PYRIMIDINE REDUCING ENZYMES IN LIVER

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ABBREVIATIONS

ATP	adenosine 5' triphosphate
AS_1, AS_2, AS_3	ammonium sulphate fractions
Bis	N,N' methylenebisacrylamide
%C	percent cross-linking
DHU	dihydrouracil
DHT	dihydrothymine
EDTA	ethylenediaminetetracetic acid
EGTA	ethyleneglycol-bis-(B-amino-ethyl ether) N,N' tetraacetic acid
HM	heavy mitochondrial fraction
M_1, M_2, M_3	heavy mitochondrial subfractions
Mg^{2+}	magnesium ions
NAD^+	nicotinamide adeninedinucleotide
NADH	nicotinamide adeninedinucleotide reduced
$NADP^+$	nicotinamide adeninedinucleotide phosphate
NADPH	nicotinamide adeninedinucleotide phosphate reduced
Nitro BT	nitroblue tetrazolium chloride
Pi	inorganic phosphate
SA	specific activity
%T	percent total gel (acrylamide +Bis)
TCA	trichloroacetic acid
v/v	volume/volume
w/v	weight/volume

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ABSTRACT

The results confirm earlier studies (30) that the enzyme dihydrouracil dehydrogenase (4,5-dihydrouracil: $NADP^+$ oxidoreductase, E.C.1.3.1.2) exists in the particulate fraction as well as the cytosol fraction of mammalian liver. Dihydrouracil dehydrogenase was partially purified from the cytosol fraction of rat liver and fractionated by disc gel electrophoresis.

A rapid and sensitive method for the location of dihydrouracil dehydrogenase after disc gel electrophoresis based on the reduction of nitroblue tetrazolium to form an insoluble dye has been developed. Semiquantitative evaluation of enzyme activity was achieved by means of densitometer tracings of stained gels. The method permits detection of enzyme activity in partially purified extracts after a minimal number of purification steps. Two enzyme bands, differing mainly in charge, were separated giving the first indication that this enzyme may possibly exist in at least 2 isoenzyme forms in liver. The substrate specificity of these bands was investigated. It was found that both bands were 2-3 times more active with dihydrothymine as substrate than with dihydrouracil in the presence of NADP+ and optimum pH of 7.4.

These results give further proof that the cytosol and particulate enzymes differ in a number of ways other than subcellular distribution.

Mitochondrial fractions containing most of the NADH-dependent uracil reductase of rat liver cells were fractionated by centrifugation in sucrose density gradients. Two procedures involving linear or discontinuous gradients were used. By both, good separation of NADH - and NADPH - dependent reductases was achieved. Marker enzyme studies supported the view that the NADH-dependent enzyme is located prinicipally in mitochondria whereas the NADPH-dependent enzyme, mainly in plasma and endoplasmic reticulum membranes. For the NADH-dependent reductase the apparent K_m for thymine at pH 7.4 was 1.39 times that found for uracil whereas for the NADPH-dependent enzyme the apparent K_m values were similar for the two substrates at this pH.

Dihydrouracil was the principal product isolated by paper chromatography from the reaction mixture containing a partially purified fraction of mitochondria, uracil and NADH at pH 7.4. This fraction also catalyzed the formation of radioactive carbon dioxide from uracil-2-^{14}C. The proportion of CO_2 formed by the mitochondria was about 10% of that formed by the original homogenate.

INTRODUCTION

The catabolism of pyrimidines to β-amino acids, ammonia and carbon dioxide through the intermediate formation of dihydropyrimidine and β-ureido acid stages has been studied by several investigators (1) (2) (3) (4) (5) (6). In mammals this pathway, shown in Figure 1, has been shown to be located primarily in the cytosol fraction of liver.

HISTORICAL BACKGROUND

(A) The Discovery of the Catabolic Pathway for Pyrimidines

The existence of a reductive pathway for pyrimidine catabolism was first suggested by Fink, Henderson and Fink (7) in 1951, following the identification of β-aminoisobutyric acid in human urine.

In 1952, *in vivo* experiments with rats by Fink et al (8) revealed that urinary β-aminoisobutyric acid increased following administration of dihydrothymine. It was implied that dihydrothymine might be an intermediate in thymine metabolism. Dihydrouracil administration resulted in detectable excretion of β-ureidopropionic acid.

In 1953, in a similar study of pyrimidine metabolism *in vitro* by rat liver slices, Fink et al (9) reported similar results. Since dihydrouracil and β-alanine had been isolated from animal tissue (10), these workers

extended their observations to include the analogous formation of β-alanine from dihydrouracil.

In 1954, Fink, Cline and Kosh (11) developed a sensitive chromatographic procedure which provided direct evidence to show that in bacterial cultures the conversion of thymine and uracil to corresponding β-amino acids was accomplished by the formation of intermediate dihydropyrimidines and β-ureido acids.

In 1955, Fink, McGaughy, Cline, and Fink (3) further postulated the existence in liver of an analogous sequence of reactions for uracil catabolism based on the rapid formation of β-alanine from dihydrouracil and β-ureido-propionic acid in liver, the natural occurence of dihydrouracil and β-alanine in animal tissues (10) and by the detection of the whole series of uracil reduction products in bacterial cultures (11).

Later in an in vitro study of the metabolism of pyrimidine reduction products, Fink et al (3) reported that rat liver slices incubated with thymine produced a small amount of β-aminoisobutyric acid but that dihydro-thymine and β-ureidoisobutyric acid were not detected. However, the latter compounds, when added in substrate amounts, were rapidly and extensively converted to β- amino-isobutyric acid.

The data reported indicated that the conversion of β-ureidoisobutyric acid to β-aminoisobutyric acid was irreversible under the experimental conditions employed. However, the reversible interconversion of β-ureidoisobutyric acid to dihydrothymine was clearly detectable while thymine appeared if dihydrothymine was used as a substrate.

