STUDIES ON ISOENZYMES OF URIDINE PHOSPHORYLASE OF RAT LIVER

by

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ABSTRACT

Uridine phosphorylase (EC 2.4.2.3) of rat liver catalyzes the reversible phosphorolysis of uridine to produce uracil and ribose-1-phosphate; hence it is both a catabolic enzyme, catalyzing the degradation of nucleosides from food or RNA breakdown, and an anabolic enzyme producing nucleosides for the 'salvage synthesis' of RNA. Amongst the other tissues studied enzyme activity was present in intestine, kidney, brain and spleen.

Subcellular distribution studies showed that 70% of the enzyme activity is in the soluble fraction (cytosol) and the rest is in the plasma membrane and mitochondria. In an earlier work the enzyme activity of crude nuclear fractions was found to be higher in regenerating compared to normal rat livers. In the present work it has been shown that the purified nuclear fractions do not have any activity and that the activity of the crude nuclear fractions is due to contaminating plasma membrane fragments.

Studies of the transferase activity of this enzyme confirms the ordered Bi Bi reaction mechanism proposed previously in this laboratory for this enzyme.

Two isoenzymes of uridine phosphorylase have been separated and purified 570 and 1400 fold respectively, and their properties compared. The first of the two isoenzymes to be eluted from DEAE Sephadex column (isoenzyme #1) has a higher Kb for uridine compared to isoenzyme #2. Isoenzyme #1 is more prominent in regenerating rat liver or young rat liver. Isoenzyme #2 seems to be more heat sensitive and even phosphate could not

afford full protection against heat inactivation as it did for isoenzyme #1. Both isoenzymes showed one protein band of active enzyme on disc gel electrophoresis. The activity of the enzyme fractions in the gel was determined by staining with triphenyltetrazolium in the presence of uridine or deoxyuridine. On aging in phosphate new bands appear; the relative mobilities (Rm) of the new bands are somewhat different for the two isoenzymes. The molecular weight of isoenzyme #1 was 110,000 and of isoenzyme #2 was 95,000. On aging in Tris-HCl buffer new, more cationic species of protein appeared which for either isoenzyme had twice the molecular weight of the unaged enzyme and were inactive when stained for activity. Both isoenzymes are composed of four subunits.

The amino acid composition of isoenzyme #1 indicates the presence of a total of 24 half cystine residues; this is in agreement with the studies with sulphydryl inhibitors which indicated the involvement of SH groups in enzyme activity. Plots of log Vmax and pKm of substrates at different pH values indicated the presence of a charged group with a pK value of about 8.0 in the enzyme substrate complex as well as 2 other groups with pK values of 6.5 and about 7.0 which were involved in either catalysis or binding. A model for the possible mechanism of phosphorolysis by uridine phosphorylase has been proposed

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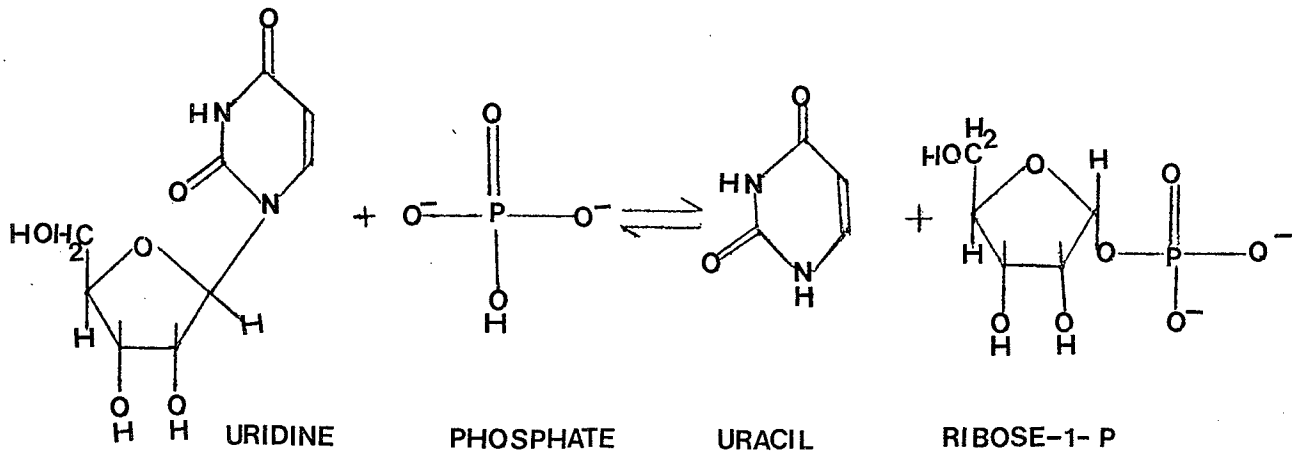
LIST OF ABBREVIATIONS

AMP, ADP, ATP	-	adenosine 5' mono-, di- and tri-phosphates.
AS_2	-	protein fraction precipitated with ammonium sulphate.
CMP, CDP, CTP	-	cytidine 5' mono-, di- and tri-phosphates.
DUR	-	deoxyuridine
DNA	-	deoxyribonucleic acid
EDTA	-	ethylene diamine tetraacetic acid
EM	-	electron micrograph
Gly-gly	-	glycylglycine
HAS_2	-	heat treated AS_2
K Pi	-	potassium phosphate buffer
Ka, Kb, Kp and Kq	-	Michaelis constants for substrate A B P and Q
Na Pi	-	sodium phosphate buffer
Pi	-	inorganic phosphate
PPi	-	pyrophosphate
PRPP	-	5-phosphoribosyl-1-pyrophosphate
R-1-P or ribose-1-P	-	ribose-1-phosphate
Rm	-	relative mobility
RNA	-	ribonucleic acid
SDS	-	sodium dodecylsulphate

TCA	-	Trichloracetic acid
TMP, TDP, TTP	-	Thymidine 5' mono-,di- and tri-phosphate
Tris	-	Tris (Hydroxymethyl) aminoethane
TdR	-	deoxythymidine
U	-	uracil
UMP, UDP, UTP	-	uridine, 5' mono di and tri phosphate
UR	-	uridine
XMP	-	xanthine 5' mono phosphate

I. INTRODUCTION

Uridine phosphorylase occupies an important amphibolic position in the metabolism of pyrimidines (Figure 1). By means of this enzyme uridine can be synthesized from uracil and ribose-1-phosphate or can be broken down to uracil and ribose-1-phosphate which then are catabolized further.



Uridine phosphorylase increases in regenerating tissues and high concentrations are found in bone marrow and intestine, which are known to have very low levels of enzymes constituting the de novo pathway (1, 2, 3). These observations point to the fact that uridine phosphorylase may have an important function in the "salvage pathway" or the "reserve pathway" of RNA synthesis from preformed derivatives.

Uridine phosphorylase has not been examined extensively with regards to its molecular structure. Partial

purification and studies of its properties have been carried out for the enzymes from E. coli (4, 5, 6), Ehrlich ascites cells (7), rat liver (8), and guinea pig intestine (9). Recently, one of the rat liver enzymes was purified 1900 fold and its kinetic properties studied (10). (Details given in section II C).

Isoenzymes for this enzyme have been reported in rat liver (8) and rat brain (11). Uridine phosphorylase is not the only nucleoside phosphorylase known, as will be apparent in the following literature review. In fact the purine nucleoside phosphorylase has been the subject of study more often and consequently more is known about this group of enzymes. However, with the little that is known about uridine phosphorylase, it promises to be an interesting enzyme for detailed study at the structural and functional level.

II. REVIEW OF LITERATURE

The fact that some tissues can utilize preformed purine and pyrimidine bases and incorporate them into nucleotides or degrade them as a preliminary to further catabolism, has been known for a long time. The nucleoside phosphorylases, so called because they catalyze the phosphorolytic cleavage of nucleosides using inorganic phosphate, catalyze a reaction which has an equilibrium constant which favours the synthesis of the nucleosides:

(1) Phosphorolysis: $\text{Nucleoside} + \text{Pi} \rightleftharpoons \text{Base} + \text{Ribose-1-PO}_4$

(2) Transfer: $\text{Nucleoside}_1 + \text{Base}_2 \rightleftharpoons \text{Base}_1 + \text{Nucleoside}_2$

Transfer activity between a base and a nucleoside seems to be associated with all the nucleoside phosphorylases known so far. Thus, this group of enzymes can affect the nucleoside pool in three ways: (1) by synthesis from base and sugar phosphate, (2) by degradation in the presence of phosphate, (3) by transfer of the sugar moiety from one base to another.

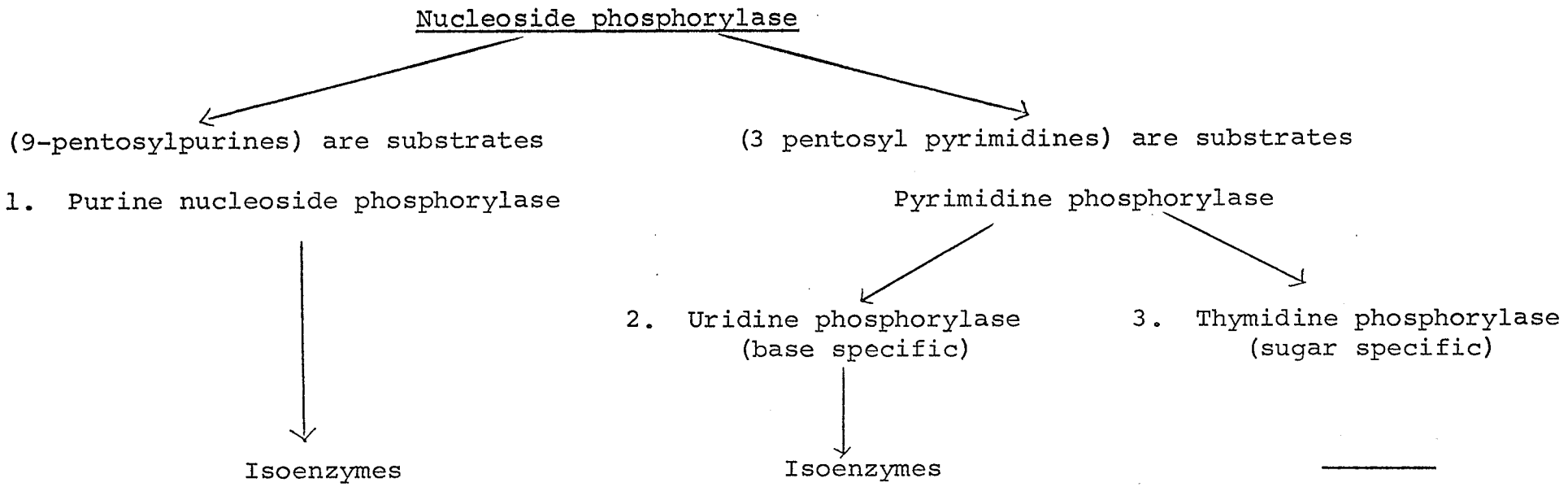
Before beginning the survey, a brief classification of nucleoside phosphorylases would be useful in following the nomenclature of enzymes used frequently throughout this

thesis. Nucleoside phosphorylases have been broadly classified into two groups depending on the nitrogen base and N-glycosidic bond of the substrate.
This is summarized on page 7.

A. Purine nucleoside phosphorylase (Purine nucleoside: orthophosphate ribosyl transferase EC 2.4.2.1).

Purine nucleoside phosphorylase has been found to occur in bacteria, vertebrate tissues and tumour cells (12). The enzyme from most sources uses inosine, guanosine as well as the respective deoxyribosides as substrates. Adenosine is phosphorolyzed to some extent by the beef liver purine nucleoside phosphorylases (13). Recently, Zimmerman et al. (14) found the partially purified rat liver enzyme and crystalline preparations of blood and spleen enzymes to catalyze a slow reaction with adenine.

1. Molecular Properties - The most extensively studied purine nucleoside phosphorylases are from human erythrocytes (15) and Bacillus cereus (16). The human erythrocyte nucleoside phosphorylase (15) was purified 7,300 fold and crystallized. It had a molecular weight of 81,000 and a constant


Nucleoside phosphorylase
(9-pentosylpurines) are substrates
(3 pentosyl pyrimidines) are substrates
1. Purine nucleoside phosphorylase
Pyrimidine phosphorylase
2. Uridine phosphorylase (base specific)
3. Thymidine phosphorylase (sugar specific)
Isoenzymes
Isoenzymes
Substrate specificity
Inosine > guanosine, also the deoxy derivatives
Uridine > Deoxyuridine
>> Thymidine
Deoxyuridine > Thymidine

ratio of activity towards inosine and deoxyinosine. This enzyme differed from the bovine spleen purine nucleoside phosphorylase in showing substrate activation by inosine, a different crystalline structure and a different specific activity.

The Bacillus cereus T enzyme had a molecular weight of 88,000 → 92,000 when obtained from the vegetative form of the bacteria (16). The spore enzyme had a molecular weight of 47,000 in the absence of phosphate and 95,000 in the presence of 10 mM phosphate. The vegetative cell enzyme was a tetramer of 4 subunits. The spore enzyme underwent a phosphate induced association from dimeric to tetrameric forms. The subunits from both sources had molecular weights of 24,000. The Km for inosine of the vegetative cell enzyme was 4.6×10^{-4} M and for the spore enzyme 7.0×10^{-4} M.

In chicken different molecular forms of purine nucleoside phosphorylase exist in the embryonic and adult livers (17); the embryonic enzyme was found to be stable compared to the adult enzyme. Isoenzymes have also been reported to be present in the muscle of Lingcod (18) but their properties have not been studied.

Isoenzymes of purine nucleoside phosphorylase have been found in a variety of human tissues including red and white blood cells, cultured fibroblasts and hair follicle extracts (19). In the latter work analysis of the isoenzyme pattern suggests that nucleoside phosphorylase has a trimeric subunit structure and a molecular weight of 84,000.

2. Kinetic Properties - The kinetic analysis of human erythrocyte purine nucleoside phosphorylase was consistant with an ordered Bi Bi* (20) reaction where the nucleoside was the first substrate to add to and purine the last product to leave the enzyme surface.

With the human skin enzyme the order of addition was reverse, thus phosphate added on first and ribose-1-P was the last product to leave (21). The bovine spleen enzyme showed an order similar to that of the erythrocyte enzyme with the difference that base and nucleoside bind to different isomeric forms of the enzyme (22). The conclusion about the order for this spleen enzyme is hard to understand especially since ribose-1-P was competitive with phosphate and base and nucleoside were non-competitive in the product inhibition studies. The reverse was true for the human erythrocyte enzyme.

* Cleland's (128) nomenclature used.

The bovine spleen enzyme (22) and other purine nucleoside phosphorylases (15) catalyzed a transfer reaction between the base and the nucleoside. This activity was not separable from the phosphorylase activity except in one instance - the enzyme of Ehrlich ascites tumour cells (23) where the two activities were separable on a sucrose gradient in the presence of 2-mercaptoethanol. The forms were also found to be interconvertible in the presence or absence of 2-mercaptoethanol.

3. Regulation and Role - Substrate activation by inosine has been found for chicken liver (24) and human erythrocyte purine nucleoside phosphorylase (15). The chicken liver (24) enzyme is inhibited by ADP (50% inhibition by 10 mM) and Na_2SO_4 (50% inhibition at 5 mM) and this inhibition increased on aging. The significance of the latter observation is not understood as yet.

Probably, one of the roles of most nucleoside phosphorylases is catabolic. However, purine nucleoside phosphorylase is an important member of the "salvage pathway" for purines. Two reviews published on this topic (25, 26) point to the fact that dependence of animal tissues on pre-formed purines is not absolute for all tissues, and it has

been established that rabbit (27), mouse as well as human blood (28), rabbit bone marrow and lymph nodes (29) cannot synthesize purines by the de novo pathway. Liver is the source of the purines for peripheral tissues as is evident in the experiments of Lajtha and Vane (29), who found lowered incorporation of C^{14}-formate into DNA purines of bone marrow following portal occlusion or partial hepatectomy. This fact is also confirmed by the work of Pritchard et al. (30) who labelled rat liver in situ by perfusion with C^{14} adenine and then restored the normal circulation; label was rapidly transfered to nonhepatic tissues, especially the red blood cell. Purine nucleoside phosphorylase of E. coli and S. typhimurium is inducible by deoxyribo-nucleosides and purine nucleosides (65).

It is not known whether the nucleoside or base is the preferred form for entry into different cells. There is evidence showing that purines enter and leave rabbit red cells as free bases rather than nucleosides (26) because p-nitrobenzyl thioguanosine prevented nucleoside entry into red blood cells but did not alter the turnover time of nucleoside pools, prelabelled with C^{14} adenine (26, 31). Hypoxanthine and xanthine seem to be the only major purine

bases released from red blood cells (32) and this release is increased by raising the oxygen level. A 20-fold increase in urinary excretion of hypoxanthine has been reported after severe muscular work (33) and deamination of AMP to IMP occurs during exercise (34) while inosine accumulates under anaerobic conditions (35). It is not known whether the muscle replenishes its depleted pool by the salvage pathway or by synthesis de novo.

Zimmerman et al. (14) using rat liver slices found that adenine 8-C^{14} was quantitatively converted to nucleosides; adenine 8-C^{14} in the incubation medium was converted to relatively large amounts of adenosine 8-C^{14} only if the medium was saturated with inosine. The relationship of these findings to a possible transport system for adenine or adenosine needs further study, but the possible involvement of phosphorylase and/or phosphorylase-like transport vehicles cannot be ruled out.

A purine nucleoside phosphorylase has been implicated in the uptake of adenosine by E. coli cell membranes (36). Adenosine is first broken down to adenine and ribose-1-P; adenine is then translocated as a nucleotide by a phosphoribosyl transferase. (Adenine + PRPP $\rightarrow$ AMP + PPi).

Ribose-1-P and adenine both inhibit the uptake of adenosine (36), while PRPP stimulates it. Isolated membrane vesicles exhibit both purine nucleoside phosphorylase and phosphoribosyl transferase activities.

Pyrimidine nucleoside phosphorylase - Pyrimidine nucleoside phosphorylases obtained from various sources indicate that this enzyme is distinct from purine nucleoside phosphorylase (12) and this group consists of at least two kinds of enzymes differing in their specificity towards substrates (37).

B. Thymidine phosphorylase (Deoxythymidine: orthophosphate deoxyribosyl transferase EC 2.4.2.4).

Thymidine phosphorylase prefers deoxyuridine to thymidine and does not catalyze the phosphorolysis of uridine; hence it is specific for the deoxyribose moiety. The partially purified (111-fold) enzyme from Bacillus stearothermophilus can also utilize uridine, though the preferred substrates are deoxyuridine followed by thymidine. Since this was an impure preparation, the possibility of having

two enzymes in the preparation cannot be totally ruled out.

1. Molecular Properties - Thymidine phosphorylase has not been purified to any great extent. The molecular weight of the 13-fold pure thymidine phosphorylase of rat liver was found to be 110,000 (10) by the gel filtration method of Andrews (38). The Bacillus stearothermophillus enzyme has a molecular weight of 78,000 (50) by the density gradient method of Martin and Ames (39).

2. Kinetic Properties - Kinetics of the partially purified rabbit intestine enzyme indicate an ordered Bi Bi reaction where phosphate must bind first to the enzyme and ribose-1-P is the last to leave (9). A similar order was found for the E.coli enzyme (41). In the case of the rabbit intestinal enzyme the order was arrived at by a study of transferase activity, which was not inhibited by high concentrations of phosphate. No product inhibition studies were done. Using human leukocyte enzyme Gallo and Breitman (42) postulated that the transferase and phosphorylase activities are the function of one protein with two different sites for deoxy-ribose-1-phosphate and deoxyuridine, primarily because the synthesis of thymidine by the transferase reaction between

thymine and deoxyuridine was not inhibited by high deoxyribose-1-phosphate concentration.

3. Regulation and Role - Human leukocyte thymidine phosphorylase is inhibited by thymine (42) and the 6-oxypurines hypoxanthine, xanthine, allopurinol and 6-mercaptopurine (43). Both transferase and phosphorylase activities are subject to substrate inhibition by thymine; the inhibition of phosphorylase activity suggests an additional inhibitory site for thymine. Purines inhibit only deoxythymidine synthesis, while thymine inhibits both the synthesis and the transfer. The presence of urea prevents the substrate inhibition due to thymine but the inhibition due to hypoxanthine is not changed (43).

Partial hepatectomy increases deoxythymidine kinase (44, 45) and deoxyribose-5-P aldolase activities (46), but decreases dihydrothymine dehydrogenase activity (47). The availability of deoxyribose-1-phosphate and thymine thus favours the synthesis of thymidine. Deoxyribose-1-phosphate and deoxyribose-5-phosphate can induce thymidine phosphorylase in E. coli (48) and S. typhimurium (49), whereas both deoxyribose-1-PO_4 and ribose-1-PO_4 can induce the B. stearothermophilus and B. cereus enzymes (50). It is not known at

present whether this is also true for some mammalian tissues. In leukocytes obtained from patients with chronic myelogenous leukemia the level of thymidine phosphorylase and deoxyribosyl transferase is lowered (51), suggesting a less significant role of "salvage pathway" in these cells. The inhibition of deoxythymidine synthesis by purine bases may provide a means for maintaining a balance between purine and pyrimidine deoxynucleotide synthesis (43). Since purine bases are normally utilized rapidly, their accumulation would indicate a block in the synthesis of purine nucleotides and thus also would stop the synthesis of pyrimidine nucleotides.

The enzyme in E. coli appears to be localized near the cell surface as judged by the fact that it is quantitatively released from the cells by osmotic shock (52, 53, 54) procedures which release a specific group of phosphatases, diesterases and nucleases into the extracellular milieu without impairing cell viability or altering the levels of other intracellular enzymes such as glucose-6-phosphate dehydrogenase, glutamic dehydrogenase and β-galactosidase. In this organism thymidine phosphorylase seems to be responsible for the uptake of thymine (52). The trans-N-deoxyribosylase, EC 2.4.2.6 (same as transferase) is also located

on the cell surface and its activity is highly dependent on the intact structure of the cells (53). This reaction requires a donor of the deoxyribosyl group other than deoxyribose-1-phosphate. However, so far there is no evidence indicating that these two activities are not the property of the same protein i.e. if the phosphorylase catalyzes the transferase reaction also.

C. Uridine phosphorylase (Uridine: orthophosphate ribosyl-transferase EC 2.4.2.3).

In 1950 Paege and Schlenk were the first to characterize this form of pyrimidine nucleoside phosphorylase from E. coli (4, 5). Since then this enzyme has also been reported in other bacteria (50), a number of vertebrate tissues like intestine, liver, spleen (37, 1, 2, 40), brain (11) and Ehrlich ascites tumor cells (7, 55).

Uridine phosphorylase purified from most of these sources shows a preference for the substrates in the following order uridine > deoxyuridine >> thymidine. Exceptions to this specificity are shown by the enzymes from bacteria. Uridine phosphorylase from E. coli is highly specific for its ribosyl moiety (4), and the enzymes from B. Stearothermophilus

catalyze the cleavage of deoxyuridine and thymidine at a faster rate than uridine (50).

1. Molecular Properties - Uridine phosphorylase has not yet been purified to homogeneity except from Ehrlich ascites tumour (7, 55) and even here its molecular properties have not been studied. The enzyme from rat liver had a molecular weight of 110,000 (10) and the 1,900-fold pure enzyme showed two bands on electrophoresis. The anionic band increased on aging. The E. coli enzyme had a molecular weight of 148,000, on density gradient centrifugation (50).

At least two forms of uridine phosphorylase were reported in regenerating rat liver (8), one of which was termed nuclear (due to higher concentration in the crude nuclear fraction) and the other cytoplasmic. Both these enzymes were inducible by uridine and cytidine.

Similarly, existence of two isoenzymes of uridine phosphorylase in rat brain has been demonstrated (11). In both these cases no difference in the substrate specificity and pH optimum was found between the two isoenzymes.

2. Kinetic Properties - Kinetics of rat liver uridine phosphorylase has been investigated intensively (10) and this

enzyme was found to have an ordered sequential reaction with phosphate adding first to the enzyme, followed by uridine. Ribose-1-phosphate is the last to leave the enzyme. Kinetics of guinea pig intestine uridine phosphorylase was studied using the transferase activity of the enzyme. Close to stoichiometric amounts of phosphate were required for the transfer between bases and the nucleosides and high concentrations of phosphate inhibited this transfer (9). Based on these observations it was concluded that similar to purine nucleoside phosphorylase, the nucleoside adds on to the enzyme first and the base is the last to leave. Complete studies using product inhibition patterns were not done hence the validity of the sequence remains questionable for the guinea pig intestine enzyme.

3. Regulation and Role - In 1957, Canellakis (56) demonstrated that rat liver slices utilized uracil-2C^{14} and incorporated it into polynucleotides; other tissues like intestinal mucosa and Ehrlich ascites tumour (57) were more active in this respect. Skold (1) found a good correlation between the enzyme levels of the "reserve mechanism" (viz. uridine phosphorylase and uridine kinase) and growth rate. In regenerating rat liver there was almost a 3-fold increase

in the level of uridine phosphorylase after 36 hours. Uridine phosphorylase levels could also be increased in normal and regenerating rat liver following an injection of cortisol, uridine or cytidine (8, 58, 59). These observations point to the role of this enzyme in the utilization of preformed uracil in the synthesis of nucleotides.

Uridine phosphorylase of Bacillus cereus was found to be inhibited by GTP, CMP, CTP, XMP, and CMP (50% inhibition with 0.15 to 0.3 mM). Co-operative inhibition was found between CMP and CTP molecules and this may be an important means of regulating the uridine nucleotide level in Bacillus cereus. The effect of these nucleotides on the synthetic reaction should be investigated for a better understanding of the role of this enzyme.

Uridine phosphorylase has not yet been implicated in the transport of nucleosides and bases across biological membranes. Jaquez (60) indicated that a passive transport was involved in the entry of the nucleosides into Ehrlich ascites cells. Since then a number of studies indicate the existence of a carrier mediated transport. Enzyme-like transport systems for nucleosides which are susceptible to inhibition by heterologous nucleosides were found for chicken,

rat and mouse cells in culture (61). In Novikoff hepatoma cells Plagemann (62) demonstrated the existence of three separate transport systems for (a) adenosine (b) uridine and cytidine (c) guanosine and inosine. These transport systems are inhibited by p-chloromercuribenzoate and competitively inhibited by each of the other nucleosides, thymidine, persantin and phenethyl alcohol. Heat treatment at 47.5^{o}C for 5' inhibited the uridine transport system only. Again uridine and cytidine transport may not be through a common carrier because they have different Km values and are inhibited to different amounts by other nucleosides, and persantin. Saccharomyces cerevisiae mutants have been isolated which have lost the capacity to transport cytidine while uridine transport is unaltered (63). Nucleoside transport in human erythrocytes was found to be due to a nonconcentrative, facilitated diffusion mechanism, where efflux of uridine or thymidine from preloaded cells could be induced by an inward flow of various nucleosides but not by purine or pyrimidine bases (64).

In conclusion it can be said that the specificities and properties of the nucleoside transport systems are very similar to those of the various enzymes (phosphorylases in particular) involved in nucleoside metabolism; characteristics

such as saturability, competition between related permeants, inhibition by specific compounds and the phenomenon of counter flow, show that transport of nucleosides is a "carrier mediated"process; the nucleoside phosphorylases have been implicated in certain instances (36, 52) but the exact role of these phosphorylases in other cases still needs to be evaluated.

III. OBJECTIVE

The review of literature brings to mind the following questions:-

(1) What is the structure of the uridine phosphorylase molecule?

(2) How is it regulated i.e. what makes it work in a particular direction and at what efficiency?

(3) Why have isoenzymes and how do they differ from each other?

(4) Is this enzyme located in some subcellular particles or is it only present in the cytosol? Could it have a role in transport of nucleosides?

It is not possible to find the full answers to all these questions in a short time but efforts have been made to answer some of these. The work embodied in this thesis can be divided as follows:-

I. Purification, properties and isoenzymes of rat liver uridine phosphorylase.

II. Transferase activity of the purified enzyme, studied for a better understanding of the kinetics.

III. Subcellular distribution of pyrimidine nucleoside phosphorylases.

IV. MATERIALS

Acrylamide - Eastman Organic Chemicals,

Adenosine 5' phosphate - Sigma Chemical Company

Alcohol dehydrogenase - Worthington Biochemical Corp.

Alkaline phosphatase (calf intestinal mucosa) - Sigma Chemical Co.

Ammonium hydroxide - Fisher Scientific Co.

Ammonium molybdate, AR - British Drug Houses

Ammonium sulphate - J.T. Baker Chemical Co.

Bromic acid, AR - British Drug Houses

Carbowax 20 M - Union Carbide

Catalase - Worthington Biochemical Corp.

Coomassie brilliant-blue R250 - Consolidated Laboratories Ltd., Weston.

Deoxy - D-Ribose-1-phosphate, dimonocylohexylammonium salt - Sigma Chemical Co.

Deoxyuridine - Sigma Chemical Co.

Deoxyuridine uniformly labelled with H^3 - New England Nuclear Corp.

Dialyzing tubing -

Ethyl acetate, AR - British Drug Houses

Ethylene diamine tetraacetic acid - Sigma Chemical Co.

Formic acid, AR - British Drug Houses

Glycine - Nutritional Biochemical Corp.

Glycylglycine - British Drug Houses

Glucose-6-phosphate (di K salt) - Sigma Chemical Co.

Histidine (free base) - Nutritional Biochemicals Corp.

Hydroxylapatite (Biogel) - BioRad Laboratories

Hydrogen peroxide - Fisher Scientific Co.

Frozen Livers from young rats - Pel Freeze Inc.

N, N'Methylene bis acrylamide - Eastman Organic Chemicals.

2-Methoxy-ethanol (Methyl cellosolve) - British Drug Houses

Molecular weight marker (non enzymic) kit - Mann Research Laboratories

p-Nitrophenol phosphate (Di sodium salt) - Sigma Chemical Co.

Omnifluor - New England Nuclear Corp.

Photoflo 200 solution - Eastman Kodak Co.

Phosphorus 32 - Atomic Energy of Canada Ltd., Ottawa.

Potassium arsenate - British Drug Houses

Potassium chloride, AR - British Drug Houses

Potassium dihydrogen orthophosphate, AR - British Drug Houses

Potassium phosphate - Matheson Coleman and Bell

α-D Ribose-1- phosphate, dimonocylohexylammonium salt - Sigma Chemical Co.

Sephadex (DEAE, G200, G150, G100) - Pharmacia

Sucrose - Schwarz/Mann Div. of B and D.

Sodium laurylsulphate - DuPont of Canada Ltd.

Sodium molybdate - British Drug Houses

Sodium dihydrogen orthophosphate - British Drug Houses

Tetrazolium Triphenyl - Mann Research Laboratories

N, N, N', N'Tetra methylethylene diamine - Eastman Organic Chemicals

Tracking dye concentrate - Canal Industrial Co.

Trizma base (Tris hydroxymethyl amino methane) - Sigma Chemical Co.

Triton X-100 (octyl phenoxy polyethoxy ethanol) - Sigma Chemical Co.

Toluene - Fisher Scientific Co.

Uracil - Sigma Chemical Co.

Uracil 2-C^{14} - Schwarz Bioresearch Inc.

Uridine - Mann Research Laboratories

Uridine uniformly labelled with H^3 - New England Nuclear Corp.

V. METHODS

A. Assay of uridine phosphorylase

1. Uridine phosphorylase activity was determined by the spectrophotomeric method of Yamada (8), where the increase in absorbance due to the formation of uracil is measured in an alkaline solution. The assay mixtures were of the following types:-

(a) Assay mixture for determination of activity in subcellular fractions, purified fractions, and for column eluates (other than those at pH 8.0) during the purification of the enzyme. The assay mixture at pH 7.4 contained in a final volume of 1.5 ml: 150 micromoles of K phosphate buffer at pH 7.4; 0.05 M K phosphate buffer at pH 7.4 and enzyme dissolved in the same buffer to make 0.5 ml and 7.5 micromoles of 2-mercaptoethanol. The reaction was started by the addition of 5.0 μmoles of pyrimidine nucleoside. After incubation at 37^{o}C for the required length of time (so that absorbance readings fell in the range of 0.1$\longrightarrow$0.3) the reaction was stopped by the addition of 0.45 ml of cold 2.12 N perchloric acid. In the control tubes the acid was added prior to the addition of substrate. The acidified mixture was then spun at 14,800 g for 10 min in a Servall

RC-2 centrifuge. Seventy μl of 10 N NaOH was then added to 1 ml aliquots of each tube and the mixtures were then read at 290 mμ for uridine and deoxyuridine and at 295 mμ for thymidine.

(b) The assay procedure used for determining activities of the eluates from columns at pH 8.0 was essentially similar to (a): the mixture had 30 μmoles of K phosphate buffer at pH 6.85, 170 μmoles of phosphate buffer at pH 7.4, 7.5 μmoles of 2-mercaptoethanol and 1.2 ml of enzyme in 0.02 M K phosphate buffer at pH 8.0. The incubation was similarly started by the addition of 5 μmoles of substrate adjusted to pH 7.0. The final pH of this mixture was also 7.4.

(c) For mixtures at different pH or in the absence of phosphate, buffers like Glycyl-glycine pH 8.9, K acetate pH 6.0, and Tris-HCl were used in concentrations to give the required pH. The exact amounts of the buffers added will be described in the results section along with individual experiments. Apart from this the assay procedure was the same as above.

(d) Formation of uridine and deoxyuridine was also measured spectrophotometrically and the reaction mixture contained 150 μmoles of Tris-HCl buffer pH 7.4,

4.2 μmoles of ribose-1-phosphate, pH 7.0, 1.0 μmole of uracil and enzyme in 0.05 M Tris. The rest of the procedure was the same as before and the decrease in absorbance in sample tubes was measured at alkaline pH.

2. Protein was determined by the method of Lowry et al. (66) for most cases except for eluants from chromatographic columns when Warburg and Christian's method (67) using optical density readings at 260 mμ and 280 mμ was utilized.

One unit of nucleoside phosphorylase (8, 10) is the amount of enzyme required to make one μmole of nucleoside or base in one hour.

3. Chloride estimation - The titration procedure of Schales and Schales (69) was followed and solutions from Sigma's chloride estimation kit were used. Mercuric nitrate (approximately 0.01 N) which ionizes to yield Hg^{++} ions, was used to titrate chloride of a standard or an unknown (0.02 ml $\longrightarrow$ 0.5 ml of the column fractions). $2Cl^- + Hg^{++} \longrightarrow HgCl_2$. Mercuric chloride does not ionize and the end point is determined by the violet colour of the indicator Diphenyl carbazone in the presence of excess Hg^{++} ions. 2-mercaptoethanol reacts with mercuric nitrate hence the amount of

mercuric nitrate needed by fractions before the gradient was started was deducted from the amount of mercuric nitrate needed by the same volume of a fraction after the gradient was started.

B. Purification of uridine phosphorylase

The method of Kraut and Yamada (10) was followed in principle except for slight modifications which provided the complete separation of two uridine phosphorylase isoenzymes. The general procedure for most preparations and modifications thereof will be described in the next 7 sections.

1. Homogenization - Livers from young rats used in the preparation of pure enzyme were bought from Pel Freez Inc. Approximately 50 rat livers were used for most preparations except in the last one when 100 rat livers were used. All the steps in the purification were carried out in the cold at 4°. The unperfused livers were thawed, cut into small pieces and homogenized in a glass homogenizer with a teflon pestle in 5 volumes of buffer A (K Pi buffer pH 7.0, 1 mM EDTA, 10 mM 2-mercaptoethanol) for each gram of liver and

the homogenate was then filtered through 4 layers of cheese cloth. The filtered homogenate was then spun at 50,000 rpm (160,000 g) for 1 hour in a 60 Ti rotor, in a Beckman ultra-centrifuge.

The supernatant was then used for ammonium sulphate fractionation.

2. Ammonium sulphate precipitation - Enough ammonium sulphate was added to give 30% saturation. The precipitate obtained (after spinning at 12,000 rpm (17,300 g) for 20 min in a Servall centrifuge) was discarded and to the supernatant more ammonium sulphate was added to give 65% saturation. The precipitate now obtained contained most of the enzyme and was recovered by spinning for 20 min as before; it was dissolved in a minimum amount of buffer A. This fraction was then used as such for heat treatment.

3. Heat treatment - Thirty ml fractions of the enzyme obtained from the previous step were put in stainless steel tubes and heated with gentle stirring in a water bath for 3 min at 50^{o}, following which they were immediately transferred to an ice bath. The precipitated protein was removed by centrifugation at 30,000 rpm for 30 min in a Beckman

Model L ultracentrifuge with Rotor no. 30. The supernatant was dialyzed overnight against 100 volumes of Buffer B (0.02 M K-Pi, pH 8.8, 1 mM EDTA, 10 mM 2-mercaptoethanol).

4. DEAE Sephadex chromatography at pH 8.0 (8) - The heat treated, dialyzed enzyme preparation was diluted with enough Buffer B to give a final protein concentration close to 15 mg/ml. This fraction was then applied to a washed, equilibrated DEAE Sephadex pH 8.0 column. Approximately ten gm of exchanger (DEAE Sephadex A-50) was used for every gm of protein applied. The column was then washed with 1.5 times the bed volume of Buffer B. A linear gradient was then started with 5.35 x bed volume of Buffer B in the mixing chamber and the same volume of 0.4 M KCl in Buffer B in the other chamber. The fractions containing the uridine phosphorylase activity were pooled and concentrated by carbowax, to a protein concentration of approximately 15 mg/ml and dialyzed against 150 volumes of Buffer C (0.02 M K-Pi, pH 7.0, 1 mM EDTA, 10 mM 2-mercaptoethanol) overnight at 4^{o}.

In the last preparation upward flow was used to minimize column packing and change in flow rate. The gradient was made shallower by the use of 0.15 M KCl and the volume

of KCl solution was 2.15 times in proportion to bed volume. These changes produced the complete separation of the two isoenzymes of uridine phosphorylase. The next three steps were carried out for each isoenzyme separately.

5. DEAE Sephadex pH 7.0 - The procedure was very similar to the first column (step 4) except that the buffer was at pH 7.0 instead of 8.0. The gradient was also of the same proportions. In two preparations the isoenzymes separated at this step.

6. Sephadex gel filtration - In preliminary experiments it was found that the combination of gels in series improved the resolution of this mixture of protein components of similar molecular weights. The Sephadex G-100, Sephadex G-150 and Sephadex G-200 were put in each of three columns which were connected in series. The last two were fitted with flow adaptors and upward flow was used to minimize column packing. The columns were washed and equilibrated with Buffer B. The enzyme was applied in a sample applicator on top of the Sephadex G-100 bed and the total volume applied was close to one hundredth of the total bed volume of all columns. The active fractions were pooled and dialyzed

against 100 volumes of Buffer D (0.02 M Pi pH 7.3, 10 mM 2-mercaptoethanol) overnight.

The quality of packing of all three columns was verified by applying 5 ml of 0.2% Blue dextran 2,000 in Buffer B.

7. Hydroxyapatite chromatography - Hydroxyapatite was washed thoroughly with Buffer D (0.02 M K-Pi, pH 7.3 and 10 mM 2-mercaptoethanol) without any EDTA, which would have complexed the Ca^{++} ions of the resin (68). The proportion of bed volume (in ml) was thrice the amount of protein (in mg) to be applied. The pooled, dialyzed enzyme from the previous gel filtration step was applied to the hydroxyapatite column, and then the column was washed with 1.5 bed volumes of Buffer D. The enzyme was eluted from the column by a linear gradient consisting of equal volumes of Buffer D and 0.25 M Pi pH 7.3 with 10 mM 2-mercaptoethanol.

The uridine phosphorylases were the first proteins to be eluted from the column. In the last preparation with isoenzyme #1 the 0.25 M Pi, pH 7.3 solution was changed to 0.15 M, which proved useful in preventing contamination of the fractions of the trailing part of peak with closely eluting proteins. The active fractions were pooled and

dialyzed against a minimum of 100 volumes of Buffer A (without EDTA) for 8 hours and stored in small aliquots. When it was necessary to store fractions in the absence of phosphate a fraction of the enzyme was dialyzed against 500 volumes of 0.05 M Tris pH 7.0, 10 mM 2-mercaptoethanol, with at least 3 changes of buffer.

8. Dialyzing and concentrating procedures

(a) Dialyzing bags were washed first with 1 mM EDTA (pH 7.0) and then finally with the buffer solution to be used. For volumes greater than 40 ml a continuous flow dialyzing apparatus was used. For smaller volumes, conical flasks or beakers equipped with magnetic stirrers and covered with parafilm were employed. The buffer surrounding the dialyzing bags was changed three or more times with fresh buffer.

(b) Concentrating procedure - With purer fractions procedures like precipitation by ammonium sulphate and ultrafiltration usually meant large loss of activity. The procedure of Setlow and Lowenstein (70), and Kohn (71) as modified by Kraut (75) was found to be useful for concentration of uridine phosphorylase samples. The dilute enzyme was poured into washed dialyzing tubing, and immersed in a

vessel full of carbowax flakes. The time required to concentrate the protein depended on the surface area of the bags and was approximately 2 hours to remove 50 mls from a bag of 3/4" diameter. After concentration the bags were washed by quickly rinsing in 0.05 M K Pi pH 7.0, 10 mM 2-mercaptoethanol a number of times until free of carbowax.

C. Gel Electrophoresis

1. pH 8.0 system - The method of Davis (72) was used, when the gel buffer was 0.37 M Tris-HCl at pH 8.9; the electrophoresis buffer was 0.05 M Tris-glycine at pH 8.3, the spacer gel buffer was 0.06 M Tris-HCl at pH 6.5. The final concentration of acrylamide was 7% for most diagnostic runs. The current was 2½ milliampere per tube. Protein was applied in a 8-10% sucrose solution containing Bromophenol blue as the tracking dye. All solution were cold and electrophoresis was performed in a cold room at 4°C. The tracking dye front was marked by inserting a piece of thin wire into the gel.

2. pH 7.0 system - The method of Williams and Reisfeld (73) was used. The separation gel buffer was 0.07 M Tris-HCl at pH 7.5, the spacer gel was 0.05 M Tris-HCl at pH 5.5 and the

electrophoresis buffer was 0.008 M Tris-barbiturate at pH 7.0. The concentration of acrylamide was 7% for most runs, unless specified otherwise. The other conditions like the sample solution, current and temperature were the same as in the pH 8.0 system.

3. Staining for enzyme activity - The method of staining developed for glycogen phosphorylase (74) can also be used for purified uridine phosphorylase. Bands of enzyme activity have been stained by the method of Mattson and Jensen in the present work. The gels were incubated in a medium containing 150 μl of 1 M potassium arsenate, pH 7.4 or 8.0, 100 μl of 0.15M 2-mercaptoethanol, 150 μl of 1 M Tris-HCl pH 7.4 or pH 8.0, 100 μl of 0.1 M uridine and water to make 3 ml. The tubes were incubated at 37^{o} for 30 min. The reaction medium was then decanted off and 3 ml of 0.5 N NaOH and 300 μl of 0.5% triphenyltetrazolium (aqueous) solution added. The tubes were then put in a boiling water bath for 3-5 min until bright red bands appeared. The solution was drained off and the gels were stored in 7% acetic acid. Simultaneous controls without substrate were run for every new preparation of enzyme. With enzyme obtained from the DEAE Sephadex pH 8.0 column no bands appeared in incubated gels in the

absence of substrate. This procedure is an adaptation of the staining method for purine nucleoside phosphorylase developed by Gardner and Kornberg (76) which is based on the method of Mattson and Jensen (77).

4. Staining for protein - The method of Chamrach (78) was used. Protein bands in the gel were stained by first fixing the bands in 12.5% trichloracetic acid (TCA) and then staining with coomassie blue (0.005%) in 12.5% TCA for about 2-6 hours. The stained gels were stored in a faintly blue solution of coomassie blue in 7% acetic acid. No destaining was required.

5. Determination of molecular weight, and separation of size and charge isomers - In 1968 Hedrick and Smith (79) published a method by which isomers of different size and charge could be distinguished. This method is particularly useful in determining the molecular weight of enzymes in crude preparations, if they can be stained specifically for their activity and also for some highly purified proteins which are available in very small amounts. In this method the logarithm of protein mobility relative to the dye front is plotted versus acrylamide gel concentration; protein

isomers of different size give a family of nonparallel lines extrapolating to a common point in the vicinity of 0% gel concentration. Charge isomeric proteins give parallel lines. Proteins which differ in both size and charge give nonparallel lines which intersect at high gel concentration. Since the slope of the line depends on the molecular weight of the particular protein, by comparison to the slopes of some standard molecular weight markers it is possible to determine the molecular weight of an unknown protein. The principle of this method has been utilized for uridine phosphorylase but the electrophoresis system used is that of David (66) and of Williams and Reisfeld (73).

6. Determination of subunit weight - The method of Weber and Osborn (80) was used for SDS gel electrophoresis. A solution of 0.1% SDS, 10% acrylamide and sodium phosphate buffer at pH 7.2 were used. Constant current was maintained at 7 mA per tube.

For staining of protein the SDS was first leached out of gels in 12.5% TCA, overnight in a shaker. The gels were then placed in a 0.005% solution of coomassie blue for about 4 hours and then stored without destaining in 7%

acetic acid. The number of bands increased on increasing the time of pre-treatment of protein with 2-mercaptoethanol and SDS to about 4 bands. The method of pre-treatment and sample application was that of Dunker and Rueckert (81).

D. Density gradient centrifugation

1. Method of Martin and Ames (39) was used with slight modifications. Solutions of 0 to 18% sucrose in 0.05 M Tris pH 7.0, 1 mM EDTA and 10 mM 2-mercaptoethanol were used. Phosphate was added to give a final concentration of 0.2 M concentration and in control samples KCl was used to replace phosphate and provide a medium of equal ionic strength. A Beckman ultracentrifuge equipped with rotor no. SW-50.1 was used, and the spinning time was either 16 hrs (with phosphate and KCl) or 6-8 hrs without phosphate or KCl. Sixteen fractions were collected by means of a drop counter.

2. Alcohol dehydrogenase was assayed by determining the amount of DPN reduced spectrophotometrically (82) at 340 mμ. Assay medium consisted of 0.5 ml of 0.32 M pyrophosphate buffer, pH 8.8, 0.15 ml of 2 M ethanol, 0.05 ml of 0.025 M NAD, enzyme and water to make a total volume of 1 ml. One

unit of enzyme activity is defined as the amount that reduces 1 μmoles of NAD/min at 25°C.

3. Alkaline phosphatase was assayed by following the hydrolysis of p-nitrophenyl phosphate at 410 mμ (83). The assay medium contained 0.5 ml of 1 M Tris-HCl buffer, pH 8.0; 10 μl of 0.105 M p-nitrophenol phosphate; enzyme and water to make a total volume of 1 ml.

One unit of enzyme activity is defined as the amount that produces 1 μmole of p-nitrophenol per hour.

Cytochrome C and myoglobin were determined separately by reading the absorbance of the solutions at 400 mμ.

E. Amino acid analysis

1. One and half mg of protein (in ammonium carbonate buffer) was put in an ignition tube with 1 ml of 6 N HCl. The contents were frozen on dry ice and evacuated by means of a vacuum pump. The tube was sealed and left in an oven at 110° for 21 hours, and then the sample was dried in a vacuum desicator with NaOH pellets, overnight. The sample was redissolved in sodium citrate buffer at pH 2.2 and applied to the columns of a Beckman-Spinco 120 C automatic amino acid analyzer.

2. For determination of cysteine and cystine performic acid oxidation was performed according to the method of Hirs (84). Half a milliliter of 30% H_2O_2 and 9.5 ml of 99% formic acid were mixed in a stoppered flask and allowed to stand for 2 hours at 25°C. This reagent and the protein solutions were cooled separately at -7° to -10° for 30 min and then were mixed in equal volumes (0.2 ml each); to this mixture 20 µl of anhydrous methanol was added. The mixture was then left at -10°C for 2.5 hours. The sample was diluted to 8 mls and lyophilized. The dried powder was suspended in another 2 mls of water and transferred to an ignition tube. This was again lyophilized and to the powder 1 ml of 6 N HCl was added. The steps from here onwards were the same as that for direct hydrolysis (see 1 above).

