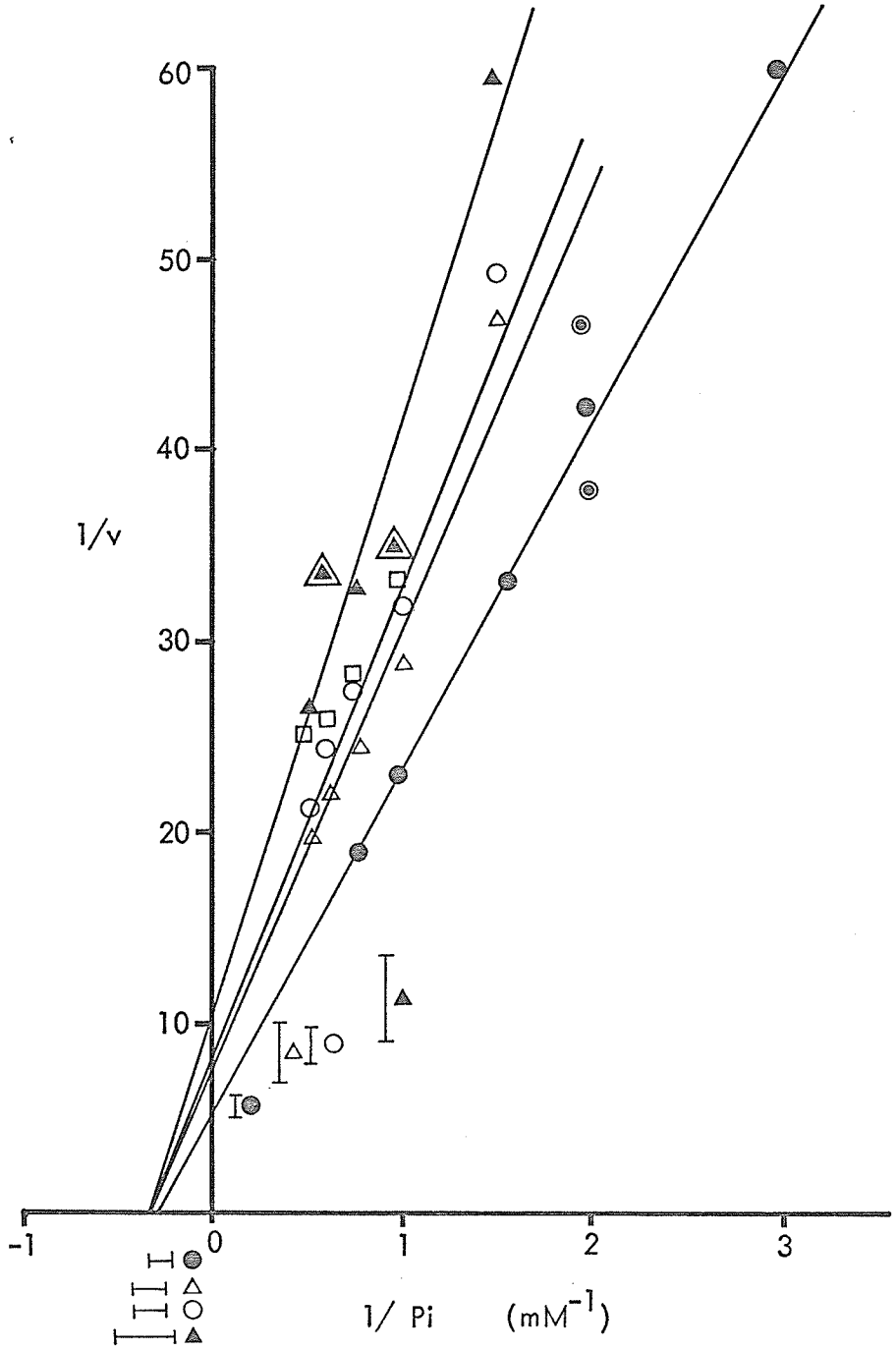
The concentration of uridine was held constant at 0.533 mM. Uracil concentrations: 0 (●), 0.121 mM (△), 0.242 mM (▲), and 0.302 mM (○). Other conditions are the same as in Figure 29.