Further proof of the pathway was presented in <u>in vivo</u> experiments by Fink (2) on excretion of pyrimidine reduction products in rats which revealed the following relationship between thymine, β-aminoisobutyric acid and the intermediate products dihydrothymine and β-ureidoisobutyric acid:

Thymine ← Dihydrothymine ⇌ β-ureidoisobutyric acid → β-aminoisobutyric acid.

Only the conversion of dihydrouracil to β-ureidopropionic acid for the related uracil series of compounds was clearly demonstrated at this time. A more rapid metabolism of β-alanine than β-aminoisobutyric acid was suggested to account for the absence of detectable urinary β-alanine after administration of uracil, dihydrouracil, or β-ureidopropionic acid.

In 1956, Fritzson (4) studied the catabolism of uracil in rat liver slices by means of ^{14}C labeled uracil, dihydrouracil, β-ureidopropionic acid or β-alanine. He demonstrated the formation of β-alanine from uracil and

by analysis of the radioactive products by paper chromatography he identified dihydrouracil and B-ureidopropionic acid as intermediates of the degradative pathway.

A similar in vivo study by Fritzson and Pihl (5) provided evidence that the conversion of uracil to dihydrouracil, B-ureidopropionic acid and B-alanine, is the principal pathway of uracil catabolism in intact rats. The conversion of dihydrouracil to B-ureiodpropionic acid was found to be the only reversible step in this process.

Fritson (4) and Canellakis (12) measured the rates of degradation of uracil-2-^{14}C, dihydrouracil -2-^{14}C, B-ureiodpropionic-2-^{14}C acid and established that the reduction of uracil to dihydrouracil was the rate limiting step in the catabolic pathway.

(B) Early Work on Intracellular Localization of the Pyrimidine Catabolic Pathway

In 1954, Rutman, Cantrow and Paschkis (6) studied the intracellular localization of the pyrimidine pathway in rat liver by measuring the release of $^{14}CO_2$ formation with only trace amounts formed in the subcellular fractions. However, the $^{14}CO_2$ formation in the supernatant fraction

greatly exceeded that of a sample of whole homogenate containing an equal amount of supernatant fraction. It was inferred that inhibitors of the supernatant activity were present in the unfractionated homogenate.

In 1955, Canellakis (1) studied the formation of $^{14}CO_2$ from uracil-2-^{14}C and thymine-2-^{14}C in rat liver slices, whole homogenate, supernatant fluid and acetone dried powder extracts of the supernatant fraction. Experimental results demonstrated that the entire enzyme system responsible for the conversion of both pyrimidines was present in rat liver. It was reported that homogenization of liver slices in Na-phosphate buffer resulted in the loss of up to 50% of enzyme activity. The activity of the 100,000 x g supernatant however, approximated that of the liver slices.

Canellakis postulated that the enzyme system for the catabolism of uracil-2-^{14}C to CO_2 resided mainly in the supernatant fraction of the rat liver homogenate. Inhibition of enzyme activity resulted upon addition of microsomes to the supernatant fraction. This lead Canellakis to suggest that the enzymic system may also be present in the various particulate fractions, but that endogenous inhibitory factors prevented measurement of its activity.

(C) Partial Purification of Dihydrouarcil Dehydrogenase

In 1956, Leon Campbell Jr. (13), was able to obtain a 27 - fold purification of dihydrouracil dehydrogenase from C. Uracilium, strain M5-2 after ammonium sulfate fractionation and repeated calcium phosphate gel treatment. This partially purified enzyme was specific for reducing uracil with the cofactor NADH; it had a pH optimum in the range of 7.0 to 7.8, its apparent K_m value was 1.4×10^{-4} M and it catalyzed the reverse reaction, (ie DHU→Uracil) in the presence of NAD^+.

In 1957, Grisolia and Cardoso (14) were successful in purifying dihydrouracil dehydrogenase from beef liver. A 20 - fold purification was achieved after repeated ammonium sulfate fractionation and gel treatment. The enzyme which showed a high specificity for uracil and thymine as substrates, catalysed a reversible reaction. The enzyme also required NADPH as a cofactor. The pH optimum was 7.4, and it was activated by sulfosalicylate and salicylate. K_m values were calculated to be 6×10^{-4}M for dihydropyrimidines and less than 3×10^{-6}M with pyrimidines.

In 1960, Fritzson (15) partially purified dihydrouracil dehydrogenase from the soluble cytoplasmic fraction of rat liver. Using uracil as the substrate and NADPH as cofactor the enzyme catalyzed the reaction reversibly, giving a ratio of 0.2 for the rate of dihydrouracil oxidation to

the rate of uracil reduction at optimal substrate concentrations. The partially purified enzyme had a pH optimum from 7.0 to 7.4 and K_m values of approximately 1.7×10^{-5} M for NADPH and less than 4×10^{-6} M for uracil.

In 1970 Godde, Agarwal and Eickhoff (16) achieved a 60 - fold purification of dihydrouracil dehydrogenase from pig liver by a fractionation method using ethacridine lactate (Rivanol) and ammonium sulfate. It was reported that the pig liver dihydrouracil dehydrogenase was NADPH specific and had an optimum pH of 7.4 to 7.6. The maximum activity was located in the supernatant fraction after centrifugation at 100,00 x g. An apparent K_m of 2.8×10^{-5} M was reported for thymine.

(D) Recent studies on pyrimidine catabolism

Recent studies of pyrimidine catabolism have involved the use of inbred strains of mice.

In 1964, Dagg, Coleman, and Fraser (17) reported that a mutation in C57Bl/6 mice resulted in a metabolic defect which affected the rate of pyrimidine degradation.