F. Assay of transferase activity

Transferase activity was measured in 2 ways: (1) between the base and the nucleoside and (2) between phosphate and ribose-1-phosphate.

1. (a) The assay mixture for nucleoside and base transfer consisted of 25 µl of 0.1 M Tris-HCl, pH 7.2; 25 µl of 1.0 M glycyl-glycine, pH 8.9; 10 µl of 0.15 M 2-mercaptoethanol; substrates

of the required specific activity (the exact amount is given with each experiment), enzyme and H_2O to make 150 μl. The pH of the final reaction mixture was 8.05. The tubes were incubated for 30 min, or as specified in the text, at 37°C and the reaction was stopped by placing the tubes in a boiling water bath for 4 minutes. Ten or 20 μl of the assay mixture were then applied to strips of Whatman No. 1 paper for chromatography.

(b) Separation of base from the nucleoside. The method of Deverdier and Potter (3) with the solvent system of Fink et al. (40) was employed. Ten or 20 μl aliquot of the assay mixture were applied to Whatman No. 1 paper and equilibrated for at least 2½ hours in the aqueous phase of the solvent system, which consisted of ethyl acetate, water and formic acid in 12:7:1 proportions by volume. In the chromatogram obtained after development with the organic phase uracil was separated from uridine or deoxyuridine but not from thymidine. The chromatograms were air dried and the spots detected under ultraviolet light. The marked areas were then cut out and the radioactivity of each was determined.

2. (a) The assay mixture for ribose-1-phosphate and phosphate exchange contained 25 μl glyclglycine buffer, pH 8.9;

25 μl 0.1 M Tris-HCl buffer, pH 7.2; 10 μl 0.15 M 2-mercapto-ethanol; 5 μl of 0.025 M ribose-1-P; 5 μl of 0.025 M potassium phosphate; 10 μl of ^{32}Pi (1:200 dilution) 0.23 μCi; enzyme and H_2O to make 150 μl. The final pH of the mixture was 8.0. Tubes were incubated at 37°C for 30 min and then the reaction was terminated by placing the tubes in a boiling water bath for 4 min.

(b) Separation of phosphate from ribose-1-phosphate:- five microliters of the reaction mixture were spotted on Whatman No. 1 paper and the paper was equilibrated for 2½ hours in an aqueous phase (a beaker containing water was placed in the chamber). The solvent system used was a modified one (85) and contained methyl cellosolve, 3 N NH_4OH and acetone in 7:3:2 proportions by volume. Acetone was used instead of methyl ethyl ketone as in the original method and was found to be more satisfactory. The separated spots of phosphate and ribose-1-phosphate were identified by spraying very lightly with a mixture of 25 ml of 4% sodium molybdate, 10 ml of 0.01 N HCl, 5 ml of 20% $HClO_4$ and 60 ml of H_2O. The strips were heated for 4 minutes at 100°. The ribose-1-phosphate spots were then cut out and placed in scintillation vials for determination of radio-activity.

3. Radioactivity determination - The radioactivity associated with the base or nucleoside spot was determined by means of a Beckman liquid scintillation counter. The area of the spot which was either determined by UV light in the case of bases and nucleosides or by the blue spot in the case of phosphate and ribose-1-phosphate, produced by the reaction with molybdate, was cut out and eluted in 1 ml of water. Half of this eluate was put in a scintillation vial, dried and to this 15 ml of standard scintillator solution (4 gms omnifluor in 1 liter toluene) was added and the radioactivity determined in a Beckman scintillation counter. Occasionally (for ^{32}Pi) the elution step was omitted and the paper containing the radioactive spot was put directly into a vial and the radioactivity determined.

G. Identification and separation of subcellular particles

The fractionation of the subcellular particles was carried out in two separate types of experiments-in one the nuclei, mitochondria, lysosomes and microsomes were separated, and in the other plasma membrane was isolated. In both the criterion of purity was the presence

or absence of the respective marker enzymes of various fractions. Glutamate dehydrogenase was the mitochondrial marker, acid phosphatase the lysosomal marker, 5'-nucleotidase was the plasma membrane marker and glucose-6-phosphatase was the microsomal marker. The procedures followed are essentially similar to those recently published by Smith and Yamada (86).

1. Subfractionation procedure for nuclei, mitochondria, lysosomes and microsomes - Perfused livers from male Holtzman rats weighing close to 200 gms were used. The livers were perfused with ice-cold 0.9% NaCl in situ and homogenized in 0.25 M sucrose, 3 mM $MgCl_2$ and 5 mM 2-mercaptoethanol. The homogenate was filtered through 4 layers of cotton gauze and the filtered homogenate was spun at 750 x g for 10 minutes. The sediment was used further for the purification of nuclei. The supernatant was spun at 3,300 x g for 10 minutes to separate the heavy mitochondrial fraction as a sediment. On further centrifugation of the supernatant at 16,300 g for 20 min a pellet was obtained which was used to purify the lysosomes. The microsomes were then obtained as a pink pellet by spinning the 16,300X g supernatant at 160,000xg for 60 min. The supernatant

from this last step is called the "cytosol". The final pellets in every case were suspended in 0.25 M sucrose, 1 mM EDTA and 5 mM 2-mercaptoethanol.

Nuclei were purified from the 750xg pellet by the method of Pogo, Allfrey and Mirsky (87). The pellet was suspended in 2.4 M sucrose, 3 mM $MgCl_2$ and 5 mM 2-mercaptoethanol with a Virtis "23" homogenizer set at 22, for 2 minutes. The suspension was then spun at 78,500 x g in Rotor no. 30 for 30 min. A small white pellet at the bottom of the centrifuge tube consisted of pure nuclei which was washed once and finally suspended in 0.25 M sucrose and 1 mM EDTA and 10 mM 2-mercaptoethanol. The thick layer floating on the top of sucrose was called debris and consisted of a mixture of plasma membranes, heavy mitochondria and possibly a few whole cells.

Lysosomes were purified from the 16,300xg pellet by the method of Sawant et al. (88). The pellet was suspended and recentrifuged at 9,500xg for 10 minutes. The 9,500xg pellet was again suspended in 50 ml of 0.45 M sucrose which formed the top of a discontinuous density gradient which consisted of 70 ml of 0.7 M sucrose as the bottom layer and 60 ml of 0.6 M sucrose in the middle.

2. Purification of plasma membranes - The method of Bosmann, Hagopian and Eylar (89) was used. In these experiments the livers were homogenized in a different medium which consisted of 0.02 M Tris-HCl buffer, pH 7.0, 0.01 M EDTA, pH 7.0 and 5 mM 2-mercaptoethanol. The homogenate was filtered through four layers of cotton gauze and spun at 4,000 x g for 15 min; the pellet from this spin was washed once with the homogenizing buffer and then suspended in 0.05 M Tris pH 7.0, 1 mM EDTA and 10 mM 2-mercaptoethanol. The 4,000xg supernatant was adjusted to 45% sucrose and this formed the bottom layer (10 ml) of a discontinuous gradient, on top of which the following were layered in order: 35% sucrose (6.5 ml), 30% sucrose (6.5 ml), 25% sucrose (5 ml), 0.05 M Tris pH,7.0 (1 ml). The tubes were then spun in a SW 25.1 swing out bucket rotor at 64,000 x g for 16 hours. The plasma membranes which formed a light pink pellet at the bottom of the tubes were resuspended, washed and stored in 0.05 M Tris 7.0, 3 mM $MgCl_2$ and 5 mM mercaptoethanol. The supernatant on top of the membrane pellet was diluted with 3 volumes of Tris-EDTA (pH 7.0) buffer and centrifuged at 70,000 x g for 1 hour. The pellet from this fraction was also suspended in 0.05 M Tris, 3 mM $MgCl_2$ and 5 mM 2-mercaptoethanol.

The method of Fitzpatrick et al. (90) was also tried with some modification for the isolation of plasma membranes and was found to be a fast method with good recovery, but there was often heavy contamination by mitochondria. Therefore, this method was combined with that of Hagopian and Eylar to obtain heavy and light membrane fragments.

The livers were homogenized in 3 volumes of ice-cold 0.25 M sucrose, 1 mM EDTA and 5 mM 2-mercaptoethanol, by means of a Potter-Elvehjem homogenizer equipped with a teflon pestle, at a rate of 16 strokes per minute. The homogenate was filtered through 4 layers of gauze and spun at 1,500xg for 10 min in a Servall RC-2 centrifuge. The supernatant was saved and used for membrane purification as before. The pellet was used for further purification and hence was suspended in 2 M sucrose, 10 mM 2-mercaptoethanol and 3 mM $MgCl_2$ (1 ml per gram of liver) and mixed with 3 strokes of homogenizer. The mixture was then spun at 13,300 g (10,500 rpm) for 10 min. The pellet consisted of nuclei and cell debris; the supernatant was diluted with 7 volumes of cold H_2O and centrifuged at 35,500xg (17,000 rpm) for 15 min. The resulting pellet had two distinct layers, pink

at the top, consisting of membranes, and brown at the bottom consisting of mitochondria. All pellets were stored in 0.25 M sucrose, 5 mM 2-mercaptoethanol and 1 mM EDTA.

3. Assay of marker enzymes

(a) Glutamate dehydrogenase - the spectrophotometric method of Beaufay et al. (91) was used. The reaction mixture contained potassium phosphate buffer (pH 7.7) 20 μmoles; nicotinamide, 30 μmoles; potassium cyanide, 0.4 moles; 2-mercaptoethanol, 5 moles; NAD 1.4 μmoles; 0.1% Triton X-100; substrate and enzyme to a total volume 1 ml. The reaction was started by the addition of 13 μmoles of glutamate. The extinction coefficient was 6.22×10^6 per mole of NADH and one enzyme unit was that quantity which catalyzed the formation of 1 μmole of NADH per hour.

(b) 5'nucleotidase - This membrane marker was assayed according to the method of Emmelot et al. (92). The assay mixture consisted of Tris buffer, pH 7.2, 50 μmoles; potassium chloride, 100 μmoles; $MgCl_2$, 10 μmoles; enzyme, substrate and water to give a final volume of 1.0 ml. The reaction was started by the addition of 10 μmoles of adenosine 5-'monophosphate, pH 7.0 and incubation was carried out for 15 min at 37^o following which 1 ml of 10%

TCA was added to stop the reaction. Inorganic phosphate in the blank and sample tubes were determined by the method of Gomori (93). One unit of enzyme was that amount which catalyzed the release of 1 μmole of inorganic phosphate.

(c) Acid phosphatase - This lysosomal marker was assayed according to the method of Bristow and Yamada (94). The assay mixture consisted of p-nitrophenol phosphate, 10.5 μmoles; acetate buffer pH 5.0, 50 μmoles; Triton X-100, 0.1% (V/V); enzyme and water to give a final volume of 1 ml. The reaction was started by the addition of substrate and incubation was done at 37° for 10 min following which 1 ml of 1 N perchloric acid was added to stop the reaction. The mixture was centrifuged at 17,000xg for 10 min and then to 1 ml aliquots of the supernatant solution 0.3 ml of 0.2 M NaOH was added. The yellow colour of the sample tubes was measured against appropriate controls (to which the substrate was added after the addition of acid) at 400 mμ in a Beckman model DU spectrophotometer. The extinction coefficient of p-nitrophenol is 4.58 per μmole. One unit of enzyme was that amount which released 1 mμmole of p-nitrophenol in one hour at 37°C.

(d) Glucose-6-phosphatase - This microsomal marker was assayed according to the method of De Duve et al. (95). The assay mixture consisted of potassium acetate buffer, pH 6.5, 1.0 μmole; histidine, pH 6.5, 7 μmoles; EDTA, pH 6.5, 1.0 μmole; enzyme, substrate and water to give a final volume of 1 ml. The reaction was started by the addition of 39.6 μmoles of glucose-6-P. The reaction was stopped by the addition of 1 ml of 10% TCA. The resulting mixture was then centrifuged at 17,500xg for ten minutes and 0.25 ml of the supernatant solution was used to determine the inorganic phosphate released during the incubation period according to the method of Gomori (93). The phosphate released by nonspecific phosphatase was corrected for by the method of Hers and Vantoff (96) with slight modifications. The tubes containing the same amount of enzyme as above were acidified to pH 5.0 with 10 μl of 1.0 M acetate buffer, pH 5.0 and heated at 37°C for five min to inactivate glucose-6-phosphatase. The other reagents were then added to these tubes and the reaction for the nonspecific phosphatase started by the addition of substrate. The rest of the procedure was the same as that with active enzyme. The values for nonspecific phosphates activity were substracted from

sample tubes. One unit of enzyme was the amount which released 1 μmole of inorganic phosphate in one hour at 37°C.

4. Electron microscopy of subcellular fractions - Some of the final pellets were fixed in buffered osmium tetroxide at pH 7.4. The fixation was followed by dehydration and embedding in a polyester resin. The sections obtained were then stained by lead citrate and examined under a Zeiss 9S electron microscope.

VI. RESULTS

A. Purification and properties

(a) Results of purification - Five different liver homogenates have been purified. The kinetics of the uridine phosphorylase catalyzed reaction have been already studied in detail (10), and then it was also shown that uridine phosphorylase, purified 1,900 fold from the cytoplasm of rat livers, separated into two bands of protein; whether both bands represented active enzyme was not determined conclusively. The object of the present work was to purify the enzyme to homogeneity and also find a method by which the enzyme activity could be detected on the gels. Prep gel electrophoresis was tried a number of times but was successful in only 2 trials out of seven giving a purification of 17-fold (1,200 fold when interposed after Step 6). Other columns which showed little success were CM cellulose, CM Sephadex and phosphocellulose. The enzyme was very unstable below pH 6.5 and at higher pH values the enzyme was not adsorbed on these columns.

Results of individual steps of purification are somewhat similar to earlier work (10) from this laboratory; results of various modification in the procedure will be

outlined below. In general the main difference from the previous work was that the volume of eluting buffer was kept strictly in proportion to the bed volume of the column, in order to get uniformly shallow gradients and reproducible results.

1. Homogenate - In spite of using unperfused livers the specific activity (μmoles of uracil formed/mg protein per hr) for uridine phosphorylase was higher than that found for resting adult liver (0.05 ⟶ 0.07) (130) and varied over the range of 0.08 ⟶ 0.17. Guroff and Rhoads (11) have shown the developmental pattern of uridine phosphorylase in rat liver (see Figure 2), and it can be seen that the highest specific activities are obtained in rats which are one month old. After this the specific activity declines to a lower plateau level. Young rat livers were chosen because they would be a better source of enzyme for purification purposes. The step of dialysis of homogenates was omitted due to inconveniences in handling large volumes, but the activity of the enzyme was determined after dialyzing small fractions of homogenate and supernatant. In Table I the purification procedure for preparation V is given as representative of the procedure carried out for all preparations.

2. Supernatant - The 160,000 g supernatant contained 60-70% of the activity of the homogenate. There was a slight increase in specific activity of uridine phosphorylase. This supernatant fraction was used directly for ammonium sulphate fractionation without storage and freezing.

3. Ammonium sulphate fractionation - The precipitate obtained between 30-60% saturation (AS_2) contained most of the activity which generally amounted to 55 to 60% of that of the original homogenate. This precipitate was then dissolved in a minimum amount of buffer to give concentration of 60 $\longrightarrow$ 80 mg protein/ml and was treated with heat at 50°C for 3 min. The heat treated sample (HAS_2) was purified almost 3-fold compared to the homogenate and the recovery was very good.

4. DEAE Sephadex pH 8.0 column - The recovery from this column was usually close to 80% and in most preparations from young rats the elution profile of uridine phosphorylase was somewhat similar to the profile obtained from regenerating rat liver (8) and showed two partially overlapping peaks of uridine phosphorylase activity (for preparation I see Figure 3). In the last preparation (V)

the complete separation of the two peaks was achieved at this step when a more shallow KCl gradient was used as can be seen in Figure 4. The isoenzymes in the 2 peaks are called #1 and #2. In this column most of the red pigments originating from the blood of the unperfused liver was excluded unadsorbed in the void volume. A minimum of 6-fold (usually as much as 14-fold) purification was obtained at this stage over the previous step and approximately 24-fold purification was obtained compared to the homogenate. Thymidine phosphorylase was completely separated at this step. The enzyme has no uridine cleaving activity and thus was easily distinguished from uridine phosphorylase.

5. DEAE Sephadex pH 7.0 column - Usually another 2-3 fold purification was achieved at this stage. Recovery was generally close to 90% (Figure 5 for isoenzyme #1 and Figure 6 for isoenzyme #2). A good deal of protein was retained on the column. Isoenzymes 1 and 2 were overlapping before this step but were separated on this column in 2 earlier preparations.

6. Sephadex gel filtration - Purification achieved in this step was 2-3 fold. The specific activity across the peak was not uniform in any preparation indicating

the presence of protein (Figure 7 for isoenzyme #1 and Figure 8 for isoenzyme #2) impurities. Gel electrophoresis also indicated the presence of other proteins.

7. Hydroxyapatite column - This was effective in purifying the enzyme another 3 fold. The specific activity across the peak of isoenzyme #1 for which a shallower KCl gradient than that used in earlier preparations, was used was fairly uniform (Figure 9). Since uridine phosphorylase is the first protein to be eluted the shallowness of the gradient decreases the contamination by other proteins in the trailing part of the peak. The peak of isoenzyme #2 had uniform specific activity except in the last few fractions where it was contaminated by closely eluting proteins (Figure 10); this finding was also confirmed by gel electrophoresis (see later). The specific activity for isoenzyme #1 was 49.39 and isoenzyme #2 126.03.

(b) Results of stability studies - These studies were done with a view to finding the optimal conditions for handling the enzymes. Enzyme preparation III (a mixture of isoenzyme #1 and #2) dissolved in 0.05 M Tris or phosphate buffer was divided into 300 µl aliquots and stored in individual test tubes. Samples of 10 µls were removed to assess the effects

of freezing and thawing, aging in the cold, heat treatment, storing at room temperatures in ammonium sulphate or in uridine.

As is evident from Table II a, phosphate at pH 7.0 was found to be essential for the stability of the enzyme at -40^{o}. Addition of ammonium sulphate, or albumin to the enzyme in Tris buffer did not prevent inactivation. Uridine in a concentration of 0.05 M was less than half as effective as phosphate.

At 0^{o} in ice the enzyme in Tris buffer was stable for about one week and under these conditions more uridine cleaving activity seemed to be lost as compared to deoxyuridine cleaving activity (Experiment A, Table II b). Freezing and thawing a number of times (8 times) (Table II b, Experiment B) or leaving at room temperature for about 6 hours (Table II b,Experiment C) also resulted in the loss of about 50% of the activity of the enzyme in Tris buffer as well as in phosphate buffer. This may be due to the oxidation of essential -SH groups in the enzyme.

In other experiments it was found that the enzyme cannot withstand lyophilization even if phosphate is present. However, since the protein concentration of the sample that

was lyophilized was 4.0 mg/ml, it is quite possible that with a higher protein concentration, the enzyme might not have been inactivated to the same extent.

The enzyme was (in preliminary studies) found to be heat sensitive in the absence of phosphate at 50° and lost about 60-80% of the activity in 15 minutes. Isoenzymes #1 and #2 were then tested for their heat sensitivity and some differences were found between the two (Figure 11). Isoenzyme #2 was more sensitive to heat denaturation; this difference was most apparent after 7 min of heat treatment. Phosphate could not afford full protection against heat denaturation to isoenzyme #2 and about 20% of the activity was lost after 30 min in the presence of phosphate.

(c) 1. <u>pH optimum of uridine phosphorylases #1 and #2</u> - The pH optimum of isoenzymes #1 and #2 were compared with uridine as the substrate for the phosphorolytic reaction. Figure 12 shows that the pH optimum for both is close to 8.2, but the curve for isoenzyme #1 is shallower compared to isoenzyme #2 indicating the probable involvement of groups with different pK values. The purity of the 2 isoenzymes fractions was comparable.

2. Vmax and Km apparent values at different hydrogen ion concentrations - Km apparent and Vmax values often provide interesting results which indicate the involvement of residues with particular pK's. The pKm (-log Km apparent) or log Vmax values have been plotted against pH by the method of Dixon (98). This method has been found to be useful for detecting groups involved in binding and catalysis, because there are only a limited number of residues in a protein molecule that have ionizable groups. Any break in the continuity of the pKm plot would indicate a change of ionization at a particular pH, of either the free substrate, free enzyme or the enzyme substrate complex thereby affecting binding. Any break in the continuity of the log V_M plot indicates ionization of the enzyme-substrate complex (at V_M all the enzyme is bound as ES). Since the nucleosides and base studied here have no ionizable groups in the pH region of 4 to 9, a bend in either plot would indicate a change in the ionized state of a residue in the enzyme molecule, and/or the phosphate molecule which has a pK_2 value of 7.2; ribose-1-P has a pK_1 of 6.7. The results obtained for uridine phosphorylase show more than one break for most substrates. The plot of pKm against pH indicated the presence of groups in the free enzyme with

a pK value of 8.4 and 6.85 involved in the binding of the substrate to the enzyme. Another group with pK values of 6.45 and/or 7.6 may also be involved in its binding. The Vmax also changes with pH and groups with a pK of 8.0, 6.45 and possibly 7.6 are involved in the enzyme substrate complex (see Figure 13).

The patterns with deoxyuridine also indicate the involvement of groups with pK's of 8.0 and 6.55 in the enzyme substrate complex and two groups with pK values of 6.85, and 7.7 are probably involved in the binding of deoxyuridine (see Figure 14).

Both isoenzyme #1 and #2 were compared in the study of the Km apparent of uridine at different pH values. Isoenzyme #1 had a broad pH range involved in both the catalysis and binding of uridine as compared to #2 enzyme although only 1 pK was detectable in the former case. For isoenzyme #2 binding of uridine probably involves a group of pK 8.1; the enzyme substrate complex involved a group of pK 7.5 and 8.05. Isoenzyme #2 had a group of pK 8.0 and 7.1 in the enzyme substrate complex (see Figure 15). Krenitsky's data on Kms of uridine and deoxyuridine (55) have been plotted and his results are in agreement with ours.

It can be concluded from these results that during the catalysis by uridine phosphorylase ionization states of groups like cysteine and histidine of the enzyme and the 2-OH of phosphate or ribose-1-P are of importance.

(d) 1. Substrate specificity - Both isoenzymes #1 and #2 used uridine, deoxyuridine and thymidine as substrates, as was found previously for other uridine phosphorylases of different tissues (7, 55), as well as of rat liver (8, 10). The order of preference for substrates is: uridine> deoxyuridine >> thymidine in a proportion of 10:6:1. On storing in 0.05 M Tris pH 7.0 the activity towards uridine decreased and therefore the ratio of activities (uridine to deoxyuridine) decreased. Actually the enzyme in 0.05 M Tris HCl pH 7.0 loses activity towards both substrates but the rate of loss for uridine is more than for deoxyuridine.

If uridine and deoxyuridine were phosphorolyzed at different active sites, then on adding the two substrates together one should get almost additive activity; on the other hand if they are phosphorolyzed at the same site competition between the two should be apparent. As can be seen in Table III, the addition of uridine and deoxyuridine

together gave a reading that was not additive but in fact was intermediate between that for uridine alone and that for deoxyuridine alone , indicating that an active site is common for both substrates, uridine being favoured over deoxyuridine at pH 7.4.

2. Michaelis constants (128) for uridine, phosphate and uracil of isoenzyme #1 and #2 - The two isoenzymes were compared for their kinetic constants. Reciprocals of initial velocity were plotted against reciprocals of substrate concentration. Any points that deviated greatly from a straight line relationship were discarded as recommended by Wratten and Cleland (112). From the remaining data, values of apparent Km and apparent V were calculated by computer (Olivetti-Underwood Programma 101). The Michaelis constants were calculated from the replots of intercepts or slopes against the reciprocal of the non-variable substrate concentration. Figure 16 shows that isoenzyme #1 has a very high affinity for phosphate, at pH 7.4 for there was little or no change in the vertical intercepts on decreasing the phosphate concentration by 15-fold. The Ka for phosphate was calculated from the vertical intercept of the replot of slopes when phosphate was varied (see Figure 18A and Figure 18B)

(which is defined by $\frac{Ka}{V}$); by substituting for V the value for Ka was found to be 0.035 mM. With isoenzyme #2 it was possible to calculate the Ka for phosphate by replot of the intercept against reciprocal of phosphate concentration and the value was found to be 0.36 mM, a much higher value than that found for isoenzyme #1 (see Figure 17). On the other hand, the Kb for uridine with isoenzyme #1 was calculated to 0.112 mM (see Figure 18) and this is higher than that for isoenzyme #2 which had a Kb for uridine equal to 0.032 mM, (see Figure 19).

An attempt was also made to calculate the values of K_q and K_p of ribose-1-phosphate and uracil respectively, at pH 7.4. Fresh isoenzyme #1 gave anomalous results (see Figure 20A). At low levels of ribose-1-P (0.03 M), or at high levels (0.2 and 0.4 M) with variable uracil the activity was low; at an intermediate concentration of ribose-1-P (0.066 mM) maximum velocity was obtained at a concentration of 0.133 mM of uracil. However, no inhibition with high uracil was observed with 0.133 mM ribose-1-P. On repeating these experiments with the same preparation of frozen enzyme, 6 weeks later, somewhat different results were obtained (Figure 20B). This preparation is known to contain several

new inactive b ands of protein compared to the fresh preparation which contained only one active band. Now, at concentrations between 0.066 mM and 0.266 mM ribose-1-P the velocity increased linearly with increasing uracil concentration (see Figure 20b) and substrate inhibition occurred at between 0.3 and 1.0 mM of uracil.

The K_p for uracil was calculated for the aged preparation of isoenzyme #1 (6 weeks old frozen) with ribose-1-P as the non-variable substrate at different concentrations of uracil. The replot of the intercepts obtained from a double reciprocal plot gave a value of K_p for uracil of 0.25 mM (see Figure 21A).

The absence of activation by ribose-1-P similar to that of the fresh isoenzyme #1 for this set of experiments may be due to loss of regulatory properties on freezing and aging. However, these observations need to be confirmed.

Isoenzyme #2 had a K_q for ribose-1-P of 0.33 mM (see Figure 23). No anomalous behaviour was observed in this case either. The K_p value for uracil could not be calculated because pure enzyme was exhausted, but at three concentrations of uracil with varying ribose-1-P, no anomalous behaviour was observed (see Figure 22).

(e) Gel electrophoresis - Analytical gel electrophoresis was used for the following purposes:- (a) diagnostic purposes to test the purity of the enzyme; (b) to see whether aging the enzyme physically alters the enzyme molecule, i.e. to test the occurrence of aggregation and/or formation of differently charged species; (c) to determine the molecular weight of enzyme band(s) by the method of Hedrick and Smith (79) using different porosity of gels; (d) to determine molecular weights after denaturing the enzyme with SDS.

1. Diagnostic - Isoenzymes #1 and #2 were analyzed at various stages of purity. Fractions obtained before the hydroxyapatite column still contained at least 3-4 protein bands although the enzyme activity was associated with only one. After purification of isoenzyme #2 by chromatography on hydroxyapatite, fractions across the peak of enzyme activity were examined by gel electrophoresis. Except for 4 fractions at the trailing edge of the activity peak, the fractions showed only one protein band (Figure 24) on staining with coomassie blue which coincided with that of the enzyme activity (Figure 25). Isoenzyme #1 was obtained also as 1 major protein band after chromatography on

hydroxyapatite (Figure 24) which was coincident with the band of enzyme activity (Figure 25). Estimation of the area under the major protein peak by means of an integrator scanner, showed that more than 80% of the protein of enzyme #1 was in the major band if it is assumed that the intensity of staining by coomassie blue is proportional to protein concentration (Figure 24).

The patterns were essentially similar in the pH 8.3 and pH 7.0 electrophoresis systems; the Rm (relative mobility) values for the isoenzymes were: #1 at pH 8.0 $\longrightarrow$ 0.33, at pH 7.0 $\longrightarrow$ 0.29; isoenzyme #2 at pH 8.0 $\longrightarrow$ 0.38, at pH 7.0 $\longrightarrow$ 0.29. The enzyme activity band was similar in Rm when uridine or deoxyuridine was the substrate (Figure 25).

In spite of the fact that isoenzyme #1 and #2 were separable on DEAE Sephadex at pH 8.0, the enzymes were not very different in their Rm values. A possible reason for this could be that the total number of charged residues is the same for both enzymes but due to the differences in some uncharged hydrophobic residues, the surface charge distribution is different in the two enzymes and since fractionation on DEAE Sephadex depends upon the charge

distribution on the surface of the protein molecules, they would thus be separated.

2. Disc gel electrophoresis of enzyme aged in Tris buffer or phosphate buffer at pH 7.0 - Enzyme fractions obtained after hydroxyapatite column when stored in Tris buffer (pH 7.0) lost most of their activity. On analyzing preparations of isoenzyme #1 or #2, aged in the Tris buffer, on electrophoresis both showed two protein bands which were almost equally stained (see Figure 25A). The Tris-aged isoenzyme #1 when reactivated with 50 mM 2-mercapto-ethanol for 180 minutes at room temperature to 25% of the original activity, also had the same two protein bands and on staining for activity, the activity was associated with only one of the bands, that which had a Rm value of 0.37 (see Figure 27 for #1).

The enzyme in phosphate buffer still retained most (70-80%) of the activity and the electrophoretic pattern was very different from that of the enzyme stored in Tris Buffer at pH 7.0. The phosphate enzyme showed one major and several minor bands above and below the major band (Figure 26B). Enzyme activity was associated with the major protein band (compare Figure 26B and Figure 28).

Figure 28 shows the densitometric tracings with isoenzyme #1 aged in phosphate buffer and it can be seen that at least four protein peaks were present and the activity was associated with the peak having a relative mobility of 0.35. Isoenzyme #2, stored in Tris and aged, had two major peaks almost identical to that of #1. Isoenzyme #2, aged in phosphate, showed 3 major peaks and here again the activity was associated with the major peak (see Figure 29). Its protein differed somewhat from that of isoenzyme #1 aged under the same conditions.

3. Determination of molecular weight of enzyme bands on electrophoresis - Electrophoresis was performed with different acrylamide concentrations giving gels of different porosity. The change in Rm would now be proportional to the molecular size; hence, on plotting Log of mobility (for convenience 100 x Log Rm x 100 is used) against % acrylamide concentration, a straight line was obtained, the slope of which was proportional to the molecular weight. The slopes of standard molecular weight markers such as alcohol dehydrogenase, apoferritin, albumin and catalase were determined and Figure 31 shows the plot of molecular weights against slope values. From this graph the molecular weight

of the slope corresponding to fraction #1 was found to be 110,000, (Figure 30), #2 was 95,000, and that of thymidine phosphorylase was 90,000. In the fraction aged in Tris buffer, of the two major peaks, the one which could be re-activated (less anionic in 7% gel) had a molecular weight of 105,000 and the anionic protein had a molecular weight of 180,000 (compare Figure 30 and 32). The species appearing in aged preparations stored in phosphate, may be aggregates or subunits of the active enzyme moiety, because the plot of Log Rm against % gel shows a converging pattern, which is typical for proteins in which association occurs (see Figure 33). Albumin gives a similar pattern (79). Figure 34 shows these enzyme aggregates above the major activity, which are still active. In this case the enzyme was the peak fraction from DEAE Sephadex pH 7.0 column stored in phosphate at protein concentration of 5.28 mg/ml.

4. Subunit weight determination using sodium dodecyl sulphate - The experiments with gels of different porosity suggested the presence of subunits in this enzyme and this was also confirmed by the results of SDS electrophoresis. On incubation in the presence of 2-mercaptoethanol and SDS for 4 hours before electrophoresis 4 bands appeared; with

both isoenzymes the molecular weights corresponding to these bands were for #1 ⟶ 100,000, 78,000, 54,000, 28,500 and those for #2 ⟶ 93,000, 70,000, 39,000, 24,500 (see Figure 35). Incubation for 8 and 16 hours in the presence of SDS and 2-mercaptoethanol complicated the results since extra minor proteins appeared. Hence, under the conditions we have used, it was not possible to totally convert the enzyme molecule into its subunits as is possible for many other enzymes; possibly freshly prepared enzyme in which aggregation was much less,might have given better results.

Urea gel electrophoresis was tried a few times and it was not possible to obtain subunits with urea in concentrations as high as 6 M.

From our results it can be concluded that uridine phosphorylase of rat liver is a tetrameric enzyme with a subunit weight close to 25,000, somewhat resembling the purine nucleoside phosphorylase of Bacillus cereus (16) and unlike the human tissue purine nucleoside phosphorylase which was suggested to be a trimeric enzyme (19).

(f) Density gradient centrifugation experiments - The term "association" will be used to imply "subunit association", while "aggregation" implies aggregation of the tetrameric

form of the enzyme. Orthophosphate was reported to cause the association of spore purine nucleoside phosphorylase of Bacillus cereus (16, 99). Since orthophosphate had a protective effect during heat treatment it was thought worth while to investigate the effect of orthophosphate and see if it did anything to the molecular weight of uridine phosphorylase. From Figures 36, 37 and 38 it can be seen that uridine phosphorylase was partially converted to its dissociated state having a molecular weight of 50,000-60,000. In the absence of phosphate in Tris buffer the enzyme had a molecular weight of 109,000. The same phenomenon was seen with both isoenzymes. The phenomenon of dissociation seems to be somewhat phosphate specific but KCl at the same ionic strength could dissociate the enzyme albeit to a smaller extent. The time required for the sedimentation of the enzyme in Tris alone and in 0.2 M phosphate or KCl (0.33 M) was different. Centrifugation times of six and half to 8 hours were required for the Tris medium and 16 hours for the others. In every case, 10 mM 2-mercaptoethanol was present. These studies could not be done in the absence of 2-mercaptoethanol, due to significant loss in enzyme activity. With the availability

of more enzyme it should be possible to detect the protein peaks even though the activity is lost. The molecular weight of the enzyme was calculated with reference to the marker proteins,(alcohol dehydrogenase, cytochrome C, alkaline phosphatase, chymotrypsinogen and haemoglobin) as was suitable for the particular run.

Why the dissociation phenomenon is only partial is not known. There is the possibility that in the presence of certain concentrations of phosphate there is an equilibrium between the dimer and the tetramer. This dissociation phenomenon would probably not be implicated in the protection of the enzyme during heat denaturation. The reverse (i.e. association increasing heat sensitivity) was true for the B. cereus enzyme (16).

(g) Amino acid analysis of isoenzyme #1 - Amino acid analysis was performed only with isoenzyme #1 since it only was available in sufficient quantities for the analysis, which was done both directly on the protein sample and also after performic acid treatment to determine the total cysteine and cystine content of the enzyme. Table IV shows the composition of isoenzyme #1, which was dialyzed in 0.05 M ammonium carbonate buffer and stored at -40°C. The moles

of the amino acid residues were calculated on the basis of the tetrameric form of the enzyme assuming a molecular weight of approximately 100,000. As can be seen in the table the enzyme molecule has at least 6 residues of 1/2 cystine per subunit and probably some of these are located at the active site, as is expected from its sensitivity towards sulfhydryl reagents and protection by 2-mercapto-ethanol (8, 10). Present studies of the variation in Km apparent of uracil with pH also indicate that a group on the enzyme with a pK close to 8 is involved in the binding of uracil and/or its catalysis (see Section IC (2)).

B. Transferase

Uridine phosphorylase, like other nucleoside phosphorylases, has been found to catalyze pentosyl transfer from a pyrimidine nucleoside to a pyrimidine base. There are two possible mechanisms for this transfer:

1. Direct not involving phosphate or ribose-1-phosphate

$$\text{Base}_1 + \text{Nucleoside}_2^* \rightleftharpoons \text{Base}_2 + \text{Nucleoside}_1^*$$

2. Indirect involving phosphate or ribose-1-phosphate as intermediates

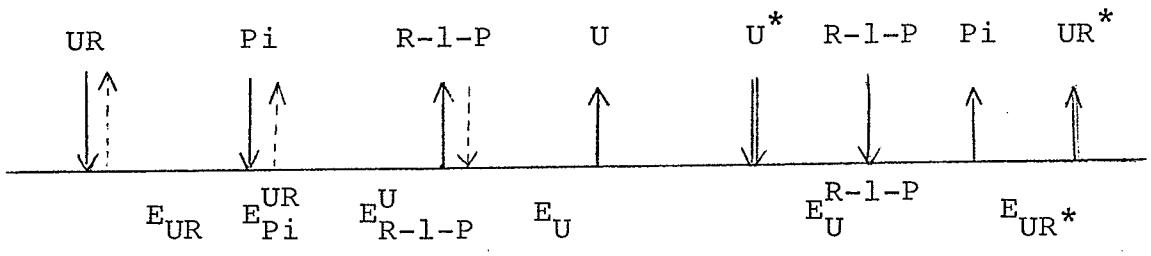
$$\text{Nucleoside}_2^* + \text{Pi} \rightleftharpoons \text{Base}_2 + \text{Ribose}^*\text{-1-P}$$

$$\text{Ribose}^*\text{-1-P} + \text{Base}_1 \rightleftharpoons \text{Nucleoside}_1^* + \text{Pi}$$

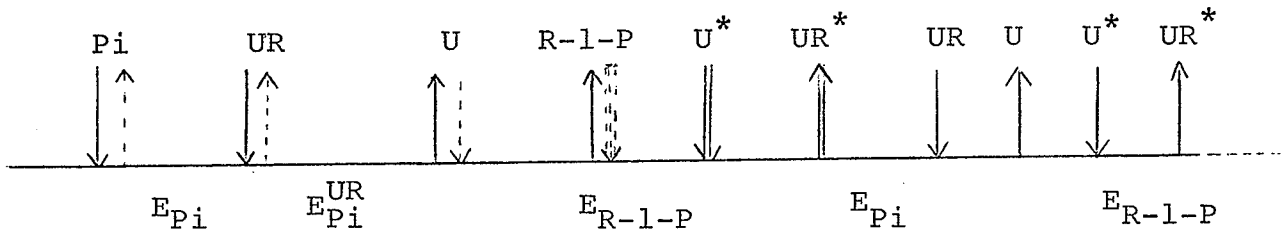
* Radioactive

It was shown previously (9, 10) that uridine phosphorylase catalyzed only the indirect transfer and thymidine phosphorylase catalyzed both indirect and direct transfers (9). In one case (9) the order of addition of substrates and release of products for uridine phosphorylase as well as thymidine phosphorylase were arrived at by studying the indirect transferase reaction (b) between nucleoside and base. The order, shown below as mechanism-1, is that proposed by Krenitsky for uridine phosphorylase of guinea pig small intestine. The basis of the mechanism was the finding that phosphate in high concentrations inhibited the transfer between the nucleoside and base; on the other hand at low concentrations, the amount of phosphate required to stimulate the transfer was stoichiometric with the amount of uracil transfered. This latter fact regarding stoichiometry is not apparent from his graph for uridine phosphorylase, although there seems to be some stoichiometry for the thymidine phosphorylase reaction in which case however, he says there is no stoichiometry. The conclusions regarding stoichiometry seem to be equivocal (9).

Mechanism-1 (Krenitsky, 9)



Mechanism-2 (Kraut and Yamada, 10)



Mechanism-2 was arrived at by initial velocity and product inhibition studies, in which phosphate was found to be competitive with ribose-1-P. This latter order was proposed for thymidine phosphorylase by Krenitsky, again based on the same argument, i.e. high concentrations of phosphate did not inhibit the transfer between base and nucleoside, and less than stoichiometric amounts of phosphate were required for optimal rates of pentosyl transfer.

The present study on transferase activity for rat liver uridine phosphorylase was done to clarify the apparent discrepancies in Krenitsky's conclusions, and to confirm mechanism-2. If mechanism-2 is operative with this enzyme

then less than stoichiometric amounts of phosphate should be required for optimum transfer and high concentrations of phosphate should inhibit the transfer. Before beginning the studies on phosphate requirement the optimum conditions and the concentrations of nucleoside and base required for maximal transfer was established. As can be seen in Figure 39A, when the concentration of uracil-2-^{14}C was 0.242 mM, maximal transfer activity was found at uridine concentrations greater than 1.0 mM. Either 1.66 mM phosphate (Figure 39A and B) or 0.033 mM ribose-1-phosphate (Figure 39B) (optimal concentrations of each) was present in this system. The Km apparent* of uridine as determined by the double reciprocal plot was found to be 5×10^{-3} M in the presence of either 1.66 mM phosphate or 0.033 mM ribose-1-phosphate.

In the experiments where uridine was held constant at a concentration of 1.32 mM and the concentration of uracil-2-^{14}C was varied (at constant specific radioactivity), maximal activity was obtained when the uracil concentration was close to 0.9 mM (Figure 40A). Again the phosphate concentration was held constant at 1.66 mM when ribose-1-P was

* Km apparent is actually a complex value being determined by a number of kinetic constants. It has been used here for convenience of expression.

absent or alternatively the latter was added to a concentration of 0.033 mM when phosphate was absent. From the double reciprocal plot shown in Figure 40B the Km apparent value for uracil were calculated to be 0.83×10^{-4} M in the presence of ribose-1-P. In the earlier study (10) uracil at a concentration of 1.2 mM has been found to inhibit the synthesis of uridine. However, in the present studies, uracil did not inhibit the transferase reaction even at concentrations greater than 2 mM and this was probably due to the presence of uridine (to be discussed later).

The transfer reaction between uridine and uracil was linear with time and enzyme concentration for the period of incubation (30 minutes) used in most of the studies (see Figure 41A and 41B). Both these studies were done in the presence of an optimal concentration of phosphate.

In order to study the effect of phosphate and ribose-1-P on the transferase reaction, the enzyme was first dialyzed against 1,000 volumes of Tris-HCl buffer, .05M pH 7.0 + 10 mM 2-mercaptoethanol for a minimum of 3 hours with at least 3 changes of the buffer. With some samples shorter times of dialysis were tried but some transferase

activity was found in these samples in the absence of exogenous phosphate; longer periods of dialysis offered no advantage and in fact on prolonged dialysis against Tris-HCl buffer there was a partial loss of activity from purified preparations, when the protein concentration was between 1 to 4 mg per ml.

In the absence of phosphate there was no transfer between the nucleoside and base. When phosphate was added in increasing amounts in the absence of ribose-1-P the velocity increased with maximal activity being attained at concentrations greater than 0.3 mM, (Figure 42A and B), thus confirming indirect transfer. The Km apparent for phosphate was 0.125×10^{-3} M.

Figure 43A illustrates transferase activity at different levels of phosphate and varying uridine concentrations in the presence of 1.32 mM uracil-2-C^{14}. The observed value of the horizontal intercept* (for the replot of intercept against phosphate concentration) was found to be 0.052×10^{-3}M. The corresponding value calculated from the kinetic analysis of the phosphorolytic reaction at pH 8.1 was 0.046×10^{-3}M (10). The 2 values are in close

* Defined as $Kii = \frac{\frac{KiP + P}{P\ KiP}}{Ka\ (\frac{KiP}{P} + 1)}$ (see Figure 43C).

agreement; however in present studies Ka (phosphate) was not calculated since the Kip in the transferase reaction was not determined. This indicates that the concentration of phosphate that is required for maximal transfer between uridine and uracil is a function of its affinity for the enzyme. The finding also supports mechanism-2 proposed for this enzyme according to which phosphate binds before the nucleoside. Once bound, phosphate can facilitate the transfer without itself being released. Very high concentrations of phosphate inhibited the transfer of uracil-2-C^{14} to uridine (see Figure 42C). This inhibition is probably due to the lack of uridine in the presence of very high concentrations of phosphate (20 mM), most of the uridine being converted to uracil by the phosphorolytic mechanism.

Ribose-1-P would replace phosphate in promoting the transfer between base and nucleoside (see Figure 44A). The level of ribose-1-P required for optimal transfer was lower than that for phosphate, under similar conditions of pH and concentration of the other substrates; about 0.1 mM ribose-1-P gave maximal transfer of uniformly labelled uridine to uracil. The Km apparent for ribose-1-P was

found to be 0.019 x 10^{-3} M from the reciprocal plot of velocity against ribose-1-P concentration. The Km apparent of phosphate is 6.58 fold greater than the Km apparent for ribose-1-P. These values seem to reflect their corresponding Michaelis constants at pH 8.0 (10); the Ka for phosphate was 0.35 mM and the K_q for ribose-1-P was 0.07 mM (Ratio Ka/K_q = 4.97). Ribose-1-P produced 50% inhibition at a 3 mM concentration (see Figure 44A); this concentration was also lower than that required for 50% inhibition by phosphate (17 mM).

Thymidine phosphorylase (partially purified from rat liver) was also studied for comparison. The transfer between uniformly labelled deoxyuridine and thymine was not inhibited with concentrations of deoxyribose-1-P up to 16 mM (Figure 44C). To verify that this difference was not due to the different substrates used the transfer between thymine and uniformly labelled deoxyuridine was studied with uridine phosphorylase; even though the activity towards these substrates was very low, the inhibition with deoxyribose-1-P (about 4.0 mM) was obvious. These observations indicate that either there is a separate site for direct transfer in thymidine phosphorylase and/or the transfer occurs

through a tightly bound deoxyribose-1-P and hence is not affected by exogenous deoxyribose-1-P.

Transfer between ribose-1-P and phosphate was studied in order to see whether any "stoichiometric" relationship exists between the requirement of uracil to the amount of P^{32} transfered to ribose-1-P. The enzyme concentration study in the presence and absence of 0.132 mM uracil indicated that no transfer occurred in the absence of uracil (see Figure 45A). The reaction rate also increased on increasing uracil concentration and at about a concentration of 0.66 mM inhibition was observed before a definite plateau of optimum activity was obtained (see Figure 45B). The results of this experiment clearly indicate that a stoichiometric relationship between the amount of P^{32} transfered to the amount of uracil required does not exist. However, the other argument used by Krenitsky (9) is probably valid - excess uracil inhibits the release of uracil from the enzyme - ribose-1-P complex and the subsequent release of ribose-1-P^{32}. Additional evidence should be obtained by using labelled R-1-P^{32} and uridine and measuring the amount of P^{32} formed, because the inhibitory action of uracil could be due to other

reasons. First uracil may bind to an allosteric site and inhibit the synthesis of nucleoside, as was reported earlier (10) or this inhibition can also occur if uracil binds to the uridine site. The latter seems to be more plausible because no inhibition with uracil was observed in the presence of uridine in the substrate concentration study for uracil in the uracil-2-C^{14} to uridine transfer (see Figure 40A). Similar findings of inhibition of thymidine synthesis by thymine have been reported by Gallo et al. (45) for thymidine phosphorylase of human leucocytes. This inhibition by thymine is overcome by thymidine during thymidine cleavage and by deoxyribose-1-phosphate during thymidine synthesis. All these and other (43) observations are best explained by the postulation of a distinct inhibitory site for thymine.

In the uracil to uridine transfer arsenate stimulated the transfer to a lesser extent than phosphate which may again indicate that bound ribose-1-phosphate is necessary for transfer; ribose-1-arsenate esters are very unstable and hence would not be as efficient as the phosphate esters. When equal amounts of arsenate and phosphate (1 mM each) were present together then the activity was more than that with arsenate alone and less than that

with phosphate alone, thus indicating the importance of ribose-1-phosphate ester in the transfer (Table X).

The results of these studies of transferase activity of uridine phosphorylase indicate that there is no stoichiometric relationship involved in the amounts of A or Q, in the P^* to B transfer, to the amount of P^* transfered; similarly the amount of P required for A^* to Q transfer is not stoichiometric. The optimal amounts of the catalytic component (catalytic components are ribose-1-P or phosphate for uridine to uracil transfer and nucleoside or base for ribose-1-P to phosphate transfer) is probably determined by its affinity for the enzyme. However, the inhibition of transferase at high concentrations may indicate the sequence of addition of substrates and release of products in an ordered reaction, as was suggested by Krenitsky.