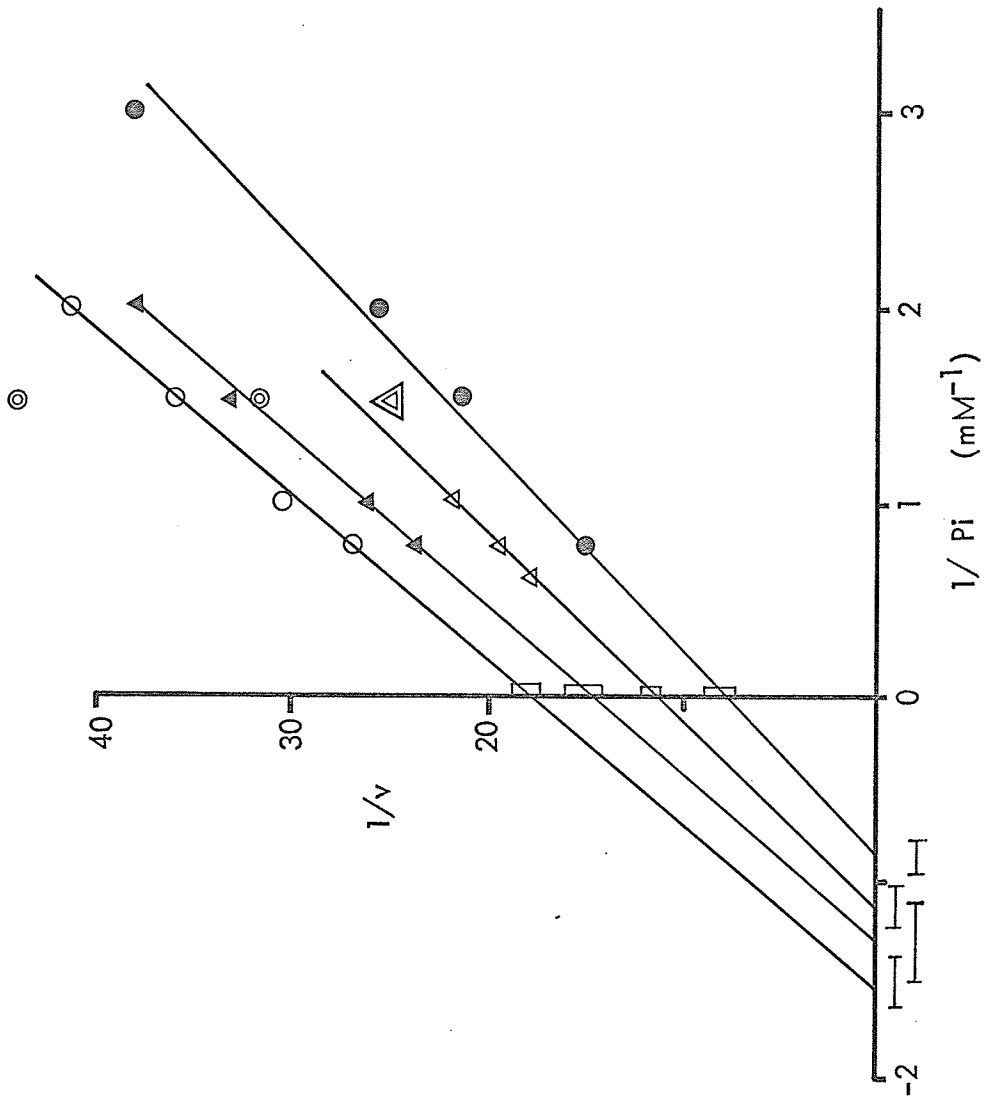
Figure 31. Product inhibition by uracil with uridine as the variable substrate at low phosphate concentration.

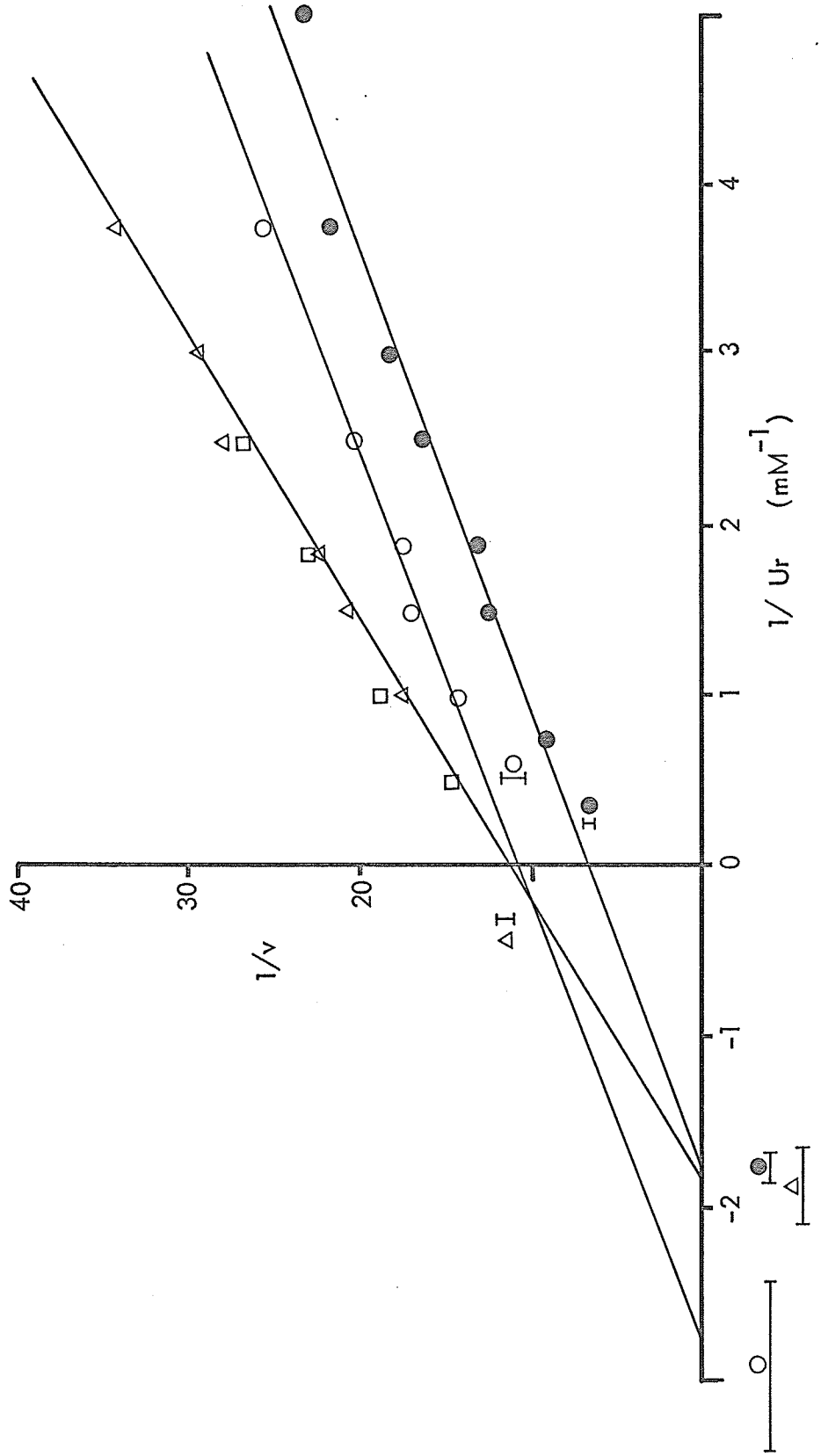
The concentration of phosphate was held constant at 0.667 mM. Uracil concentrations: 0 (●), 0.242 mM (○), 0.362 (△), and 0.484 mM (□). Other conditions are the same as in Figure 29.