It was reported that Rf/J mice were capable of converting injected uracil - 2 - ^{14}C to $^{14}CO_2$ at a rate three times that of C57Bl/6 mice. Using uracil - 2 - ^{14}C, dihydro-uracil - 2 - ^{14}C or β-ureidopropionic - 2 - ^{14}C acid as the

substrate, determining the release of $^{14}CO_2$ in crude liver extracts, it was reported that all three enzymes in the pathway were diminished 3 - 8 fold in the C57Bl/6 relative to RF/J.

The in vitro studies reported by these workers indicated that the rate limiting step is the first reaction in the sequence in agreement with that found in rats by Canellakis (12).

On the basis of their experiments, Dagg et al. have postulated that pyrimidine metabolism was regulated by two alleles at a single locus with codominant inheritance properties. The mutation affected the rate of pyrimidine degradation in a manner which involved the activities of the three enzymes in the pathway.

It has been suggested (18) that if the mutation affects the entire operon in the classical bacterial sense it would be one of the first clear indications in higher organisms of a single mutation affecting in a coordinate manner a number of enzymes in a single metabolic pathway.

In 1970, Sanno, Holzer and Schimke (18) examined the mechanism for the metabolic defect reported by Dagg (17) by assaying separately the three enzymes involved in pyrimidine degradation in mouse liver of mouse strain RF/UP from which RF/J was derived.

Separation of the three enzymes, dihydrouracil

dehydrogenase, dihydropyrimidinase and β-ureidopropionase, was accomplished by diethylaminoethylcellulose chromatography. The ability to separate the three enzymes indicated to these workers that the metabolic defect could not have resulted from a mutation in a single protein with multiple enzymatic activities.

Sanno et al. confirmed Dagg's findings that uracil-2-^{14}C administered to the intact strains of RF/J and C57Bl/6 mice showed the same three fold difference in the rate of $^{14}CO_2$ evolution as did the crude extracts of livers of these strains. They reported, however, that only the β-ureidopropionase activity is different in the two strains. It was noted that in order to maintain optimal enzyme activity there was a requirement for the maintenance of conditions of sulfhydryl reduction. Of the three enzymes involved in pyrimidine degradation, Sanno et al. reported that only β-ureidopropionase was found to be activated by dithiothreitol. They concluded from the low activity that β-ureidopropionase was the rate-limiting step for the overall degradation of pyrimidines to β-alanine in liver of both mouse strains.

The nature of the mutation affecting β-ureidopropionase activity appeared to these workers to be structural since the partially purified enzyme from C57Bl/6 was more thermolable and had a decreased apparent K_m for substrate.

(E) Tetrazolium Salts

Tetrazolium salts, first synthesized in 1894 (19), are water soluble, colorless compounds which on reduction yield deep red, water insoluble pigments known as formazans.

The stability of the colored formazans and their solubility in water suggested the possiblity of using tetrazolium salts as hydrogen acceptors in developing histochemical methods for demonstrating a variety of enzymes with the aid of suitable substrates.

In 1949, Kun and Abood (20) used a triphenyltetrazolium chloride to do color estimations of succinic dehydrogenase activity. A preference for a dark blue rather than a red pigment for microscopic studies lead Rutenberg, Gofstein and Seligman (21) in 1950 to prepare a new ditetrazolium salt which yielded a blue diformazan on reduction. Rutenberg noted the effectiveness of this tetrazolium salt on detecting succinic dehydrogenase activity in biological systems.

Nachlas and coworkers (22) (23) reported that nitroblue tetrazolium chloride (Nitro BT) was a salt of better solubility and had improved pigment qualities and a higher redox potential.

In 1956, Farber and Breeding (24) demonstrated that in histochemical preparations, phenazine methosulfate served as an intermediate electron carrier between the

reduced enzyme and the tetrazolium salt, Nitro BT. Nachlas and coworkers (25) have reported similar results.

Nitro BT with phenazine methosulfate as an intermediate hydrogen acceptor have been used to study lactate (26) succinate (25) and malate (28) dehydrogenase activities. The enzymes could be located after starch gel (27), agar gel (29) or polyacrylamide gel (28) electrophoresis.

The Purpose

Studies conducted by Smith and Yamada (30) have shown that the first enzyme involved in pyrimidine catabolism, dihydrouracil dehydrogenase (4,5 - dihydrouracil: $NADP^+$ oxidoreductase, E.C. 1.3.1.2), exists in the particulate fraction as well as the cytosol fraction of mammalian liver. The cytosol and particulate enzymes differ in a number of ways other than in subcellular distribution. Dihydrouracil dehydrogenase catalyzes a reversible reaction and is specific for $NADP^+$ (1,14,30,48,49), although in the current work it has been shown that NAD^+ is a cofactor at high nonphysiological pH values. Barret et al (49) have concluded that the same enzyme acts on both substrates.

In contrast to dihydrouracil dehydrogenase, NADH as well as NADPH are cofactors at physiological pH for uracil reductase fractions (30). ATP plus Mg^{2+} inhibit enzyme activity and the reaction catalyzed is essentially nonreversible.

The work presented in this thesis is a continuation of the work reported by Smith and Yamada (30). The following are its objectives:

A) to clarify the location and numbers of particulate enzymes.

B) to determine whether or not the complete catabolic

pathway for pyrimidines is present in rat liver mitochondria.

C) to investigate the role of thymine as substrate for NADH dependent reductase

D) to investigate the role of dihydrothymine as substrate for NAD^+ dependent dehydrogenase.

E) to study the various forms of the supernatant enzyme.

MATERIALS AND METHODS

Materials

Dihydrouracil (Sigma grade), dihydrothymine (Sigma grade), uracil (Sigma grade), thymine (Sigma grade), NADH (Sigma grade), NAD^+ (Sigma grade), NADPH (Sigma grade), $NADP^+$ (Sigma grade), adenosine triphosphate (disodium salt), Sigma 104 phosphatase, disodium salt (Sigma grade), Trizma HCl (reagent grade) Trizma base (reagent grade), ethylene-diamine tetraacetic acid (Sigma grade), p-dimethylamino-benzaldehyde, β-ureidopropionic acid, β-ureidoisobutyric acid, β-alanine, β-aminoisobutyric acid, p-nitrophenol phosphate (disodium salt), Triton X-100, and adenosine -5' - phosphate, type III were purchased from the Sigma Chemical Company.