C. Tissue distribution and subcellular localization of pyrimidine phosphorylases

Six rat tissues were analysed for the relative cleaving activity of uridine, deoxyuridine and thymidine (see Table V). Amongst, brain, kidney, spleen, testes, heart, liver and intestine, the specific activity of

uridine cleavage was highest for intestine, heart and kidney. Testes, spleen,brain, and liver had about similar specific activities, but in terms of total uridine cleaving activity heart, testes and brain are of relatively little importance. Kidney, spleen and liver seem to be the important sites of uridine cleavage. Since only a portion of the small intestine was analyzed, total units are not given but most probably it is also an important site of uridine cleavage. Thymidine cleaving activity seems to be present only in the liver and possibly the intestine in significant amounts; in others tissues and the thymidine cleaving activity may be due to uridine phosphorylase. Deoxyuridine cleaving activity may be contributed by the two enzymes, uridine phosphorylase and thymidine phosphorylase (assuming that all the enzymes are similar to the liver enzymes in specificity). The distribution pattern of deoxyuridine cleavage follows that of uridine cleavage, the specific activities being highest in liver, intestine and kidney. Spleen and brain have some activity but it is not significant in terms of total activity.

Other mammalian tissues in which uridine cleaving activity has been reported are mouse Ehrlich ascites cells,

rat and calf thymus (83, 100), rabbit bone marrow (1, 2) and human fibrosarcoma (37).

Most studies on nucleoside phosphorylase were done with the cytosol fraction, where the major part of the activity is localized. Purine nucleoside phosphorylase has also been localized in the nucleolus of Starfish oocyte (113).

Yamada in 1968 (8) reported the occurrence of an isoenzyme in the crude nuclear fraction of rat liver.

Rat liver was used for fractionation and the subcellular localization of the pyrimidine cleaving activities. The procedure of Sawant et al. (88) with some modification (86) was used, and marker enzymes had the distribution shown in Table VI, and agrees well with other studies. There is some degree of cross contamination in most fractions except in the pure nuclei and lysosomes. The nuclear fraction was fairly free of all the four markers (glucose-6-phosphatase, for microsomes, 5'-nucleotidase for plasma membranes, glutamate dehydrogenase for mitochondria and acid phosphatase for lysosomes) studied. Pure lysosomal fraction showed only 2.6% recovery of acid phosphatase but a 23 fold increase in specific activity.

The heavy mitochondrial fraction though mainly consisting of mitochondria had a good deal of contamination from plasma membranes and lysosomes. The fraction termed light mitochondria was really a lysosomal fraction containing plasma membranes and microsomes but few mitochondria. This fraction had 30% of the acid phosphatase, 17% of glucose-6-phosphatase, 14% of 5'-nucleotidase and 7% of glutamate dehydrogenase. The supernatant fraction is obtained after removing the pellet at 160,000 g, and hence in these sets of experiments this fraction contains the microsomes apart from the contamination with 25% of the total acid phosphatase produced from the lysed lysosomes. This supernatant fraction also has 32% of the plasma membranes as indicated by the presence of 5'-nucleotidase. The fraction termed debris is the fraction left floating on the top after the pure nuclei have been spun down in 2.4 M sucrose. This fraction consists of heavy mitochondria comprising 33% of the total glutamate dehydrogenase, almost half (44%) of the 5'-nucleotidase, and may also contain some unbroken cells (see Electron micrograph no. III).

The results of distribution of pyrimidine nucleoside phosphorylases in these fractions indicated the absence

of any activity in the pure nuclei, microsomes and pure lysosomes. The major portion (70%) of uridine, thymidine and deoxyuridine cleaving activity was located in the cytosol or the 160,000 g supernatant. The next highest activity was located in debris which primarily consisted of crude plasma membranes. The mitochondrial fraction also contained about 8% of the total uridine cleaving activity. In this procedure of fractionation the plasma membrane fragments contaminate almost all the fractions and have a higher concentration in the "debris". Since the "debris" fraction had a fair amount of uridine cleaving activity, the purification of plasma membrane was undertaken to see if this activity is associated with the plasma membrane fragments (Table VII).

The plasma membranes were isolated by the procedures of Bosmann, Hagopian and Eylar (89). The purification of the membranes was only 6.7 fold compared to 150 fold purification of the HeLa cell membranes. This difference may be due to the differences in tissues used. Similar methods of purification using liver tissue for preparing plasma membranes gave only 11 fold enrichment of 5'-nucleotidase (101). The membrane fragments prepared

by this procedure showed an enrichment of glucose-6-phosphatase (almost 3 fold). The presence of this enzyme activity may be either due to its localization in the plasma membrane as is also claimed by others (102, 103, 104, 105), or may be due to the contamination with endoplasmic reticulum (the microsomal fraction) as is suspected by some (106, 107) (see Table VIII). Glucose-6-phosphatase activity is probably due to a non-specific phosphatase of the plasma membrane (108, 109). The plasma membrane fragments are relatively free of mitochondria, as seen by the presence of only 0.89% of glutamate dehydrogenase activity.

Uridine phosphorylase activity was found to be associated with pure plasma membrane fragments. Approximately 17% of the total uridine phosphorylase was found to be associated with plasma membrane fragments, in which deoxyuridine and thymidine cleaving activity could not be detected in high enough levels, in freshly prepared membranes (see Table IX). However, on solubilization with Triton X-100 deoxyuridine cleaving activity was detectable. The effect of 8.7 M glycerol and Triton X-100 on various fractions showed that both mitochondria and plasma membrane fractions show an increase in activity on solubilization. The enzyme

in the plasma membrane fragments may be present in the vesicles as has been shown for E. coli thymidine phosphorylase (49, 51).

The membraneous structures seemed to be preferentially solubilized by Triton X-100 compared to glycerol which is known to solubilize and stabilize the mitochondrial enzymes better (98, 110).

The electron microscopic pictures of various fractions confirm to the enzyme activity patterns. The plasma membrane fraction (see EM no. I) with the desmosome granules and vesicles can be seen to be relatively free of mitochondria. There may be some rough endoplasmic reticulum also present as indicated by the densely stained particles. The fraction which was above the plasma membrane fraction appears to be rich in bile canaliculi (see EM no. II); it has some lysosomes and plasma membranes as seen from enzyme activities of 5'-nucleotidase and acid phosphatase. The "debris" fraction in which a significant portion of uridine phosphorylase (15%) activity is present was seen under the electron microscope to contain mitochondria, plasma membranes and some rough endoplasmic reticulum (EM no. III).

With present methods of fractionation and identification it is not possible to distinguish very definitively between the plasma membranes and the rough endoplasmic reticulum; many enzymes are distributed in both the structures, like cytochrome C reductase and glucose-6-phosphatase as mentioned earlier.

The uridine phosphorylase localization pattern resembles the distribution pattern of adenine phosphoribosyltransferase very much (111). Uridine phosphorylase is probably present in the vesicular structures associated with the membrane structures and also in the mitochondria, (where RNA metabolism does occur) apart from the major portion (about 70%) being in the cytosol.

VII. DISCUSSION

1. Purification - There have been several attempts to purify uridine phosphorylase. The enzyme of Ehrlich ascites cell has been purified up to 250 fold and was shown to be homogenous upon moving boundary electrophoresis (55). Pontis et al. (7) had also obtained a preparation from the same source of about 300 fold purification. In neither study was there any indication of isoenzymes. The best purification so far achieved was 1,900 fold of a cytoplasmic uridine phosphorylase of rat liver with a specific activity of 144 units per mg protein with uridine as a substrate (10). In the present work this purification procedure of Kraut and Yamada (10) was followed with some slight modification. This method separates uridine phosphorylase from thymidine phosphorylase and is also effective in separating the isoenzymes of uridine phosphorylase. The purified enzyme fraction obtained (10) resembled that of isoenzyme #2 of present studies. Many other procedures were tried in pilot experiments for purifying the rat liver enzymes. CM Sephadex, phosphocellulose and preparative disc electrophoresis were either not effective or effective to the extent that they could not substitute for any of the

steps of the previous method (10). Preparative disc electrophoresis may be useful if ways of stabilizing the enzyme on this system are more fully explored; otherwise this method can be used only in the final stages of purification because of the limitation in the quantity of protein that can be applied. Preparations of low specific activity are thus difficult to identify in the dilute eluting fractions. The first DEAE Sephadex pH 8.0 column separated the two uridine phosphorylase isoenzyme better than that achieved previously because a shallower gradient and upward flow was used. The upward flow counteracted to some extent the gel contraction produced by an increasing salt gradient. In two other instances the isoenzymes were almost completely separated on DEAE Sephadex at pH 7.0 upon elution with a shallow (0 $\longrightarrow$ 0.2 M KCl) gradient. Another advantage of using shallower gradients was that there was less variation in specific activity across the peak with the result that most of the activity could be pooled thus improving recovery of the enzyme. The recovery of uridine phosphorylase by comparable methods in a previous study in this laboratory was 8.9% (10) and in the present work was close to 27%. In the last step of purification with a

hydroxyapatite column, a shallower gradient eliminated contamination from proteins that eluted close to the enzyme. The fact that thymidine phosphorylase was not contaminating the fractions of uridine phosphorylase was evident from the fairly constant ratio of thymidine to uridine cleaving activity in different stages of purification after the DEAE Sephadex pH 8.0 column, and this agrees fairly well with the earlier study (10).

The relative proportions of isoenzymes #1 and #2 determined the final specific activity, since there seems to be close to a 2.6 fold difference in the specific activity of enzyme #1 compared to enzyme #2; this would be an important factor in determining the final specific activity of a preparation having the two types of isoenzyme present. The specific activity of the Ehrlich ascites cell enzyme was 106 and 60 in two different preparation (7, 55) both of which were purified to electrophoretic homogeneity. The specific activity of the 1,900 fold purified enzyme from rat liver cytoplasm was 144 (10). No other pyrimidine nucleoside phosphorylase has been purified to this extent.

As mentioned previously in the review of literature isoenzymes have been reported for (1) purine nucleoside

phosphorylase of Bacillus cereus in both the spore and vegetative cells, chicken liver - adult and embryonic (2), uridine phosphorylases of rat brain and rat liver. Amongst these only the enzymes of Bacillus cereus have been characterized to some extent. They were shown to be interconvertible. However, the molecular weight of the two enzyme forms was the only criterion used. This does not seem to be the case for the isoenzymes of uridine phosphorylase of rat liver. In present studies, the fact that the two enzymes were separable on ion exchange chromatography but had similar Rm values in disc gel electrophoresis at pH 8.0 or pH 7.0, suggests that there is a difference in the surface distribution of charge, due to differences in the conformation of the protein molecules (which may be due to differences in position or substitution of a few amino acid residues).

2. Stability - Uridine phosphorylase was found to be fairly stable at -40° for 60 days in previous studies from this laboratory (10). In another study (11) rat brain uridine phosphorylase lost about 30% activity on storage at -20°C or on dialysis at 0° in dilute Tris buffer at pH 7.3. In the present work phosphate was found to stabilize

the enzyme greatly. In Tris buffer even at -40°C most of the activity was lost after one week with repeated freezing and thawing, upon storage at room temperature and upon heating at 50°C for about 15 min to 30 min. Although 2-mercaptoethanol was present during these stability studies it did not afford full protection, even though at higher concentrations it could partially reactivate a preparation that had totally lost all activity, to give 25% of the original activity. It is possible that the inactivation by all the procedures listed above is due to the oxidation of essential -SH group(s) in the active site. Phosphate affords protection during these procedures. Uridine could protect partially during inactivation by heat or on prolonged storage. This observation agrees with that for the rat brain enzyme (11), but is opposite to the observations on purine nucleoside phosphorylase of Bacillus cereus spore (99). In addition, the enzyme of vegetative B. cereus was equally heat sensitive in Tris or phosphate. Uridine cleaving activity seems to be lost more than deoxyuridine cleaving activity, when the enzyme is stored in Tris (pH 7.0) and assayed at pH 7.4 (Table II b), and this observation reminds one of Pontis et al.'s work (7) in which

acid precipitation was used to obtain deoxyuridine cleaving activity devoid of uridine cleaving activity. It is not apparent from the latter work whether uridine cleaving activity is separated or lost,leaving activity towards deoxyuridine only. Looking at their figures it appears that the recovery of deoxyuridine cleaving activity is about 86.37% and that of uridine cleaving activity 47.4%. The presence of a contaminating thymidine phosphorylase cannot be entirely excluded in this preparation, in spite of the fact that the enzyme was electrophoretically pure and the substrate specificity was very similar to that of the purified cytoplasmic rat liver enzyme.

3. Substrate specificity - The substrate specificity of the two isoenzymes was similar to that found for the cytosol enzyme of rat liver (10) and Ehrlich ascites cells (7). The enzyme cleaved uridine in preference to deoxyuridine at pH 7.4 and both these substrates were phosphorylated at the same or overlapping sites as is evident from the results of Table III. Relative loss of uridine or deoxyuridine cleaving activity on heat treatment at different pH values (similar to that of Pontis et al.) has not been done in the present work. However, uridine and deoxyuridine cleaving

activity was tested after heat treatment for 20 min at pH 7.4 and pH 8.0. At both pH values the loss in activity towards uridine or deoxyuridine was similar and thus differs from the observations of Pontis et al. (7). Also from studies with sulphydryl inhibitors such as iodosobenzoate (10) the degree of protection from inhibition with o-iodosobenzoate was in the following order uridine > deoxyuridine > phosphate, for the cytoplasmic uridine phosphorylase. Besides, uridine protected deoxyuridine cleaving activity at pH 8.1 from inhibition by DTNB and similarly deoxyuridine could protect uridine cleaving activity from inhibition by DTNB also at pH 8.1 (by 51.75%) (126), although to a lesser extent. These observations can only be explained on the basis of a common active site or overlapping sites for both uridine and deoxyuridine.

Thymidine is also cleaved by the purified cytoplasmic uridine phosphorylase of rat liver at about one tenth the rate of uridine at pH 7.4 (10). In the present work, similar studies have not been done with thymidine, mostly because it required 10 times as much of the pure enzyme, and the amount of the latter was the limiting factor. The optimum pH for thymidine cleavage was found

to be 5.8 for the cytoplasmic rat liver enzyme (10). There is, however, no reason to believe that thymidine is not phosphorylated at the same or overlapping sites as uridine or deoxyuridine because constant ratios of activities towards the 3 substrates were reached during the latter steps of purification (10).

4. pH optimum of uridine phosphorylase isoenzymes and the Km apparent and Vmax at different pH values - pH optimum of both isoenzymes #1 and #2 was close to 8 but isoenzyme #1 had a steeper curve compared to isoenzyme #2, indicating possible involvement of groups with a wide range of pKs.

pKm apparent values (with the other substrate saturating) were plotted according to the method of Dixon (98). Km apparent values at different pHs for uridine and deoxyuridine, from a table of an earlier work (55) using Ehrlich ascites cell has been plotted by the method of Dixon (98) for comparison. In general there are at least three prominent groups with pKs close to 6.8, 7.6 and 8.0 involved in either the binding or catalysis of uracil, 2 of which (pK 6.8 and 8.0) also appeared in studies of uridine or deoxyuridine. The plots with ribose-1-P and phosphate would have greatly helped to complete the

picture; however, with the present data it would not be very unwise to say that there are ionizing groups in the enzyme substrate complex of enzyme and uridine or deoxyuridine which have pK values of 6.6 and 8.0. These may correspond to a histidyl group and a cysteinyl residue of the enzyme since there are no groups in the nucleosides or base which have similar pK values. The pK of 7.6 holds a number of possibilities. One is that even though the phosphate concentration (0.1 M) is greater than its Km value, it can still influence the catalysis or binding of the other substrate if the ratio of monoanion to dianion is the determining factor. The ratio of monoanion of phosphate to its dianion was found to be close to unity at pH 7.2 (114). The other is that a second cysteinyl group with a lower pK value is involved.

5. Kinetic constants - The kinetic constants of the two isoenzymes have been compared and although data is not available for comparing all the values, the following facts are apparent. Isoenzyme #1 needs greater concentrations of uridine (Kb, 0.112 mM) to function in the phosphorolytic direction, compared to #2 enzyme which has a lower Kb value (0.032 mM). The Ka for phosphate seems to indicate the

reverse on a glance at the figures (#1 Ka 0.035 mM, #2 Ka 0.36 mM). The difference in Pi levels in normal and regenerating or fast growing tissues, and also its intracellular distribution is not known. It is possible that phosphate is never a limiting factor but if the level of phosphate is low in these tissues (similar to the dormant spores (115)) a lower Ka value for isoenzyme #1 (which seems to be present in very young rat liver or regenerating rat liver (8)) would ensure the binding of phosphate on all occasions, and the level of uridine would then be the determing factor for the reaction to proceed or not. The same is the case with purine nucleoside phosphorylase of B. cereus (16), except that the Ka value for inosine is not as much different for the spore and the vegetative enzymes. The apparent Km values of uridine for uridine phosphorylase of Ehrlich ascites cell at the same pH was 0.76 mM (7) and 0.13 mM (55) in two different studies suggesting that 2 different enzymes from the same source were being studied. The cytoplasmic uridine phosphorylase had a Kb value of 0.28 mM for uridine at pH 8.1 (10). The Kb and Ka values for isoenzymes #1 and #2 have not been determined at pH 8.1, hence comparing the values from the present work is not of much significance.

The K_q and K_p values for isoenzymes #1 and #2 need more scrutiny. The K_q for ribose-1-P using #2 enzyme was 0.33 mM and is higher than the value of 0.07 mM reported for the cytoplasmic enzyme (10) at pH 8.1. Some anomalous results were obtained when the K_q for ribose-1-P with isoenzyme #1 was determined, as has already been described in the results section. Even though these appear anomalous at first sight they may be very important in the regulation of uridine phosphorylase. The activating effect of low concentrations (0.66 and 0.133 mM) of ribose-1-P may be the regulating factor for the enzyme to function in the synthetic direction when the need for uridine in rapidly growing tissues is great. Beyond 0.133 mM ribose-1-P the enzyme cannot be activated (probably by a change in the state of association) and obeys the normal Michaelis-Menton type of relationship. Uracil is known to inhibit the synthesis of uridine by 50% at 1.2 mM concentration at pH 8.1 (10), when the concentration of ribose-1-P was 0.143 mM or higher. At 0.066 mM ribose-1-P inhibition started to occur at 0.3 mM uracil (10). In present studies both isoenzymes were inhibited by uracil. The aged isoenzyme #1 had a K_p of 0.25 mM for uracil, which may be very different for the fresh enzyme.

6. Gel electrophoresis

(a) Diagnostic - Moving boundary electrophoresis showed the uridine phosphorylase of Ehrlich ascites cells to be present in one band (7, 55); disc gel electrophoresis in phosphate buffer showed the cytoplasmic rat liver enzyme albeit purified 1,900-fold to have two bands of protein (10). The results of present work may explain the difference in these two studies. Freshly purified rat liver uridine phosphorylase has one band of protein coincident with activity whether dissolved in Tris or phosphate buffers but on aging the Tris enzyme gave two prominent bands; aging in phosphate produced at least 2 more minor bands apart from the major band. The freshly dialyzed enzyme in Tris usually had slightly less relative mobility compared to the enzyme in phosphate. There could be several reasons for this:

(i) The phosphate enzyme has a more negative charge because of the bound phosphate groups.

(ii) The Tris enzyme may have a different shape which slows down the molecule on the gel sieve.

(iii) The molecular weight of the two could be different.

Amongst these three reasons, (iii) seems to be invalid because the molecular weight of the active Tris enzyme was found to be almost the same as the molecular weight of the phosphate enzyme, both by density gradient centrifugation and gel electrophoresis. This leaves (i) and (ii), one or both of which may be right, and it is not possible to differentiate between the two at the moment. Phosphate binding to uridine phosphorylases is believed to be loose since filtration on a dextran gel column removed most of the radioactivity (9). The same is also true for purine nucleoside phosphorylase (16). Acrylamide gels do not have similar sieving properties as dextran gels and hence the phosphate on the enzyme may still be bound to the enzyme during electrophoresis. Besides uridine phosphorylase of rat liver may be different from purine nucleoside phosphorylase of B. cereus and uridine phosphorylase of guinea pig intestine in its phosphate binding properties.

(b) Aging patterns on disc gel electrophoresis, revealed the fact that phosphate not only has a protective action on the activity of the molecule but is also in some way responsible for maintaining its form; whether the latter property is totally responsible for the former is not known

yet. By this method molecular weight determinations varied by ± 5%, and as can be seen for the present work interpretation of results by this technique, as well as many other techniques can be complicated by proteins undergoing association or dissociation processes (79). In spite of these drawbacks this method is still the fastest available for small amounts of protein for molecular weight determination.

(c) Subunit determination - The rat liver enzymes appear to be tetramers having a subunit weight close to 25,000 for #2 and 28,500 for #1. This is similar to the B. cereus purine nucleoside phosphorylase (16) but unlike the human purine nucleoside phosphorylase (19). Even though the electrophoretic pattern on gels without SDS show a fair degree (> 80%) of homogeneity both at pH 7.0 and pH 8.0, the SDS gel electrophoretic pattern in the presence of 2-mercaptoethanol indicates that there may be some slight contaminating proteins of molecular weights close to 100,000. However, these minor species may very well be differently charged enzyme molecules having a different number of reduced or oxidized -SH groups, thereby affecting their shape and the amount of SDS taken up. Even after 4 hours

of incubation in the presence of 2-mercaptoethanol why it was not possible to convert all of the enzyme molecules into monomers is not known at present. It is possible that the phosphate in the gel buffers may have some effect in spite of the SDS. On the addition of 0.1% SDS to the assay medium 25-30% of the activity was still detectable with the fresh enzyme.

7. Density gradient centrifugation - In two papers, the use of density gradient centrifugation for the study of purine nucleoside phosphorylase has brought forth interesting results. Engelbrecht and Sadoff (99) found that the spore enzyme of Bacillus cereus underwent a phosphate induced association from dimers to tetramers whereas the vegetative cell enzyme was insensitive (16). However, we failed to find any aggregation in the presence of phosphate concentrations of 0.05 M, 0.1 M and 0.2 M. The main peak still was the tetramer (as is the vegetative enzyme): however a small fraction of activity did appear in the lower molecular weight region, the significance of this has to be found out. In another paper in which purified Ehrlich ascites cell purine nucleoside phosphorylase was used, evidence for the conversion of phosphorolytic

activity into transfer activity in the absence of 2-mercaptoethanol was obtained; a number of peaks were obtained indicating aggregation and the facile interconversion of the molecular forms. Similarly in the presence of EDTA no transferase activity was observed but phosphorolysis remained unaffected (23). Uridine phosphorylase has not been studied under these conditions, since the transferase activity of this enzyme was weak and such a kind of study would involve a lot of enzyme; however it is known that in the absence of 2-mercaptoethanol the enzyme loses both activities rapidly and EDTA does not affect the phosphorolysis reaction. Isoenzymes #1 and #2 did not behave very differently during these set of experiments, with or without phosphate. The property of interconversion of transfer and phosphorylase activity seems to be true for the Ehrlich ascites cell purine nucleoside phosphorylase only, since this has not been found to be true for the human erythrocyte enzyme (116).

8. Amino acid analysis - So far only one other nucleoside phosphorylase has been studied for its amino acid composition and this is the purine nucleoside phosphorylase of B. cereus (99). This enzyme has been found to have a molar ratio of

11 cysteine, 2 half cysteine and 44 histidine residues. It had twice the amount of acidic residues compared to the basic residues (19). Similarly in the present study isoenzyme #1 has been found to have 156 acidic residues (Asp. and glu.) compared to only 84 basic residues. The cysteic acid content was 24 residues per molecule of uridine phosphorylase which is very similar to the total cysteine residue content of purine nucleoside phosphorylase. However, the histidine content differs very much and uridine phosphorylase has only 12 residues per molecule. The amino acid analysis gives an idea of the presence or absence of certain residues possibly involved in the mechanism of action of the enzyme.

9. Transferase activity of uridine phosphorylase - All the nucleoside phosphorylases studied so far catalyze the exchange between base and nucleoside. In the case of purine nucleoside phosphorylase there is only one report (23) on the separation and interconversion of transferase and phosphorylase activity; in all other cases (15, 22, 116), the phosphorylase activity was inseparable from the transferase activity and the ratio of transferase to phosphorylase activity was very low (< 0.01). Transferase activity was

markedly decreased in the case of a highly purified crystalline enzyme from human erythrocyte (15). The physiological significance of the transferase activity for the purine nucleoside phosphorylase enzyme is probably to maintain a balance between inosine, guanosine and their deoxy-derivatives in the nucleoside pool. Thus, the following reactions can occur through the purine nucleoside phosphorylase.

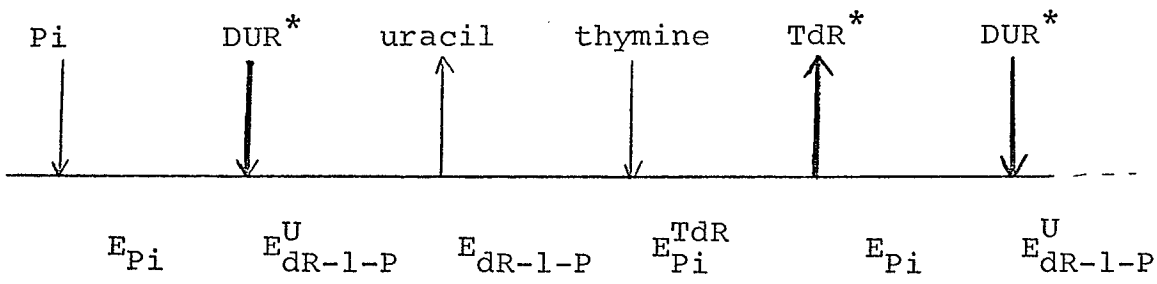
(i) Inosine + Guanine $\rightleftharpoons$ Guanosine + Hypoxanthine

(ii) Hypoxanthine + Deoxyguanosine $\rightleftharpoons$ deoxyinosine + guanine

Since adenine is used very slightly by this enzyme and has a very high Km, in most cases, the transfer of adenine may not be of any physiological significance (14).

No phosphate requirement for the transferase activity of purine nucleoside phosphorylase was established by Abrams *et al.* (117); similarly the exchange between ribose-1-P and P_i^{32} does not require the presence of purines (118). However, the transfer reaction of rat liver purine nucleoside phosphorylase is stimulated by inorganic phosphate (117). The purine nucleoside phosphorylase probably catalyze a direct as well as an indirect transfer.

Thymidine phosphorylase also catalyzes a transfer which is stimulated by phosphate. The ratio of phosphorylase to transferase activities has not been reported. Gallo and Breitman (42) proposed that the two activities belong to the same enzyme molecule, but to two different active sites, since thymidine synthesis from thymine and deoxyuridine is not inhibited by deoxyribose-1-P. Besides these two sites, the enzymes seem to have a third site for substrate inhibition by high concentrations of thymine; deoxyuridine is competitive for this site. However, the possibility that this 3rd site is the same as the thymidine site to which thymine can bind to produce inhibition, cannot be ruled out. An alternate explanation for the lack of inhibition by deoxyribose-1-P, would be that the enzyme has a tightly bound deoxyribose-1-P and the following reaction sequence.



This now means that tightly bound deoxyribose-1-phosphate is being used over and over again and hence exogenous deoxyribose-1-P is not effective as an inhibitor. Transfer activity of thymidine phosphorylase may be of much physiological significance because it catalyzes the following reaction for the synthesis of thymidine.

Thymine + deoxyuridine $\rightleftharpoons$ Thymidine + uracil

Under conditions when thymidine requirements are increased, it can be supplied by the above reaction even in absence of deoxyribose-1-P. However, this is only a speculation. The cell has a number of enzymes which interact with these compounds as substrates or products; hence, which enzyme reaction will actually be responsible for synthesizing thymidine will depend on many factors like Kms, etc., e.g. deoxyuridine may be broken down by uridine phosphorylase to form uracil and deoxyribose-1-P in preference to the transfer reaction, and the deoxyribose-1-P formed may then be used in the synthesis of thymidine by thymidine phosphorylase.

Uridine phosphorylase also catalyzes the transfer reaction but this is totally dependent on phosphate or ribose-1-P. The transfer reactions catalyzed by uridine

phosphorylase are probably not of any physiological significance and are of interest only in understanding the mechanism of action of the enzyme. Thus, in the reactions:

(i) Uracil* + Uridine $\rightleftharpoons$ Uridine* + Uracil

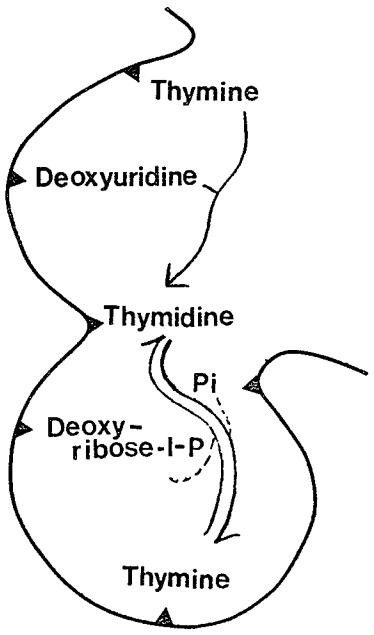
(ii) Uracil* + Deoxyuridine $\rightleftharpoons$ Deoxyuridine* + Uracil

and (iii) Ribose-1-P + Pi* $\rightleftharpoons$ Ribose-1-P* + Pi

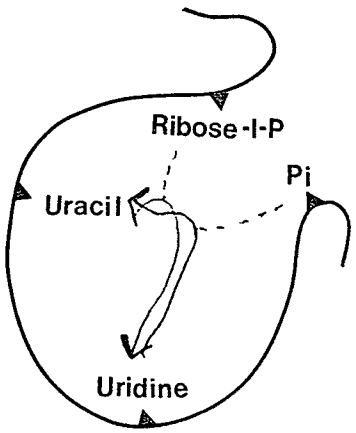
There is no net production of a new species of a compound and thus, there is no effect on the nucleoside pool. One possible role of any physiological importance could be the transfer between pseudouridine and uracil to form uracil and uridine. It is not known if the anomalous (c-c) linkage of pseudouridine would allow it to be used at all in the transfer reaction, and this seems to be unlikely. Uridine phosphorylase does catalyze the transfer between thymine and deoxyuridine to produce thymidine but this reaction is very slow and is probably not of any physiological significance. Uridine phosphorylase also differs from thymidine phosphorylase in having a common active site for both the phosphorylase and transferase activity. This conclusion has been arrived at by the same reasonings as that of Gallo and Breitman (42). The alternative explanation

* Radioactive

for thymidine phosphorylase, (as mentioned in the results section) could be that this enzyme has one site but the order is similar to mechanism-2 as was found for uridine phosphorylase of present studies, and a tightly bound deoxyribose-1-phosphate is involved. The transfer between uracil and uridine was totally inhibited by very high concentrations of phosphate or ribose-1-phosphate. If the binding site were different then the transfer activity would not be inhibited by high concentrations ribose-1-P or phosphate, as is the case for mechanism-1 proposed for thymidine phosphorylase. The following diagram illustrates the different binding sites proposed for the 2 enzymes.



THYMIDINE PHOSPHORYLASE(42)



URIDINE PHOSPHORYLASE

Uridine to uracil transfer was stimulated more by phosphate than arsenate (this observation agrees with Krenitsky's findings), thus indicating that the transfer occurs through a bound ribose-1-P intermediate or a bound ribose-1-arsenate in the presence of arsenate. There was no transfer between $^{32}P_i$ and ribose-1-P but transfer was detected only on the addition of uracil. The uracil requirement was not stoichiometric with the number of molecules of ribose-1-P transfered. The results are complicated due to substrate inhibition by uracil (10) (due to binding at the uridine site or on allosteric site), (see Figure 45b). This finding confirms mechanism-2 in which inhibition by uracil is expected, when it is present in more than optimal concentrations.

Both with Pi and ribose-1-P the inhibition was total at high concentrations. This indicates that the mechanism is only ordered without any randomness, because with a partly random mechanism the inhibition would be only partial as was shown for yeast alcohol dehydrogenase (119) and malate and lactic dehydrogenase (120, 119). Probably, the best way to distinguish between the two orders of reaction (Krenitsky's order resembles the Iso Theorell Chance) is by binding studies, as was suggested by Cleland (128).

Extensive binding studies have not been performed as yet; however with the available data on initial velocity, product inhibition and transferase it appears that the rat liver uridine phosphorylases catalyzes an ordered Bi Bi reaction with phosphate binding first.

10. Subcellular distribution of pyrimidine nucleoside cleaving enzymes - Knowledge of subcellular distribution is often helpful in understanding the physiological importance of the enzyme or the reaction pathway, e.g. the occurrence of a fraction of the total cell DNA polymerase in mitochondria, raised the question of partial or full autonomy of mitochondrion as an organelle. Almost 50% of the purine nucleoside phosphorylase was found in the crude nuclear fraction of calf liver and kidney (122). In rat heart the crude nuclear fraction had almost 4 times as much activity as the soluble fraction; in horse liver twice as much was found in the nuclei compared to the soluble fraction. On the other hand, calf intestinal mucosa and thymus have more activity in the soluble fraction. In another study purine nucleoside phosphorylase was found to be concentrated in the nucleoli of starfish oocyte (113). Thymidine phosphorylase was localized near the cell surface

of E. coli (49, 51). Apart from these few findings there has not been any systematic study of the subcellular distribution of nucleoside phosphorylases. Amongst other closely related enzymes uracil reductase has been mostly localized in the mitochondria and plasma membranes (123). Nucleoside diphosphatases and nucleoside monophosphatases have been localized in the plasma membranes (127). The results of present work indicate that a large portion of uridine cleaving activity is present in the cytosol (close to 70%) and the rest of it is in the mitochondria and membrane systems (viz. plasma membranes and perhaps endoplasmic reticulum). The thymidine cleaving activity may be present in such low amounts so as to have escaped detection. Deoxyuridine cleaving activity appeared only after solubilization with Triton X-100. It would be a little too premature to implicate this uridine phosphorylase in the transfer of nucleosides and bases across the membrane; nevertheless it would not be out of place to speculate that uridine phosphorylase is involved along with a pyrimidine phosphoribosyl transferase, similar to that proposed for purine nucleoside (adenosine) transfer (111, 124) or by itself in a process similar to that postulated for thymidine

phosphorylase (52). Since nucleoside diphosphatase and 5' nucleotidase have been definitely localized in the plasma membranes (108, 127), it is not very surprising to find a part of the nucleoside phosphorylase activity in the membranes. The enzyme which follows uridine phosphorylase has also been localized in part at least in the plasma membranes (123).

11. Distribution in different tissue - The present results are in agreement with some of the previously published work on tissue distribution. Thus, Skold found high activity of uridine cleaving enzyme in intestine and bone marrow (1). Yamada (2) also found very high specific activities for uridine cleavage in bone marrow and intestinal mucosa of rabbit and in both these tissues the uridine cleaving activity was greater than the deoxyuridine cleaving activity (ratio of UR/dUR, 5.18 and 1.97 respectively) in contrast to liver which had more deoxyuridine cleaving activity. This difference is probably due to the presence of thymidine phosphorylase in liver which has a greater reactivity with deoxyuridine. In the present work (bone marrow was not analyzed) intestine, kidney and heart had the high specific activities; amongst these only in kidney and heart was the

specific activity of uridine cleavage greater than that for deoxyuridine cleavage. It is not known at the moment if the properties and types of the pyrimidine phosphorylases from different tissues are the same. A comparative study of the kinetics and physical properties of these enzymes from different tissues of the same animal should give some idea of the mode of nucleoside metabolism in these tissues.

12. Model for the mechanism of phosphorolysis by uridine phosphorylase - A model for the mechanism of action of uridine phosphorylase is proposed based on observations of substrate specificity, optimum pH, effect of sulphydryl inhibitors and protection against these inhibitors by various substrates.

The essential features of this model as outlined in Figure 46 are:

(a) The active site of the enzyme has 3-SH groups essential for full activity. Indirect evidence for this was obtained from studies with sulphydryl inhibitors (10).

(b) One histidyl group is involved in the binding of the hydroxy group of carbon 2 of ribose in the case of uridine at pH 8.0 (see Figure 46A).

(c) At pH 8.0 (which is the pH optimum for uridine) uridine is bound to the active site at two other points by hydrogen bonds between R-SH groups (cysteine) of the enzyme and the o = c of uridine. These points of attachment not only orient the β-N glycosidic bond of uridine for subsequent attack by the -OH of phosphate, but also help in the withdrawal of electrons making the nitrogen somewhat less nucleophilic. This situation also prevails at pH 6.5. Alternately, at pH 8.0 hydrogen bonding may occur between the N_3-H group of the substrate and the $R\text{-}S^-$ group of the enzyme. This situation can orient the molecule very differently (see Figure 46C) depending upon the degree of ionization of this -SH group in the enzyme.

(d) The phosphate group may also be bound to a third -SH group of enzyme (10) at pH 8.0 possibly by a hydrogen bond; however, it can also hydrogen bond with -NH of an imidazole group, and the latter site may be preferred at pH 6.5, when phosphate does not protect the enzyme from inhibition by o-Iodosobenzoate (10). At pH 8.0 there was about 19% protection by phosphate.

(e) The presence of the OH group in the 2-C' of ribose has a stabilizing effect on the 1 carbon to N bond

(129). Once this OH group is hydrogen bonded to an imidazole - N of the enzyme, at pH 8.0, the stability of C-N bond is thereby decreased. At pH 6.5 the imidazole group is charged and not available for hydrogen bonding and hence the C-N bond is less susceptable to uridine as compared to deoxyuridine; stereochemically also, uridine may be less favoured at pH 6.5 than deoxyuridine due to the presence of an awkward unbonded -O- in the 2-C' position; this latter case is especially true if the orientation of the phosphate moiety is also changed.

(f) The cleavage of uridine may be regarded as a direct displacement reaction at 1-C' of ribose. "In direct displacement reactions, the incoming group hits the carbon under attack on one side while the leaving group departs from the opposite side" (121), and this type of attack can lead to the formation of an α linkage in the ribose-1-phosphate molecule, in contrast to the β linkage in the nucleoside.

(g) The 1-C' of ribose of the weakened β-glycosidic N bond is attacked by the oxygen of the HPO_4^-, thus forming the phosphomonoester (1-C' - O-P) bond. The mechanism of the glycosidic bond cleavage is similar at both pH 8.0 and

pH 6.5. The difference in specificity at the two pH values is due to the absence of hydrogen bonded OH at the 2-C' of uridine at pH 6.5 and may also be due to the unsuitable stereochemical orientation of glycosidic bond of uridine.

(h) In addition this model may help to explain the observations of inhibition with o-iodosobenzoate and the protecting effects of various substrates. Uridine can almost completely (90.4%) protect the enzyme from the inhibitory effects of this sulphydryl reagent, at pH 8.0. This can be explained if one visualizes the uridine to be attached at 3 points, 2 of which involve -SH and the other of which involves imidazole of the active site; under these conditions the third SH group in the interior is also protected. Deoxyuridine binds to only 2 SH groups and the 3rd SH is not protected. Hence, under these conditions there is still 57% inhibition. The observations with phosphate have been mentioned before (see point (d) above).

(i) The observation that the same active site cleaves uridine and deoxyuridine fits nicely in the proposed model and the possible explanation for the preference of the substrates at the two different pH values is reasonable.

(j) Amino acid analysis confirms the presence of at least 24 cysteine, and 12 histidine residues per mole of enzyme.

VIII. CONCLUSIONS

1. Uridine phosphorylase isoenzymes have been separated and purified.
2. The two isoenzymes have the same molecular weight and probably the same or close enough total charge, but differ in surface distribution of charge and/or shape of the molecule.
3. Both enzymes are tetrameric, and the active forms of each have 110,000 (for #1) and 95,000 (for #2) molecular weights.
4. Isoenzyme #2 is more heat sensitive compared to #1 and both are protected by phosphate, (especially #1) during heat denaturation at 50°C.
5. Kb for uridine with #1 is higher than that of #2. #1 has a very low Ka for phosphate compared to #2. Both isoenzymes #1 and #2 are inhibited by uracil at high concentrations, and isoenzyme #1 is probably also activated by low concentrations of ribose-1-P.
6. Uridine phosphorylase of rat liver does not undergo phosphate induced aggregation, similar to purine nucleoside phosphorylase of Bacillus cereus. In the presence of phosphate there probably is some kind of

equilibrium between the dimeric and the tetrameric forms of the enzymes.

7. On aging in Tris both isoenzymes aggregate into a species which has almost twice the molecular weight of the active enzyme tetramer and this new species cannot be reactivated with 2-mercaptoethanol. The new inactive enzyme molecules differs from the active enzymes both in charge and molecular weight.

8. Upon aging in the presence of phosphate the isoenzymes remain in their active forms but minor amounts of new species with higher and lower mobilities appear. Densitometric patterns of aged fractions in different gel concentrations are too complex for any meaningful analysis, other than that the major active peak of each isoenzyme has a molecular weight of the tetramer, and the other new species are related by molecular weight, i.e. they are either aggregates or dissociated subunits of the enzyme tetramer.

9. Uridine phosphorylase catalyzes transfer between a base and nucleoside only in the presence of phosphate or ribose-1-P, both of which inhibit the transfer at high concentrations. The transfer between $^{32}P_i$ and

ribose-1-P is catalyzed only in presence of uracil, and both activities (transfer and phosphorolysis) seem to be functions of the same active site.

10. Most of the pyrimidine nucleoside cleaving activity is localized in the cytosol but some activity is also localized in plasma membranes, heavy mitochondria, as well as in other membrane structures. Uridine cleaving activity was the only one detectable in significant amounts in purified unsolubilized plasma membranes.

11. A model for uridine phosphorylase has been proposed which takes into account the different pH optima of deoxyuridine and uridine phosphorylase activities as well as the inactivation by sulphydryl inhibitors and the protection from inhibition by uridine, deoxyuridine and phosphate.

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TABLE I

PURIFICATION OF URIDINE PHOSPHORYLASES #1 AND #2

Fractions	Volume	Uridine Cleaving Activity*	Recovery (%)	Specific Activity*	Purification	Deoxyuridine Cleaving Activity*	Thymidine Cleaving Activity*	Ratios of activities Ur/dUr	Ratios of activities Ur/Tm
1. Homogenate	4945	13,115	100%	0.09	1	17,357	6,880	0.76	1.91
Supernatant	4050	8,898	67.83	0.11	1.22	10,532	3,334	0.85	2.66
2. AS_2	473	7,660	58.39	0.15	1.68	10,726	2,762	0.71	2.77
3. HAS_2	433	6,675	50.89	0.24	2.78	8,957	2,829	0.75	2.36
4. DEAE Sephadex pH 8.0									
#1	1525	3,396	25.89	2.12	24.65	2,165	392	1.57	8.67
#2	1080	2,097	15.99	3.88	45.12	1,387	513	1.51	4.09
5. DEAE Sephadex pH 7.0									
#1	270	2,774	21.15	2.67	31.02	1,807	276	1.54	10.04
#2	155.5	1,898	14.47	6.78	78.86	1,109	165	1.71	11.53
6. Sephadex gel									
#1	29.0	2,655	20.24	11.03	128.26	1,670	206	1.60	12.87
#2	22.5	1,715	13.07	22.74	264.5	980	165	1.75	10.39
7. Hydroxyapatite									
#1	18.2	2,067	15.76	49.39	574.31	1,321	324	1.57	6.38
#2	53.8	1,492	11.37	126.04	1,465.53	898	139	1.66	10.75

* One unit is the amount of base formed in μmoles per hour, and specific activity refers to units per mg.

TABLE II (a)

STABILITY OF ENZYME DURING STORAGE

Freshly prepared enzyme had the following units# of activity with uridine as the substrate; in 0.05 M Pi, pH 7.0 → 2.160; in 0.05 M Tris, pH 7.0 → 2.089; lyophilized in the presence of 0.05 M Pi, pH 7.0 → 0.668.

Aged -	Activity 6 weeks at -40°C (units)	8 weeks at -40°C (units)
1. In 0.05 M Pi pH 8.0	1.96	1.49
2. In 0.05 M Tris pH 7.0	0	0
3. In 0.05 M Tris pH 7.0 and 1/2 sat. $(NH_4)_2SO_4$ or albumin	ND*	0.19
4. In 0.05 M Tris pH 7.0 and 0.05 M uridine	0.48	0.44
5. In 0.05 M Pi pH 7.0 + 1/2 $(NH_4)_2SO_4$	2.13	1.25
6. In 0.05 M Pi pH 7.0 + 13.33 mg/ml Albumin	2.16	0.94

100 µl enzyme preparation BIII, (60 µg protein).

All preparations contained 10 mM 2-mercaptoethanol.

* ND No data

\# One unit of enzyme activity is that amount which catalyzed the formation of 1 µmole of free base per hour.

TABLE II (b)

STABILITY OF PURIFIED URIDINE PHOSPHORYLASE

Experiment A			Experiment B		Experiment C	
Ratio of $\frac{UR}{dUR}$ Activity	Age of enzyme in 0.05 M Tris-HCl pH 7.0 at $0^{o}C$	Number of Freezings & Thawings	Activity	Time at room temperature	Activity	
$\frac{0.400}{0.275} = 1.454$	Fresh	0	Pi 0.145 Tris 0.107	0	Pi 0.147 Tris 0.092	
$\frac{0.255}{0.200} = 1.159$	Approximately 10 days	1	Pi 0.140 Tris 0.100	1 hour	Pi 0.137 Tris 0.087	
$\frac{0.192}{0.192} = 1.0$	14 days	2	Pi 0.135 Tris 0.085	2 hours	Pi 0.127 Tris 0.072	
*$\frac{0.545}{0.620} = 0.879$	19 days	4	Pi 0.085 Tris 0.047	4 hours	Pi 0.100 Tris 0.077	
*$\frac{0.322}{0.470} = 0.685$	22 days	8	Pi 0.095 Tris 0.037	6 hours	Pi 0.080 Tris 0.047	

Activity here denotes optical density readings at 290 $m\mu$.

* The same amount of enzyme (43 μgms of protein) was used for all except in * when a different preparation was used.

TABLE III

	Substrate	Amount mM	OD readings at 290 mμ for activity
1.	Uridine	0.33	0.495
		0.166	0.505
2.	Deoxyuridine	0.33	0.410
		0.166	0.360
3.	Uridine + Deoxyuridine	0.33 + 0.33	0.477
		0.166 + 0.166	0.472

Assay procedure as described in methods

TABLE IV

AMINO ACID COMPOSITION OF #1 ENZYME

Amino Acid Residue	Molar Ratio Ala = 1	Moles of amino acids per mole of enzyme and of subunit		
		per subunit	per mole enzyme	
Lysine	0.815	15	60	
Histidine	0.252	3	12	
Arginine	0.460	6	24	
Cysteic acid*	0.294	6	24	
Aspartic acid	1.173	18	72	
Threonine	0.616	9	36	
Serine	0.701	12	48	
Glutamic acid	1.316	21	84	
Proline	0.568	9	36	
Glycine	1.586	24	96	
Alanine	1.0	15	60	
Valine	0.787	12	48	
Methionine*	0.196	3	12	
Isoleucine	0.737	12	48	
Leucine	1.214	18	72	Total residue weight
Phenylalanine	0.529	9	36	94,698

* Determined after performic acid treatment.

Average values of two experiments.

Tryptophan and tyrosine not determined.

TABLE V

DISTRIBUTION OF PYRIMIDINE NUCLEOSIDE PHOSPHORYLASE ACTIVITY IN TISSUES OF RAT

Tissue	Uridine cleaving activity		Thymidine cleaving activity		Deoxyuridine cleaving activity	
	Total unit*	Sp. Act.	Total unit	Sp. Act.	Total unit	Sp. Act.
Brain	10.84	0.06	3.54	0.01	7.81	0.06
Kidney	45.48	0.15	6.35	0.02	22.82	0.10
Spleen	56.52	0.09	1.36	0.01	9.93	0.04
Testes	10.39	0.04	1.06	0.01	9.26	0.05
Heart	11.16	0.15	2.37	0.02	7.38	0.08
Liver	109.52	0.06	110.90	0.06	208.58	0.12
Small Intestine	‡	0.99	-	0.10	-	1.44

Average of 3 or more experiments except for intestine.