Figure 32. Product inhibition by uracil with uridine as the variable substrate at high phosphate concentration.

The concentration of phosphate was held constant at 6.67 mM. Uracil concentrations: 0 (●), 0.121 mM (○), 0.242 mM (△), and 0.362 mM (□). Other conditions are the same as in Figure 29.


60
50
40
30
20
10
1/v
-1
0
1
2
3
1/ Pi (mM^{-1})


40
30
20
1/v
0
1
2
3
-2
1/ Pi (mM^{-1})


40
30
20
1/v
ΔI
0
1
2
3
4
1/ Ur (mM^{-1})
-1
-2

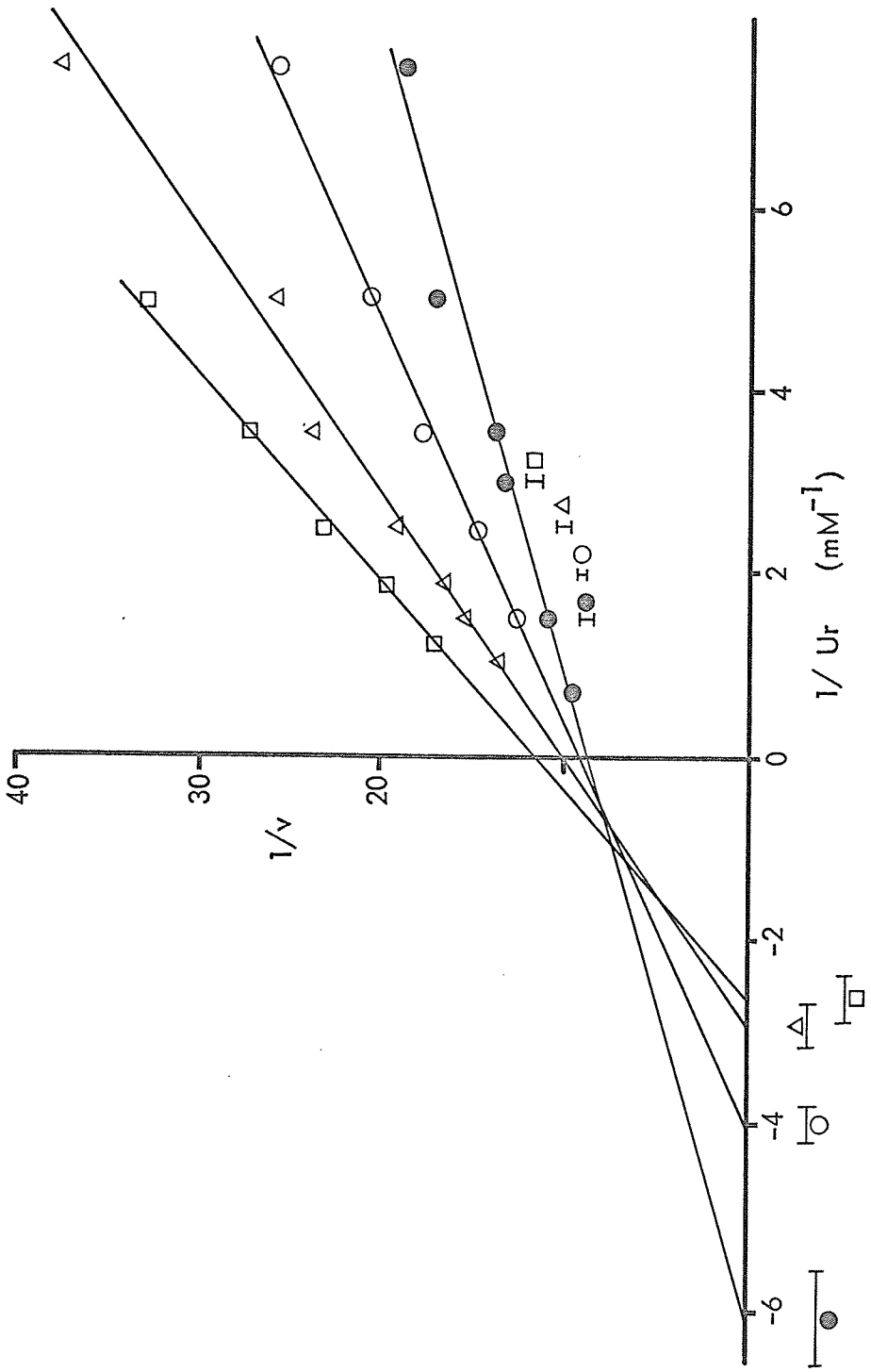

40
30
20
1/v
0
2
4
6
-2
-4
-6
1/ Ur (mM^{-1})

Figure 33. Product inhibition by ribose-1-phosphate with phosphate as the variable substrate at low uridine concentration.