Glycerol (Analar), dipotassium hydrogen orthophosphate (Analar), potassium dihydrogen orthophosphate (Analar), magnesium chloride, nitroblue tetrazolium, tertiary butyl alcohol, 2,4,6 collidine, and ammonium molybdate were purchased from British Drug Houses Limited.

Sucrose (special enzyme grade), ammonium sulfate (special enzyme grade), bovine serum albumin, phenazine methosulfate, and uracil-2-^{14}C were purchased from Schwarz/Mann.

Acrylamide, N,N' methylene bis acrylamide, and N,N,N',N' tetramethylethylenediamine were purchased from Eastman Organic Chemicals.

Glucose-6-phosphate and monosodium glutamate were purchased from the Nutritional Biochemical Corporation.

Hydroxide of Hyamine 10-X was purchased from Packard. Photoflo 200 solution was purchased from Eastman Kodak. Omnifluor was purchased from New England Nuclear Corporation. Sephadex G-100 was purchased from Pharmacia. Toluene (spectroanalyzed grade) was purchased from Fisher Scientific Company. RDS-J tracking dye concentrate was purchased from Canalco Industrial Company. Coomassie brillant blue R 250 was purchased from Consolidated Laboratories Limited.

Preparation of Solution

All solutions were prepared using deionized glass-redistilled water and it was included in the incubation mixtures when necessary. Unless otherwise specified all solutions except NaCl and sucrose solutions were adjusted to pH 7.0. The sucrose and buffer solutions were stored in a refrigerator at 2^0 for a week and all other solutions were stored at -80^0 until they were required for use.

Preparation of Liver Homogenates for Subcellular Fractionation Studies

Male Holtzman rats weighing 200 to 350 grams, were decapitated, their livers perfused *in situ* with ice cold 0.9% NaCl, removed and weighed. All subsequent procedures were done in a cold room at $2\text{-}4^0$. The livers were homogenized in 8 volumes of 0.25 M sucrose - 3mM $MgCl_2$ - 5mM β-mercaptoethanol by means of a Potter- Elvehjem apparatus equipped with a Teflon pestle. The homogenate was filtered through eight layers of cotton gauze (28 x 24 mesh).

Preparation of Subcellular Fractions

Intracellular constituents were separated by a modification of the method of Sawant, Shibko, Jumta and Tappel (31) as shown in Figure 2.

A crude nuclear fraction was sedimented from the homogenate by centrifugation at 750 x g for 10 minutes. The resulting supernatant fraction was centrifuged at 3,300 X g for 10 minutes, to sediment the heavy mitochondria. Light mitochondria and lysosomes were sedimented at 16,300 X g for 20 minutes and microsomes at 160,00 X g for 60 minutes to leave a final supernatant fraction (S-160). Each particulate fraction was washed once with sucrose-Mg+, recentrifuged, and then suspended in 0.25 M sucrose - 1mM EDTA (pH 7.0) - 5mM β - mercaptoethanol. The washings were combined with their respective supernatant fractions.

A refrigerated Sorvall RC-2 centrifuge with fixed angle SS-34 rotor was used for preparation of the crude nuclear, heavy and light mitochondria fractions while a Beckman L2-65B centrifuge with a 50.1 rotor was used to obtain the microsomal and supernatant fractions.

Dialysis of Fractions

After preparation, a portion of each subcellular fraction was diaylzed for 16 hours against 100 times its volume of 0.05 M phophate buffer (pH 7.0) - 1mM EDTA - 5mM β - mercaptoethanol, in a continous flow dialyzing apparatus (Model B, Oxford Laboratories).

Storage of Enzyme Fractions

All fractions were stored in ice during preparation and then frozen for storage at -78^{0} in 10 - 15 ml aliquots. Frozen aliquots were thawed only once before use.

Identification of Subcellular Fractions

Fractions were identified by the presence or absence of marker enzymes: Glutamate dehydrogenase for mitochondria and 5' nucleotidase for plasma membranes. The activities of the marker enzymes were determined prior to freezing.

Protein Estimations

Protein was determined by the method of Lowry, Rosebrough, Farr and Randall (32) with crystallized bovine serum albumin as the reference standard.

Enzyme Assays

Glutamate Dehydrogenase

The method of Beaufay, Bendall, Baudhuin and De Duve (33) was used. The reaction mixture contained potassium phosphate buffer (pH 7.7) 20 µmoles; nicotinamide, 30 µmoles; β-mercaptoethanol, 5 µmoles; NAD, 1.4 µmoles; 0.1% (v/v) Triton X-100; glutamate and enzyme to a total volume of 1 ml. The increase in optical density was followed at 340 nm at 25^0 in a Beckman D.B.G. recording spectophotometer with the reference cell containing all the components of the system except glutamate. Triton X-100 was used to solubilize the enzyme. The reaction was started by the addition of glutamate, (13 µmoles). 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. Specific activity is the number of enzyme units per milligram of protein.

5' Nucleotidase

The method of Emmelot, Bos, Benedetti, and Rumke (34) was used. The assay mixture consisted of Tris buffer, pH 7.2, 50 µmoles; potassium chloride, 100 µmoles; $MgCl_2$, 10 µmoles; tissue preparation, 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 10 to 15 minutes at 37^0 after which the reaction was terminated by the addition of 1 ml 10% TCA. The resulting mixture was centrifuged at 17,500 X g in a Sorvall RC-2 at 0^0 for 15 minutes and 1 ml of the supernatant was taken for the determination of inorganic phosphate according to the method of Gomori (35).