* Has been calculated for one rat (weighing 200-250 gms), usually 2 or more rats used.

‡ Only a portion of intestine was used.

TABLE VI

DISTRIBUTION OF MARKER ENZYMES IN VARIOUS FRACTIONS

Fractions	5' Nucleotidase		Glutamate Dehydrogenase		Acid Phosphatase		Glucose-6-phosphatase	
	% Act.	Sp. Act.	% Act.	Sp. Act.	% Act.	Sp. Act.	% Act.	Sp. Act.
Homogenate	100	2.14	100	2.45	100	2.42	100	3.47
Heavy mito.	15.83	5.59	44.15	19.74	14.05	0.46	8.57	1.89
Light mito.	14.02	7.21	7.78	2.95	30.24	4.03	17.34	8.73
Supernatant	32.50	1.86	2.68	0.22	25.04	1.18	28.01	2.04
Debris	44.23	3.52	33.44	2.89	17.50	0.47	28.38	2.91
Purified Nuclei	0	0	0.07	1.07	0.2	0.16	1.16	1.85
*Lysosomes	1.06	4.92	0	0	2.61	54.62	0.24	16.25

Averages of 2 or more experiments except for *

% Act. = Percentage of total activity.

Sp. Act. = Specific activity.

TABLE VII

DISTRIBUTION OF PYRIMIDINE NUCLEOSIDE CLEAVING ACTIVITIES IN THE VARIOUS FRACTIONS

Fractions	Uridine cleaving activity		Thymidine cleaving activity		Deoxyuridine cleaving activity	
	% Act.	Sp. Act.	% Act.	Sp. Act.	% Act.	Sp. Act.
Homogenate	100	0.07	100	0.08	100	0.16
Heavy Mito.	8.38	0.08	3.93	0.10	6.47	0.11
Light Mito.	4.95	0.09	4.52	0.05	7.56	0.18
Debris	16.84	0.06	13.17	0.07	15.64	0.14
Supernatant	72.76	0.20	70.27	0.21	71.42	0.39
Microsomes	0.2%	0	0.63	0.01	1.47	0.04

Pure nuclei and lysozomes had less than 1% of pyrimidine nucleoside cleaving activity.

Averages of two to four experiments.

% Act. = Percentage of total activity.

Sp. Act. = Specific activity.

TABLE VIII

DISTRIBUTION OF MARKER ENZYMES IN FRACTIONS

Fractions	5' Nucleotidase		Glutamate Dehydrogenase		Acid Phosphatase		Glucose-6-phosphatase	
	Sp. Act.	% Act.	Sp. Act.	% Act.	Sp. Act.	% Act.	Sp. Act.	% Act.
Whole homogenate	2.02	100	1.74	100	1.71	100	2.19	100
4000 x g pellet	2.03	39.88	3.91	84.74	2.38	48.26	2.31	37.69
4000 x g supernatant	2.03	64.05	0.18	6.65	1.28	44.22	2.54	68.97
70,000 g pellet	3.27	8.20	0.09	0.26	1.92	3.00	7.09	9.69
70,000 g supernatant	2.07	14.50	0.20	4.89	1.54	30.09	1.05	26.60
Membrane fraction	13.48	43.66	0.11	0.89	1.70	7.72	13.30	27.13

Sp. Act. = Specific activity

% Act. = Percentage of total activity

Average of two experiments.

TABLE IX

DISTRIBUTION OF PYRIMIDINE PHOSPHORYLASES

Fractions	Uridine cleaving activity		Thymidine cleaving activity		Deoxyuridine cleaving activity	
	Sp. Act.	% Act.	Sp. Act.	% Act.	Sp. Act.	% Act.
Whole homogenate	0.05	100	0.05	100	0.93	100
4000 x g pellet	0.02	13.13	0.02	17.54	0.02	13.52
4000 x g supernatant	0.07	84.02	0.06	76.70	0.10	66.36
70,000 g pellet	*	*	*	*	*	*
70,000 g supernatant	0.10	78.00	0.07	61.97	0.12	46.28
Membrane fraction	0.57	17.44	*	*	*	*

Sp. Act. = Specific activity

% Act. = Percentage of total activity

* = Very low activity

Average of two experiments

TABLE X

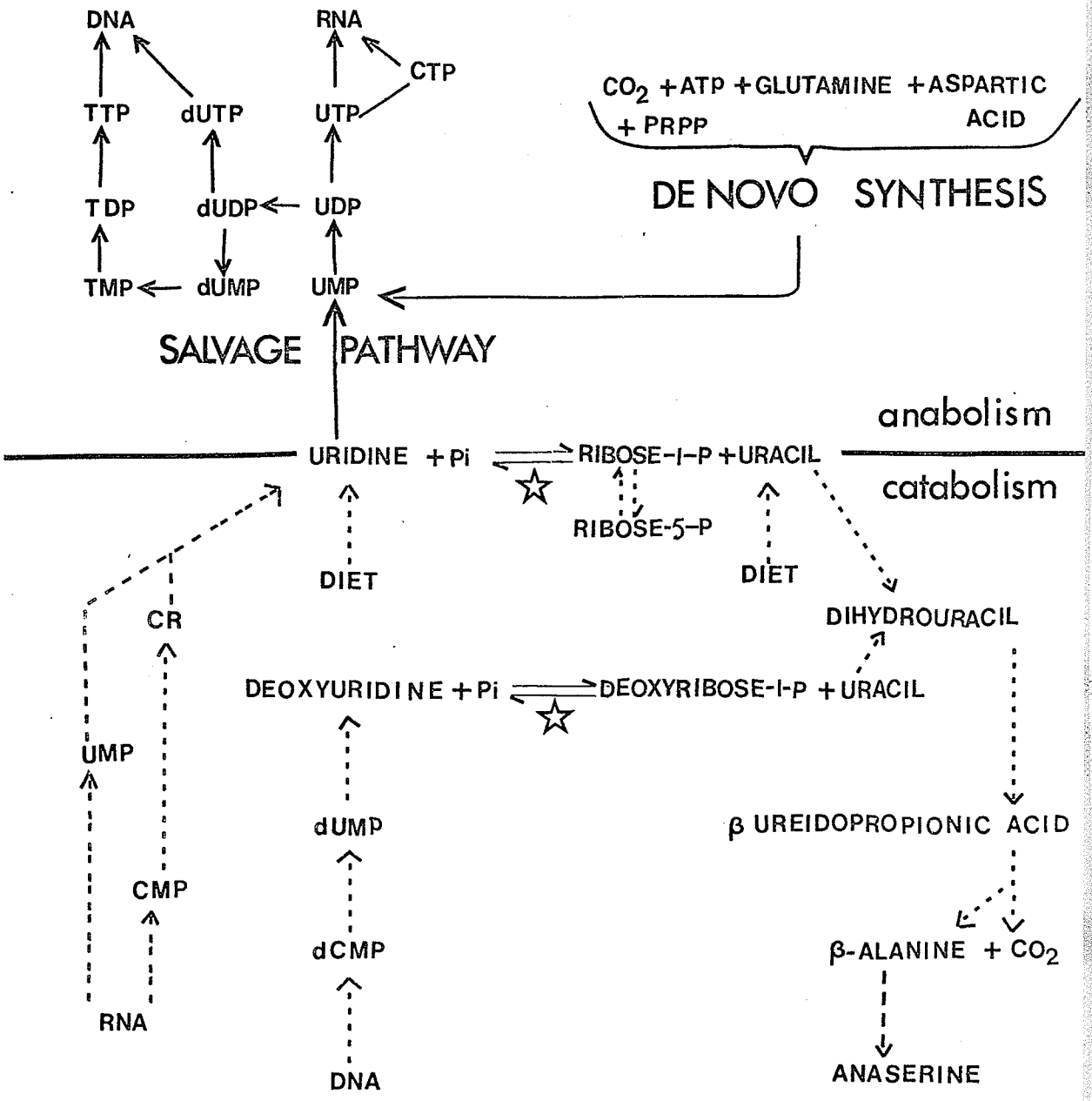
EFFECT OF PHOSPHATE AND ARSENATE
ON TRANSFER ACTIVITY

Reaction	ADDITIONS			
	None	Phosphate	Arsenate	Phosphate + Arsenate
		1 mM	1 mM	1 mM+1mM
$U^{C^{14}} + UR \rightleftharpoons UR^{C^{14}} + U$	36.4*	685.5	199.5	346.5
$U^{C^{14}} + dUR \rightleftharpoons dUR^{C^{14}} + U$	17.2	93.2	97.2	153.2

* Units in mμmoles per ml per hour.

FIGURE - 1

URIDINE PHOSPHORYLASE AND PYRIMIDINE METABOLISM IN LIVER.



☆ URIDINE PHOSPHORYLASE

FIGURE 2

Changes in the specific activity of uridine phosphorylase in liver of Sprague Dawley rats during development

This graph is from the work of Guroff and Rhoads (44). Specific activity is defined as the nmoles of uridine cleaved per mg protein of liver homogenate per hour at pH 7.4 and 37°C.

FIGURE - 2

SPECIFIC ACTIVITY OF URIDINE PHOSPHORYLASE DURING DEVELOPMENT IN THE LIVER OF SPRAGUE DAWLEY RATS.

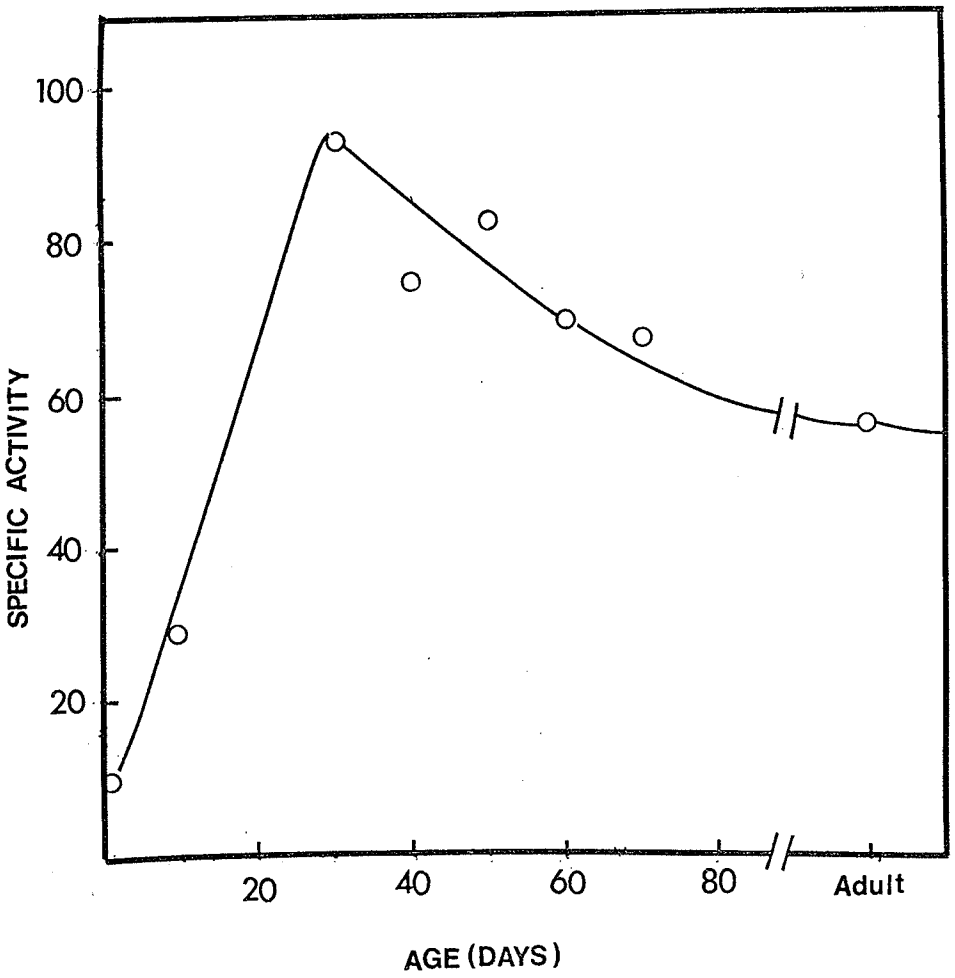


FIGURE 3

Separation of pyrimidine nucleoside phosphorylases by chromatography on DEAE Sephadex pH 8.0 (preparation I).

U P = uridine phosphorylase, the two peaks correspond to isoenzyme #1 and isoenzyme #2; Recovery 87.94%.

T P = thymidine phosphorylase.

The horizontal arrows indicate fractions that were pooled together.

Column type - K 25/45 (Pharmacia)

Bed volume - 147 ml.

Flow rate - 38 ml/hour

Sample - 95 ml of sample from purification step 3, containing 1.35 g protein with 328 units of uridine cleaving activity.

Gradient - Linear consisting of 800 ml of buffer (0.02 M K Pi, pH 8.0, + 1 mM EDTA + 10 mM 2-mercapto-ethanol) and 800 ml of 0.5 M KCl in the same buffer.

Units and protein expressed per fraction in this and the following 7 figures.

FIGURE -3

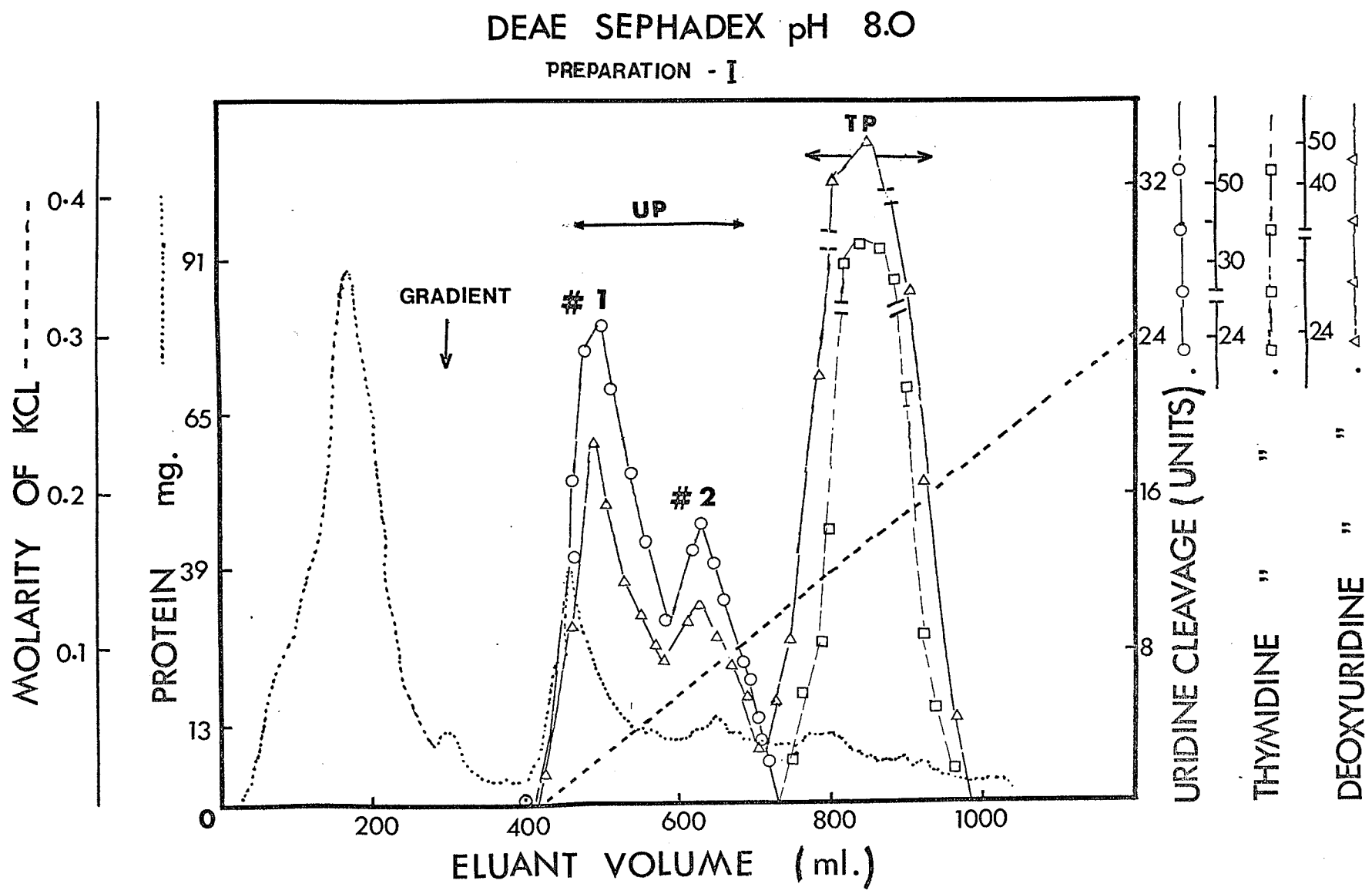

DEAE SEPHADEX pH 8.O
PREPARATION - I
MOLARITY OF KCL
0·4
0·3
0·2
0·1
PROTEIN mg.
91
65
39
13
0
UP
GRADIENT
1
2
TP
200
400
600
800
1000
ELUANT VOLUME (ml.)
URIDINE CLEAVAGE (UNITS).
32
24
16
8
THYMIDINE " "
50
30
24
DEOXYURIDINE " "
50
40
24

FIGURE 4

Separation of isoenzymes by chromatography on DEAE Sephadex pH 8.0, preparation V.

Upward flow was used; #1 and #2 correspond to isoenzymes #1 and #2 of uridine phosphorylase with uridine as substrate. Recovery of activity was #1 50.88% and #2 31.42%. Horizontal arrows indicate fractions that were pooled for subsequent purification.

Column type - K 50/100 (Pharmacia)

Bed volume - 1.57 liters

Flow rate - 30 ml/hour

Sample - 1.49 liters of dialyzed heat-treated AS_2 from purification step 3 containing 27.71 g protein and 6630 units of uridine phosphorylase activity.

Gradient - Linear consisting of 4 liters of buffer (0.02 M K Pi buffer, pH 8.0 + 10 mM 2-mercaptoethanol + 1 mM EDTA) and 4 liters of 0.2 M KCl in the same buffer.

FIGURE - 4

DEAE SEPHADEX pH 8.0

PREPARATION V

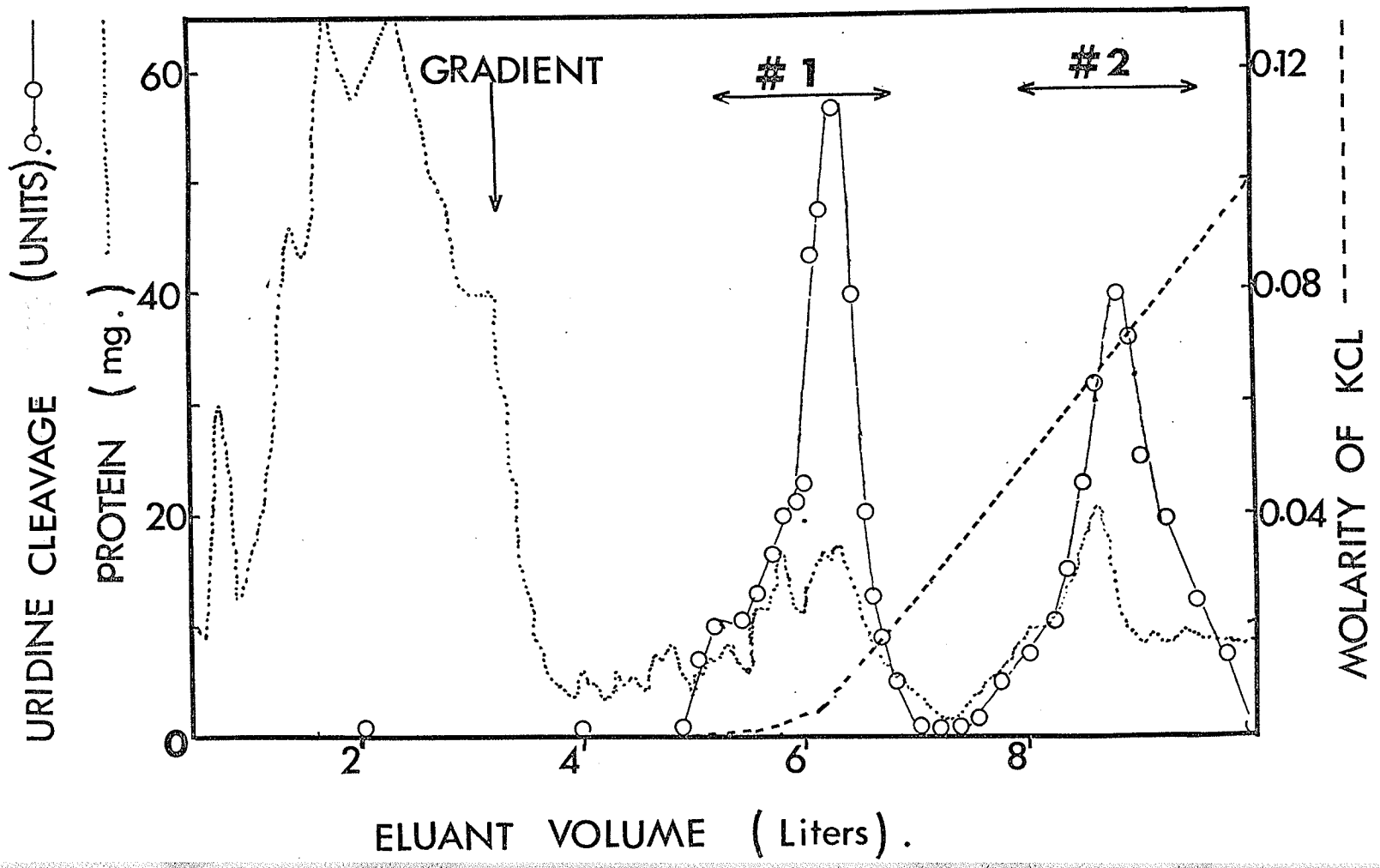


FIGURE 5

Elution profile of isoenzyme #1, preparation V from DEAE Sephadex, pH 7.0 column.

Uridine was used as substrate in the assay of column fractions; the horizontal arrow indicates the fractions that were pooled, and recovery was 81.67%.

Column type - K 25/45 (Pharmacia)

Bed volume - 1.96

Flow rate - 28 ml/hour

Sample - Isoenzyme #1 fraction from step 4 contained 1.42 g protein with 2946 units of uridine phosphorylase activity.

Gradient - Linear consisting of 500 ml of buffer (0.02 M K Pi, pH 7.0 + 10 mM 2-mercaptoethanol + 1 mM EDTA) and 500 ml of 0.2 M KCl in the same buffer.

FIGURE - 5

DEAE SEPHADEX pH 7

PREPARATION V

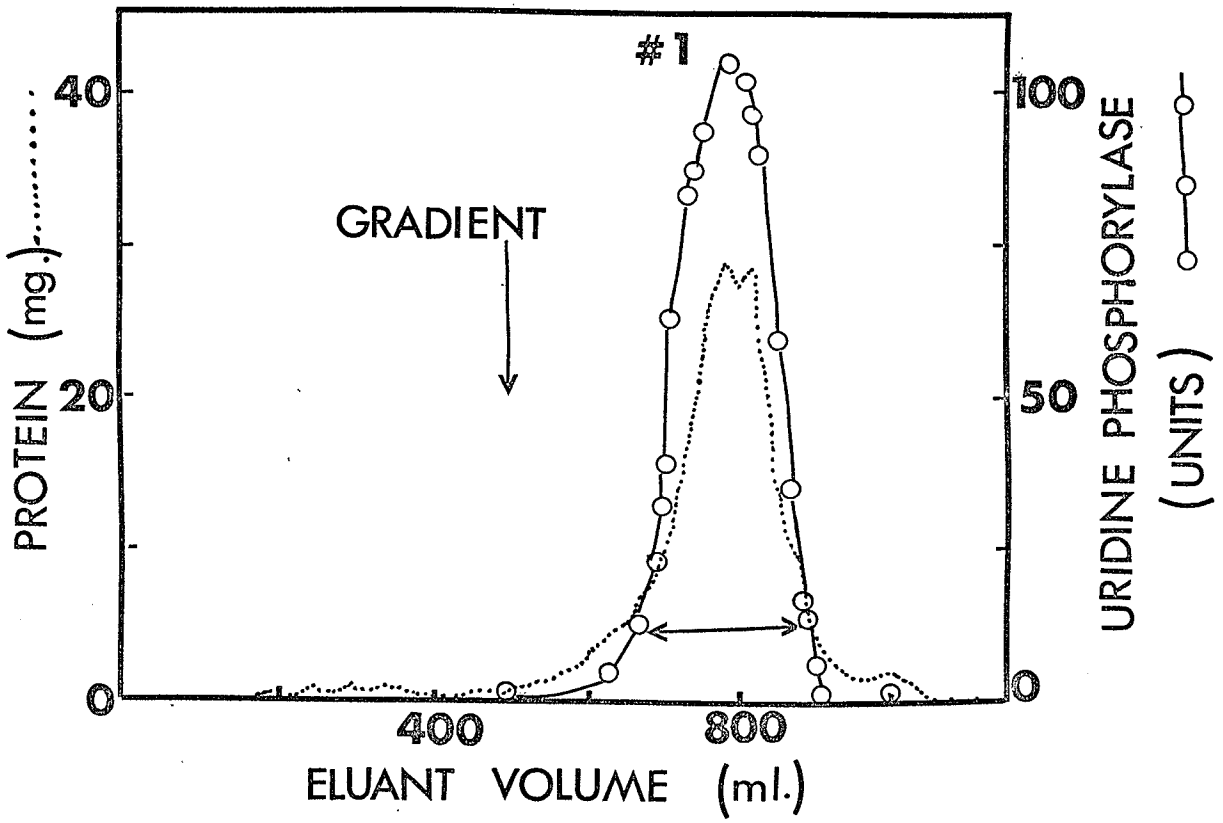


FIGURE 6

Elution profile of isoenzyme #2, preparation V from DEAE Sephadex, pH 7.0 column.

Uridine was used as the substrate in the assays of column fractions; the horizontal arrow indicates the fractions that were pooled for subsequent purification. Recovery of activity was 90.51%.

Column type - K 25/45 (Pharmacia)

Bed volume - 171 ml

Flow rate - 26.5 ml/hour

Sample - Isoenzyme #2 from step 4 contained 5.42 g protein with 2077 units of uridine phosphorylase activity.

Gradient - Linear consisting of 500 ml of buffer (0.02 M K Pi, pH 7.0 + 10 mM 2-mercaptoethanol + 1 mM EDTA) and 500 ml of 0.15 M KCl in the same buffer.

FIGURE - 6

DEAE SEPHADEX pH 7

PREPARATION V

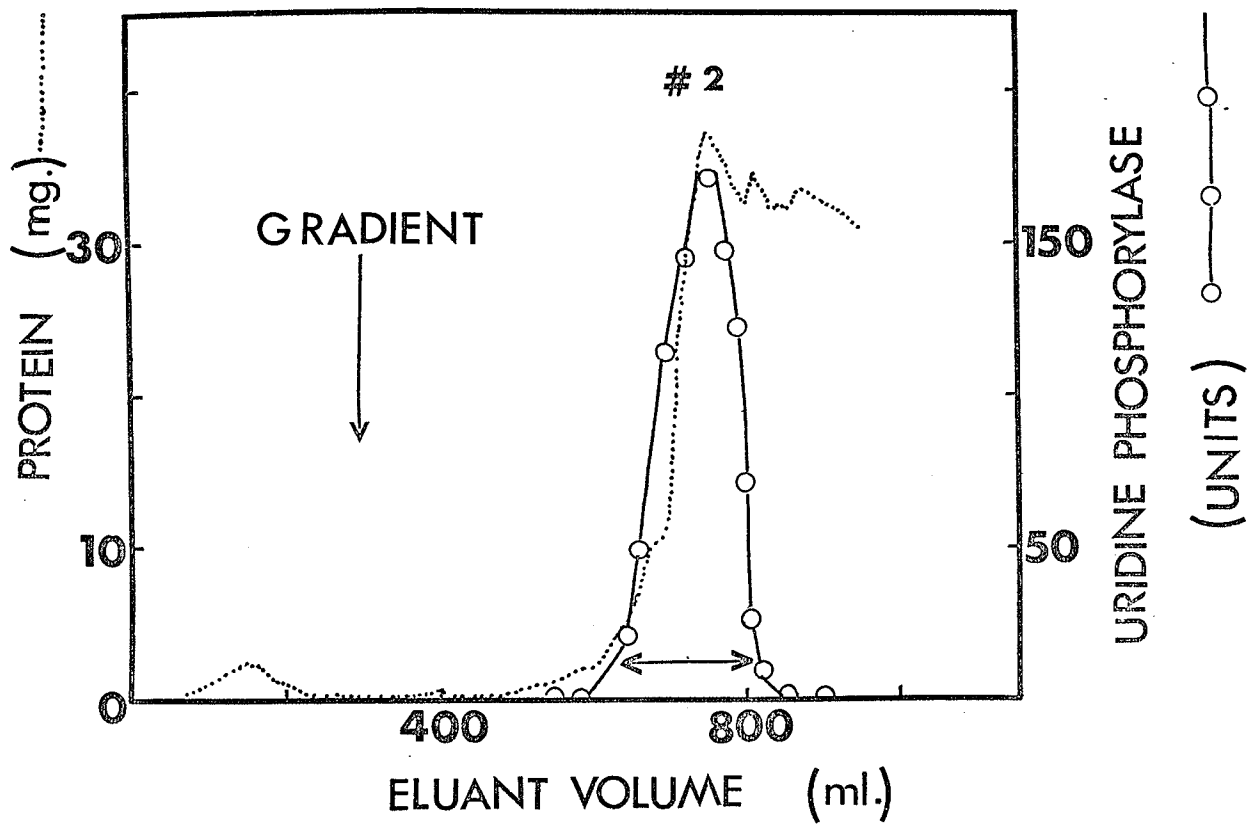


FIGURE 7

Sephadex gel filtration of isoenzyme #1, preparation V.

Uridine was used as the substrate in the assays of column fractions. Column (1), (2) and (3) were connected in series by means of Pharmacia flow adapters and upward flow was used. Five ml of 0.2% blue dextran 2000 solution in 0.02 M K Pi, pH 8.0 + 1 mM EDTA + 10 mM 2-mercaptoethanol was used. The elution buffer was 0.02 M K Pi, pH 8.0 + 1 mM EDTA + 10 mM 2-mercaptoethanol. The horizontal line indicates the fractions that were pooled for subsequent purification. Recovery was 95.72%.

Column types - (1) K 25/45 (2) 25/100 (3) 25/45 (Pharmacia) in series.

Bed volume - 171 ml + 367.5 ml + 171.5 ml = 710.5 ml

Sephadex gels - (1) G-200 (2) G-150 (3) G-100

Flow rate - 10 ml/hour

Sample - 9 ml of isoenzyme #1 fraction from step 5 contained 1.04 g protein and 2774 units of uridine phosphorylase activity.

FIGURE - 7

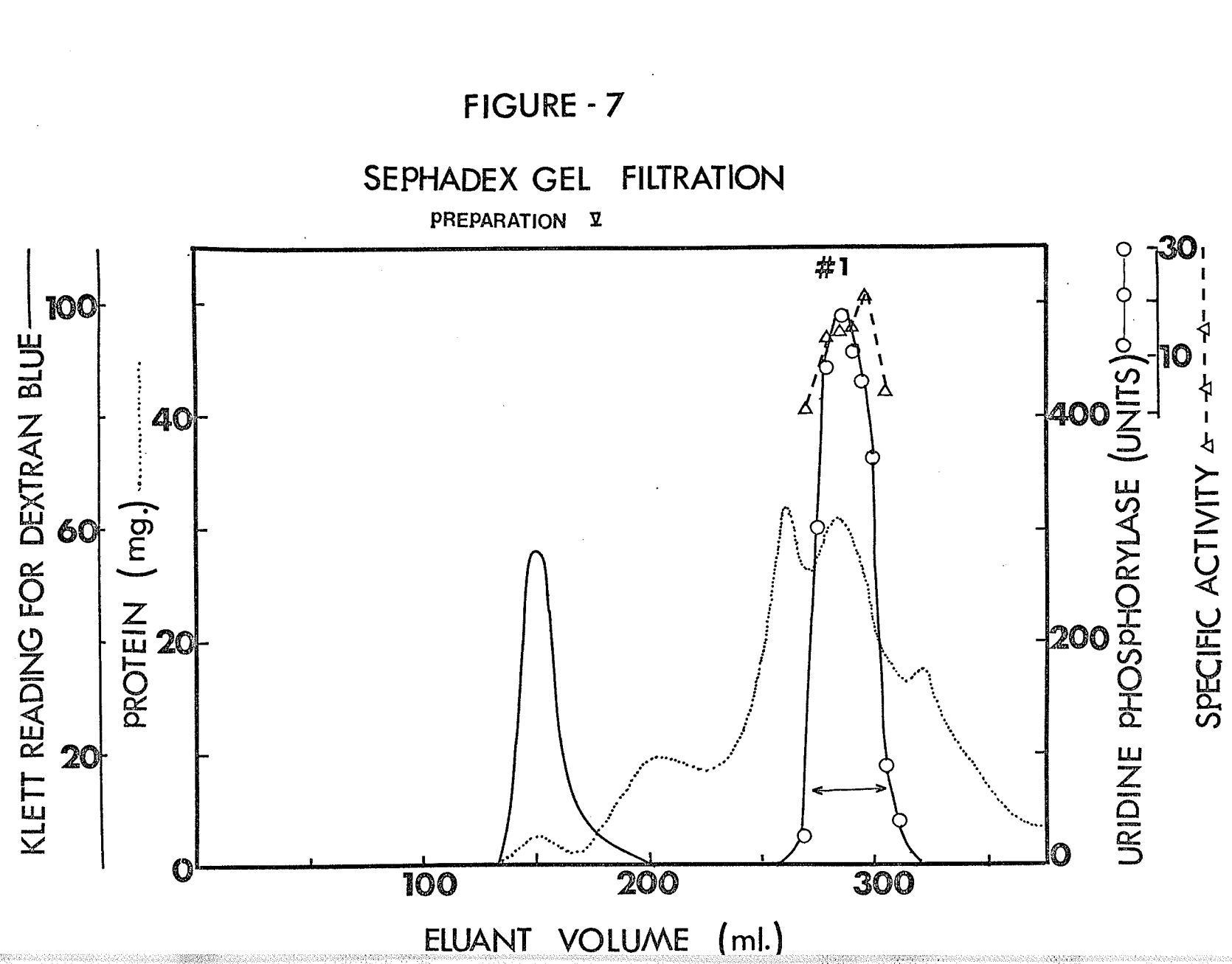

SEPHADEX GEL FILTRATION
PREPARATION V
#1
KLETT READING FOR DEXTRAN BLUE
PROTEIN (mg.)
URIDINE PHOSPHORYLASE (UNITS)
SPECIFIC ACTIVITY
ELUANT VOLUME (ml.)
100
60
20
40
0
100
200
300
400
200
0
30
10

FIGURE 8

Sephadex gel filtration of isoenzyme #2 preparation V.

Uridine was used as the substrate in the assays for the column fractions. Column (1), (2) and (3) were connected in series by means of Pharmacia flow adaptors and upward flow was used. Five ml of 0.2% blue dextran 2000 solution in 0.02 M K Pi, pH 8.0 + 1 mM EDTA + 10 mM 2-mercaptoethanol was used. The elution buffer was 0.02 M K Pi, pH 8.0 + 1 mM EDTA + 10 mM 2-mercaptoethanol. The horizontal arrow indicates the fractions that were pooled for subsequent purification. Recovery was 90.32%.

Column types - (1) 25/100 (2) K 25/45 (3) K 25/45 in series.

Bed volume - 367.5 + 171.5 + 171.5 = 710.5

Sephadex gels - (1) G-200 (2) G-150 (3) G-150

Flow rate - 6 ml/hour

Sample - 4.2 ml of isoenzyme #2 fraction from step 5 contained 0.28 g protein with 1898 units of uridine phosphorylase activity.

FIGURE - 8

SEPHADEX GEL FILTRATION

PREPARATION V

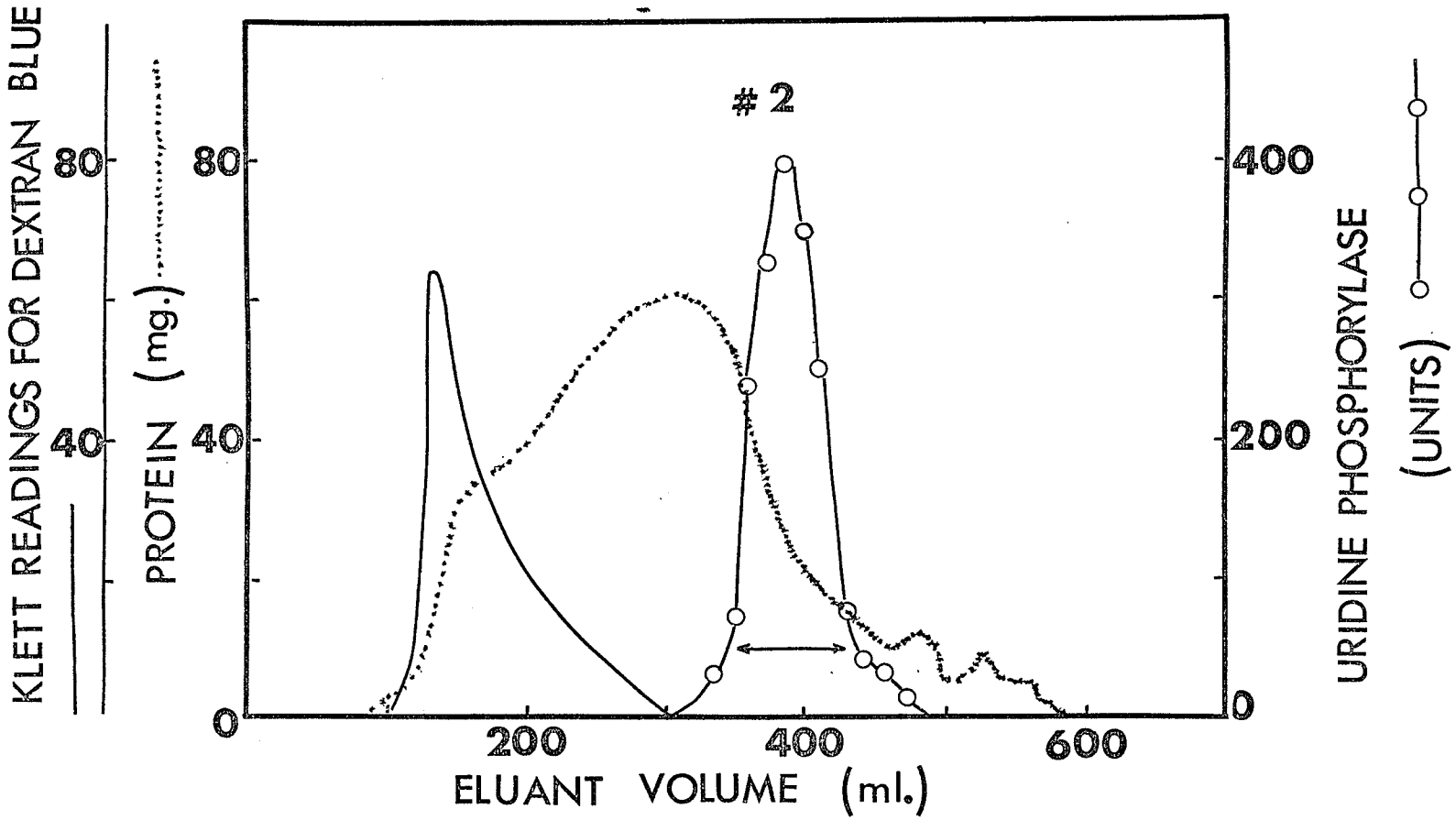


FIGURE 9

Chromatography of isoenzyme #2, preparation V on hydroxyapatite, pH 7.3.

Uridine was used as the substrate in the assays of column fractions. The horizontal line indicates the fractions that were pooled. Recovery was 77.86%.

Column type - K 25/45 (Pharmacia)

Bed volume - 152 ml

Flow rate - 25 ml/hour

Sample - 22 ml of isoenzyme #2 fraction from step 6 containing 75 mg protein with 1676 units of uridine phosphorylase activity.

Gradient - Linear consisting of 500 ml of 0.02 M K Pi, pH 7.3 + 10 mM 2-mercaptoethanol and 500 ml of 0.2 M K Pi, pH 7.3 + 10 mM 2-mercaptoethanol.

FIGURE - 9

HYDROXYAPATITE pH 7.3

PREPARATION V

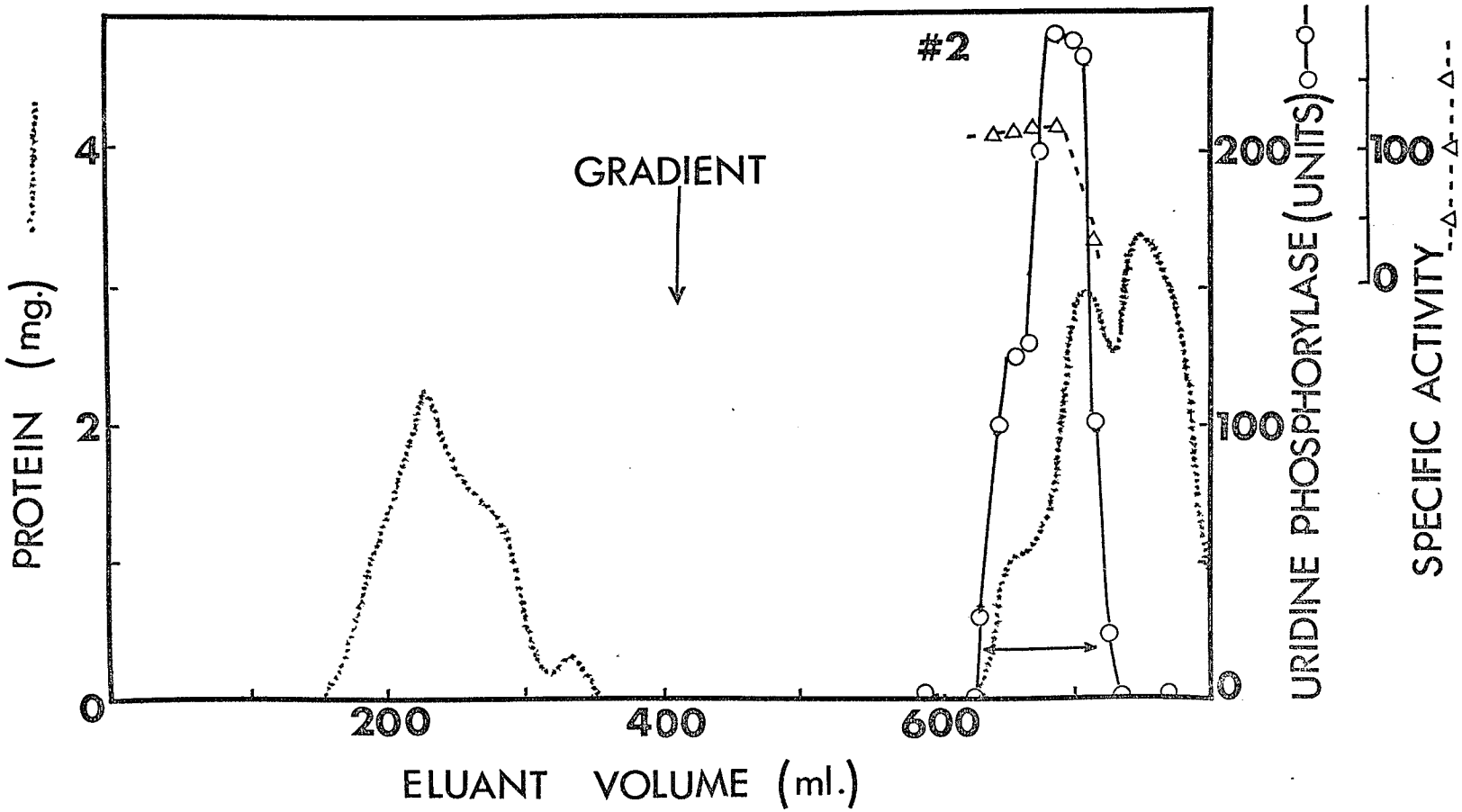


FIGURE 10

Chromatography of isoenzyme #1, preparation V on hydroxyapatite pH 7.3.

Uridine was used as the substrate in the assays of the column fractions. The horizontal arrows indicate the fractions pooled. Recovery was 86.99%.

Column type - K 25/45 (Pharmacia)

Bed volume - 137.2 ml

Flow rate - 22 ml/hour

Sample - 28.5 ml of isoenzyme #1 from step 6 contained 236.5 mg protein with 2609 units of uridine phosphorylase activity.

Gradient - Linear consisting of 500 ml of 0.02 M K Pi, pH 7.3 + 10 mM 2-mercaptoethanol and 500 ml of 0.15 M K Pi, pH 7.3 + 10 mM 2-mercapto-ethanol.

FIGURE - 10

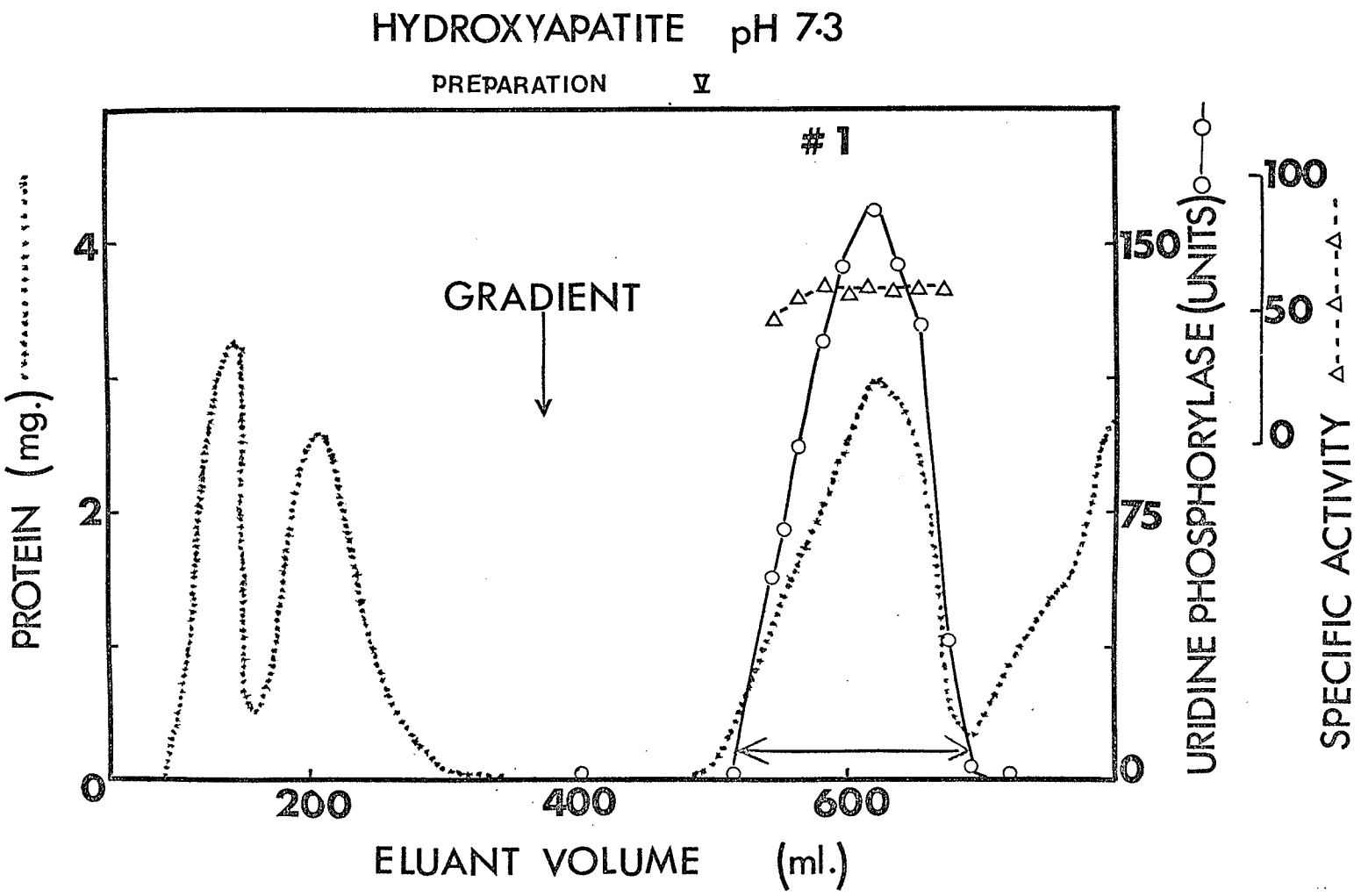

HYDROXYAPATITE pH 7.3
PREPARATION V
#1
GRADIENT
PROTEIN (mg.)
URIDINE PHOSPHORYLASE (UNITS)
SPECIFIC ACTIVITY
ELUANT VOLUME (ml.)
0
2
4
200
400
600
0
75
150
0
50
100

FIGURE 11

Stability of isoenzymes at 50°C.