The experimental procedure for inhibition of uridine cleavage at pH 8.1 with preparation C_f (20 to 40 μg protein) is described in section IV B 2. The concentration of uridine was held constant at 0.266 mM. Ribose-1-phosphate concentrations: 0 (●), 0.438 mM (○), and 0.830 mM (△).

Figure 34. Product inhibition by ribose-1-phosphate with phosphate as the variable substrate at high uridine concentration.

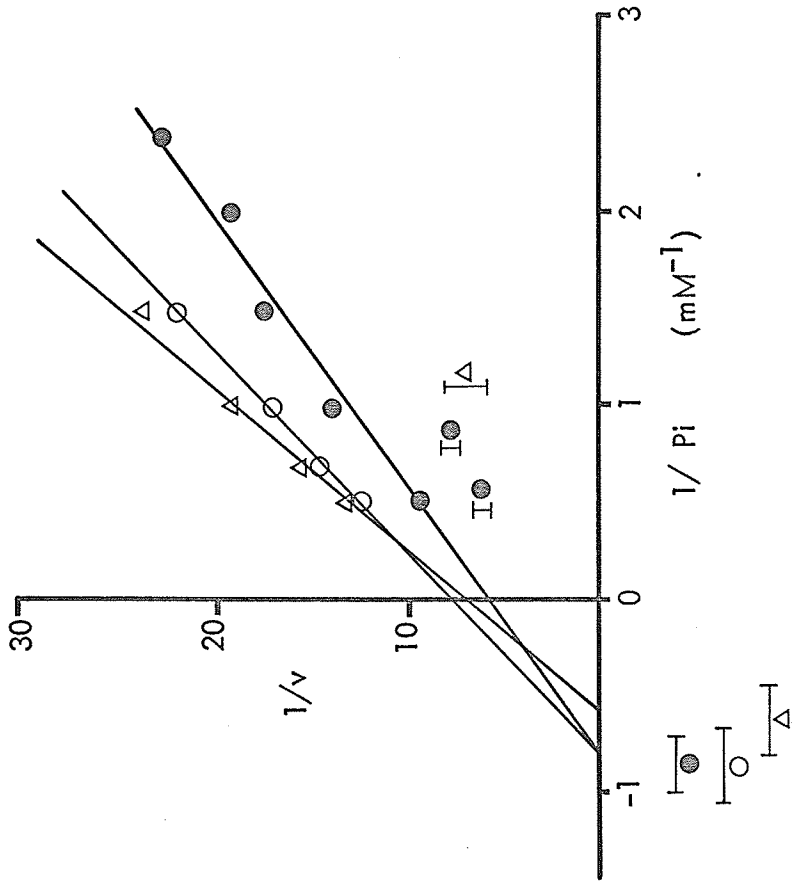
The concentration of uridine was held constant at 3.33 mM. Ribose -1-phosphate concentrations: 0 (●), 0.292 mM (△), 0.438 (○), and 0.730 mM (□). Other conditions are the same as in Figure 33.

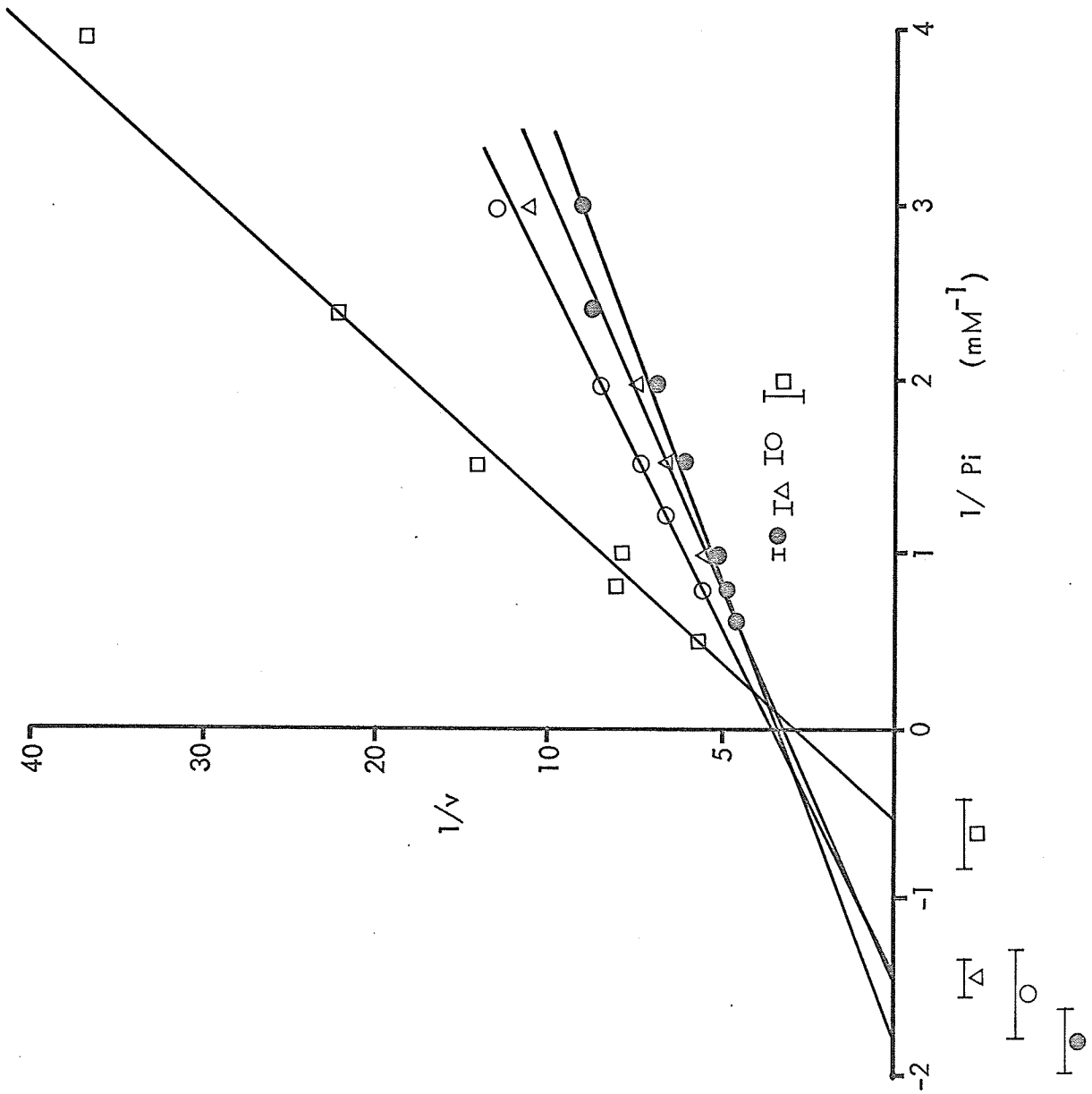
Figure 35. Product inhibition by ribose-1-phosphate with uridine as the variable substrate at low phosphate concentration.