Control tubes were incubated without substrate which was added immediately after the reaction was stopped. Values obtained for controls were substracted from the test values. All assays were done in duplicate.

A unit of enzyme activity is defined as that quantity which catalyzes the release of 2 µmoles of inorganic phosphate per hour. Specific activity is the number of enzyme units per milligram of protein.

Pyrimidine Reductase

The enzyme activity was assayed spectrophotometrically according to the procedure of Yamada (36). Pyrimidine reductase activity was measured by determining the decrease in absorbance at 290 nm or 295 nm resulting from the conversion of pyrimidine to dihydropyrimidine in the presence of the cofactor NADH or NADPH. The extinction coefficients per μmole of substrate reduced are 14.84 and 17.74 for uracil in the presence of NADPH and NADH respectively; and 13.28 and 16.26 for thymine in the presence of NADPH and NADH respectively.

The standard assay mixture contained the following in a final volume of 1.5 ml: potassium phosphate buffer (pH 7.5), 250 μmoles; β-mercaptoethanol, 7.5 μmoles; NADH or NADPH (pH 6.0), 0.25 μmoles; uracil or thymine (pH7.0), 0.2 μmoles; and enzyme made 5.4 M with glycerol (unless specified otherwise) and diluted to 0.5 ml with 0.05 M potassium phosphate buffer (pH 7.0) - 5 mM β-mercaptoethanol. The final pH of the incubation mixture was 7.5. The reaction was started by the addition of substrate and tubes were incubated for 10 minutes at 37^0. The reaction was terminated by placing the tubes in ice and adding 0.45 ml of 2.12 M perchloric acid. The mixtures were centrifuged at 17,500 X g for 10 minutes at 0^0 in a Sorvall RC-2 centrifuge. A 1 ml aliquot of each supernatant

was removed and added to 0.07 ml of 10 N NaOH.

The optical density of the resultant solution was read at 290 nm when uracil was used as the substrate or at 295 nm when thymine was used. Controls were incubated in the presence of coenzyme but without substrate which was added immediately after the addition of perchloric acid. All assays were done in duplicate.

One enzyme unit is defined as that quantity which catalyzes the formation of 1 µmole of dihydropyrimidine per hour. Specific activity is the number of enzyme units per milligram of protein.

Dihydropyrimidine Dehydrogenase

Enzyme activity was assayed by the spectrophotometric procedure of Yamada (30, 36). Dihydropyrimidine activity was measured by the increase in absorbance at 290 nm with dihydrouracil or 295 nm with dihydrothymine resulting from the conversion of dihydropyrimidine to pyrimidine in the presence of NAD+ or NADP+. The extinction coefficients per μmole of product formed are as follows: 14.94 and 17.74 for uracil in the presence of NADPH and NADH respectively, and 13.16 and 16.26 for thymine in the presence of NADPH and NADH, respectively.

The standard assay mixture contained the following in a final volume of 1.5 ml: potassium phosphate buffer (pH 7.5), 250 μmoles; β-mercapotethanol, 7.5 μmoles; ATP (pH 7.0), 15 μmoles; $MgCl_2$, 15 μmoles; enzyme diluted to 0.5 ml with 0.5 M potassium phosphate buffer, pH 7.0, NAD^+ or $NADP^+$, pH 8.0, 1.5 μmoles; and dihydrouracil or dihdyrothymine pH 7.0, 15 μmoles. The reaction was initiated by addition of substrate and the tubes were incubated for 10 minutes at 37^0. The reaction was terminated by placing the tubes in ice and adding 0.45 ml of 2.12 M perchloric acid. Following centrifugation of the mixture at 17, 500 X g in a Sorvall RC-2 at 0^0 for 10 minutes, a 1 ml aliquot of the supernatant was added to 0.25 ml of 1 M EDTA, pH 10.8. To this mixture 0.07 ml of 10 M NaOH was added.

Controls were incubated in the presence of coenzymes but without substrate which was added immediately after the addition of perchloric acid. All assays were done in duplicate.

One enzyme unit is defined as that quantity which catalyzes the formation of 1 μmole of pyrimidine per hour. Specific activity is the number of enzyme units per milligram of protein.

Purification of Heavy Mitochondria

Further purification of the heavy mitochondrial fraction (HM) was obtained through centrifugation procedures utilizing either a linear sucrose denisty gradient (procedure 1) or a discontinous sucrose gradient (procedure 2).

Procedure 1. In early experiments a modification of the procedure of Nass (44) was used. Three milliliters HM fraction were layered on 25.5 ml of a sucrose density gradient linear from 1.91 to 0.5 M sucrose and centrifuged for 1 1/2 hours at 70,000 X g in a Spinco S.W. 25.1 swing out bucket rotor. Fractions of 1.5 ml were then collected.

Procedure 2. The discontinous sucrose density gradient utilized by Sarazala et al (37) was modified, to achieve larger yields of comparable purity in a shorter time.

The HM fraction obtained from the subcellular fractionation procedure as outlined in Figure 2 was suspended in 0.25 M sucrose - 1mM EDTA (pH 7.0) - 5mM β - mercaptoethanol. 1.2 ml of HM fraction were applied on top of discontinous sucrose gradients neutralized with 1M Tris buffer (pH 7.0) and having the following composition: 0.5 ml 20% sucrose, 0.5 ml 25% sucrose, 0.5 ml 30% sucrose, 0.75 ml 35% sucrose, 2.8 ml 45% sucrose, 2.75 55% sucrose and 2.0 ml 65% sucrose (Figure 4). The gradient tubes were spun in a SB 283 rotor of an International Model B60 preparative ultracentrifuge at 130,000 X g for 30 minutes.

Following centrifugation three distinct layers were visible. Each layer was withdrawn from the gradient tubes with the aid of a syringe equipped with a U shaped needle. The layer above the mitochondrial band M_1 was withdrawn followed by the mitochondrial containing layer M_2 and the lower layer M_3.