TRIS - 23 μg of isoenzyme #1 or 4.4 μg of isoenzyme #2 was preincubated in 500 μl of 0.05M Tris-HCl buffer, pH 7.0 at 50° in stoppered test tubes for the times specified following which the samples were immediately immersed in an ice bath. The rest of the ingredients required for the assay of uridine phosphorylase activity were then added to each tube. The rest of assay procedure was as described in "Methods".

Pi - Similarly for heat treatment in the presence of phosphate, either 23 μg of isoenzyme #1 or 4.4 μg of isoenzyme #2 were preincubated in 500 μl of 0.05 MKPi, pH 7.0 in glass stoppered test tube at 50° for the times specified. The rest of the procedure was same as in the samples pre-incubated with Tris.

Uridine used as substrate.

FIGURE - 11

HEAT TREATMENT OF ISOENZYMES AT 50°C

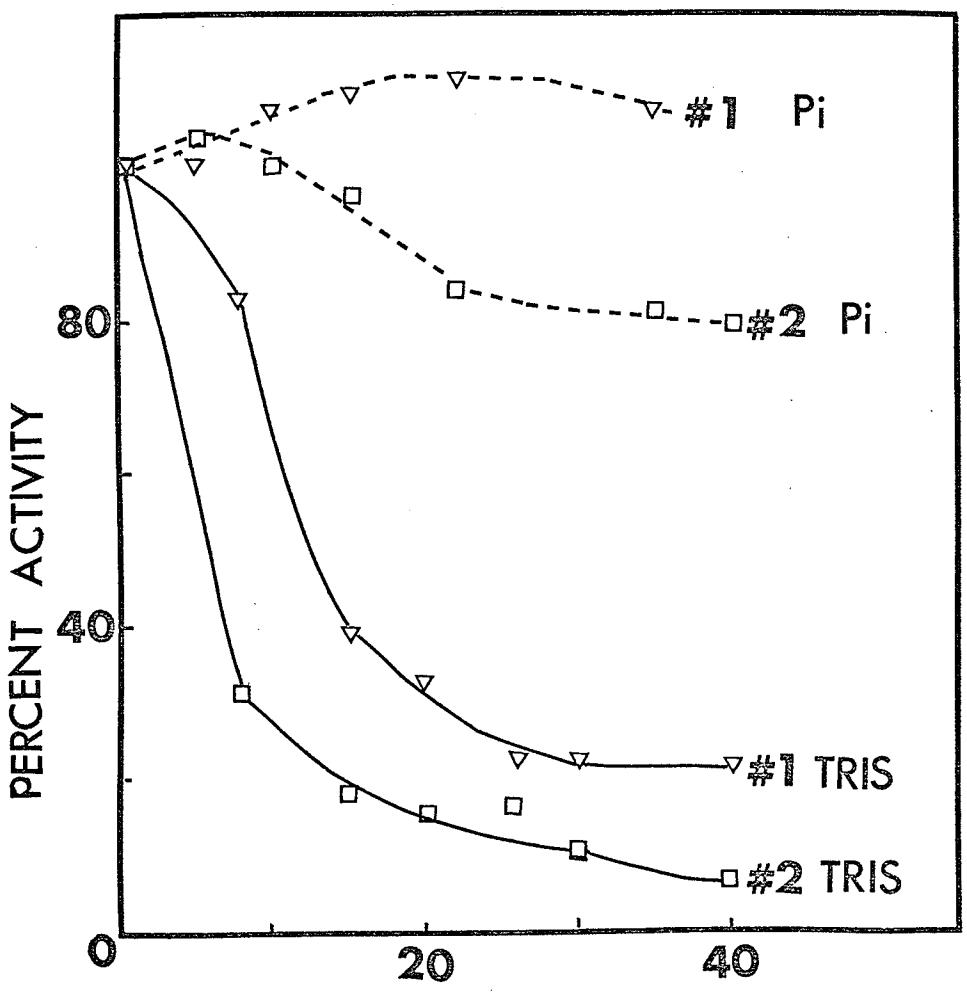


FIGURE 12

pH optimum of isoenzyme #1 and isoenzyme #2.

Either 8.4 μg of isoenzyme #1 or 8.8 μg of isoenzyme #2 was used for assaying uridine cleavage at all pH values. The assay system consisted of 150 μl of 1 M K Pi adjusted to the required pH value, as well as either 150 μl of 1M acetate buffer (for pH values of 6.0 $\longrightarrow$ 6.5) or tris-HCl buffer (for pH 7.0 and above) or Tris-HCl buffer in addition to 150 μl of 1 M glycyl glycine (for pH values of 8.0 $\longrightarrow$ 9.5).

Incubation was for 1 hour at 37^{o} and the rest of the procedure was the same as that described in the Methods section.

FIGURE - 12

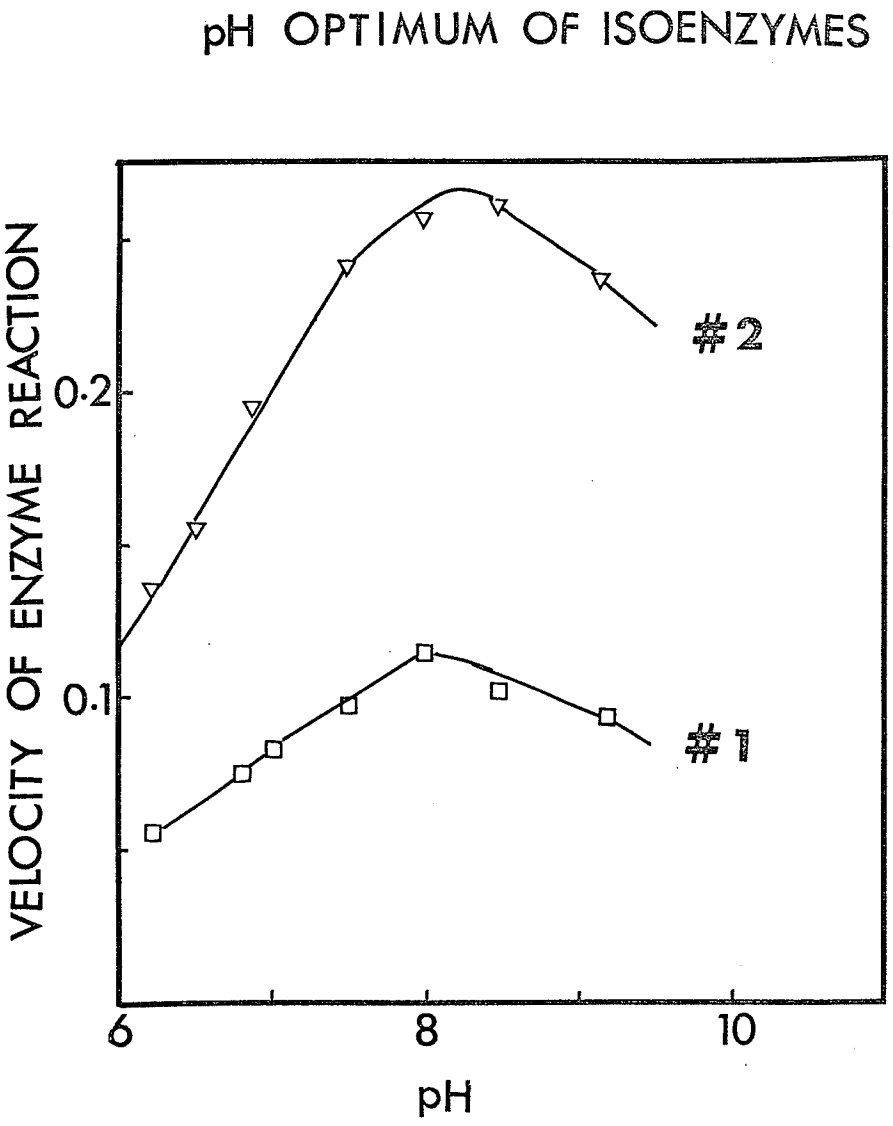
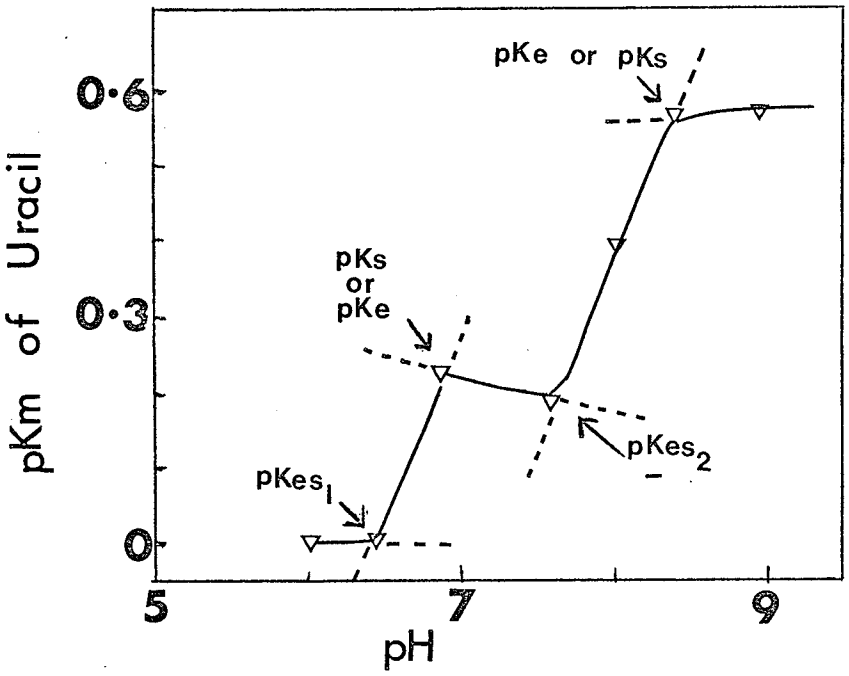

pH OPTIMUM OF ISOENZYMES
VELOCITY OF ENZYME REACTION
0.2
0.1
#2
#1
6
8
10
pH

FIGURE-13


pKe or pKs
pKs
or
pKe
pKes1
pKes2
pKm of Uracil
0·6
0·3
0
5
7
9
pH

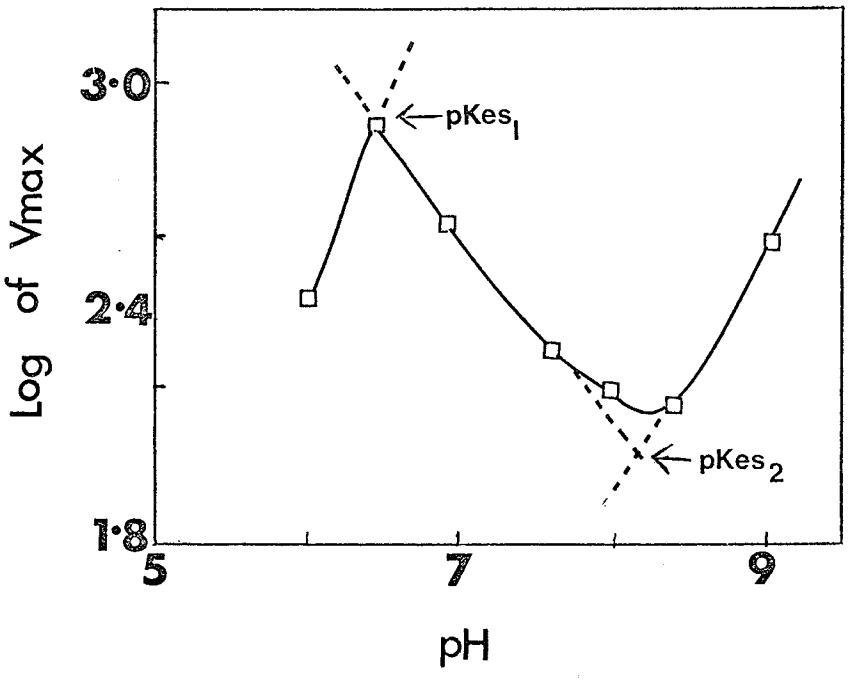

pKes1
pKes2
Log of Vmax
3·0
2·4
1·8
5
7
9
pH

FIGURE 13

pKm and log Vmax at different pH values; calculated from double reciprocal plots of initial velocity against variable uracil concentration and saturating levels of ribose-1-phosphate (0.533 mM).

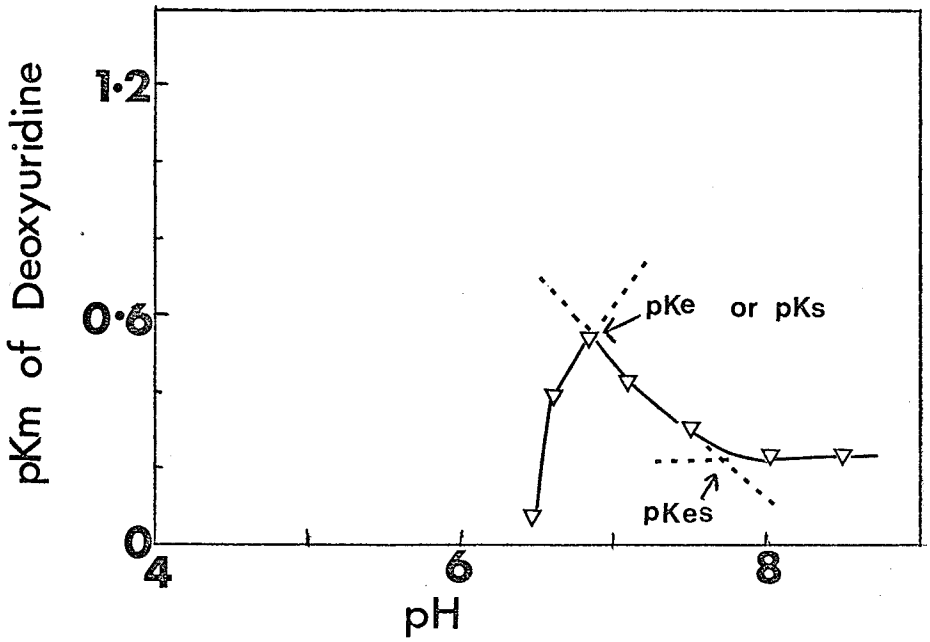
The standard assay system was adjusted to the required pH by either 150 μl of 1 M acetate buffer (pH 6 to 6.5) or 150 μl of 1 M Tris-HCl (pH 6.8 to 9). Fifty μl of glycyl-glycine was also present at pH values above 8.0. The uracil concentration was varied over the range of 0.033 mM to 0.5 mM at each pH value. The same amount of isoenzyme #1 (43 μg protein) was present in every case.

The dotted lines are extrapolations of the slopes of the bends in the graph. The point of intersection is taken as the value for pKe, pKes or pKs where:

pKes - pH at which an enzyme substrate complex is half ionized.

pKe, pKs - pH at which a group in the enzyme or substrate is half ionized.

FIGURE - 14


pKm of Deoxyuridine
1·2
0·6
0
4
6
8
pH
pKe or pKs
pKes

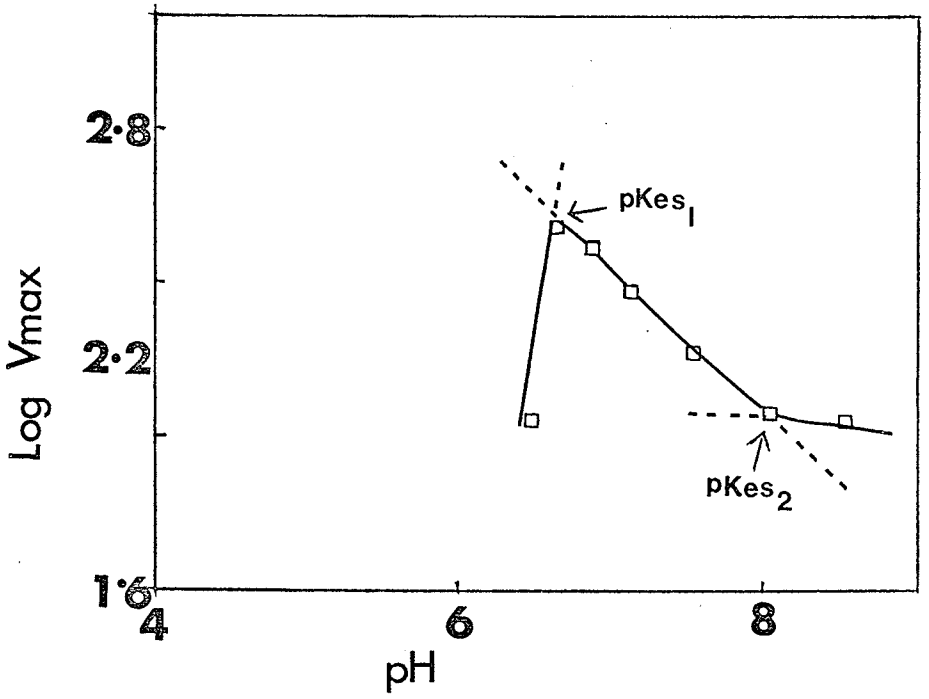

Log Vmax
2·8
2·2
1·6
4
6
8
pH
pKes1
pKes2

FIGURE 14

pKm and log Vmax at different pH values; calculated from double reciprocal plots of initial velocity against variable deoxyuridine concentration and saturating levels of phosphate (0.116 M).

Deoxyuridine was varied over the range of 0.33 mM to 0.66 mM. The same amount of isoenzyme #2 (6.6 μg of protein) and 0.116 M K phosphate was present in every case. Adjustment of pH and the rest of the procedure was as described for Figure 13.

FIGURE -15

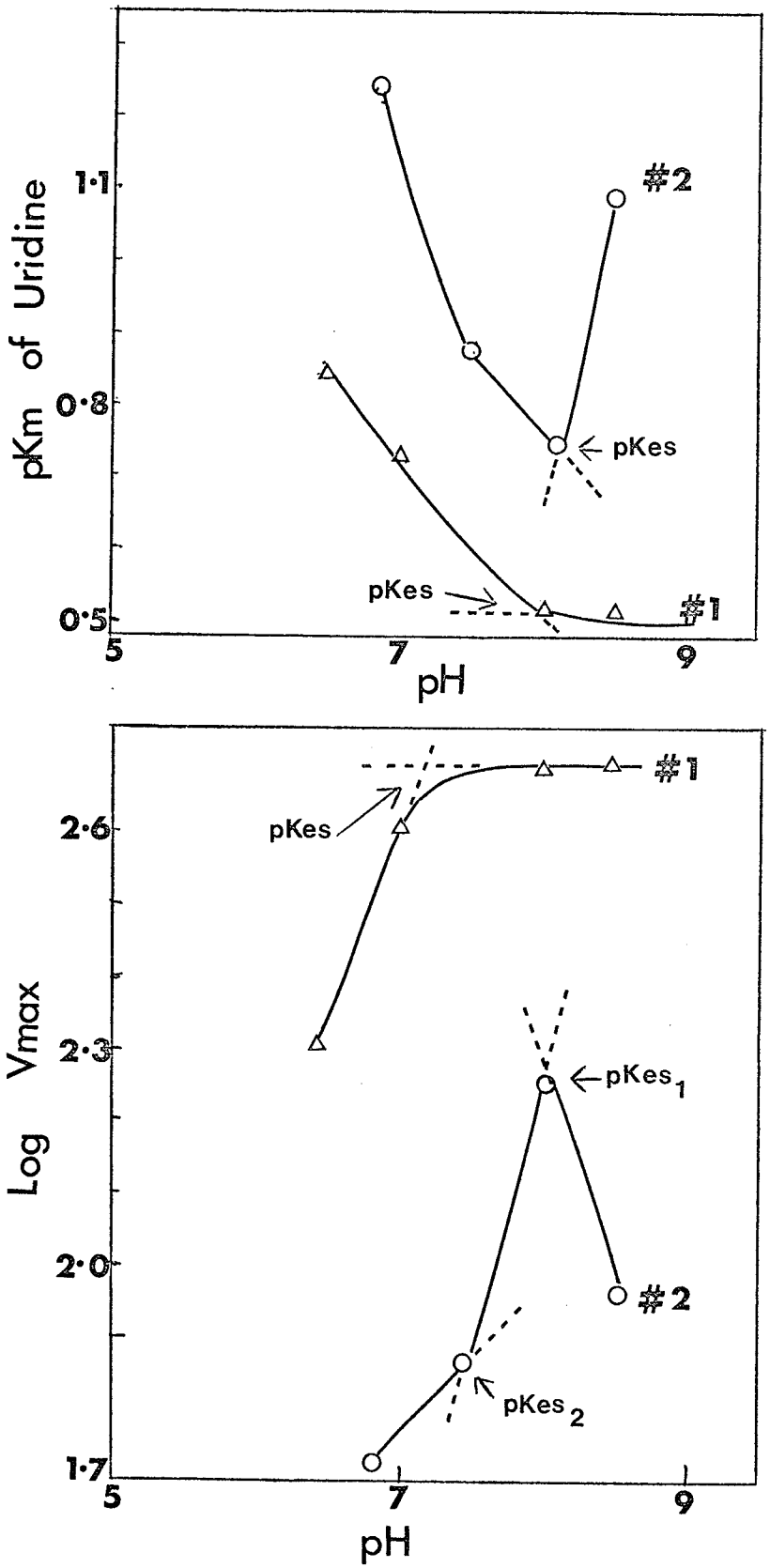

pKm of Uridine
1·1
0·8
0·5
#2
pKes
pKes
#1
5
7
9
pH
Log Vmax
2·6
2·3
2·0
1·7
#1
pKes
pKes1
#2
pKes2
5
7
9
pH

FIGURE 15

pKm and log Vmax at different pH values; calculated from double reciprocal plots of initial velocity against variable uridine concentration and saturating levels of phosphate (0.116 M).

The procedure was similar to that described for Figure 14. The uridine concentration was varied from 0.016 mM to 3.33 mM. 6.6 μg of isoenzyme #2 and 34.5 μg of isoenzyme #1 was used for every test.

FIGURE-16

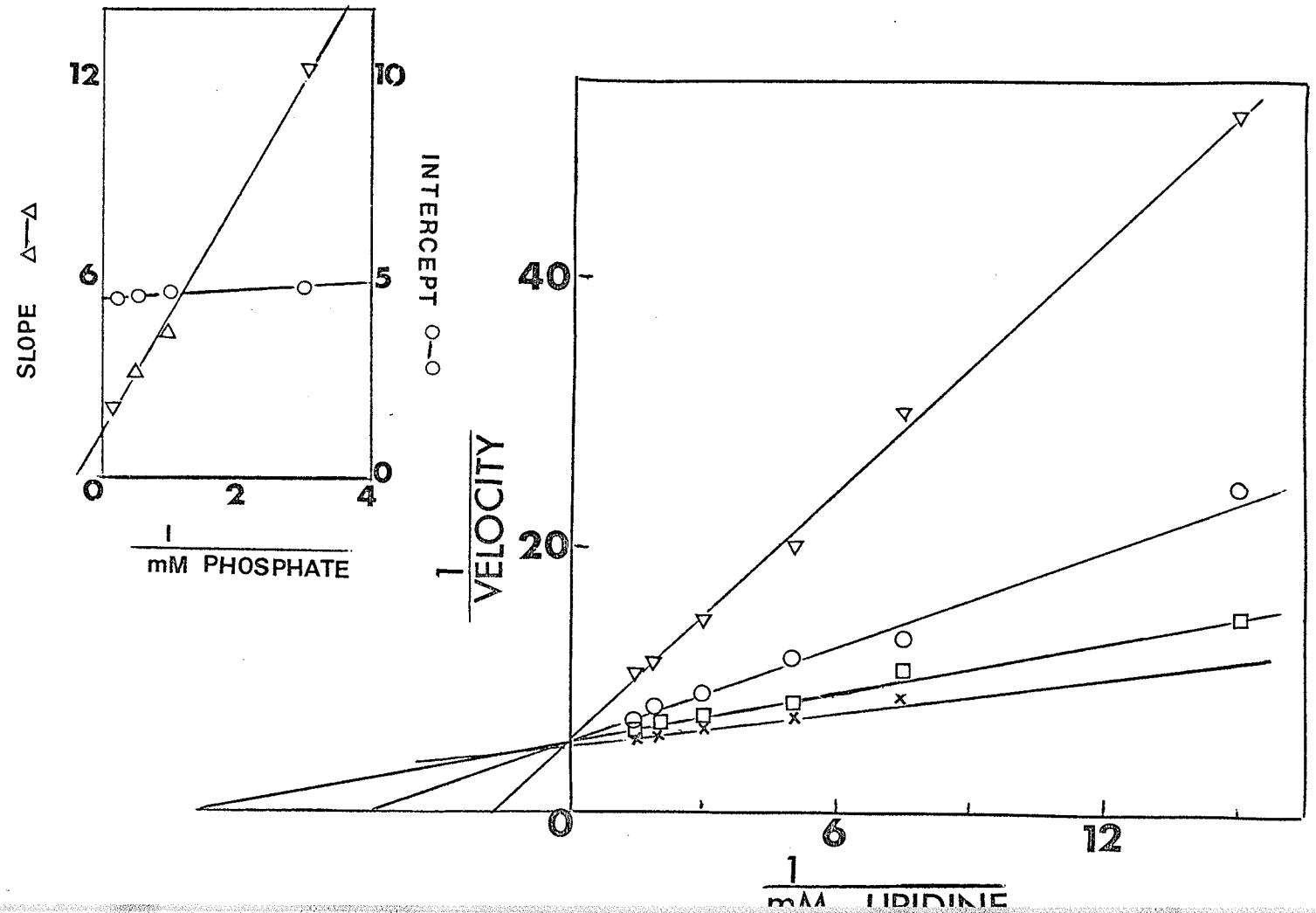

SLOPE Δ—Δ
INTERCEPT ○—○
12
6
0
10
5
0
2
4
1
mM PHOSPHATE
1
VELOCITY
40
20
0
6
12
1
mM URIDINE

FIGURE 16

Initial velocity plot of isoenzyme #1 with uridine as the variable substrate and phosphate as the non-variable substrate at pH 7.4.

The standard assay medium contained isoenzyme #1 purified 574 fold from step 7, (34.5 μg protein) in 1.5 ml along with 0.1 M Tris-HCl buffer, pH 7.4 and the other ingredients as described in Methods. Uridine was varied from 0.066 mM to 0.66 mM at different levels of phosphate which were 0.33 mM (△———△——△), 0.99 mM (O——O——O—), 1.99 mM (-□———□——□—) and 5.0 mM (×—— ×—— ×).

FIGURE - 17

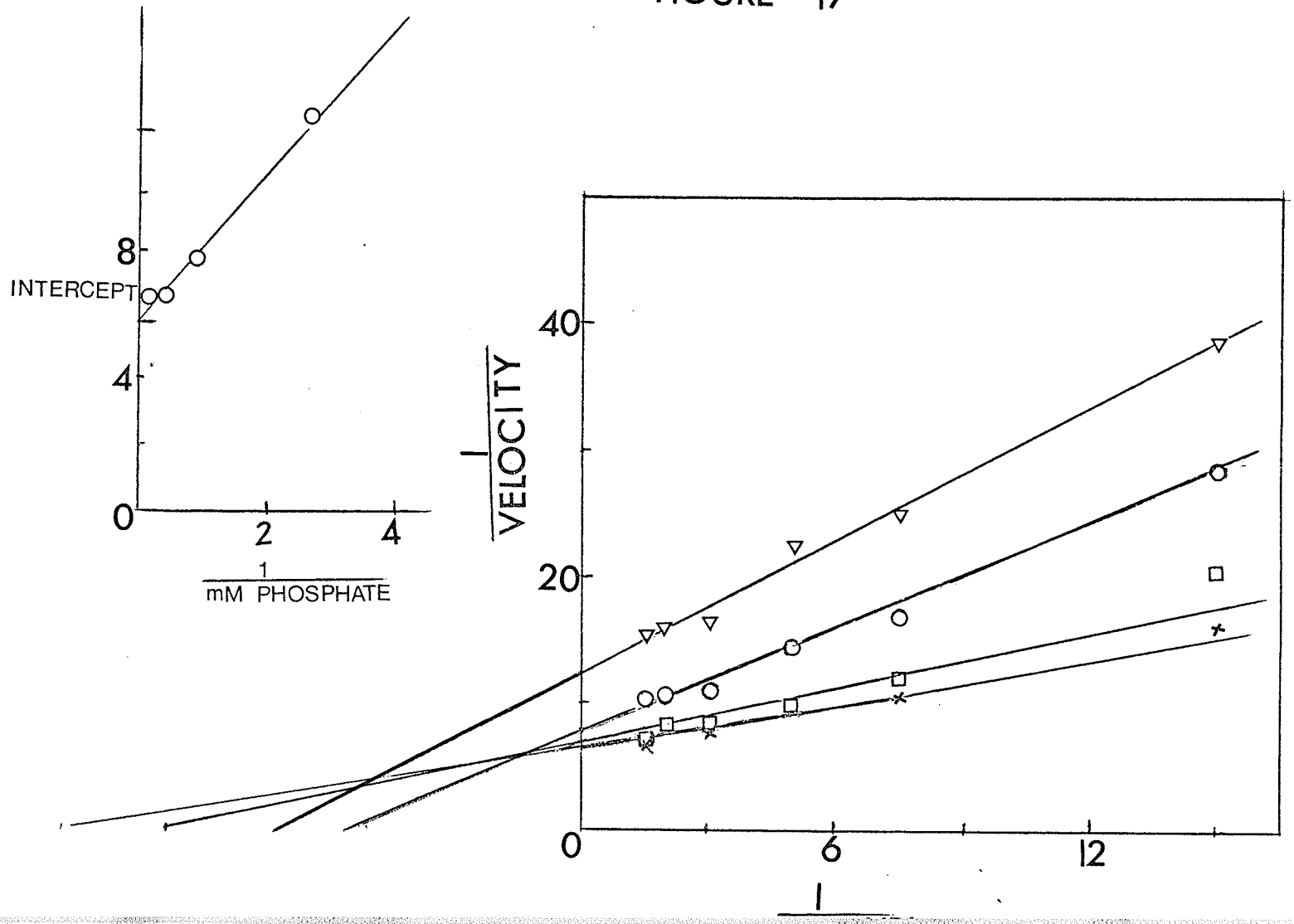


FIGURE 17

Determination of Ka of phosphate with isoenzyme #2 at pH 7.4.

The standard assay medium contained isoenzyme #2 (purified 1400 fold) from step 7 (6.6 μg protein) and 0.1 M tris-HCl buffer pH 7.4, along with the other ingredients described in Methods section. Uridine was varied in the range of 0.066 mM to 0.66 mM concentration at different levels of phosphate, which were 0.33 mM (▽——▽——▽——), 0.99 mM (O——O—), 1.99 mM (□——□——□—) and 5.0 mM (×——×——×). Ka of phosphate was calculated from the replot of intercept against reciprocal of phosphate concentration.

FIGURE - 18 A

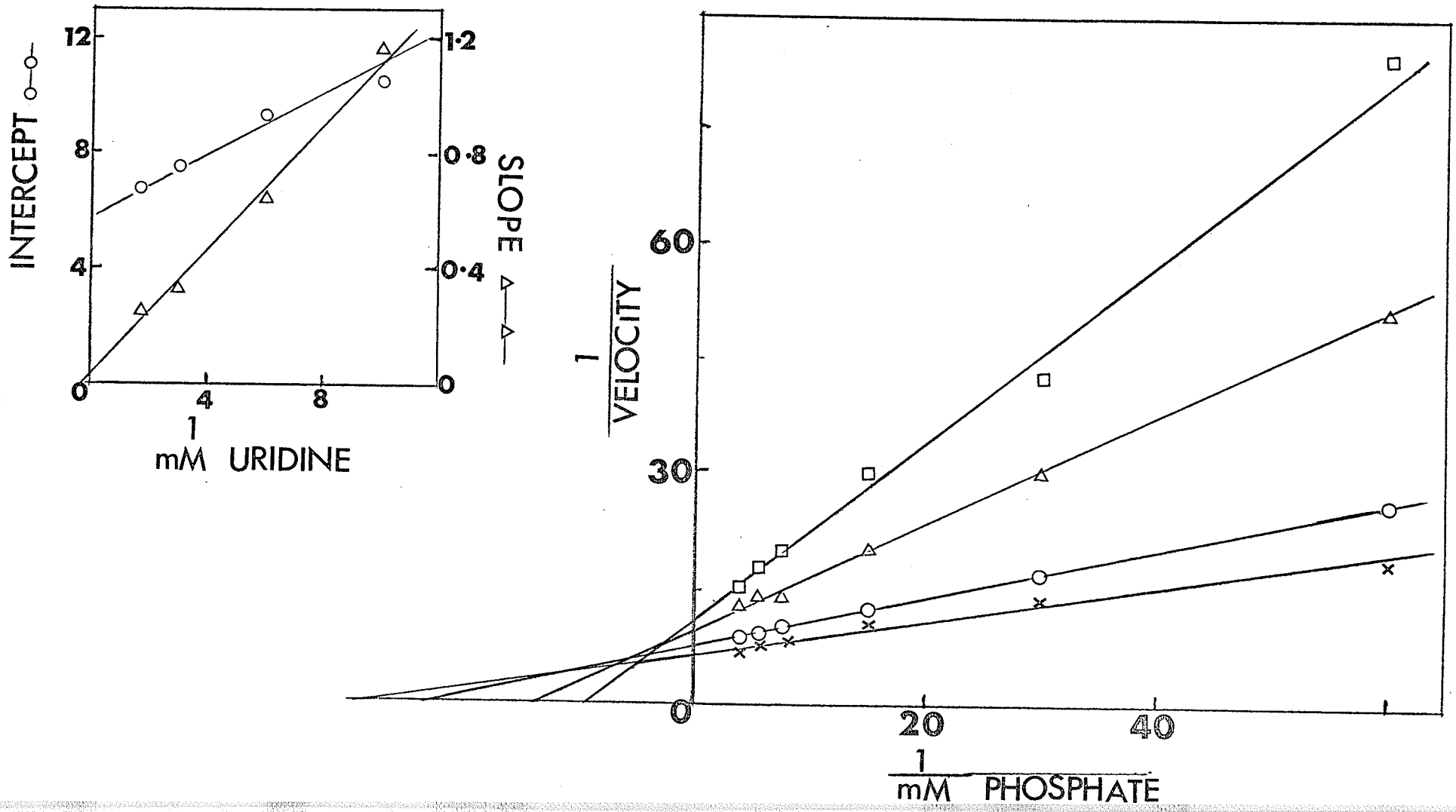

INTERCEPT
SLOPE
12
8
4
0
1·2
0·8
0·4
0
4
8
1
mM URIDINE
1
VELOCITY
60
30
0
20
40
1
mM PHOSPHATE

FIGURE 18 A and B

Determination of Kb of uridine and Ka of phosphate for isoenzyme #1 at pH 7.4.

A - The standard assay medium contained isoenzyme #1 (purified 574 fold) from step 7, (34.5 μg protein) and 0.1 M Tris-HCl buffer, pH 7.4 along with other ingredients described in the methods section. Phosphate was varied over a concentration range of 0.166 mM to 2.5 mM at different levels of uridine which were 0.1 mM (□——□——□—), 0.166 mM (△——△——△—), 0.33 mM (○—○—○→ and 0.66 mM (×——×——×).

The replot of intercept against the reciprocal of the uridine concentration gave Kb of uridine (horizontal intercept) and the V under the conditions of the experiment.

The replot of the slope against reciprocal of the uridine concentration, gave Ka/V at the vertical intercept and from this it was possible to calculate the Ka of phosphate.

B - Derivation of Ka from the replot of slopes from the double reciprocal plot of initial velocity against phosphate concentration.

FIGURE 18 B

Rate equation for ordered Bi Bi when P = o, Q = o, A = phosphate, B = uridine.

$$v = \frac{V_1 \; A \; B}{Kia \; Kb + Ka \; B + Kb \; A + A \; B}$$

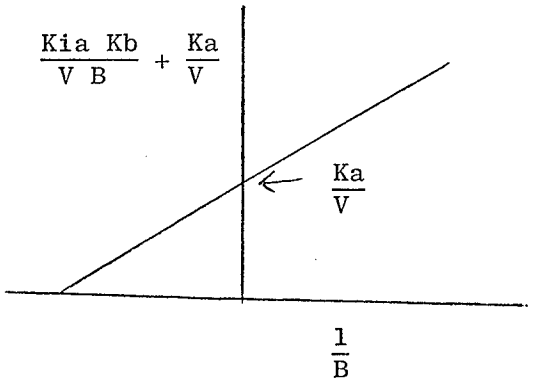
$$\frac{1}{v} = \frac{Kia \; Kb}{V \; A \; B} + \frac{Ka}{V \; A} + \frac{Kb}{B \; V} + \frac{1}{V}$$

1. Plot $\frac{1}{v}$ against $\frac{1}{A}$

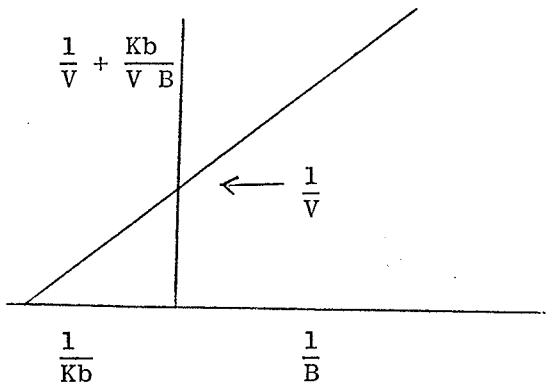
$$\frac{1}{v} = \left(\frac{Kia \; Kb}{V \; B} + \frac{Ka}{V} \right) \quad \frac{1}{A} + \left(1 + \frac{Kb}{B} \right) \frac{1}{V}$$

slope intercept

Plot slope against $\frac{1}{B}$



Plot intercept against $\frac{1}{B}$



2. Plot $\frac{1}{v}$ against $\frac{1}{B}$

$$\frac{1}{v} = \frac{1}{B} \left(\frac{Kia \; Kb}{V \; A} + \frac{Kb}{V} \right) + \left(\frac{Ka}{A} + 1 \right) \frac{1}{V}$$

slope intercept

Intercept in this case did not change significantly.

FIGURE - 19

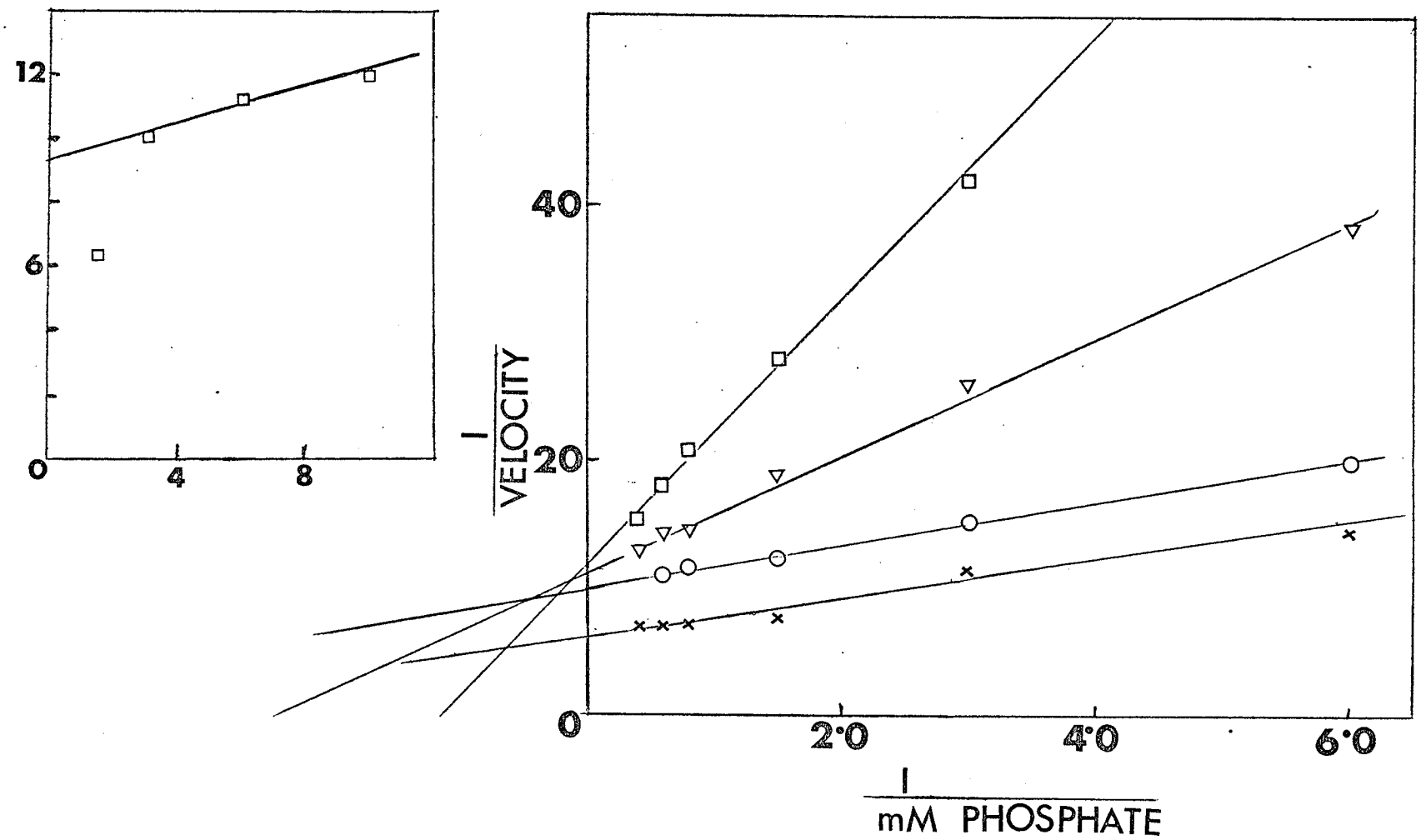


FIGURE 19

Determination of Kb of uridine for isoenzyme #2 at pH 7.4.

The standard assay medium contained isoenzyme #2 (purified 1400 fold, 6.6 μg protein) and 0.1 M tris-HCl buffer, pH 7.4 along with the other ingredients described in the methods section. Phosphate was varied in a concentration range of 0.166 mM to 2.5 mM at different levels of uridine which were 0.1 mM (□—□—□—), 0.166 mM (△—△—△—) 0.33 mM (O—O—O—) and 0.66 mM (×—×—×). The Kb value of uridine for isoenzyme #2 was calculated from a replot of intercepts against the reciprocal of uridine concentration.

FIGURE - 20 A

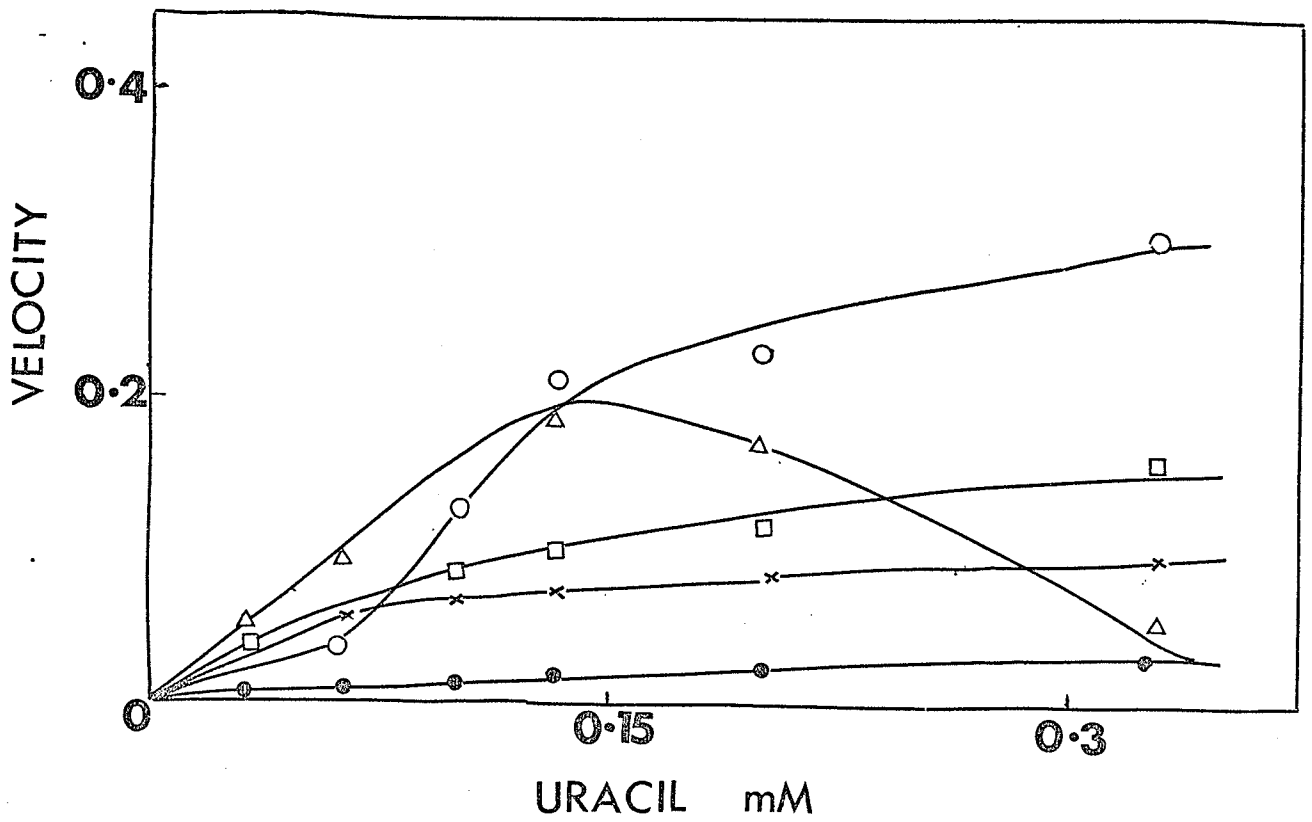


FIGURE 20 A

Initial velocity studies with variable uracil at different levels of ribose-1-P.

The standard assay medium had 574 fold pure isoenzyme #1 from step 7 (23 μg protein) in 1 M tris-HCl buffer pH 7.4 along with other ingredients described in the methods section. Uracil concentration was varied from 0.033 mM to 0.33 mM at different levels of ribose-1-P which were 0.033 mM (●——●——●—), 0.066 mM(△——△——△-), 0.133 (○—○—○→), 0.2 mM (×——×——×) and 0.4 mM (□——□——□—).