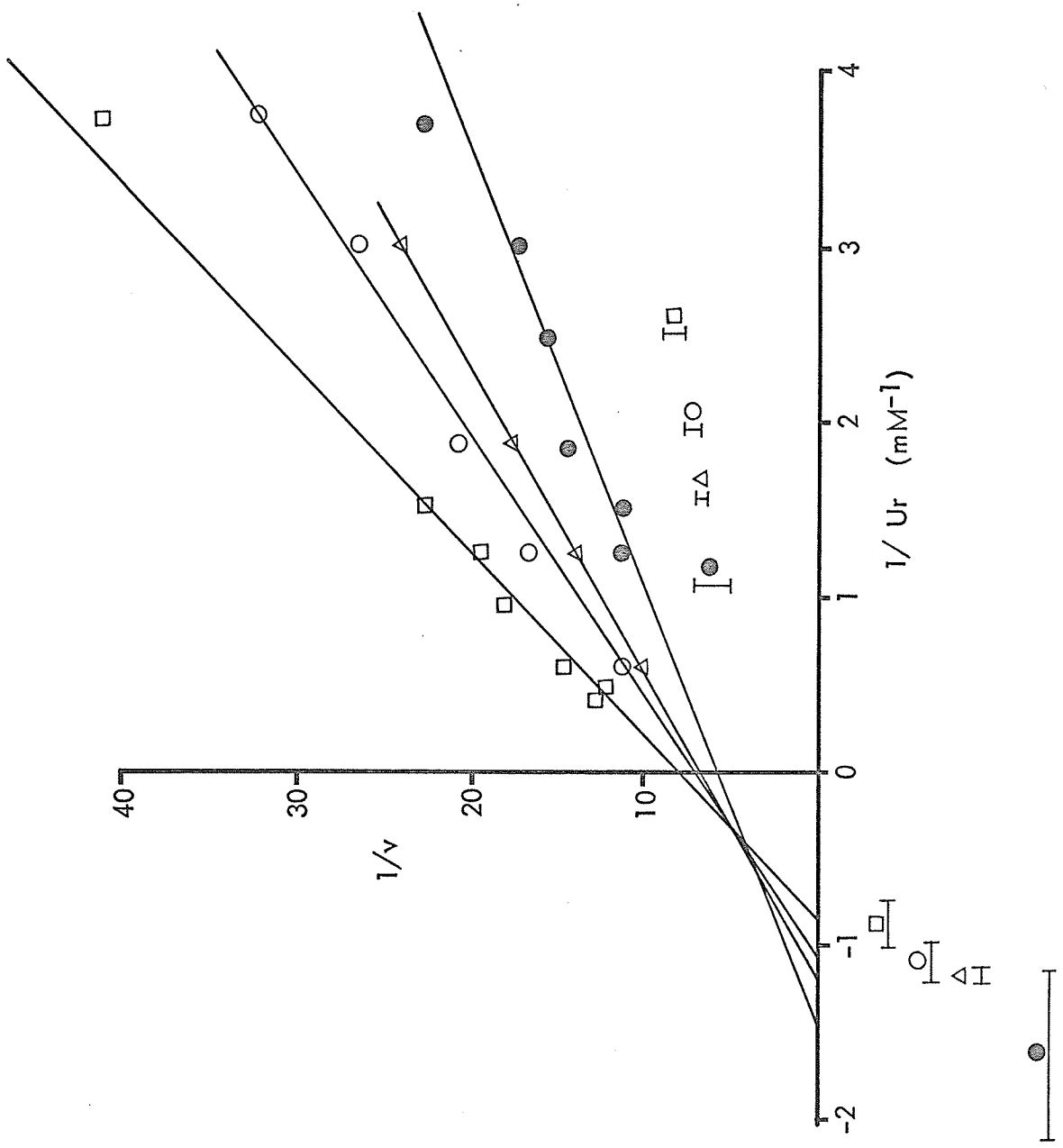
The concentration of phosphate was held constant at 0.667 mM. Ribose-1-phosphate concentrations: 0 (●), 0.292 mM (△), 0.438 mM (○), and 0.730 mM (□). Other conditions are the same as in Figure 33.