In early experiments the mitochondrial containing layer M_2 was centrifuged at 35,000 X g in a SS - 34 rotor of a Sorvall RC - 2 centrifuge. The resultant mitochondrial pellet was resuspended in 0.25 M sucrose - 1mM EDTA (pH 7.0) - 5mM β-mercaptoethanol while the supernatant was added to the M_1 fraction. This step was discontinued in later experiments due to the inability to obtain a compact mitochondrial pellet.

All fractions were dialyzed routinely for 16 hours at $2\text{-}4^0$ in either 0.005 M potassium phosphate buffer (pH7.0) - 5mM β - mercaptoethanol - 1 mM EDTA (pH 7.0) or when 5' nucleotidase was being assayed in buffer containing Tris HCl in place of potassium phosphate.

Each fraction was assayed for the following enzyme activities; glutamate dehydrogenase, 5' nucleotidase, NADH - linked uracil reductase, NADH - linked thymine reductase, NADPH - linked uracil reductase and NADPH - linked thymine reductase as described under "Materials and Methods".

Radioactive Uracil - 2 - ^{14}C Studies

Paper Chromatography of Reaction Products

To the standard assay mixture for NADH - linked uracil reductase was added 0.6 μmole of uracil - 2 - ^{14}C (0.0035 μCi). In these experiments, enzyme fractions were not suspended in glycerol because it interfered with the separation of reaction products. Duplicate tubes were incubated at 37^0 for 30 minutes. The reaction was stopped by boiling for 3 minutes. Control tubes contained no enzyme which was added just prior to boiling. The tubes were centrifuged at 16,000 X g for 20 minutes in a Sorvall RC - 2B refrigerated centrifuge. Fifty micro-liter aliquots (representing 300 - 500 cpm per strip) were applied to Whatman No. 1 filter paper strips (1.8 X 38 cm) which were developed by descending chromatography in collidine saturated with water as described by Fritzson (5). The solvent was permitted to travel 35 cm beyond the point of application of the sample.

Staining Methods of Detection

A similar procedure to that of Fink, Cline, McGaughy and Fink (3) was employed to detect the biological conversion of pyrimidines to β - amino acids through the intermediate dihydropyrimidine and β - ureido acid stages.

Detection of Pyrimidines and β - amino acids

The chromatograms were air - dried and then viewed under an ultraviolet lamp (2537 A^0) to mark the location of the pyrimidines. β - Amino acids were then detected by spraying the chromatograms with ninhydrin and developing the spots by drying the chromatograms in an oven at 80^0 for 10 minutes. β - Amino acids must be detected prior to spraying with acidic p - dimethylaminobenzaldehyde since most of the ninhydrin color is destroyed by this procedure.

Detection of Dihydropyrimidines and Ureido Acid Compounds

Dihydropyrimidines and β - ureido acids were detected by noting the color development with p - dimethylamino-benzaldehyde by the following procedure:

1) Dihydropyrimidines were converted to β - ureido acids by uniformly spraying the chromatograms, once the solvent had evaporated, with 0.5 N NaOH and allowing the chromatograms to dry for 30 minutes.
2) The chromatograms were then sprayed with an acidic p - dimethylaminobenzaldehyde solution containing: 1 g p - dimethylaminobenzaldehyde, 10 ml concentrated HCl, 100 ml ethanol and dried for a 10 - 15 minute period in an oven at 100 - 110^0 or allowed to hang in a ventilated room for 2 to 6 hours depending upon the apparent rate of color development in the spots and in the reagent background. The color spots were

viewed in transmitted light to facilitate the outlining of spots. It should be noted that the rate of color development is affected by the humidity and hence the variation in the developing time required.

Detection of Radioactivity

After the development of the chromatograms, the chromatograms to be used for the detection of radioactivity were cut into 1 cm segments. Each segment was eluted with 500 µl of water overnight at 25^0 and then 15 ml of toluene - based scintillation fluid containing 0.4% (w/v) omnifluor and 30% ethanol were added. Radioactivity was counted in a Beckman LS - 250 scintillation spectrometer at an efficiency of 80%.

Detection of Labelled Carbon Dioxide

A modification of the method of Canellakis (1) was used. Purified mitochondrial fractions (M_2) were incubated in a final volume of 3 ml, in the following (in µmoles): potassium phosphate buffer (pH 7,4), 75; uracil, 0.7143 (0.1 µCi); NAD+, 0.4; absolute ethanol, 100 µl; 30 µg alcohol dehydrogenase (Nutritional Biochem.) and M_2 (6 mg protein). Duplicate samples were incubated at 37^0 in a shaker bath in 10 ml Erlenmeyer flasks equipped with rubber wells (Kontes Co.) to which 0.3 ml hyamine hydroxide was added to absorb radioactive CO_2. Flasks were

preincubated for 15 minutes prior to the addition of radioactive uracil. After incubation for 30 minutes, 0.3 ml of 30% trichoroacetic acid was added to stop the reaction. Flasks were incubated for an additional hour to ensure the complete absorption of CO_2. To scintillation vials containing toluene - based scintillation fluid (4 g omnifluor per liter toluene), 0.2 ml hyamine hydroxide from the wells of the reaction flasks was added. Radioactivity was determined as before. Appropriate controls lacking M_2 were included.

Partial Purification of Supernatant Enzyme

a) Ammonium Sulfate Fractionation

Liver homogenate was prepared as described previously. The homogenate was centrifuged at 150,000 X g for 1 hour in a Spinco Model L4 preparative ultracentrifuge at 2^0 and the supernatant fraction was dialyzed in 100 volumes of 0.05 M potassium phosphate buffer (pH 7.0) - 5mM β-mercaptoethanol - 1 mM EDTA (pH 7.0) at 2^0 overnight.