FIGURE - 20 B

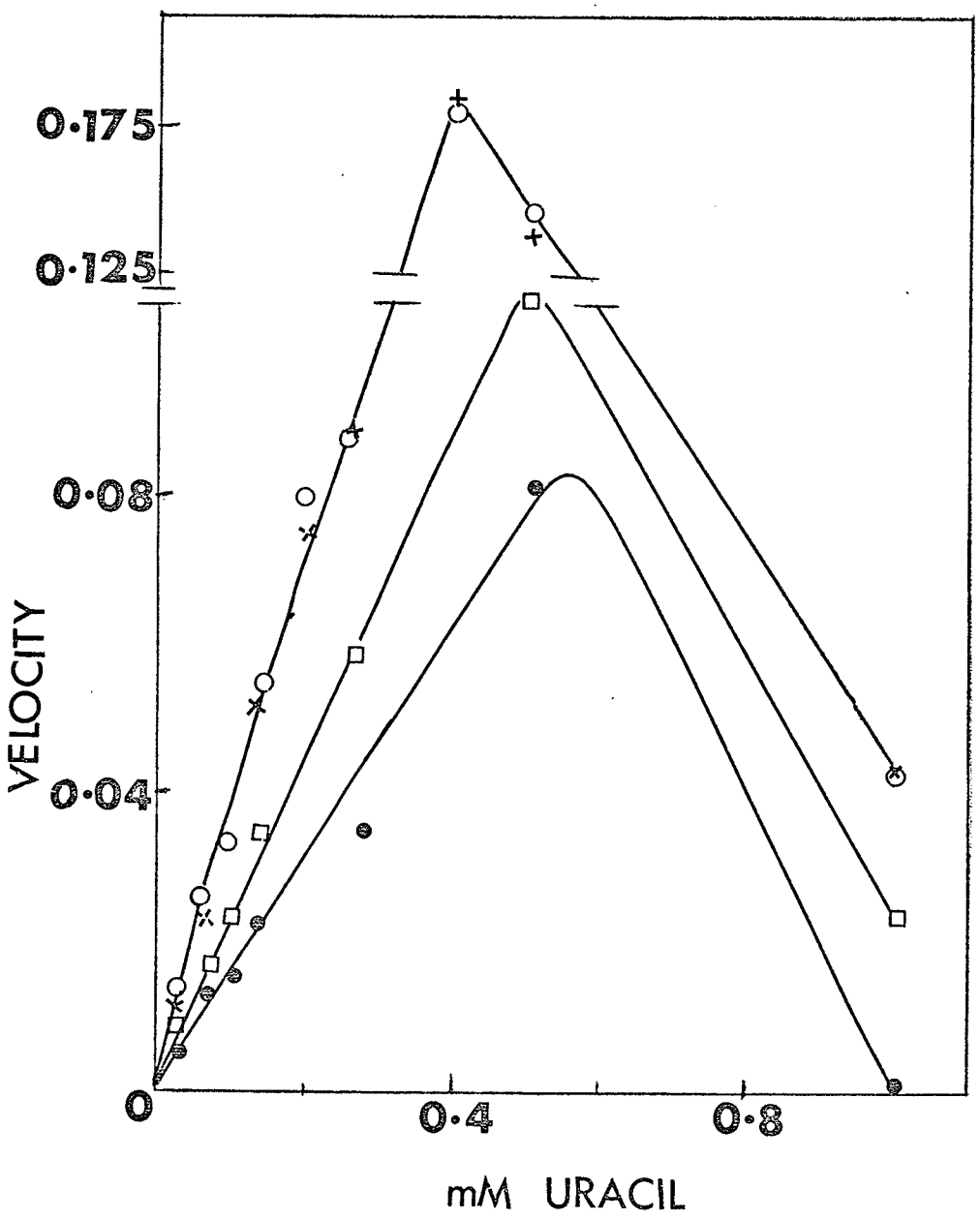


FIGURE 20 B

Initial velocity studies with variable uracil at different levels of ribose-1-P.

This experiment was similar to that described in Figure 20 A, however, the enzyme (#1) used was from preparation V that had been stored frozen for 6 weeks.

Ribose-1-P concentrations were .066 mM (●—●—●), 0.133 mM (□—□—□), 0.2 mM (×—×—×) and 0.266 mM (○—○—○).

FIGURE - 21

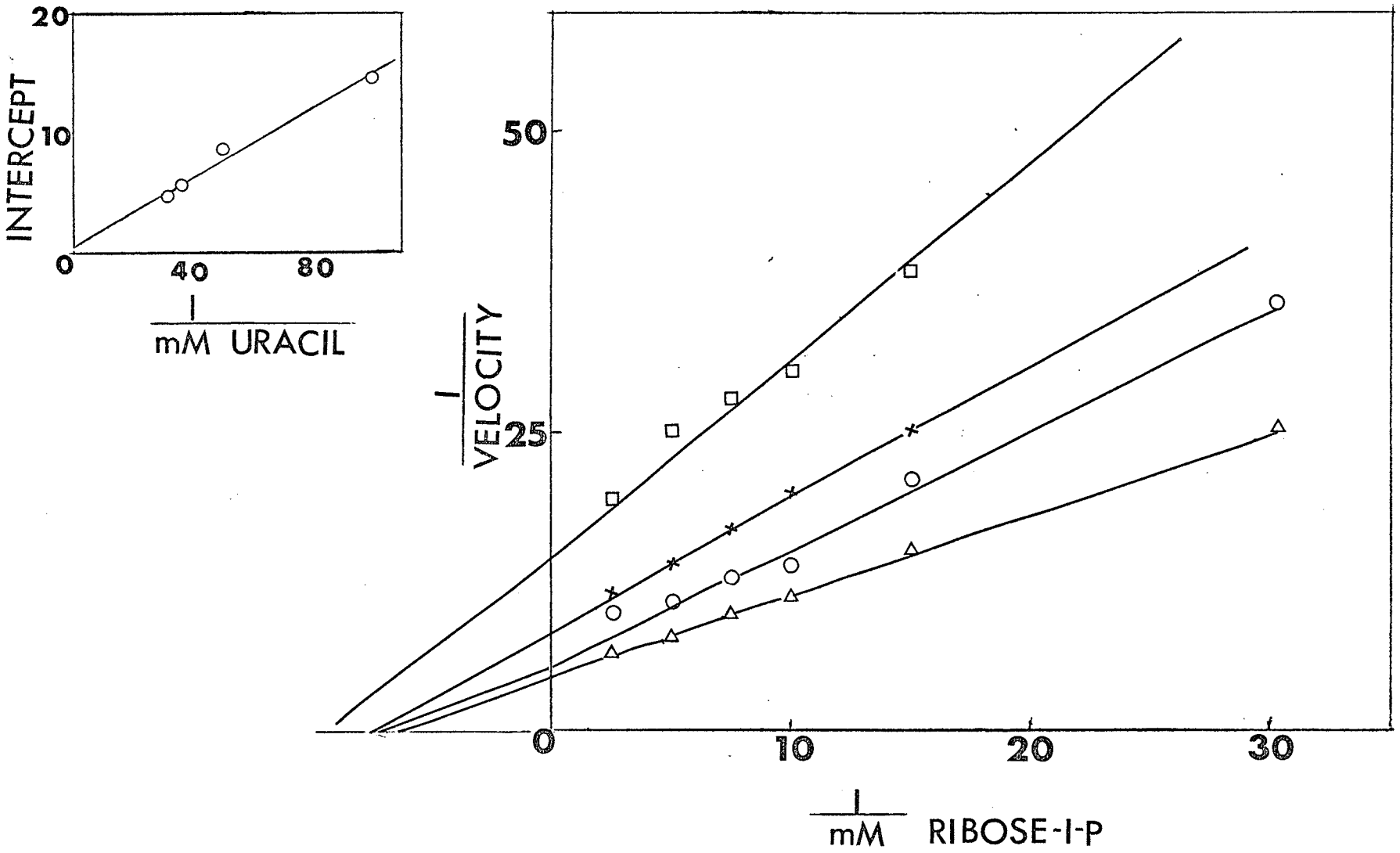

20
10
0
40
80
INTERCEPT
1
mM URACIL
50
25
1
VELOCITY
0
10
20
30
1
mM RIBOSE-1-P

FIGURE 21

Determination of Kp of uracil for isoenzyme #1 at pH 7.4.

The standard assay medium had 574 fold pure, 6 weeks old, frozen isoenzyme #1 (46 µg protein) in 0.1 M tris-HCl buffer, pH 7.4 along with other ingredients described in method section. The ribose-1-P concentration was varied over the range of .033 mM to 0.4 mM at different levels of uracil which were 0.01 mM (□——□——□—), 0.02 mM (×——×——×), 0.026 mM (○——○——○) and 0.033 mM(△——△——△—). Kp of uracil was calculated from the replot of intercepts against uracil concentration.

FIGURE - 22

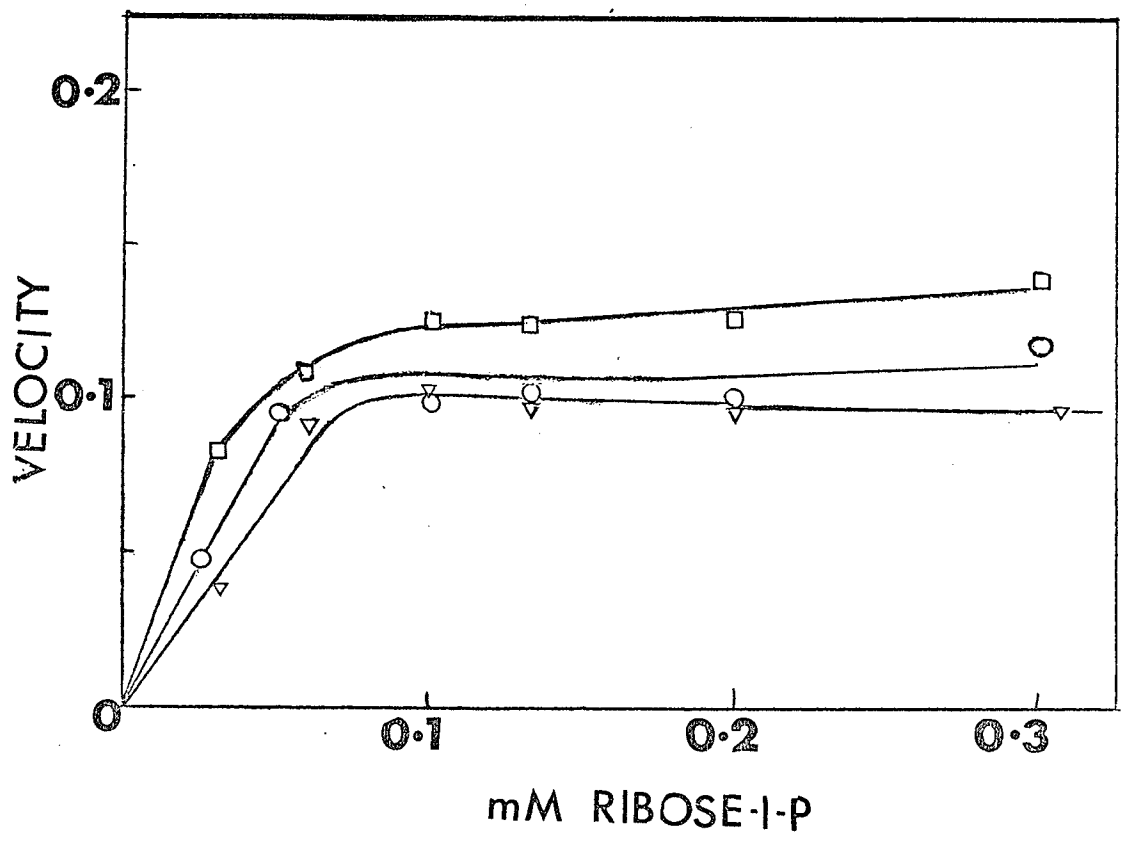
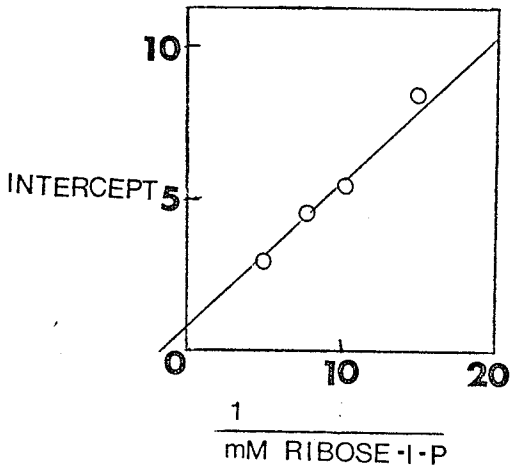


FIGURE 22

Plot of initial velocity against ribose-1-P concentration at different levels of uracil with isoenzyme #2.

The standard assay medium contained isoenzyme #2 purified 1400 fold from step 6 (3.3 μg protein) in 0.1 M tris-HCl buffer along with other ingredients described in the methods section. Ribose-1-P concentration was varied in the range of 0.033 mM to 0.5 mM at different levels of uracil which were 0.1 mM (△——△——△—), 0.2 mM (□—□—□—) and 0.166 mM (O—O—O-).

FIGURE -23


10
INTERCEPT
5
0
10
20
1
mM RIBOSE-1-P

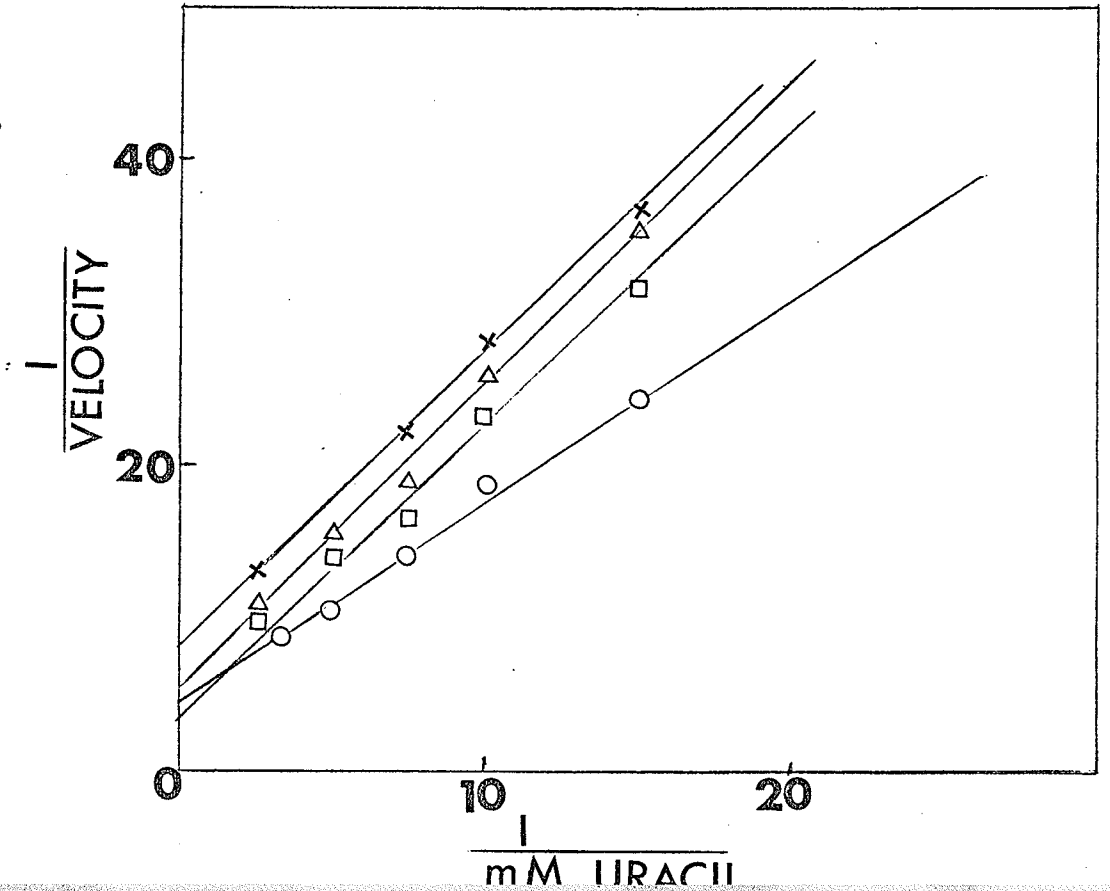

40
1
VELOCITY
20
0
10
20
1
mM URACIL

FIGURE 23

Determination of Kq of ribose-1-P for isoenzyme #2.

The standard assay medium contained isoenzyme #2 purified 1440 fold, (4.4 μg of protein) in 0.1 M tris-HCl buffer pH 7.4 along with other ingredients described in the methods section. Uracil was varied from 0.033 mM to 0.33 mM at different levels of phosphate which were 0.066 mM (×——×——×), 0.1 mM (△——△——△), 0.2 mM (□——□——□) and 0.133mM (O——O——O).

Kq of ribose-1-P was calculated from a replot of intercepts against ribose-1-P concentration.

FIGURE - 24

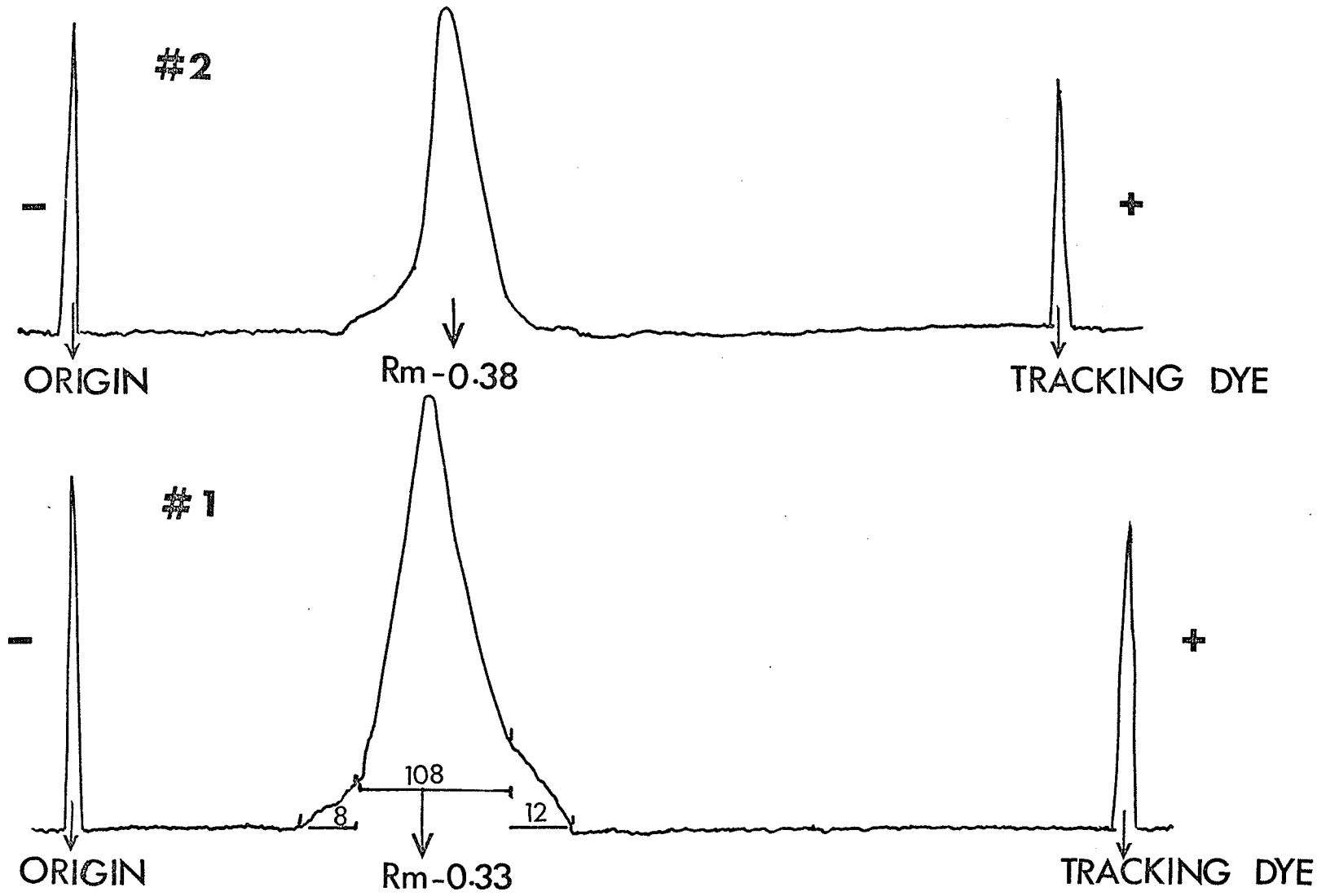

#2
-
+
ORIGIN
Rm-0.38
TRACKING DYE
#1
-
+
108
8
12
ORIGIN
Rm-0.33
TRACKING DYE

FIGURE 24

Densitometric tracings of protein bands after disc gel electrophoresis at pH 8.3 in 7% gel.

Electrophoresis was performed according to the procedure described in methods. For isoenzyme #1 the sample contained 15.6 μg protein from step 7 preparation V in 60 μl of 4% sucrose + 0.04 M K Pi buffer, pH 7.0 + (diluted 20 fold) tracking dye concentrate isoenzyme #2. For isoenzyme #2 the sample contained 21.1 μg protein from step 7 preparation V in 50 μl along with other ingredients described for isoenzyme #1.

Proteins on the gel were fixed in 12.5% Trichloracetic acid and stained with 0.005% solution of coomassie blue.

The junction between the spacer and separation gel was marked by a piece of wire after the gel was placed in the holder for densitometric tracings in a Joyce Loebl Chromoscan with red filter and cam C.

Relative mobility (Rm) is the ratio of the distance of the peak from the origin to the distance of the distance of the tracking dye front. The area under the peak was determined by an integrator and is given by the number on the horizontal line under each peak.

FIGURE 25

Detection of enzyme activity after disc gel electrophoresis with 7% gel at pH 8.3 or pH 7.0.

The samples applied were the same as those described for isoenzyme #1 in Figure 24. The gels were stained for enzyme activity as described in the methods section.

a - uridine as substrate

b - deoxyuridine as substrate

Electrophoresis at pH 8.3

c - uridine as substrate

d - deoxyuridine as substrate

Electrophoresis at pH 7.0

FIGURE - 25

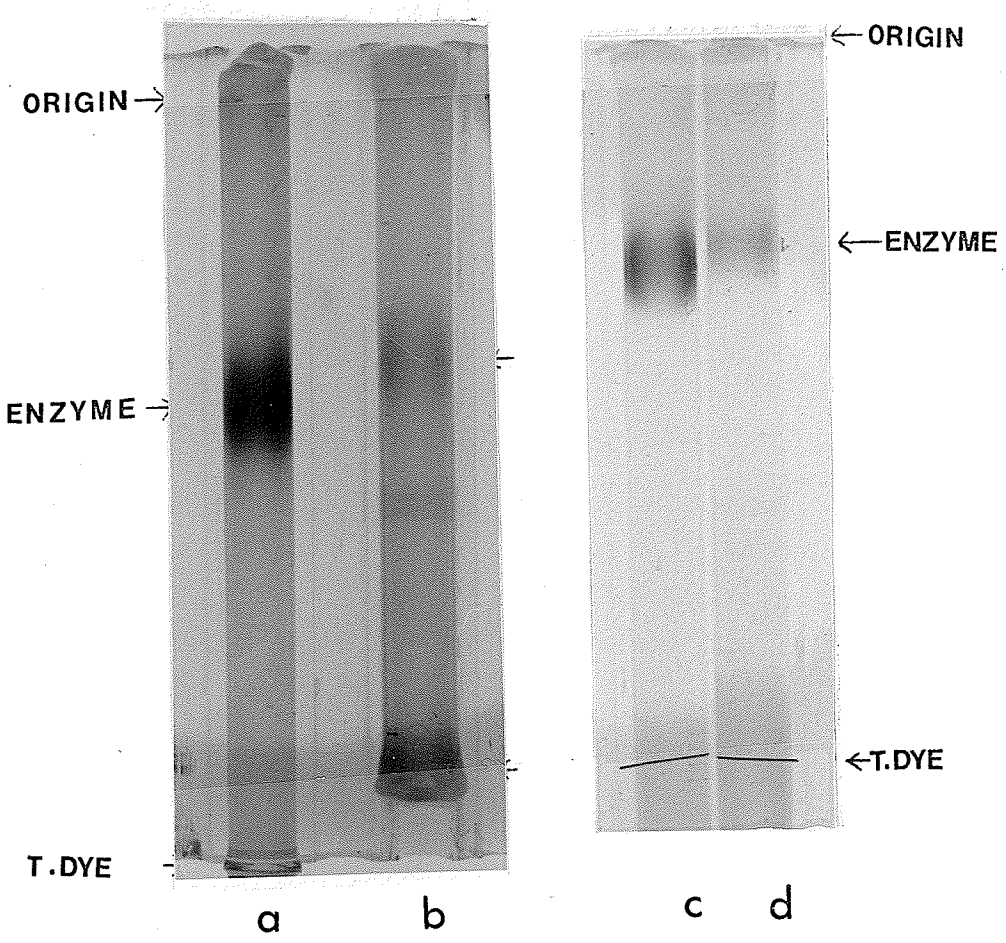


FIGURE 26

Detection of protein bands of aged enzyme preparations after disc gel electrophoresis at pH 8.3 in 7% gel.

The procedure was similar to that described in Figure 24.

Isoenzyme #1 - Samples contained 26.45 μg of protein from step 7, preparation V, which had aged at -40°C (a) 0.05 M K phosphate buffer pH 7.0 for 7 weeks or in (b) 0.05 M Tris-HCl buffer pH 7.0 for 8 weeks.

Isoenzyme #2 - Samples contained 48 μg of protein from step 7, preparation V which had aged at -40°C (c) 0.05 M K phosphate buffer pH 7.0 for 7 weeks or in (d) 0.05 M Tris-HCl buffer pH 7.0 for 8 weeks.

The protein bands on the gels were fixed in 12.5%. Trichloracetic acid and stained with 0.005% coomassie blue.

A - Fresh isoenzyme #1.

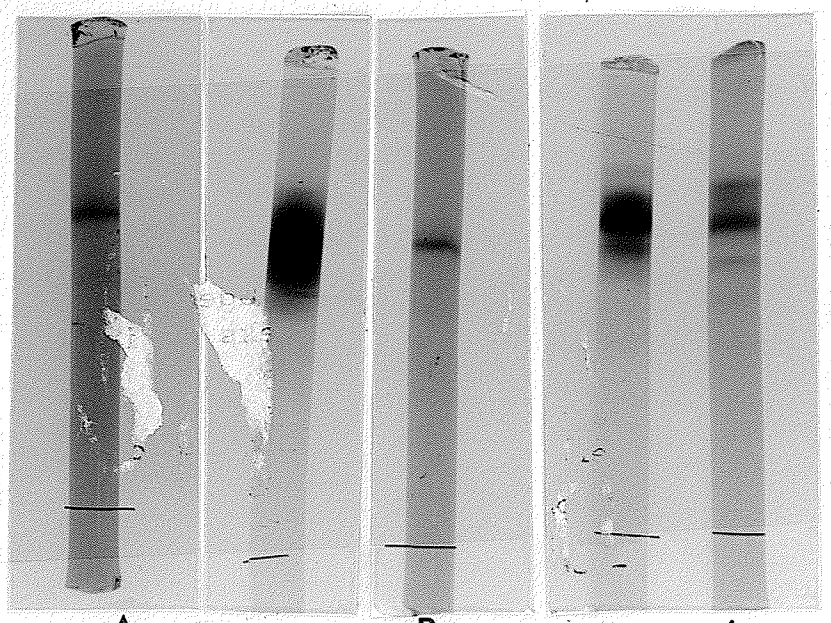
B - Fresh isoenzyme #2.

T - Fresh isoenzyme #1 in Tris buffer, 10 % gel.

b' and d' - Electrophoresis at pH 7.0.

c' - #2 enzyme 1 week old.

FIGURE - 26



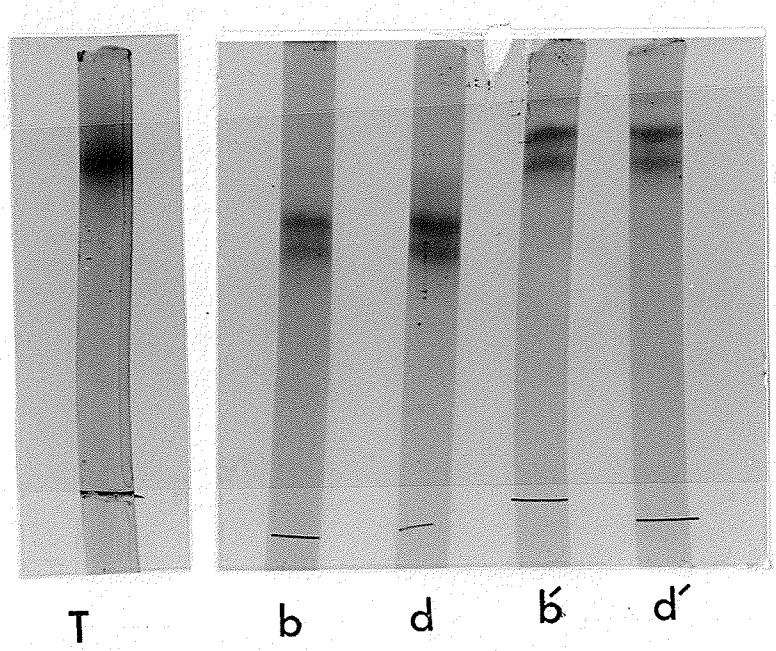


FIGURE 27

Densitometer tracings of isoenzyme #1 aged in 0.05 M Tris-HCl pH 7.0.

Isoenzyme #1 fraction from step 7, preparation V was aged in 0.05 M Tris-HCl buffer pH 7.0 at -40°C for 8 weeks when all the activity was lost. A portion of this enzyme was reactivated at room temperature with 0.075 M 2-mercaptoethanol in 0.05 M K Pi (pH 7.0) for 90 min. Samples contained 115 µg protein of reactivated enzyme, when the gels were stained for activity with uridine as substrate or 46 µg of protein of the aged enzyme when stained with coomassie blue for the protein bands.

Electrophoresis was performed at pH 8.3 with 7% gel as described for Figure 24. The blue filter and Cam C were used for obtaining tracings of gels stained for activity while the red filter and Cam C were used for gels stained with coomassie blue for proteins.

(i) Stained for activity.

(ii) Stained for proteins.

FIGURE - 27

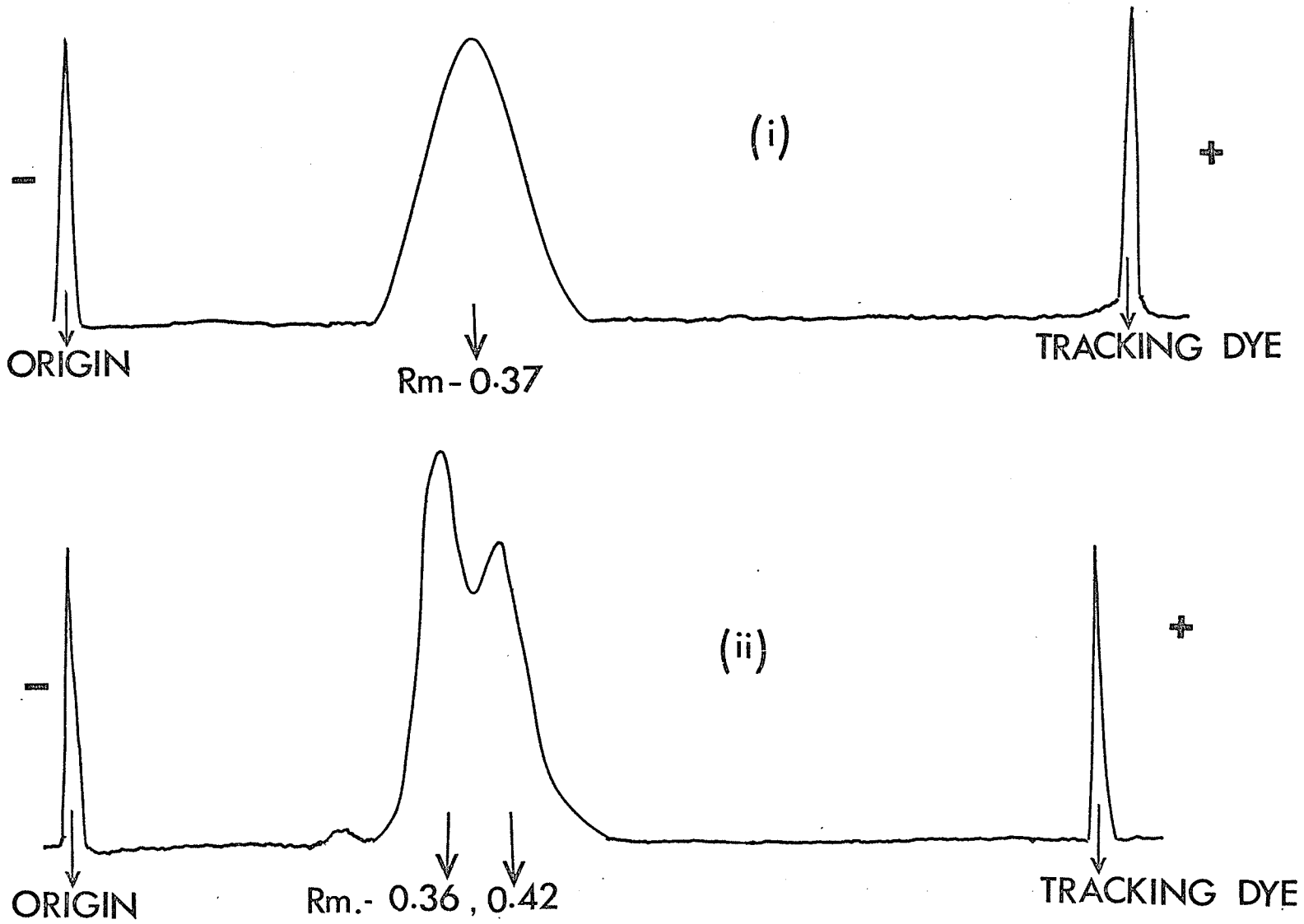

(i)
-
+
ORIGIN
Rm - 0·37
TRACKING DYE
(ii)
-
+
ORIGIN
Rm.- 0.36 , 0.42
TRACKING DYE

FIGURE 28

Densitometer tracings of isoenzyme #1 aged in 0.05 M K phosphate buffer.

Isoenzyme #1 fraction from step 7 preparation V was aged for 7 weeks at -40°C in 0.005 M K phosphate buffer pH 7.0. Samples contained 46 μg protein of this aged preparation per gel.

Electrophoresis was performed with 7% gel at pH 8.3, as described for Figure 24.

Uridine was the substrate when the gels were stained for activity and coomassie blue was used for staining of protein bands.

Filters and Cams were as described in Figure 27.

FIGURE - 28

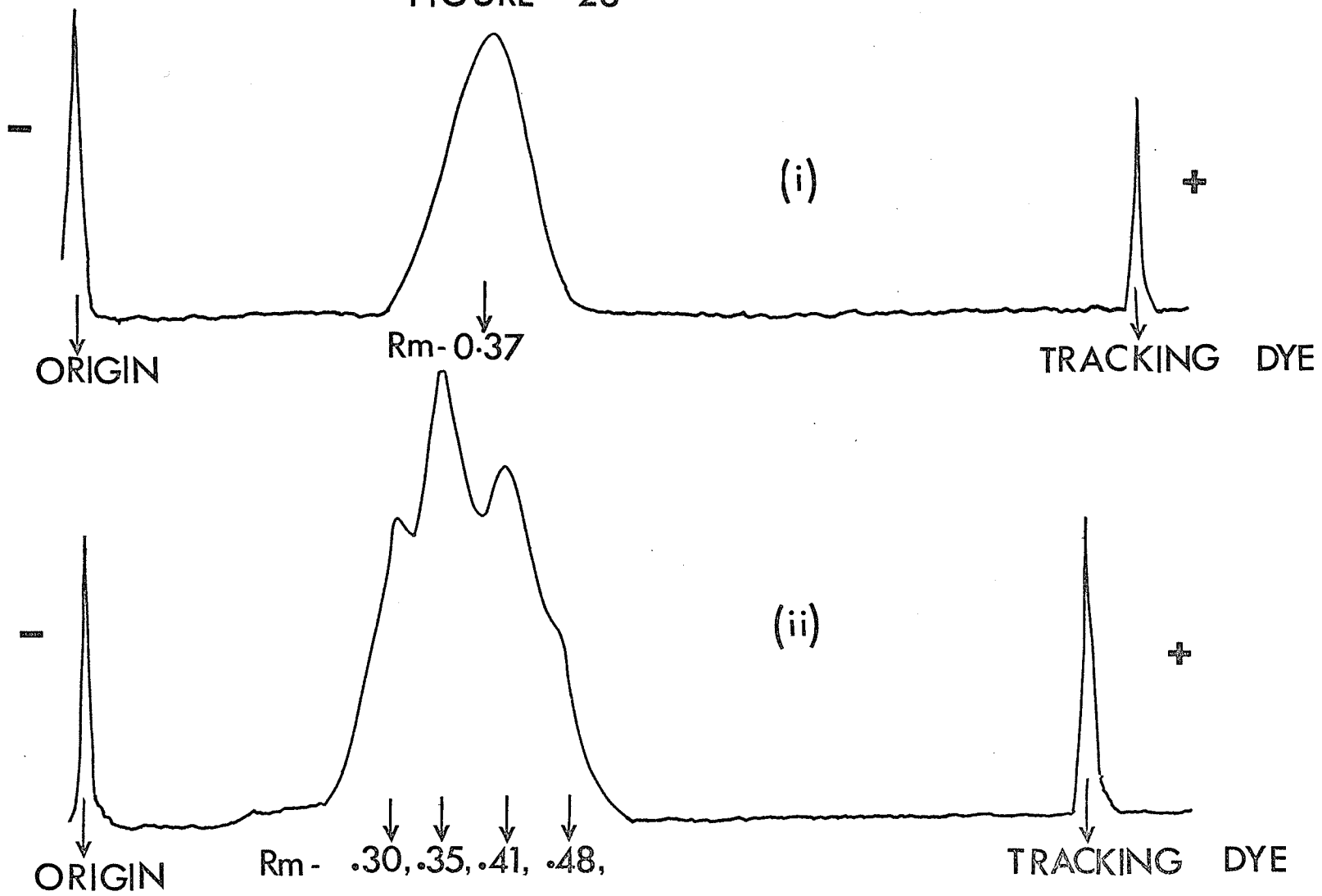

(i)
ORIGIN
Rm- 0·37
TRACKING DYE
(ii)
ORIGIN
Rm - ·30, ·35, ·41, ·48,
TRACKING DYE

FIGURE 29

Densitometer tracings of isoenzyme #2 aged in 0.05 M K phosphate buffer pH 7.0.

Isoenzyme #2 fraction from step 7 preparation V was aged at -40°C for 1 and 7 weeks. Samples contained 48 µg protein from this fraction. Electrophoresis was performed at pH 8.0 with 7% gel as described for Figure 24.

(i) stained for activity with uridine as substrate, enzyme aged for 7 weeks.

(ii) stained for protein with coomassie blue, enzyme aged for 7 weeks.

(iii) stained for protein with coomassie blue, enzyme aged for 1 week.

FIGURE -29

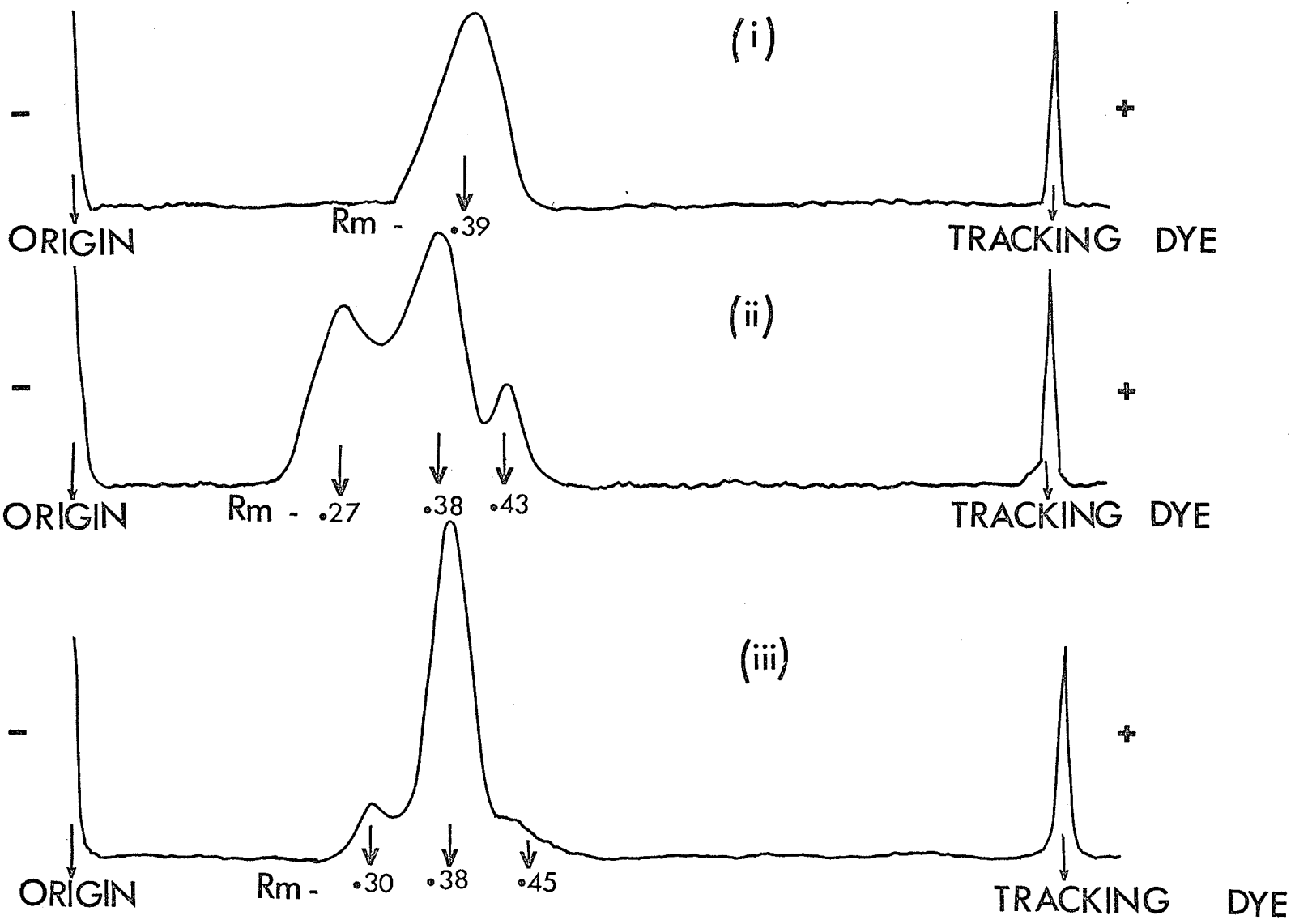

(i)
-
+
ORIGIN
Rm - •39
TRACKING DYE
(ii)
-
+
ORIGIN
Rm - •27
•38
•43
TRACKING DYE
(iii)
-
+
ORIGIN
Rm - •30
•38
•45
TRACKING DYE

FIGURE 30

Effect of gel concentration on relative mobilities (Rm) of uridine phosphorylase isoenzymes and thymidine phosphorylase.

Electrophoresis was performed with different acrylamide concentrations at pH 8.3.

Samples applied to the gels were either 220 µg protein of isoenzyme #1 from step 4, or 23.6 µg protein of isoenzyme #2 from step 4 or 276 µg protein of thymidine phosphorylase from step 4.

All gels were stained for activity with either uridine as substrate for isoenzymes #1 and #2 or thymidine as substrate for thymidine phosphorylase.

The slope of the plot for each enzyme fraction shows the relationship between log Rm and gel concentration.

FIGURE- 30

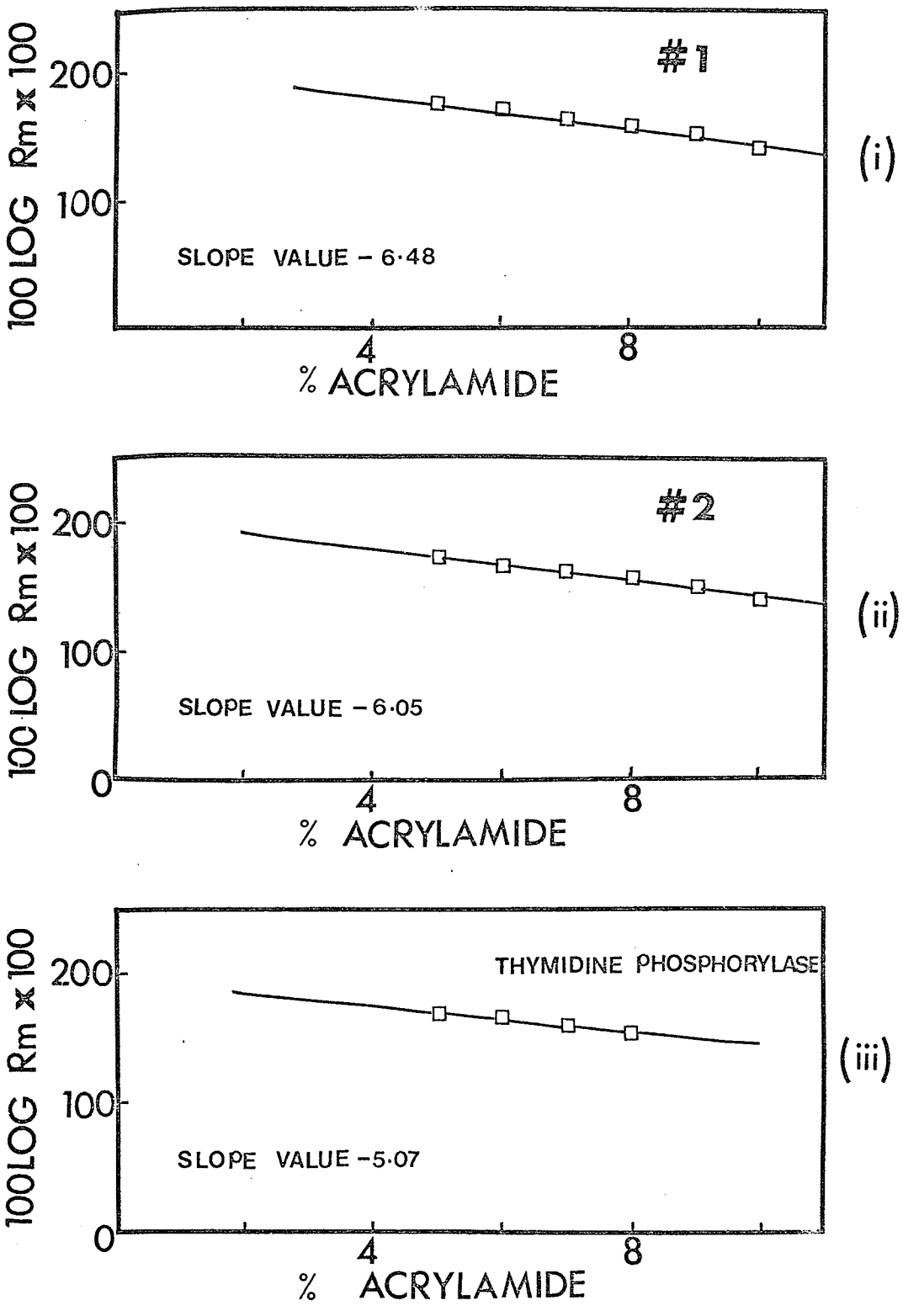

100 LOG Rm x 100
200
100
#1
SLOPE VALUE – 6·48
4
8
% ACRYLAMIDE
(i)
100 LOG Rm x 100
200
100
0
#2
SLOPE VALUE – 6·05
4
8
% ACRYLAMIDE
(ii)
100LOG Rm x 100
200
100
0
THYMIDINE PHOSPHORYLASE
SLOPE VALUE –5·07
4
8
% ACRYLAMIDE
(iii)

FIGURE 31

Relationship between the slope values (for log Rm of marker proteins at different gel concentrations) and molecular weight.