Figure 36. Product inhibition by ribose-1-phosphate with uridine as the variable substrate at high phosphate concentration.

The concentration of phosphate was held constant at 6.67 mM. Ribose-1-phosphate concentrations: 0 (●) and 0.730 mM (○). Other conditions are the same as in Figure 33.


30
20
10
0
1
2
3
-1
1/v
1/ Pi (mM^{-1})


40
30
20
10
5
0
-1
-2
1
2
3
4
1/v
1/ Pi (mM^{-1})
I
I
I
I


40
30
20
10
1/v
0
1
2
3
4
-1
-2
1/ Ur (mM-1)

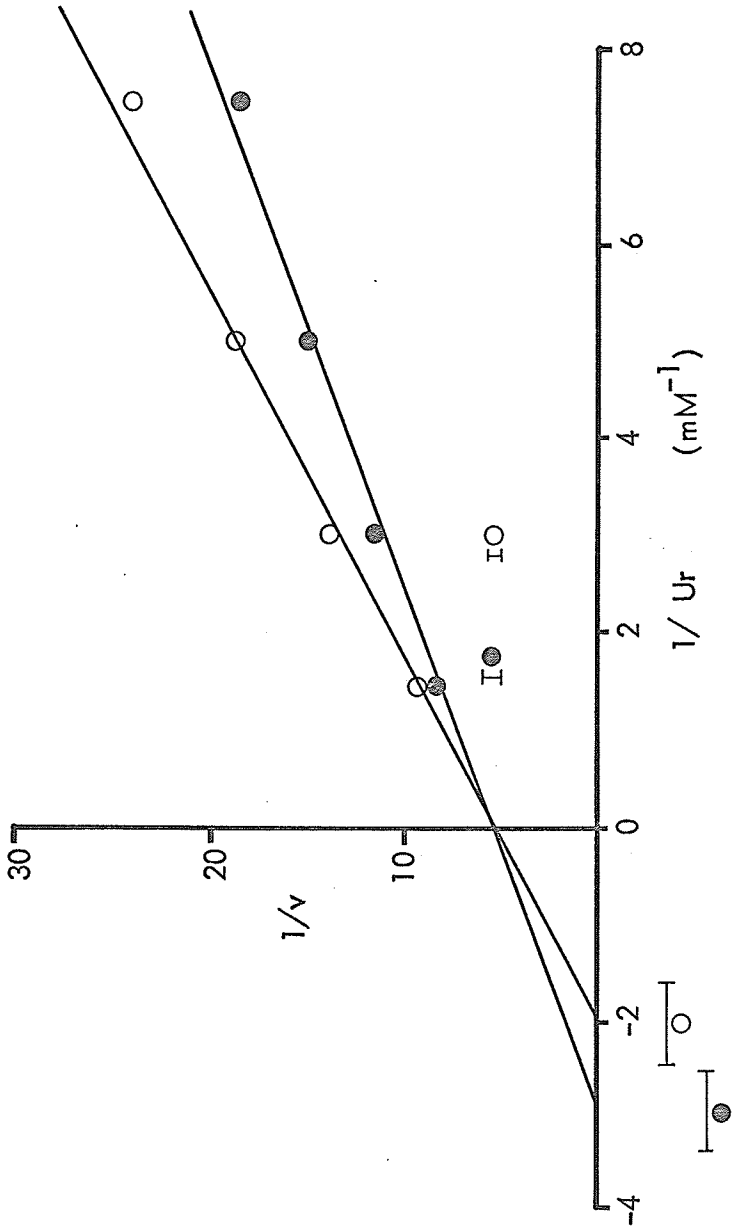
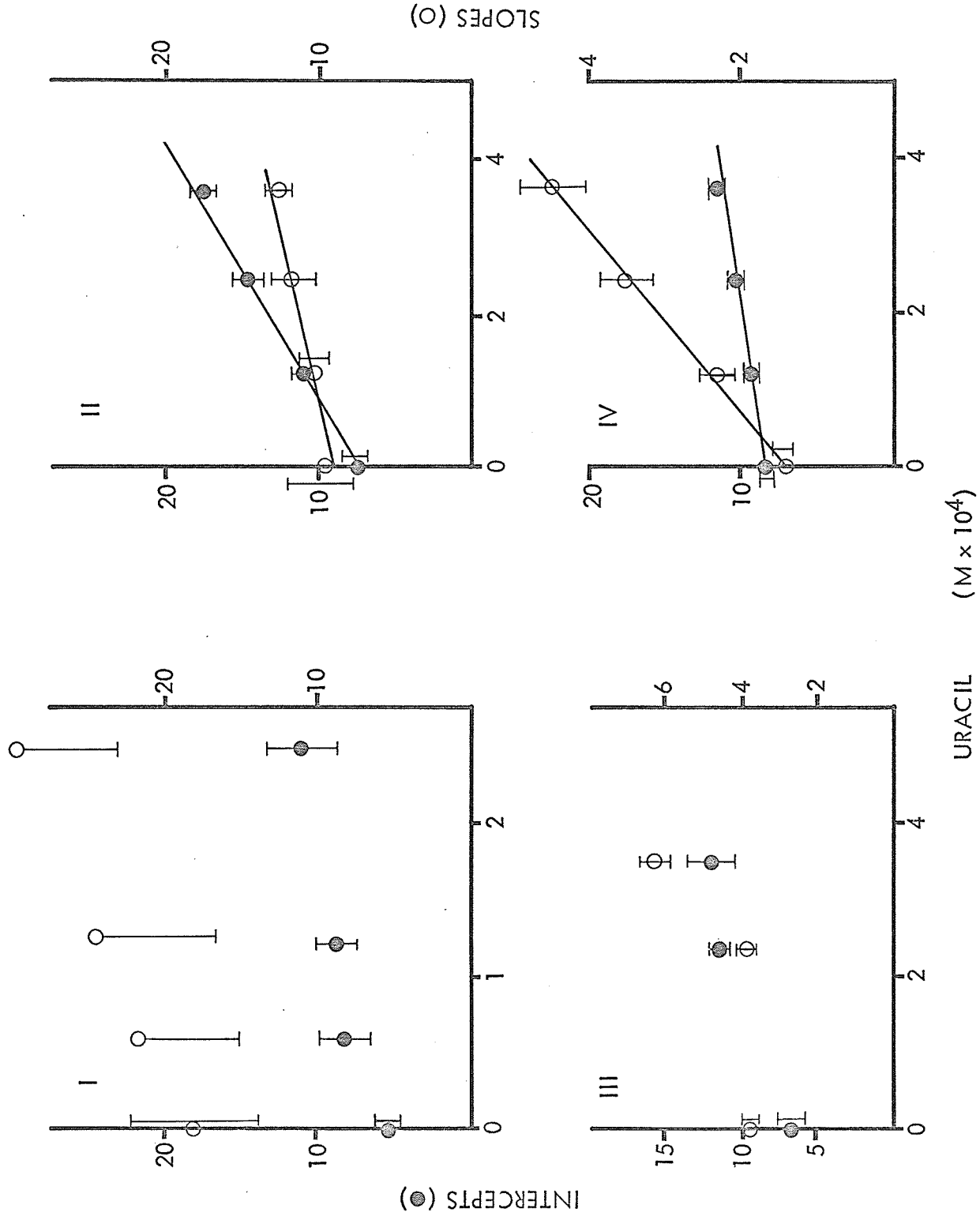