Enzyme protein was precipitated from the dialyzed supernatant by the addition of ammonium sulfate (0.7g/10 ml = 10% saturation) ammonium sulfate was added slowly with constant stirring to 30% saturation at 0^0. The mixture was stirred slowly for 15 minutes and centrifuged at 12,000 X g for 20 minutes to obtain a precipitate designated AS_1. The procedure was repeated with the resulting supernatant by an increase in the ammonium sulfate to 60% to obtain a second precipitate designated AS_2. A third precipitate AS_3 was obtained by an increase in the ammonium sulfate in the AS_2 supernatant to 80% final saturation.

The three precipitates AS_1, AS_2 and AS_3 were each dissolved in a minimum volume of 0.05 M phosphate buffer pH 7.0 - 5mM β-mercaptoethanol and dialyzed as before.

The AS_2 fraction routinely provided a 4 to 5 fold purification.

b) Sephadex Column Chromatography

Prpearation of Gel

Twenty grams of Sepadex G-100 was allowed to swell in 0.02 M phosphate (pH 7.3) - 10 mM β-mercaptoethanol buffer in a cold room at 4^0 for three days. During this time the supernatant liquid and fines were decanted and replaced four times with two liter aliquots of the above buffer.

Packing the Column

The gel slurry was then poured into a glass column of 2.5 cm diameter to a height of 90 cm (bed volume, 240 ml). The first few cm were allowed to settle by the force of gravity alone. The outlet was then opened and the final pressure head was gradually increased to its final pressure. After the column was packed with the slurry, a sample applicator was added and the column was equilibrated with two bed volumes of 0.02 M phosphate (pH 7.3) - 10 mM B-mercaptoethanol buffer.

Determining the Void Volume

A 5 ml aliquot of 0.2% dextran blue in phosphate buffer was applied to the column and 4 ml fractions were collected and read in a Klett colorimeter with a filter of 650 nm. The total volume was 104 ml.

Application and Collection of Sample

A 25 ml aliquot of an AS_2 fraction containing 53 units of dihydrouracil NAD+ linked dehydrogenase activity was

placed on the sample applicator. Four milliliter fractions were collected in a cold room at 2^0 at a flow rate of 30.96 ml per hour and washing with buffer was continued until the optical density of the effluents was reduced,at 260 and 280 mμ, to low, constant levels.

Determination of Protein Concentration

Protein concentration of each fraction was determined by the method of Warburg and Christian (38) from the optical density readings at 260 and 280 mμ.

Disc Gel Electrophoresis

A) Solutions and Gels

The nomenclature of Davis (39) is used throughout.

Solutions A and B - were prepared according to the method of Williams and Reisfeld (40).

Solution A- contained 48 ml 1 N HCl, 6.85 g Tris, 0.046 ml N,N, N',N' tetramethylethylenediamine (TEMED) and water to 100 ml. The pH of the solution was 7.5.

Solution B- contained 39 ml 1 M $H_3 PO_4$, 4.95 g Tris, 0.46 ml TEMED and water to 100 ml. The pH of the solution was 5.5.

Solution C- was prepared according to the method of Deitz and Lumbrano (41). It contained 30 g acrylamide, 0.8 g N,N'methylenebisacrylamide (Bis) and water to 100 ml.

Solution D, E and ammonium persulfate - were prepared according to the method of Davis (39).

Solution D- contained 10.0 g acrylamide, 2.5 g Bis and water to 100 ml.

Solution E- contained 4 mg riboflavin and water to 100 ml.

Ammonium persulfate- contained 0.14 g ammonium persulfate and water to 100 ml.

Electrophoresis buffer

Tris - glycine buffer at pH 8.3 (39) was used.

routinely. The buffer contained 6.0 g Tris base and 28.8 g glycine dissolved in water to 1 liter.

In a few experiments, Tris - veronal at pH 7.0 (40) (5.52 g veronal and 1 g Tris base) or Tris - maleate (11.85 g Tris - maleate and 5.07 g Tris base per liter), pH 7.0, was used in place of Tris -glycine.

Separating Gel (small pore)

Two volumes of A, 4 volumes of C, 2 volumes of water adn 8 volumes of ammonium persulfate were mixed to give a gel of 7.7% total concentration (T) (monomers + cross linking agent (42)) with 2.6% cross-linking (C). To 25 ml of this solution 6.25 ml. of water were added to form a 6.16% gel with 2.6% cross-linking. Other gels of different % T but constant C were prepared by appropriate dilution with water.

Spacer Gel (large pore)

The spacer gel was prepared according to the procedure of Davis (39). It contained 1.0 ml solution B, 2.0 ml solution D, 1 ml solution E and 4 ml 40% (w/v) sucrose solution. The pH of the spacer gel was 5.5.

Tracking Dye

Canalco tracking dye concentrate (RDS-J) was used.

Staining Solution

(A) Enzyme staining solution: The standard reaction mixture used to assay dihydrouracil dehydrogenase activity, but lacking β-mercaptoethanol because it would reduce the

tetrazolium salt, was incorporated into the staining solution as follows: 2.5 ml nitroblue tetrazolium chloride (1 mg/ml), 0.25 ml phenazine methosulfate (1 mg/ml) and 1.0 ml of 0.1 M NaCl were included in the standard reaction mixture to give 10 ml of staining solution (sufficient for staining 4 gels). Dihydrouracil, coenzymes and ATP were prepared weekly and stored frozen at -20^0; buffers and salt solutions were prepared weekly. Nitroblue tetrazolium and phenazine methosulfate were prepared monthly and stored in the dark at $2 - 4^0$.

(B) Protein staining solution: Protein stain was freshly prepared by a 1:20 dilution in 12.5% TCA of a 1% (w/v) aqueous stock solution of Coomassie blue.