The electrophoresis was performed at pH 8.3 at different gel concentrations for each standard molecular weight marker. In these experiments either 43 μg of apoferritin, 50 μg of catalase, 43 μg of gamaglobulin, 71 μg of bovine serum albumin or 5 μg of alcohol dehydrogenase in approximately 8% sucrose and tracking dye concentrate diluted 20 fold were used.

The slope values were determined as in Figure 30 after staining the gels for protein with coomassie blue in all cases except for alcohol dehydrogenase when the enzyme band was stained for enzyme activity with tetrazolium blue.

The standard curve was obtained by plotting the slope values of marker proteins against their molecular weights.

FIGURE - 31

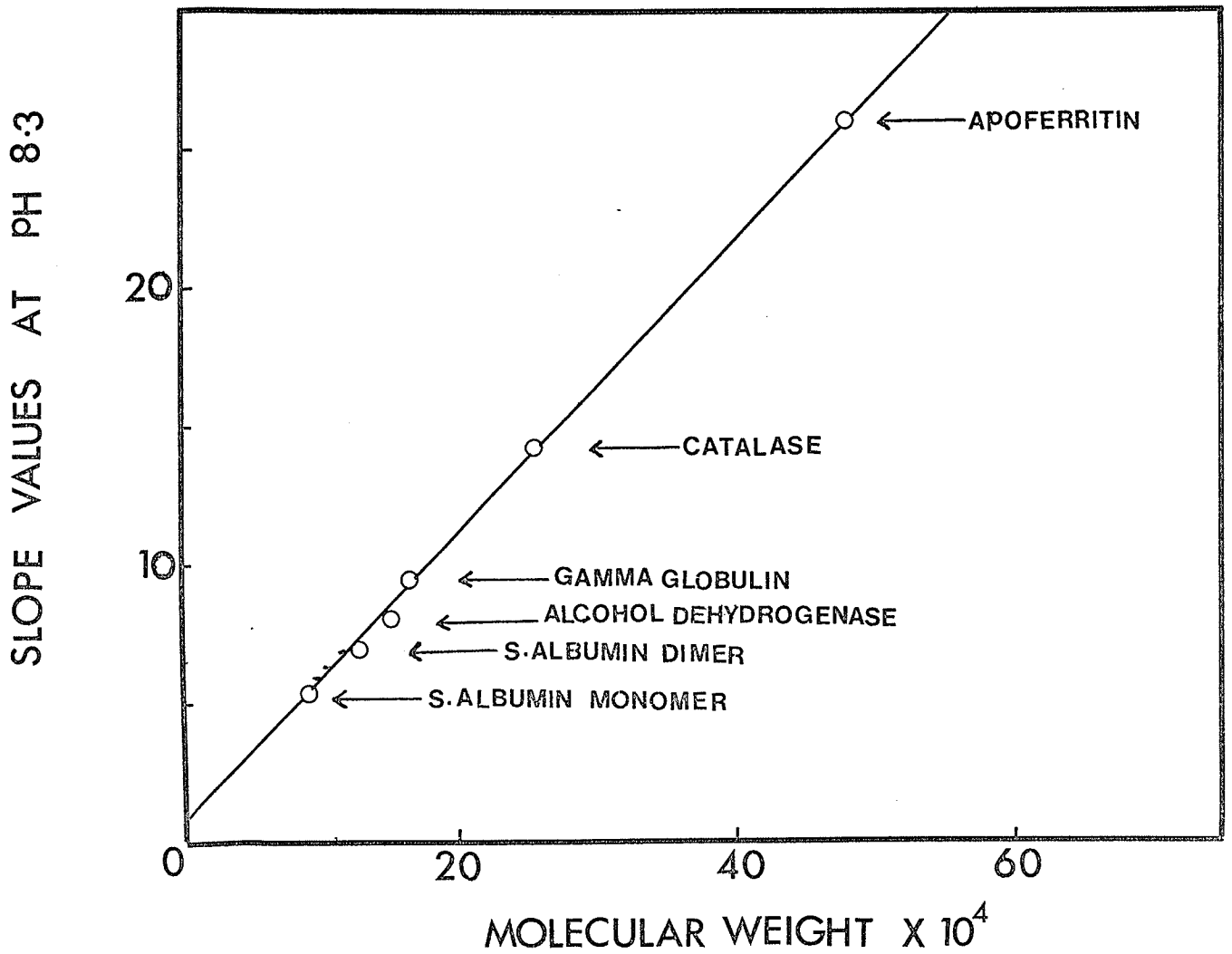
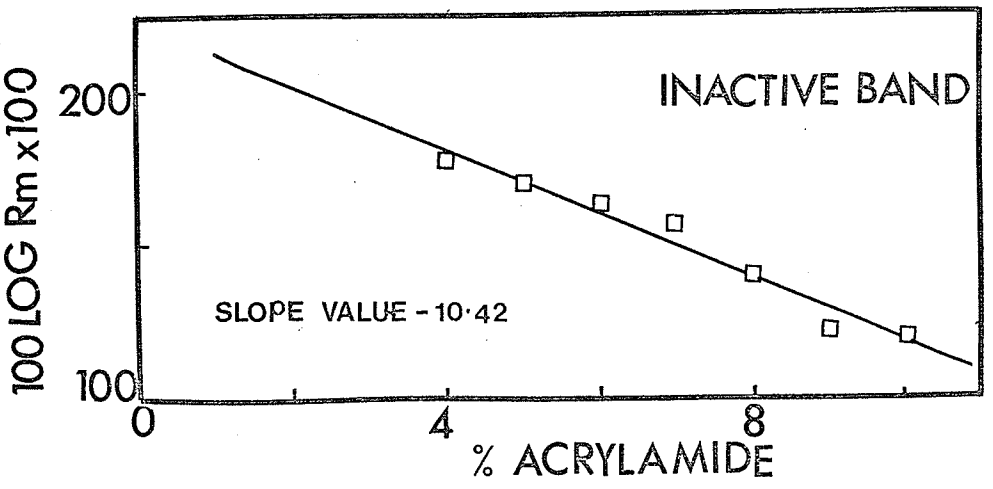

SLOPE VALUES AT PH 8.3
20
10
0
20
40
60
MOLECULAR WEIGHT X 10^4
APOFERRITIN
CATALASE
GAMMA GLOBULIN
ALCOHOL DEHYDROGENASE
S.ALBUMIN DIMER
S.ALBUMIN MONOMER

FIGURE 32

Determination of molecular weight of protein bands obtained on aging the isoenzyme in 0.05 M Tris-HCl buffer at pH 7.0.

Electrophoresis was performed at pH 8.3 with different gel concentrations using 26.45 μg protein of isoenzyme #1 fraction from step 7, preparation V, aged in 0.05 M Tris-HCl buffer at pH 7.0 for 8 weeks at -40^{o}C. The protein bands were stained with coomassie blue and their molecular weights were calculated from the standard plot (Figure 31).

FIGURE -32


INACTIVE BAND
100 LOG Rm x100
200
100
SLOPE VALUE - 10·42
0
4
8
% ACRYLAMIDE

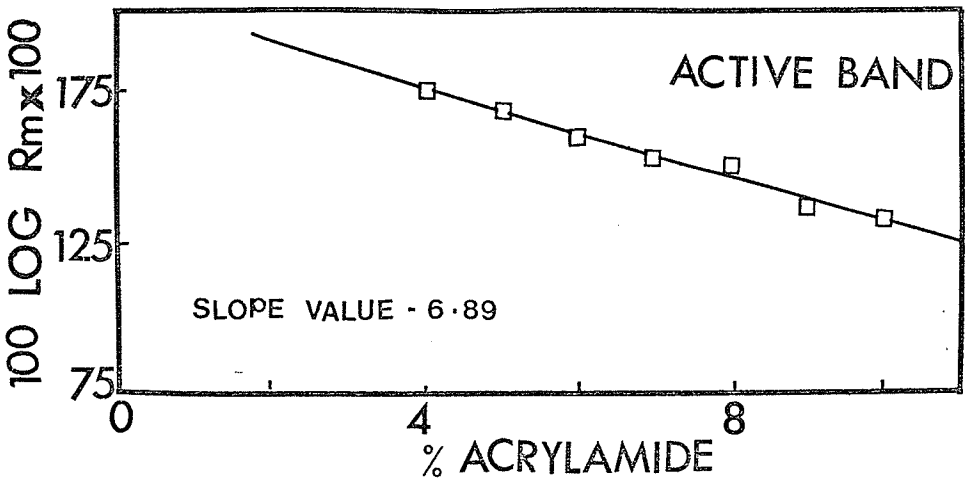

ACTIVE BAND
100 LOG Rm x100
175
125
75
SLOPE VALUE - 6·89
0
4
8
% ACRYLAMIDE

FIGURE 33

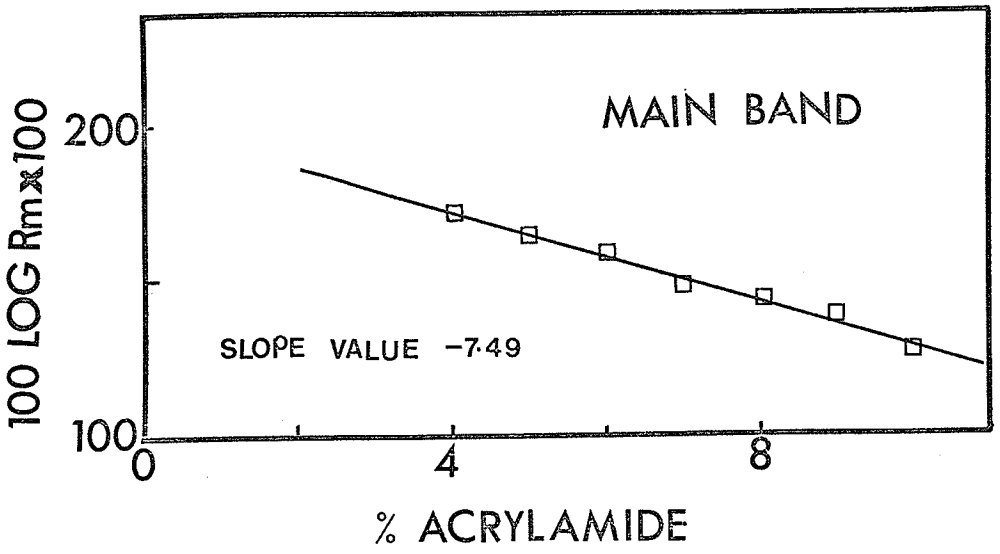
Determination of molecular weights of protein bands obtained on aging in 0.05 M K phosphate buffer at pH 7.0.

Electrophoresis was performed at pH 8.3 with different gel concentrations using 26.45 μg protein of isoenzyme #1 fraction from step 7, preparation V, aged in 0.05 M K phosphate buffer at pH 7.0 for 7 weeks at -40°C. The protein bands were stained with coomassie blue and their molecular weights determined from the standard plot (Figure 31).

FIGURE -33

ISOENZYME #1AGED IN

PHOSPHATE BUFFER PH 7



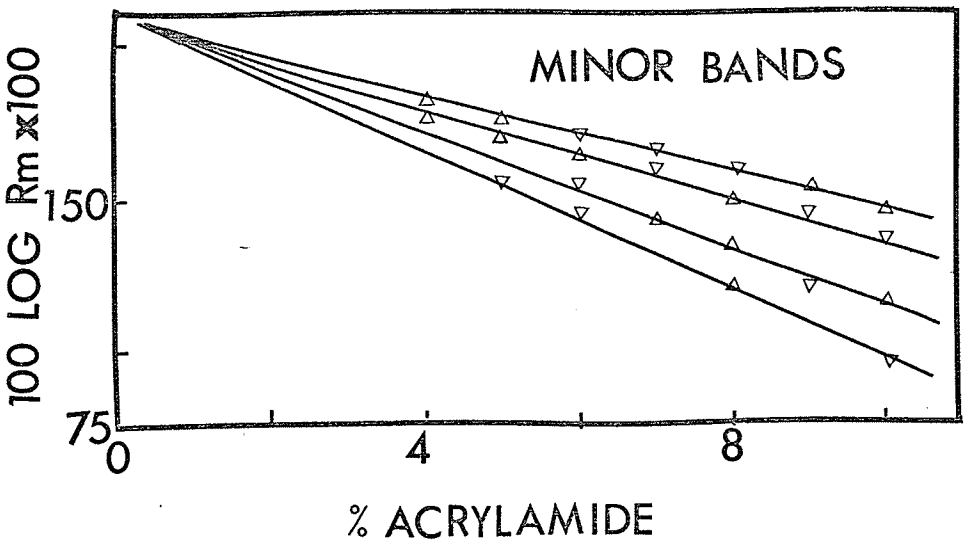


FIGURE 34

Appearance of active aggregates of uridine phosphorylase stored in 0.05 M K phosphate pH 7.0 at 0°C for 1 week.

Electrophoresis was performed at pH 8.3 with 4 to 8% gel. The samples contained 229 µg of protein from step 5, preparation III and contained both isoenzymes 1 and 2 stored in 0.05 M K phosphate buffer pH 7.0 at a protein concentration of 5.28 mg/ml at 0°C. The gels were stained for enzyme activity with uridine as substrate.

FIGURE -34

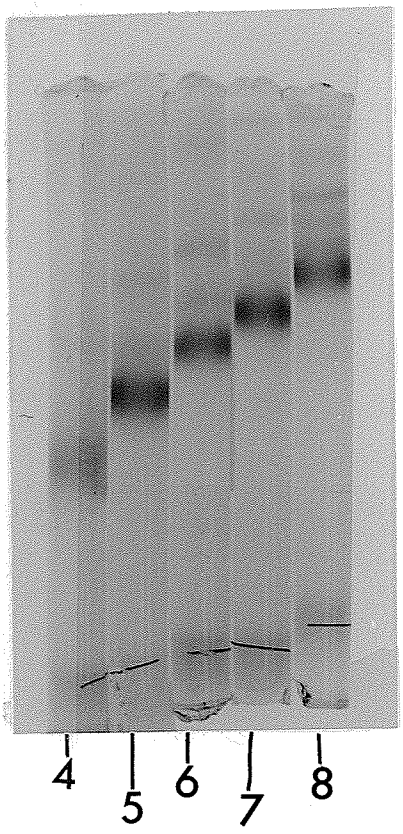


FIGURE 35

SDS gel electrophoresis and the molecular weights of subunits.

Electrophoresis was performed at room temperature with 10% acrylamide gel at pH 7.2 containing 0.1% SDS.

Sample treatment - 215 μl of each molecular weight standards (1 mg protein/ml) was treated with 25 μl of 10% SDS + 5 μl 2-mercaptoethanol (or H_2O) and 5 μl of 1 M Na Pi buffer pH 7.2 in a total volume of 250 μl. The mixture was incubated for 4 hours at 37^{o}C with mild shaking. Following incubation 25 μl of the above mixture + 1 drop of glycerol + 5 μl of 2-mercaptoethanol + 50 μl of 0.05 M Na Pi buffer pH 7.2 with 0.1% SDS was applied to gel columns.

At the same time, 215 μl of isoenzyme #1 (2.3 mg protein/ml) or 215 μl of isoenzyme #2 (4.8 mg/ml) were treated identically and similar aliquots were applied to gels in duplicate. Another set of isoenzyme #1 and #2 was treated identically except that 2-mercaptoethanol was omitted from the incubation mixture and also from the samples applied.

The gels were fixed in 12.5% trichloracetic acid overnight and then stained with coomassie blue.

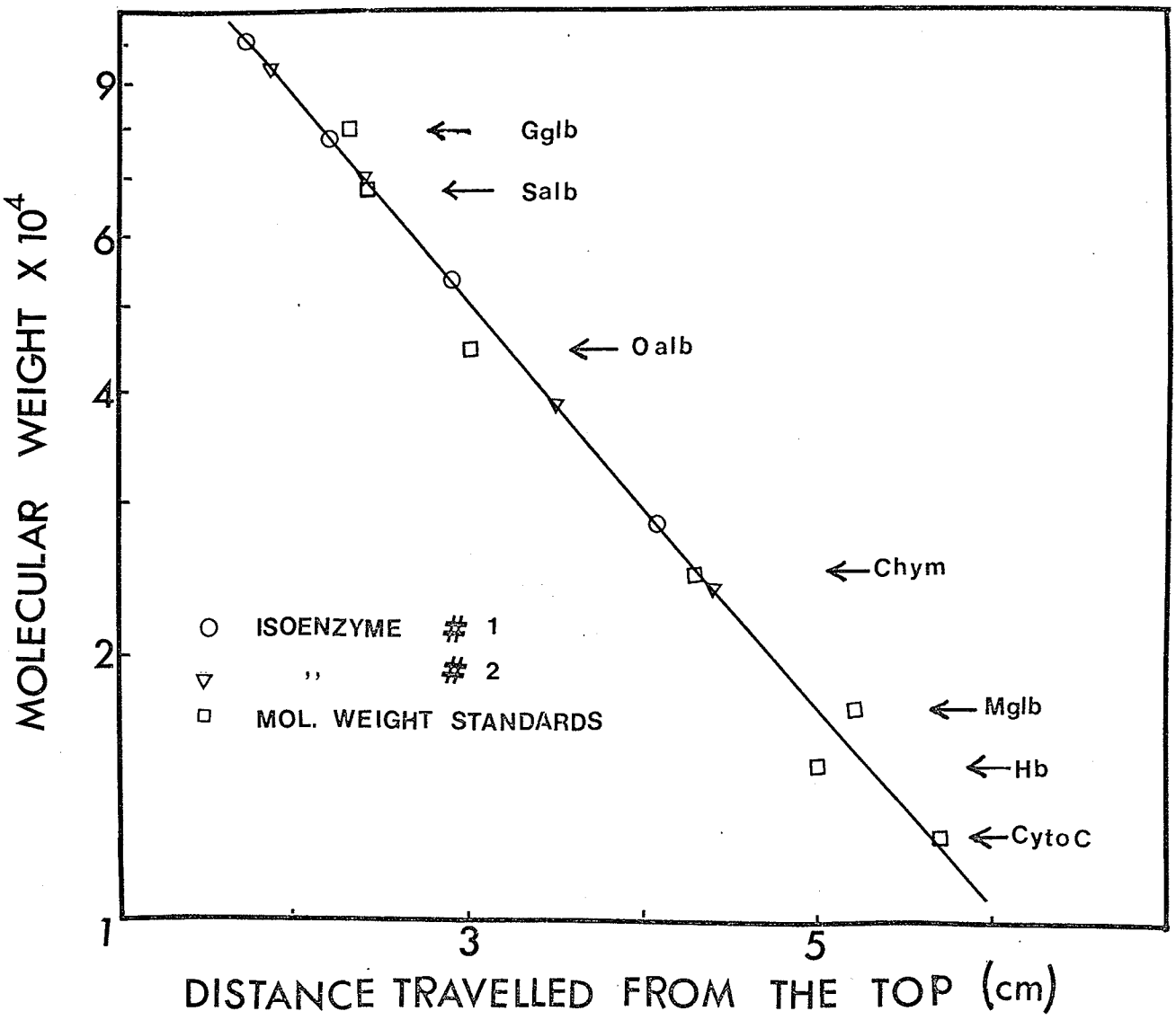
Gglb, gammaglobulin; Salb, serum albumin; Oalb, ovalbumin; Chym, chymotrypsinogan; Mglb, myoglobin; Hb, haemoglobin; Cyto C, cytochrome C.

◀-Incomplete destaining,

O-Mercaptoethanol absent,

M- " present.

FIGURE - 35



4

O M

O M

#1 #2

FIGURE 36

Density gradient centrifugation of isoenzyme #1 in the presence of 0.33 M KCl and 0.05 M Tris-HCl buffer pH 7.0.

The gradient was linear from 0 to 18% sucrose (in 0.33 M KCl + 0.02 M 2-mercaptoethanol + 0.05 M Tris-HCl pH 7.0 + 1 mM EDTA). Either 200 μl of enzyme in 0.05 M Tris-HCl buffer or 200 μl of standard (either 2 mg myoglobin, 2 mg Cytochrom C or 250 μg alkaline phosphatase) were layered on each 5 ml gradient. The tubes were centrifuged for 16 hours in a SW50.1 swing out bucket rotor and 16 fractions were collected by means of a drop counter.

ALPH — alkaline phosphatase → assayed for activity with p-nitrophenyl phosphate as substrate.

UP — uridine phosphorylase → assayed for activity with uridine as substrate.

MGLB — myoglobin → determined by absorbance at 450 mμ.

CYTO C — Cyto chrome C → determined by absorbance at 450 mμ.

FIGURE - 36

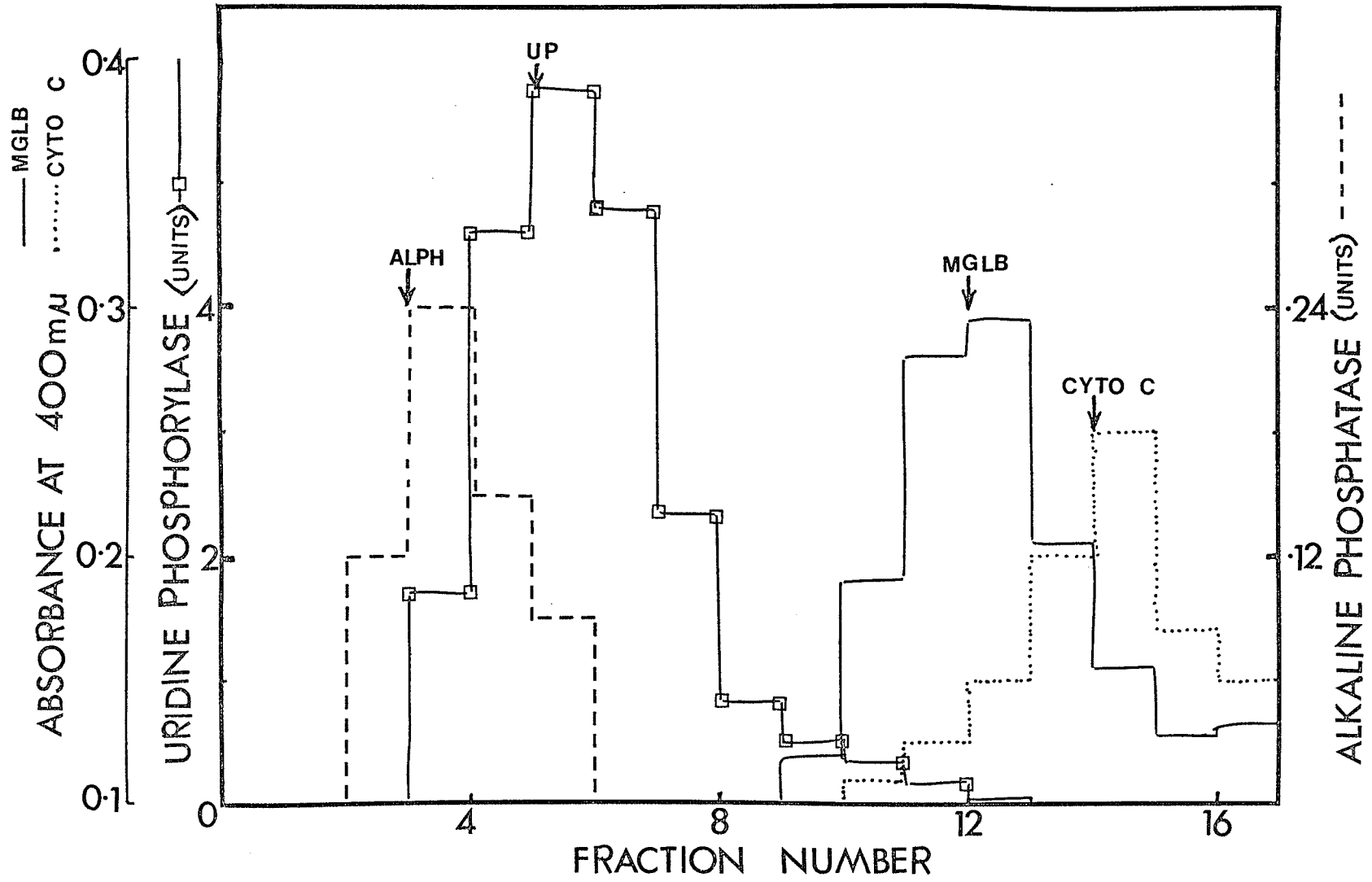

MGLB
CYTO C
ABSORBANCE AT 400mμ
0·4
0·3
0·2
0·1
URIDINE PHOSPHORYLASE (UNITS)
4
2
0
UP
ALPH
MGLB
CYTO C
4
8
12
16
FRACTION NUMBER
·24
·12
ALKALINE PHOSPHATASE (UNITS)

FIGURE 37

Density gradient centrifugation of isoenzyme #1 in the presence of 0.2 M K phosphate buffer pH 7.0.

The gradient was linear from 0 to 18% sucrose (in 0.2 M K Pi pH 7.0 + 0.02 M 2-mercaptoethanol + 0.05 M Tris-HCl, pH 7.0 + 1 mM EDTA). Either 200 μl of uridine phosphorylase in 0.05 M K Pi buffer, pH 7.0 or with 200 μl standard (either 250 μg alkaline phosphatase, or 13.4 μg alcohol dehydrogenase, or 2 mg chymotrypsinogen) were layered on each 5 ml gradient. The tubes were centrifuged for 16 hours in a SW 50.1 swingout bucket rotor following which 16 fractions were collected by means of a drop counter.

ALDE — alcohol dehydrogenase → assayed for enzyme activity with ethanol as substrate.

ALPH — alkaline phosphatase → assayed for activity with p-nitrophenyl phosphate as substrate.

UP — uridine phosphorylase → assayed for activity with uridine as substrate.

CHMO — chymotrypsinogen → determined by Warburg and Christians method (67).

FIGURE -37

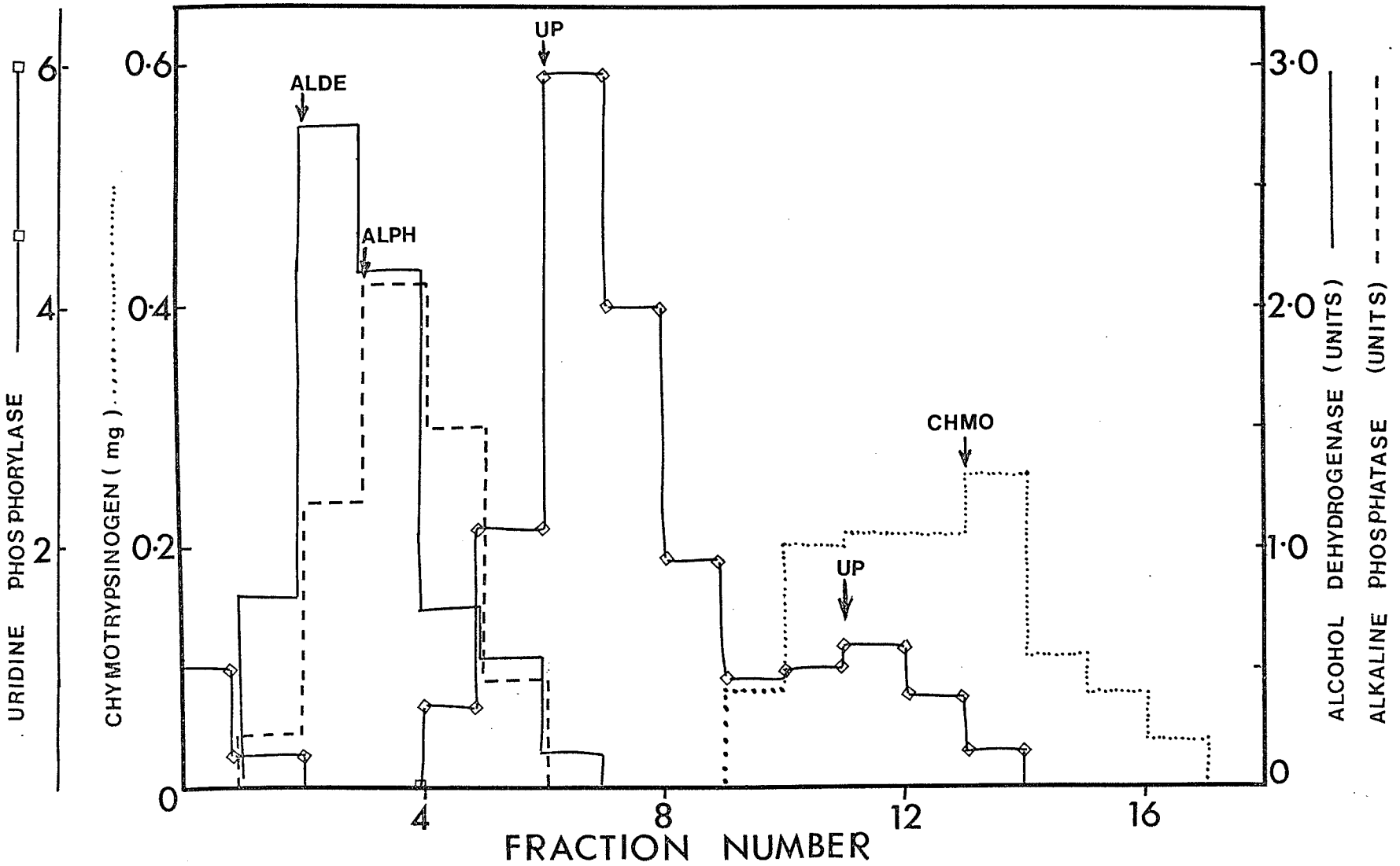

URIDINE PHOSPHORYLASE
CHYMOTRYPSINOGEN (mg)
ALCOHOL DEHYDROGENASE (UNITS)
ALKALINE PHOSPHATASE (UNITS)
ALDE
ALPH
UP
UP
CHMO
FRACTION NUMBER
0
0·2
0·4
0·6
1·0
2·0
3·0
2
4
6
4
8
12
16

FIGURE 38

Density gradient centrifugation of isoenzyme #1 in Tris-HCl buffer, pH 7.0.

The gradient was linear from 0 to 18% sucrose (in 0.05 M Tris-HCl buffer, pH 7.0 + 0.02 M 2-mercaptoethanol + 1 mM EDTA). Either 200 μl of uridine phosphorylase in 0.05 M Tris-HCl buffer, pH 7.0 or 200 μl of standard protein (either 250 μg alkaline phosphatase or 13.4 μg alcohol dehydrogenase or 1 mg of haemoglobin) were layered on each 5 ml gradient. The tubes were centrifuged for 16 hours in a SW 50.1 swingout bucket rotor following which 16 fractions were collected by means of a drop counter.

ALPH — alkaline phosphatase ⟶ assayed for activity with p-nitrophenyl phosphate as substrate.

UP — uridine phosphorylase ⟶ assayed for activity with uridine as substrate.

Hb — Haemoglobin ⟶ determined by absorbance at 400 mμ.

FIGURE -38

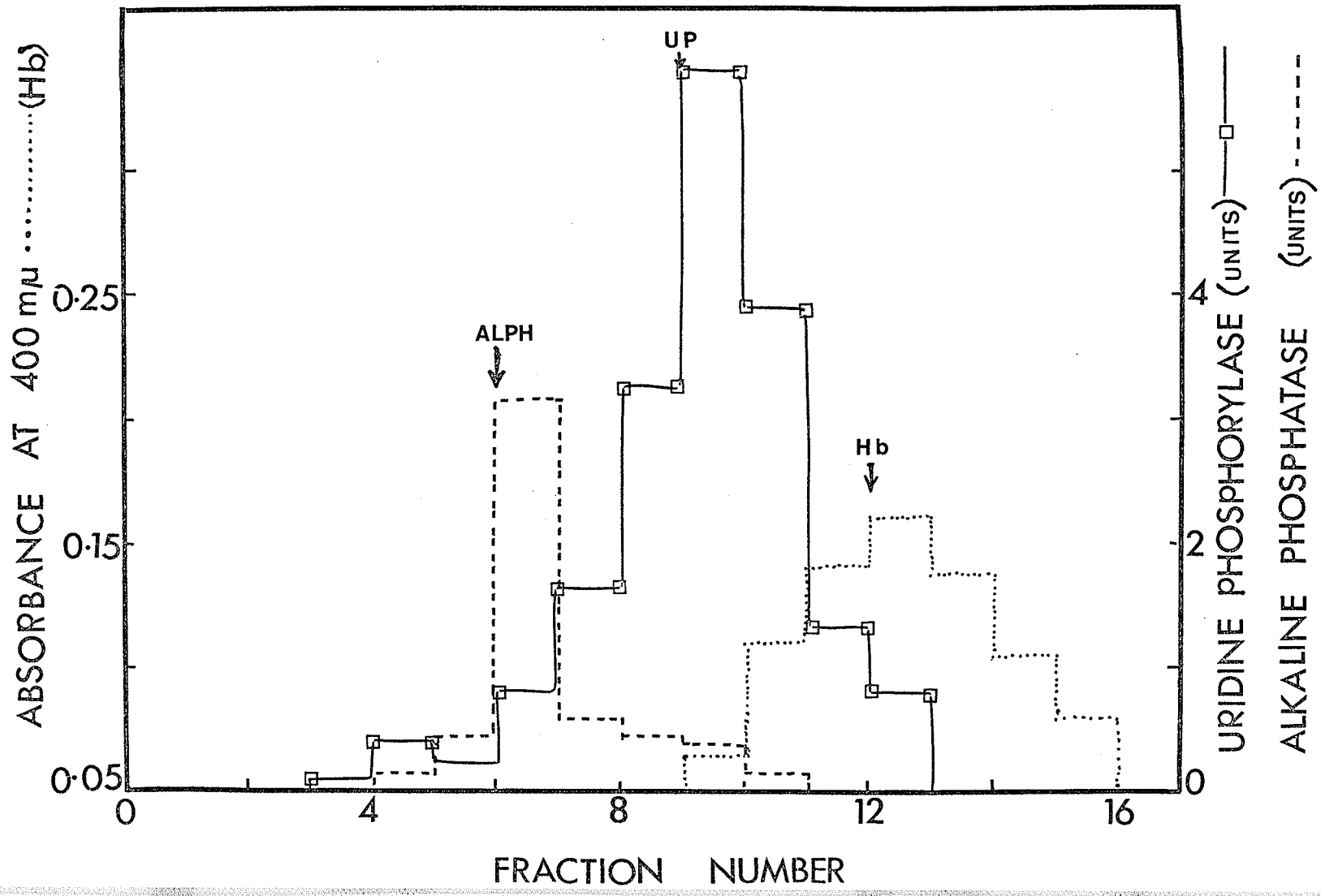

UP
ALPH
Hb
ABSORBANCE AT 400 mμ .....(Hb)
0·25
0·15
0·05
0
4
8
12
16
FRACTION NUMBER
URIDINE PHOSPHORYLASE (UNITS)
ALKALINE PHOSPHATASE (UNITS)
4
2
0

FIGURE 39

A - Uridine concentration study in the transferase reaction between uracil 2-C^{14} and uridine.

The standard assay medium contained 0.101 μmoles of uracil 2-C^{14} (0.06 μCi), 0.008 μmoles of K Pi (pH 7.0) and 9.5 μg enzyme protein from step 7, preparation IV (isoenzyme 1 and 2 both present). Incubation was for 30 minutes at 37°C.

B - Double reciprocal plot of velocity against uridine concentration, when 1.6 mM phosphate (Δ——Δ——Δ-) was present in the assay system, or when 0.033 mM ribose-1-P and 2.0 mM uracil (O——O——O-) was present.

FIGURE -39

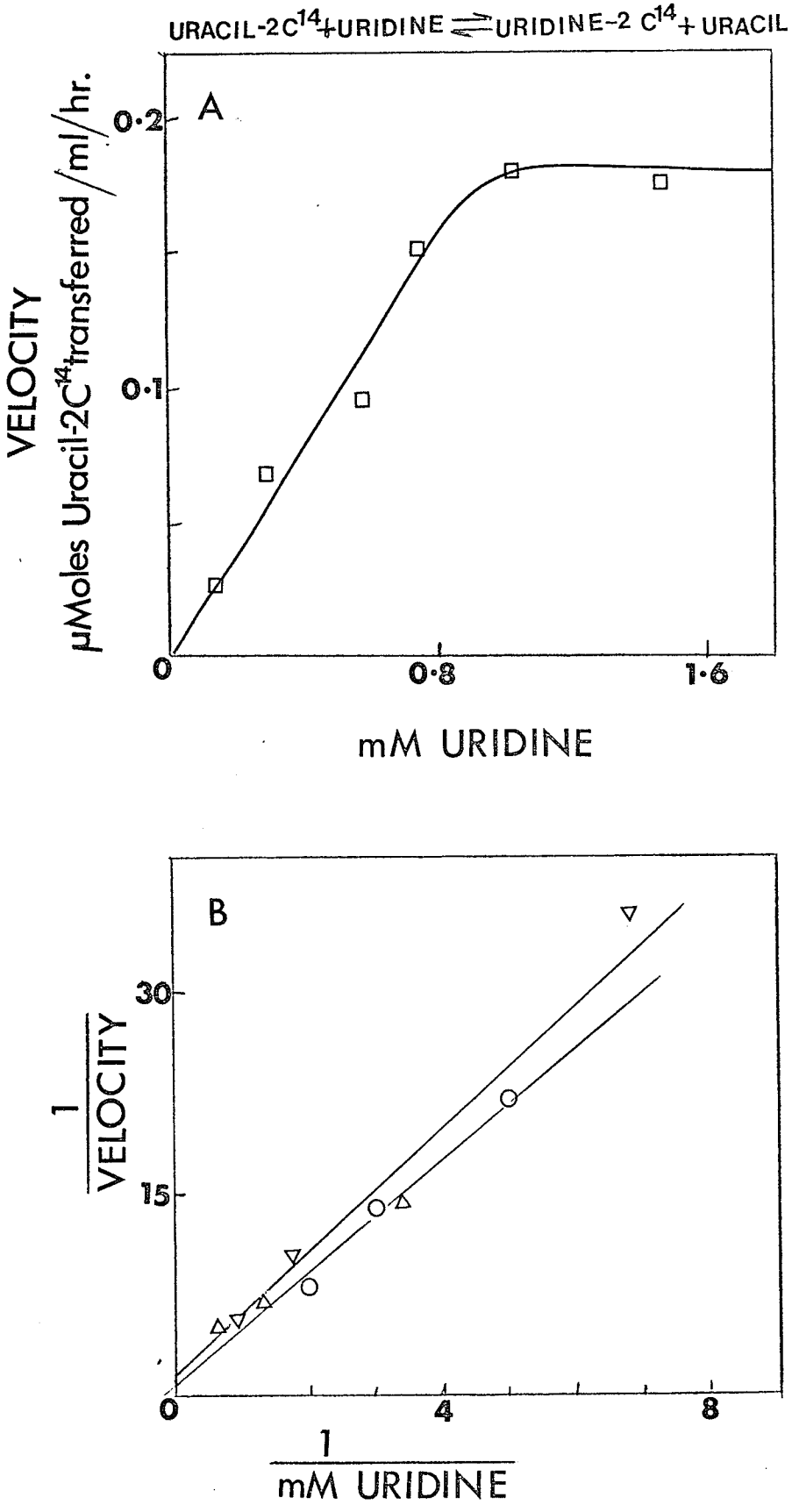

URACIL-2C14+URIDINE ⇌ URIDINE-2 C14+ URACIL
A
VELOCITY
µMoles Uracil-2C14transferred /ml/hr.
0·2
0·1
0
0·8
1·6
mM URIDINE
B
1/VELOCITY
30
15
0
4
8
1/mM URIDINE

FIGURE-40

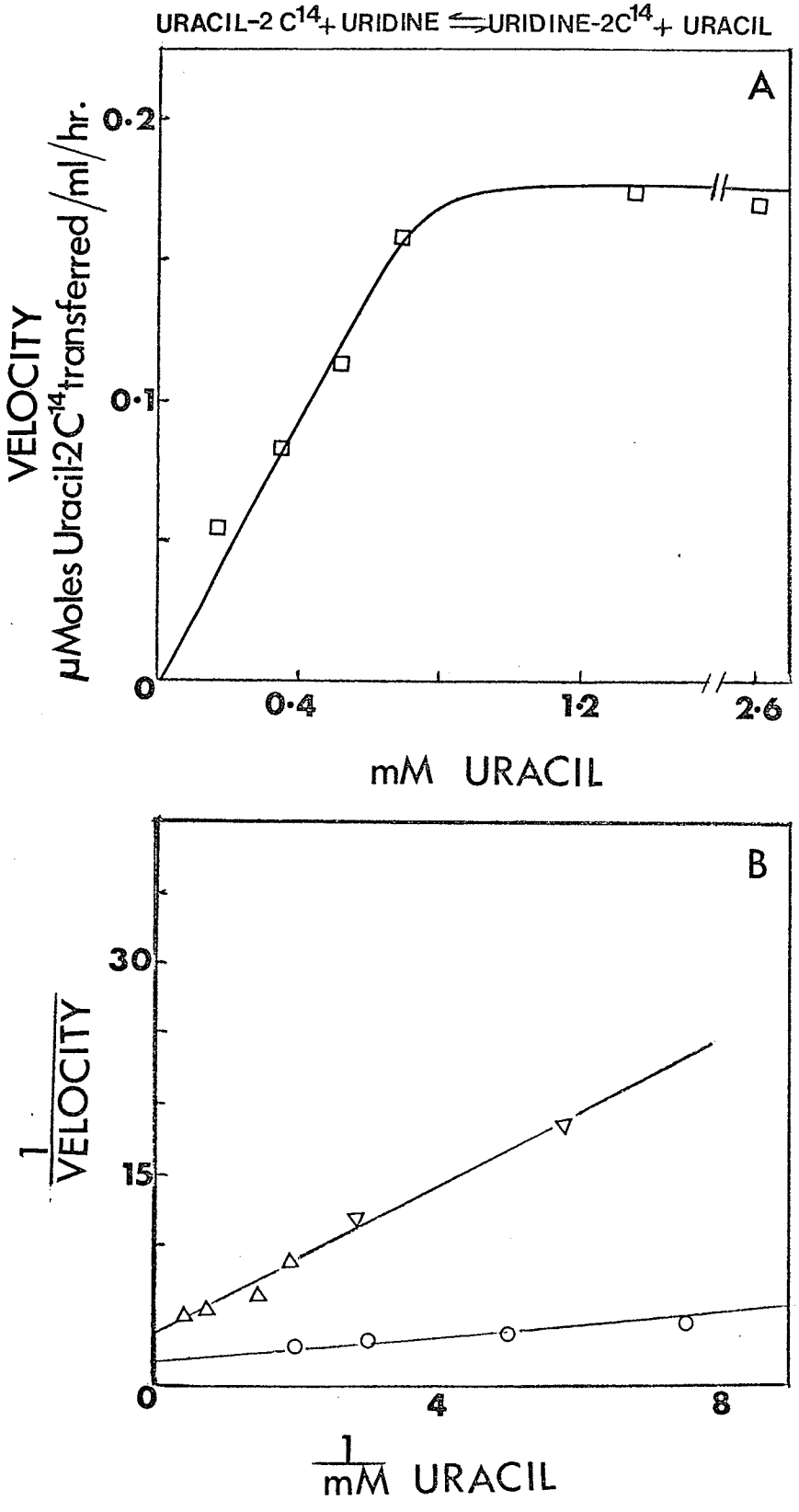

URACIL-2 C14 + URIDINE ⇌ URIDINE-2C14 + URACIL
A
VELOCITY
μMoles Uracil-2C14 transferred /ml/hr.
0·2
0·1
0
0·4
1·2
2·6
mM URACIL
B
1/VELOCITY
30
15
0
4
8
1/mM URACIL

FIGURE 40

A - Uracil concentration study in the transferase reaction between uracil 2-C^{14} and uridine.

The standard assay medium contained 0.199 μmoles of uridine, variable uracil-2-C^{14} of constant specific activity (0.19 34 μCi per μmole uracil) and 9.5 μg enzyme protein from step 7 preparation IV (isoenzyme 1 and 2 both present). Incubation was for 30 minutes at 37°C.

Independent background radioactivity had to be determined in the uridine spot for every concentration of uracil used.

B - Double reciprocal plot of velocity against uracil concentration, when 1.66 mM K phosphate pH 7.0 (▽——▽——▽) or 0.033 mM ribose-1-P (○——○——○) and 2.0 mM uridine was present.

FIGURE 41

A - Enzyme concentration study of the transferase reaction.

The standard assay medium contained 0.301 μmole uracil-2-C^{14} (0.06 μCi), 0.199 μmole uridine and 0.025 μmole of K phosphate buffer pH 7.0. The enzyme fractions used contained 4.75 μg, 7.12 μg, 9.5 μg, 11.87 μg and 14.25 μg protein from step 7, preparation IV in which both isoenzyme 1 and 2 were present. Incubations were for 30 minutes at 37°C. See methods section for other details.

B - Time study of the transfer between uracil and uridine.

The standard assay medium contained 0.199 μmole of uridine, 0.101 μmole of uracil-2-C^{14} (0.06 μCi) and 9.5 μg enzyme protein from step 7, preparation IV, in which both isoenzyme 1 and 2 were present.

Incubations were at 37°C for different lengths of time.

FIGURE-41

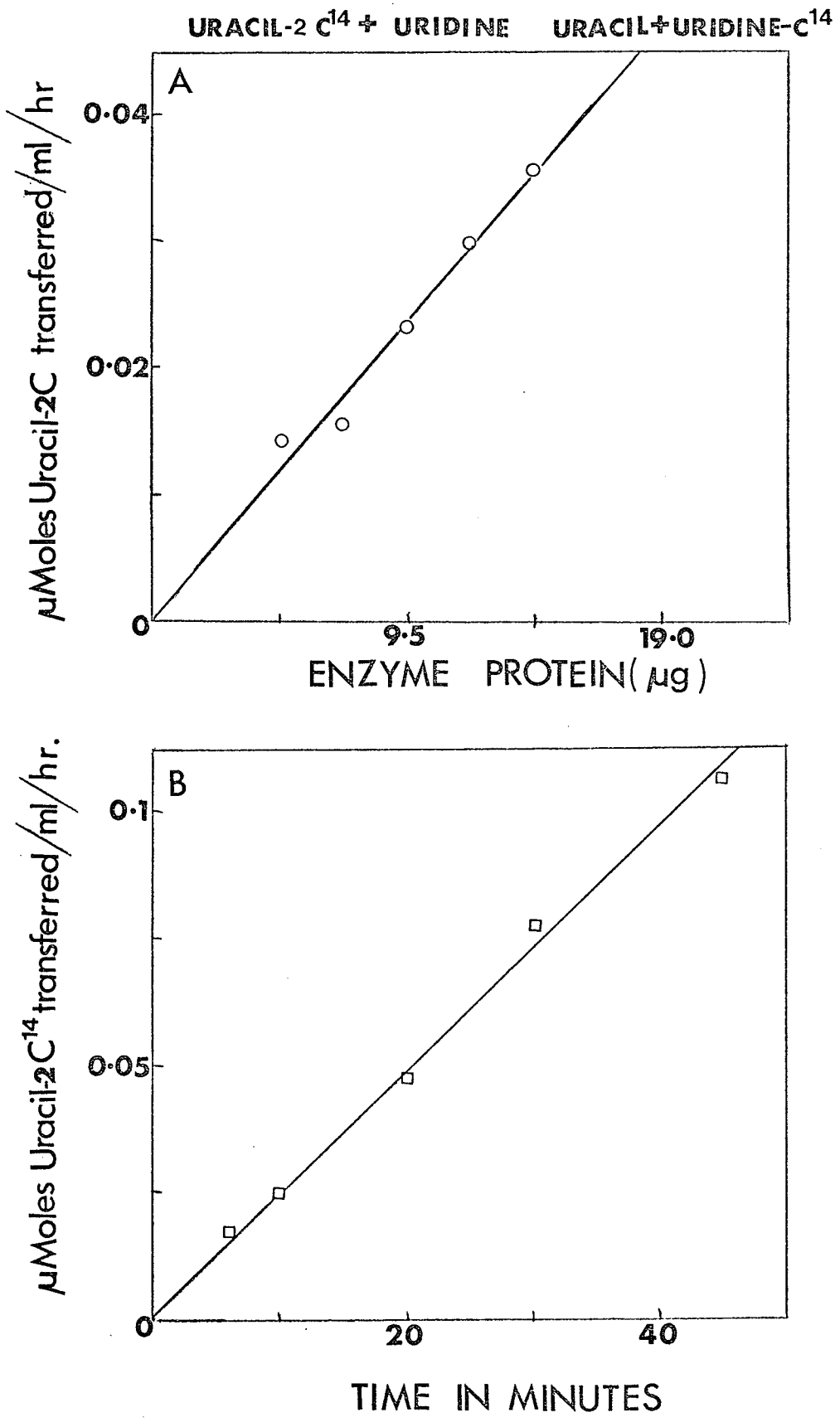

URACIL-2 C14 + URIDINE
URACIL+URIDINE-C14
A
0·04
0·02
0
µMoles Uracil-2C transferred/ml/hr
9·5
19·0
ENZYME PROTEIN(µg)
B
0·1
0·05
0
µMoles Uracil-2C14 transferred/ml/hr.
20
40
TIME IN MINUTES

FIGURE 42

A - Effect of phosphate concentration on the transfer between uracil and uridine.