30
20
10
1/v
0
2
4
6
8
1/ Ur (mM^{-1})
-2
-4
I
IO

Figure 37. Replots of slopes and intercepts in the product inhibition study with uracil as the inhibitor.

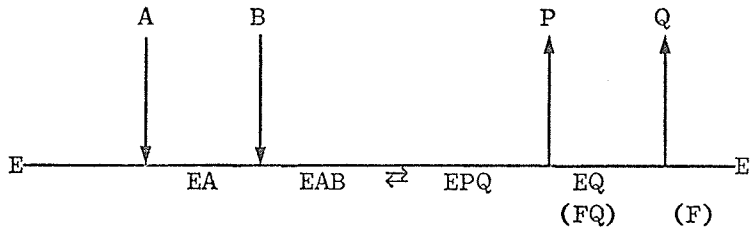
The slope (○) and intercept (●) replots are from Figure 29 (I; low uridine, variable phosphate), Figure 30 (II; high uridine, variable phosphate), Figure 31 (III; low phosphate, variable uridine), and Figure 32 (IV; high phosphate, variable uridine). The X-axis intercept of the slope and intercept replots give K_{is} and K_{ii}, respectively (Table XXI).

Figure 38. Replots of slopes and intercepts in the product inhibition study with ribose-1-phosphate as the inhibitor.

The slope (○) and intercept (●) replots are from Figure 33 (V; low uridine, variable phosphate), Figure 34 (VI; high uridine, variable phosphate), and Figure 35 (VII; low phosphate, variable uridine).

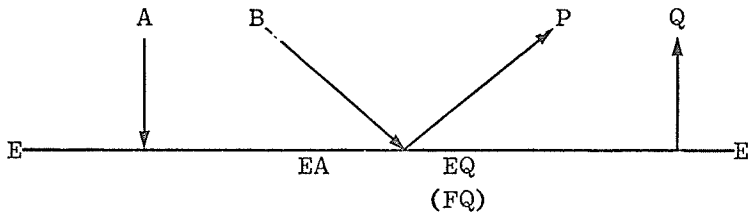

SLOPES (O)
INTERCEPTS (●)
URACIL (M x 10⁴)
I
II
III
IV

SLOPES (○)
INTERCEPTS (●)
RIBOSE - 1 - PHOSPHATE (M × 10^4)
V
VI
VII



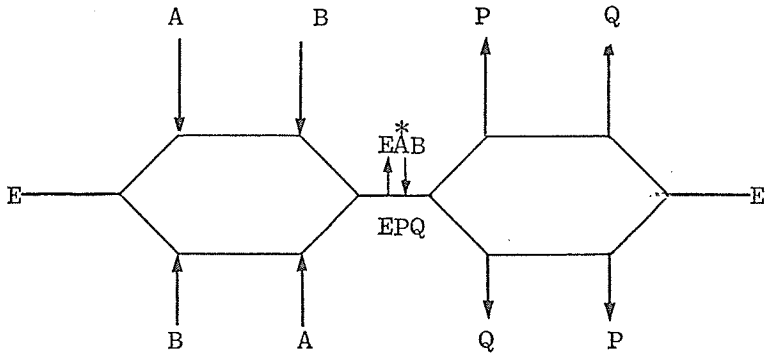
(5) ORDERED Bi Bi

(iso ordered Bi Bi if EQ and E are changed to FQ and F, as shown).



(6) THEORELL-CHANCE

(iso Theorell-Chance mechanism if EQ and E are changed to EQ and F, as shown).



(7) RAPID EQUILIBRIUM RANDOM Bi Bi

*(rate-limiting)

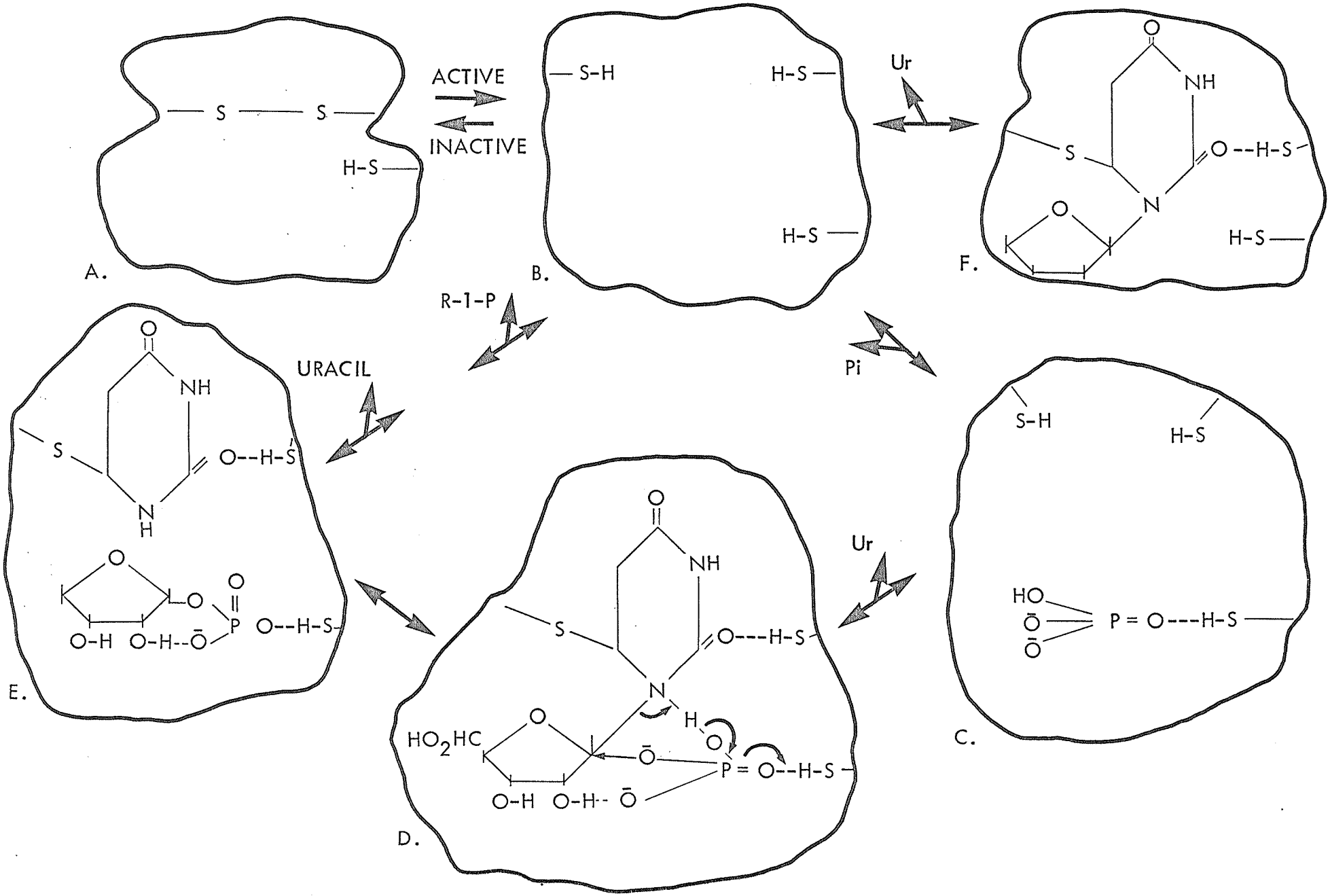
(Random Bi Bi if EAB $\rightleftharpoons$ EPQ is not rate limiting).

Figure 39.

Possible sequential mechanisms for uridine phosphorylase.

Figure 40. Model for the phosphorolysis of uridine.

An equilibrium exists between enzyme forms A (oxidized) and B (reduced). Phosphate combines with Form A. Uridine then adds on to C to give D (transition state). Form E results after cleavage of the nucleoside linkage. A possible inactive enzyme-substrate complex is illustrated by F.


A.
S
S
H-S
ACTIVE
INACTIVE
B.
S-H
H-S
H-S
Ur
F.
NH
O
S
O--H-S
N
O
H-S
R-1-P
Pi
URACIL
E.
NH
S
O--H-S
N
H
O
O
O
P
O-H
O-H--O
O--H-S
D.
NH
O---H-S
S
N
H
O
HO2HC
O
P = O--H-S
O-H
O-H--O
Ur
C.
S-H
H-S
HO
O
O
P = O---H-S