The standard assay medium contained 0.199 μmole uridine, 0.299 μmole uracil (0.06 μCi), 19 μg enzyme protein from step 7, preparation IV, which had both isoenzymes 1 and 2 present. The phosphate concentration ranged from 0.0266 mM to 0.399 mM.

B - Double reciprocal plot of velocity against phosphate concentration. The assay conditions are as described in A above.

C - Effect of high concentrations of phosphate on the transfer between uracil and uridine.

The assay conditions were as described in A except that the phosphate concentration ranged from 16.6 mM to 166.6 mM.

FIGURE - 42

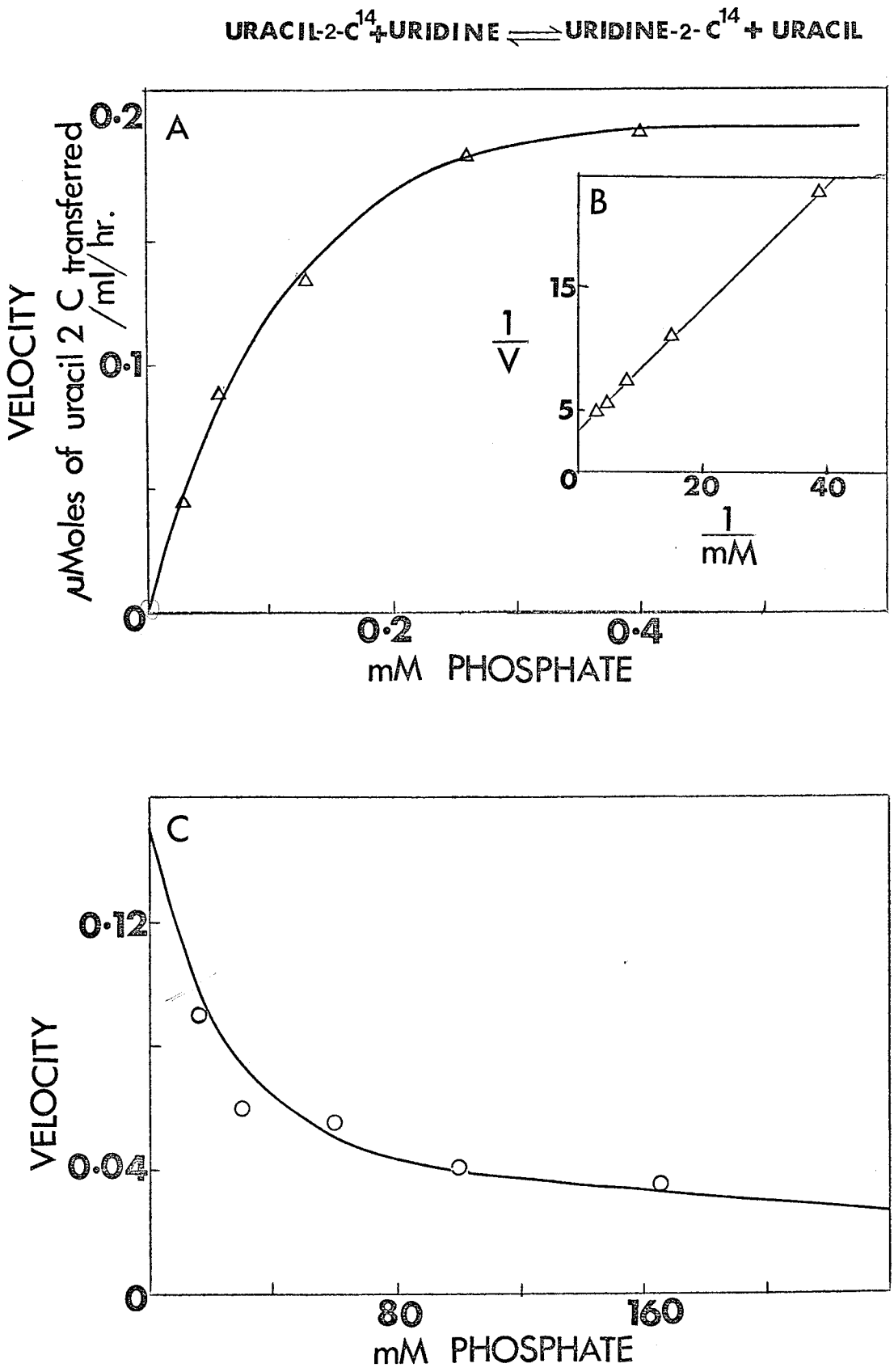

URACIL-2-C14 + URIDINE ⇌ URIDINE-2-C14 + URACIL
A
B
C
VELOCITY
μMoles of uracil 2 C transferred /ml/ hr.
0·2
0·1
0
0·2
0·4
mM PHOSPHATE
1/V
15
5
0
20
40
1/mM
0·12
0·04
0
80
160
mM PHOSPHATE

FIGURE 43

Transfer between uracil-2-C^{14} and uridine at different levels of phosphate and variable uridine.

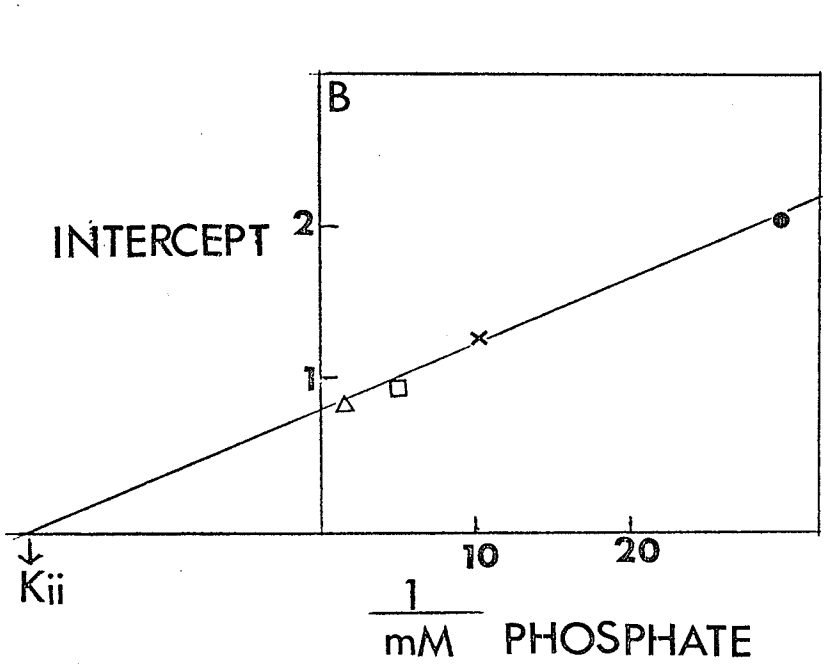
A - Double reciprocal plot of velocity against uridine concentration. The standard assay medium contained 0.202 μmole uracil-2-C^{14} (0.1 μCi), 4.6 μg protein of isoenzyme #1 fraction obtained after step 7, preparation V; uridine concentration was varied from 0.066 mM to 2.0 mM, at different levels of phosphate which were 0.033 mM (●—●—●), 0.1 mM (×—×—×), 0.2 mM (—□—□—□) and 0.66 mM (▽—▽—▽—).

B - Replot of vertical intercepts against phosphate concentration.

C - Derivation and definition of horizontal intercept (Kii) of replot in kinetic terms. Calculated value of Kii was obtained for phosphorolysis from Ka and Kip values published previously (10).

FIGURE -43

URACIL-2 C^{14} + URIDINE $\rightleftharpoons$ URIDINE -2 C^{14} + URACIL



$$\frac{1}{K_{ii}} = \frac{1}{18 \cdot 92} = 0 \cdot 052 \text{ mM}$$

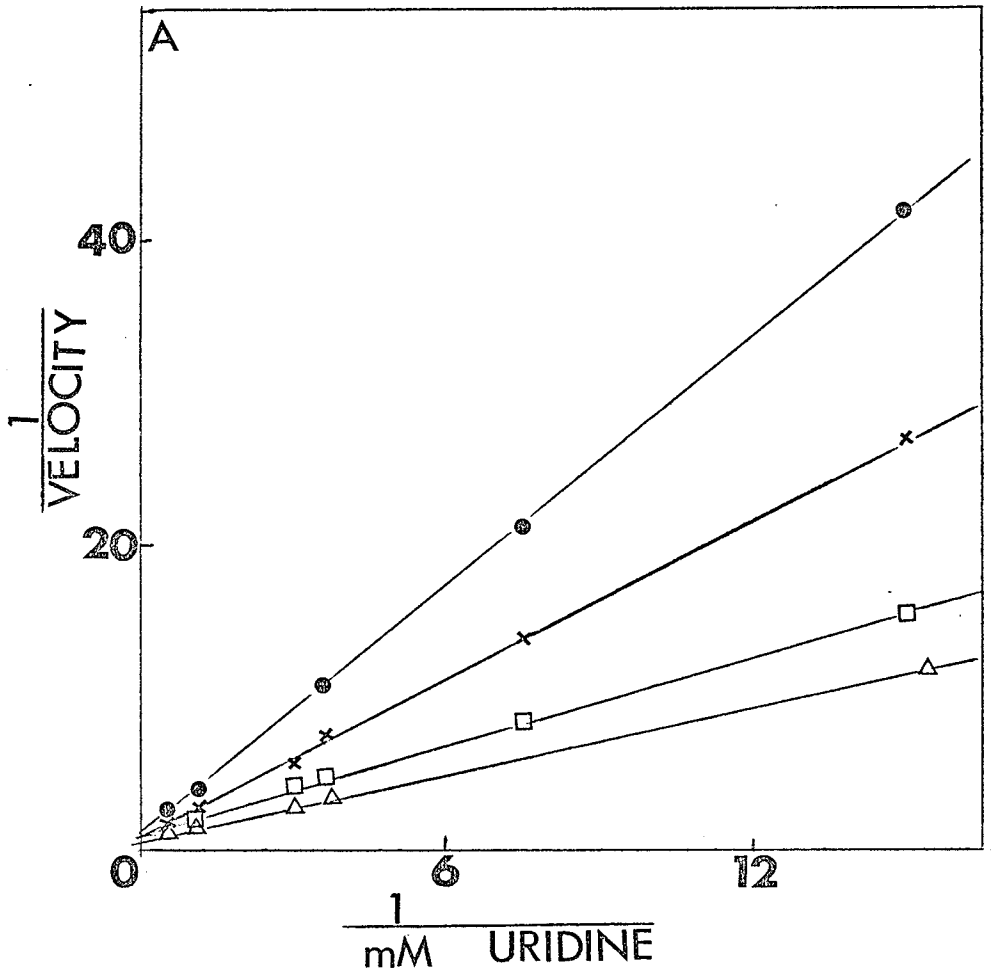


FIGURE 43 C

Rate equation for ordered Bi Bi (128)

$$v = \frac{V_1 V_2 (A B - \frac{P Q}{Keq})}{Kia\ Kb\ V_2 + Kb\ V_2\ A + Ka\ V_2\ B + V_2\ A\ B + \frac{Kq\ V_1\ P}{Keq} + \frac{Kp\ V_1\ Q}{Keq}}$$

$$+ \frac{V_1\ P\ Q}{Keq} + \frac{Kq\ V_1\ A\ P}{Kia\ Keq} + \frac{Ka\ V_2\ B\ Q}{Kiq} + \frac{V_2\ A\ B\ P}{Kip} + \frac{V_1\ B\ P\ Q}{Kib\ Keq}$$

P^* [Uracil 2-C^{14}] $\longrightarrow$ B [Uridine] transfer, with Q (Ribose-1-P) = 0.

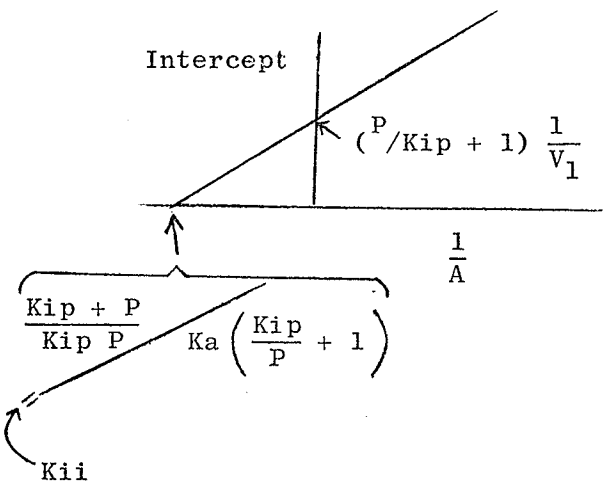
$$v = \frac{V_1 V_2 A B}{Kia\ Kb\ V_2 + Kb\ A\ V_2 + Ka\ B\ V_2 + A\ B\ V_2 + \frac{A\ B\ P\ V_2}{Kip} + \frac{Kq\ V_1\ P}{Keq} + \frac{Kq\ V_1\ A\ P}{Kia\ Keq}}$$

$$\frac{1}{v} = \frac{Kia\ Kb}{V_1\ A\ B} + \frac{Kb}{V_1\ B} + \frac{Ka}{V_1\ A} + \frac{1}{V_1} + \frac{P}{Kip\ V_1} + \frac{Kq\ P}{A\ B\ Keq\ V_2} + \frac{Kq\ P}{Kia\ Keq\ B\ V_2}$$

$$\frac{1}{v} = \frac{1}{B}\left(\underbrace{\frac{Kia\ Kb}{V_1\ A\ B} + \frac{Kb}{V_1} + \frac{Kq\ P}{A\ Keq\ V_2} + \frac{Kq\ P}{Kia\ Keq\ V_2}}\right) + \left(\underbrace{\frac{Ka}{A} + 1 + \frac{P}{Kip}}\right)\frac{1}{V_1}$$

Plot $\frac{1}{v}$ against $\frac{1}{B}$ $\longrightarrow$ slope and intercept

Replot Intercept against $\frac{1}{A}$ (A is phosphate)



At pH 8.1 for uridine phosphorylase (10) for phosphorylsis

Ka (phosphate) = 0.349 mM

Kip (uracil at low phosphate) = 0.315 mM

∴ Calculated intercept value at 1.322 mM uracil (P) will be 0.046

FIGURE 44

A - Effect of ribose-1-P on the transfer between uracil and uniformly labelled uridine.

The standard assay medium contained 0.299 μmole of uracil + 0.2 μmole of uridine-^{3}H (0.25 μCi), and 19 μg of enzyme protein from step 7, preparation IV which contained both isoenzymes 1 and 2. The ribose-1-P concentrations were from 0.0166 to 16 mM.

B - Double reciprocal plot of velocity against ribose-1-P concentration.

C - Effect of deoxyribose-1-P on the transfer between deoxyuridine and thymine.

The standard assay medium contained 0.5 μmoles of uniformly labelled deoxyuridine (0.25 μCi), 0.3 μmole of thymine and 19 μg of enzyme protein from step 7, preparation IV which contained both isoenzymes 1 and 2 (O——O——O). The same assay medium was used for the assay with thymidine phosphorylase fraction after step 4 (Δ——Δ——Δ—).

FIGURE - 44

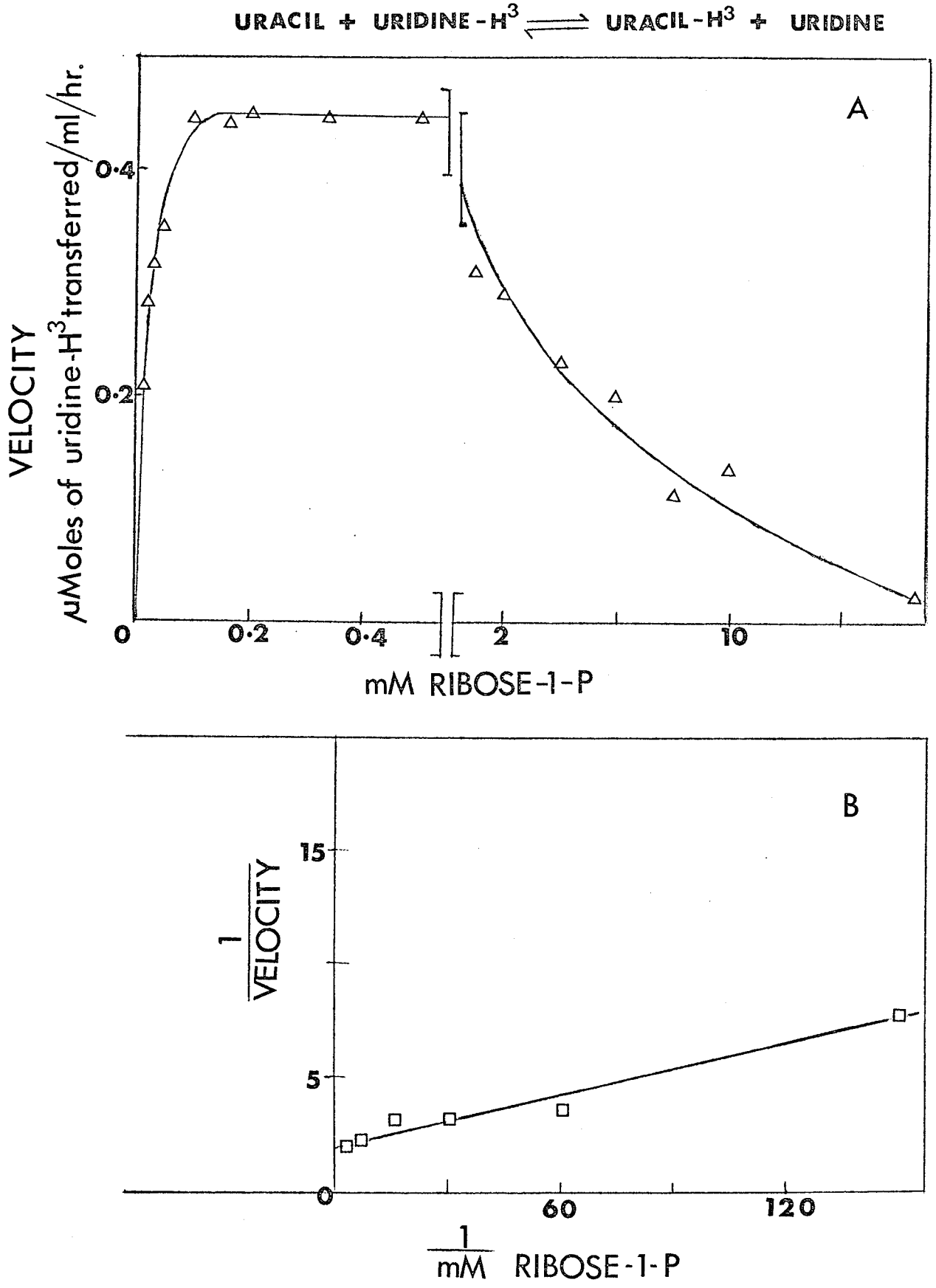

URACIL + URIDINE-H3 ⇌ URACIL-H3 + URIDINE
A
VELOCITY
μMoles of uridine-H3 transferred/ml/hr.
0·4
0·2
0
0·2
0·4
2
10
mM RIBOSE-1-P
B
1/VELOCITY
15
5
0
60
120
1/mM RIBOSE-1-P

FIGURE - 44 C

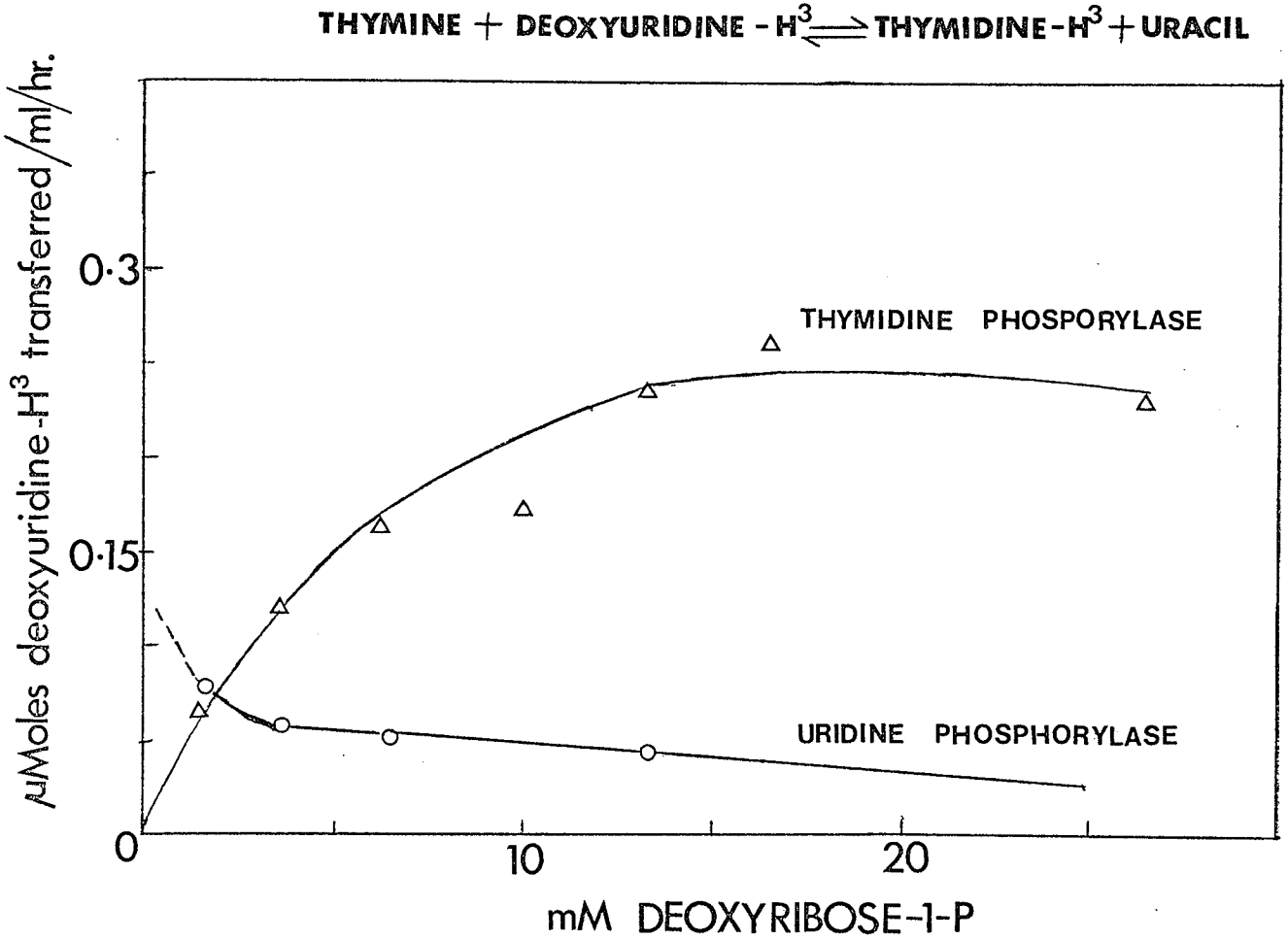
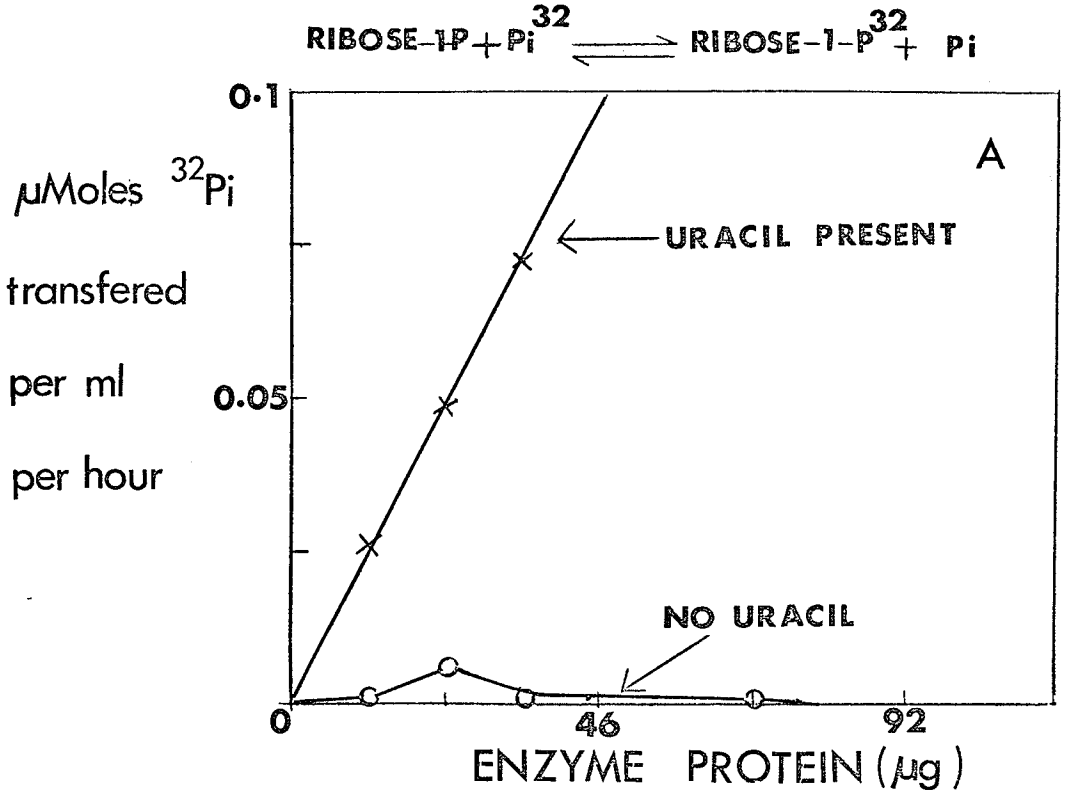

THYMINE + DEOXYURIDINE - H3 ⇌ THYMIDINE - H3 + URACIL
µMoles deoxyuridine-H3 transferred/ml/hr.
0·3
0·15
0
10
20
mM DEOXYRIBOSE-1-P
THYMIDINE PHOSPORYLASE
URIDINE PHOSPHORYLASE

FIGURE -45


RIBOSE-1P + Pi 32 ⇌ RIBOSE-1-P 32 + Pi
A
μMoles 32Pi transfered per ml per hour
0·1
0·05
0
URACIL PRESENT
NO URACIL
46
92
ENZYME PROTEIN (μg)

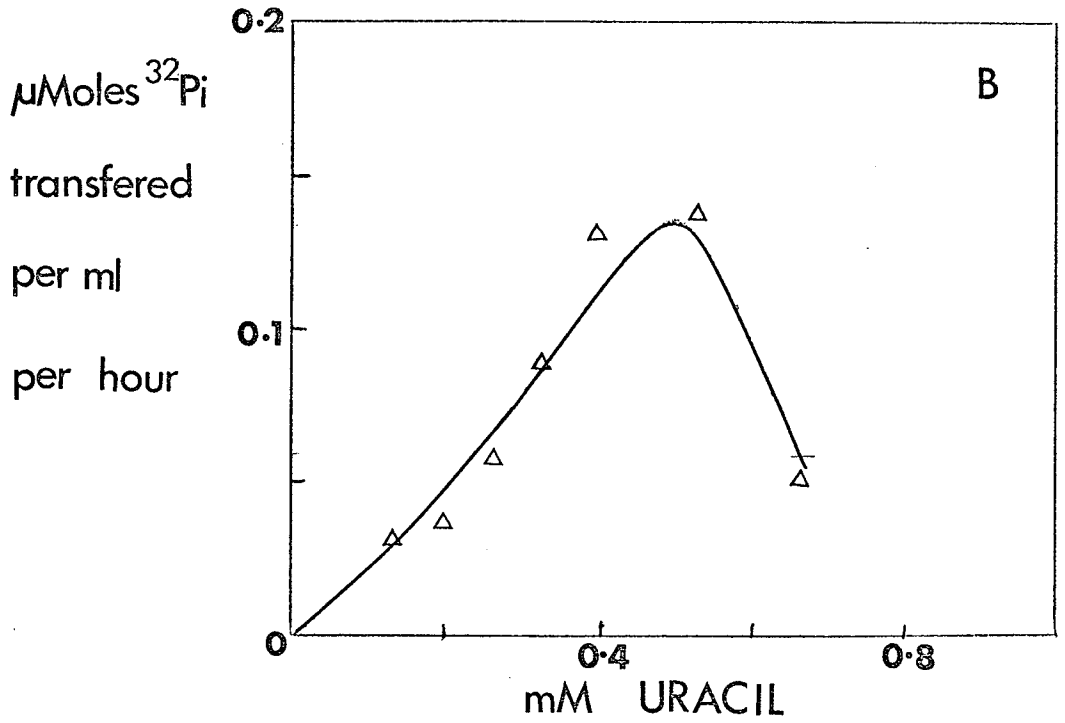

B
μMoles 32Pi transfered per ml per hour
0·2
0·1
0
0·4
0·8
mM URACIL

FIGURE 45

Effect of uracil on transfer between ^{32}Pi to ribose-1-P.

A - Enzyme concentration study in the presence or absence of uracil.

The standard assay medium contained 0.5 μmole (0.23 μCi) of ^{32}Pi, 0.5 μmole of ribose-1-P and 0.02 μmole of uracil (×——×——×) or no uracil (O——O——O) as well as 11.5 μg to 69 μg of protein of isoenzyme #1 fraction obtained after step 7, preparation V.

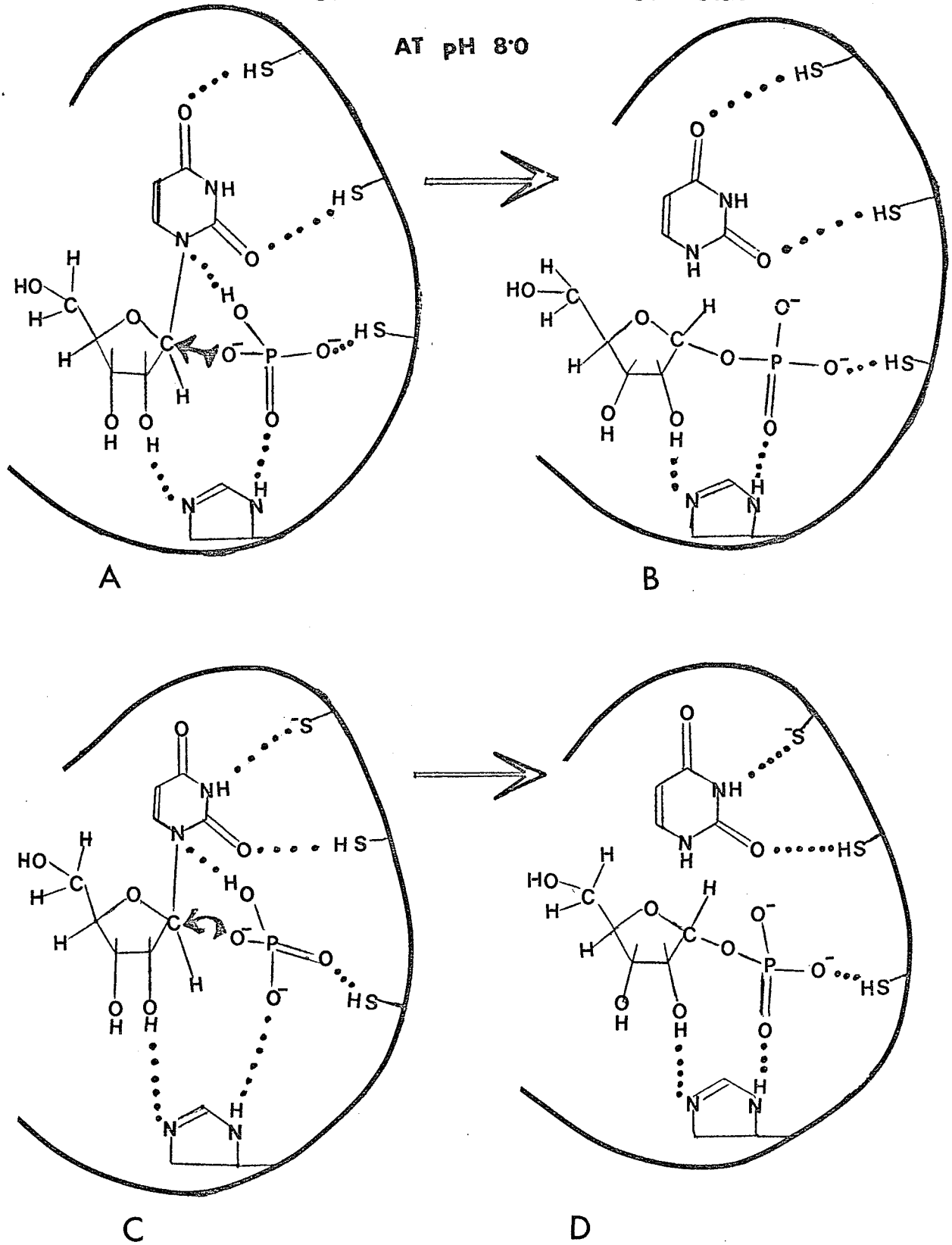
B - Effect of variable uracil on ^{32}Pi to ribose-1-P transfer.

The standard assay medium contained 0.25 μmole (0.23 μCi) of ^{32}Pi, 0.25 μmole of ribose-1-P and 46 μg of protein from isoenzyme #1 fraction after step 7, preparation V. Uracil concentration was varied from 0.133 mM to 0.66 mM.

FIGURE-46

MECHANISM OF PHOSPHOROLYSIS OF URIDINE

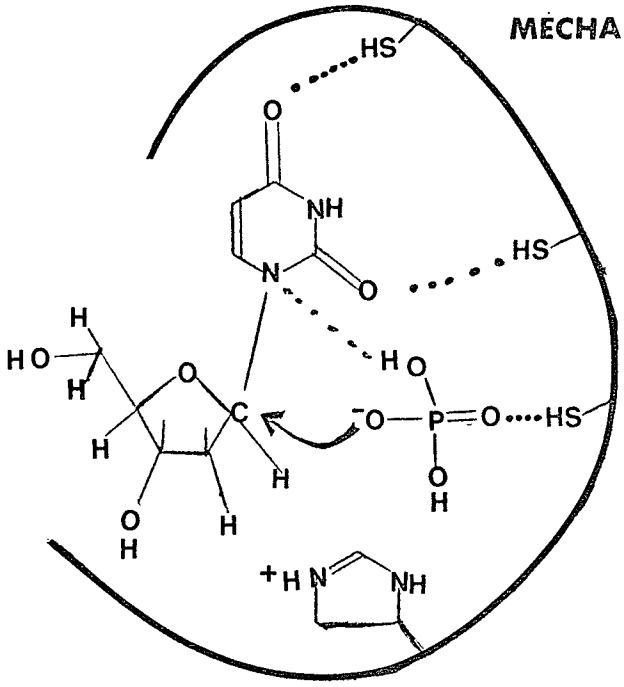
AT pH 8·0



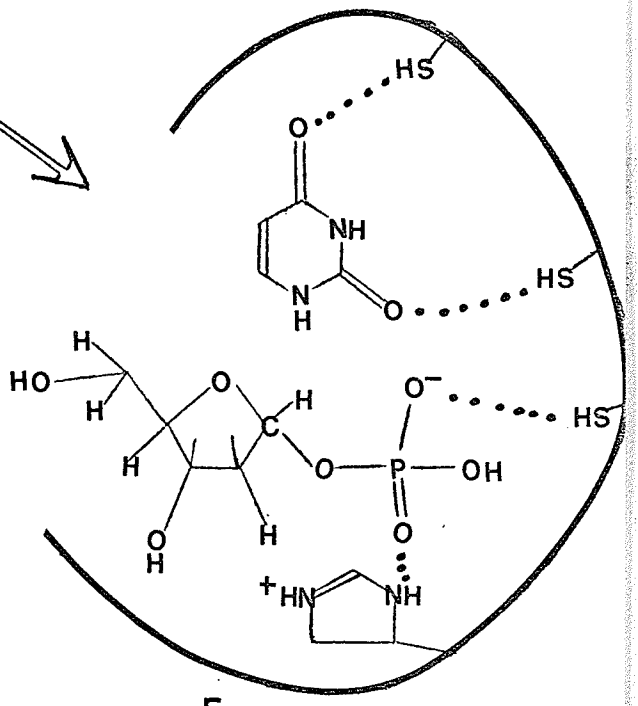
ALTERNATE BINDING SITE

FIGURE - 46

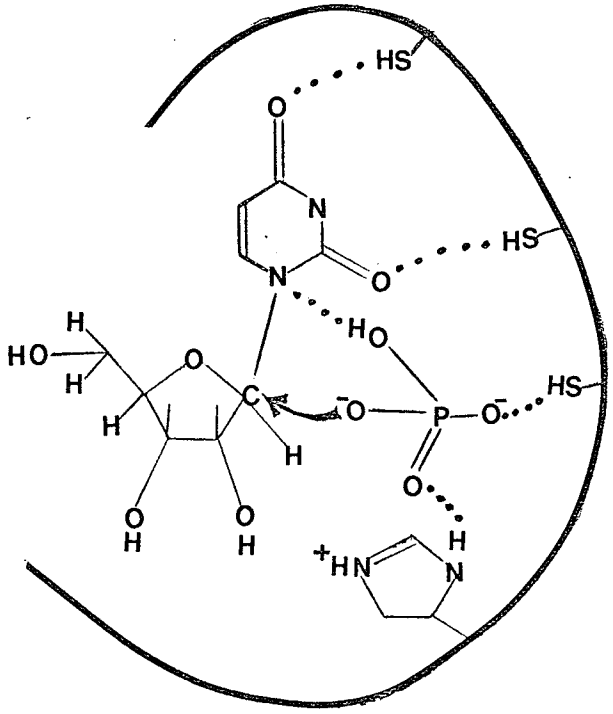
MECHANISM OF PHOSPHOROLYSIS BY URIDINE PHOSPHORYLASE AT pH 6.5



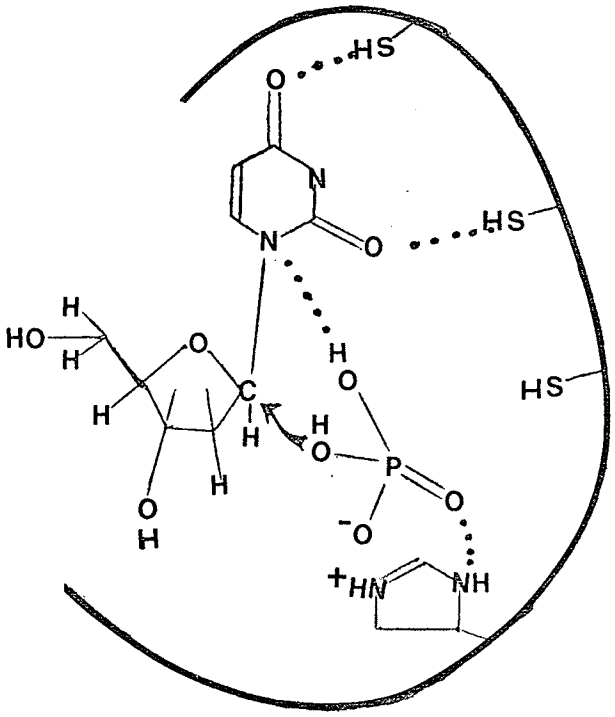
E DEOXYURIDINE



F



G URIDINE

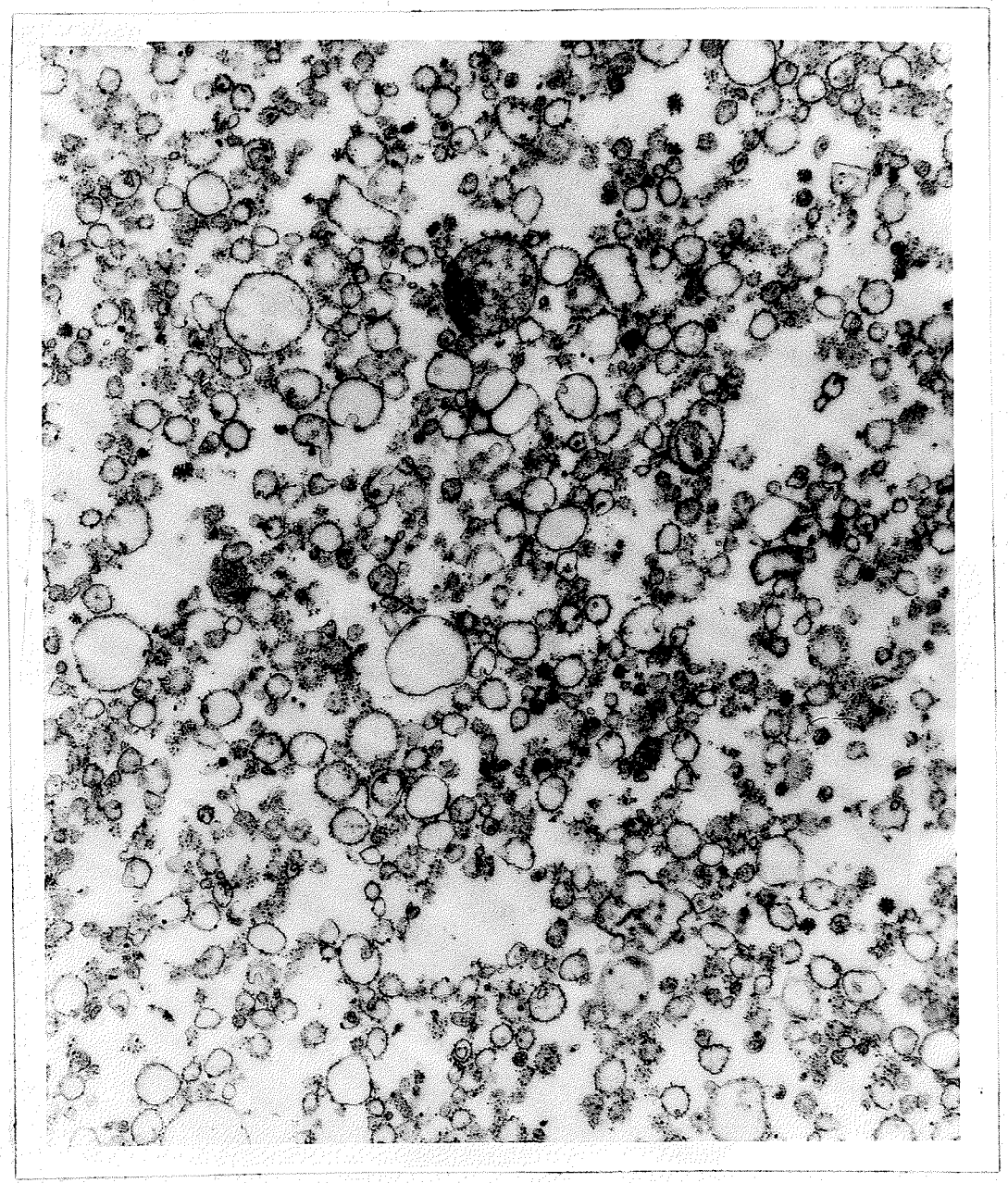


H DEOXYURIDINE (ALTERNATE BINDING)

ELECTRONMICROGRAPH I

PLASMA MEMBRANE FRACTION

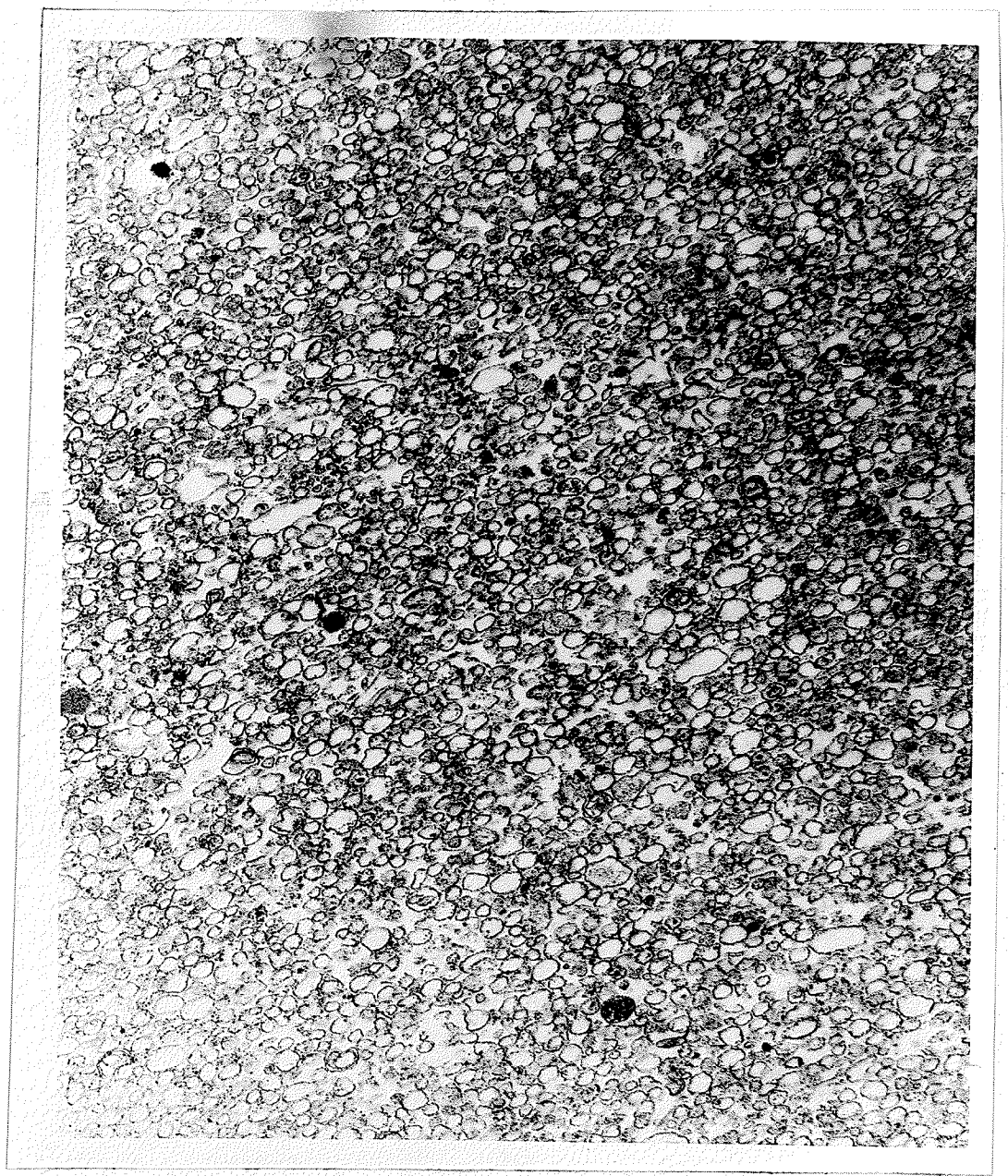
MAGNIFICATION 20,250 x



ELECTRONMICROGRAPH II

BILE CANALICULI (70,000 x g pellet)

MAGNIFICATION 12,500 x



ELECTRONMICROGRAPH III

DEBRIS FRACTION

MAGNIFICATION 20,250 x