Disc Gel Electrophoresis Procedure

The procedure of Davis (39) was followed. Preparation of gels and electrophoresis were performed in a cold room at $2 - 4^0$. All tests were in triplicate; glass tubes of 63 mm length and 5 mm internal diameter were used. To each tube, 0.15 to 0.2 ml of 40% sucrose was added in place of sample gel, overlaid with 0.15 ml of spacer gel and subsequently filled with separating gel (6.16% T unless specified otherwise).

The tubes were inserted into a Canalco Model 12 electrophoresis apparatus. An aliquot of 15 to 100 µl of

sample (containing partially purified enzyme preparation, 15 µl of tracking dye concentrate, 100 µl of 82% sucrose and electrophoresis buffer to make 1.0 ml) was added to each tube above the spacer gel. Each aliquot consisted of from 100 to 450 µg of enzyme protein (0.02 - 0.07 units).

Electrophoresis was performed at 2.5 mA per tube until the tracking dye had migrated 40 - 50 mm. The position of the tracking dye was marked with a short piece of wire. The gels were then transferred to 75 X 100 mm culture tubes and covered with 2.3 ml of staining solution. Incubation was at 37^0 in the dark. Control gels were incubated in the absence of dihydrouracil, dihydrothymine or coenzyme.

Gels were stained within 3/4 hour for the higher protein loads but took up to 2 hours for the lower. Gels were then washed with tap water and stored in 7.5% acetic acid at 2 - 4^0. A Joyce - Loebl chromoscan with disk integrator attachment was used for densitometer tracings of the stained gels.

Protein Staining Procedure

The procedure of Chrambach, Riesfeld, Wychoff and Zaccari (43) was used.

After the electrophoretic fractionation, protein fixation of the polyacrylamide gels was brought about by suspending the gels in a 10 to 40 fold volume of 12.5% TCA

preferably with agitation, for 30 minutes. Protein bands were then located by immersing the gel in a Coomassie blue staining solution for 30 minutes to 1 hour. The gel was transferred into a 10% TCA solution and photographed within 48 hours. It was noted that band intensity increases in the succeeding 48 hour period. Gels were stored in 7% acetic acid solution.

Molecular Weight Determination

The method of Hedrick and Smith (46) was used to determine the molecular weight of the protein associated with bands located by disc gel electrophoresis. A standard plot of the relationship between total polyacrylamide gel concentration and the log Rm prepared by Bose and Yamada (58) was used. Proteins of known molecular weight used included apoferritin, catalase, gamma-globulin, alcohol dehydrogenase, albumin monomer, ovalbumin and chymotrypsin. Gel concentration ranged from 4 to 9%. The molecular weight of the protein bands was determined by comparing the value of the slope of the resulting linear plot of Log Rm and total gel concentration (Figure 13) to slope values of the above marker proteins.

Detection of an Enzyme Band Associated with the Conversion of Pyrimidines to Dihydropyrimidines

In an attempt to stain the reverse reaction, pyrimidine to dihydropyrimidine, uracil or thymine and NADH or NADPH

replaced dihydrouracil or dihydrothymine and NAD^+ or $NADP^+$ in the enzyme staining solution described previously. Location of enzyme activity was detected by the absence of stain.

RESULTS

Intracellular Distribution of Enzymes

Marker Enzymes

The distribution of the marker enzymes glutamate dehydrogenase and 5' nucleotidase among the subcellular fractions of rat liver homogentate is shown in Table 1A.

The homogenate was prepared as described under "Materials and Methods" and the intracellular constituents were separated by the differential centrifugation procedure outlined in Figure 2.

The data in Table 1A indicates that 63% of the glutamate dehydrogenase activity was present in the heavy mitochondrial fraction which also had the highest specific activity (2.86).

5' nucleotidase activity appeared to be spread fairly evenly between the microsome-supernatant (33.4%), crude nuclei (32.7%) and heavy mitochondria (31.7%). The highest specific activity for 5' nucleotidase was found in the light mitochondria (3.21) indicating contamination of this fraction by plasma membranes.

The distribution of the marker enzymes is similar to the distribution obtained by Smith and Yamada (30) in rat liver except that their per cent recovery of 5' nucleotidase activity in the heavy mitochondria was lower.

Pyrimidine Reductase

The distribution of NADPH - linked uracil reductase, NADH - linked uracil reductase among the subcellular fractions of rat liver homogenate is shown in Table 2.

The homogenate was prepared as described under "Materials and Methods" and the intracellular constituents were separated by differential centrifugation according to the procedure outlined in Figure 2.

From Table 2 it is seen that over 51% of the NADPH-linked uracil reductase activity and 57% of the NADPH - thymine reductase activity is distributed between the heavy and light mitochondrial fractions with the highest specific activity in the light mitochondrial fraction in each case.

On the other hand 47.7% of the NADH - linked uracil reductase and 51% of the NADH - linked thymine reductase activity was found in the heavy mitochondrial fraction which also had the highest specific activity.

The activity distribution pattern for the pyrimidine reductases supports the results of Smith and Yamada (30) who suggested that the localization of the catabolic pathway in the particulate fractions as reported earlier by others (1, 6).

Pyrimidine Dehydrogenase

The partial purification of pyrimidine dehydrogenase activity associated with either cofactors, NAD+ or NADP+, of either substrate, dihydrouracil or dihydrothymine, is summarized in Table 4.

The supernatant contained a two fold increase in specific activity in each case following centrifugation of the homogenate at 150,000 X g for one hour. Further purification by ammonium sulfate treatment as described in the "Material and Methods" section was conducted. The results revealed that enzyme protein precipitated between 30 - 60% saturation routinely provided a purification of four fold compared to homogenate activity. Further purification of liver extracts beyond step 3 using Sephadex G - 100 increased purification by an additional two fold.

Purification of Heavy Mitochondria

Procedure I : Linear Sucrose Density Gradient

Figure 3 shows the H.M. fraction by centrifugation in a linear sucrose density gradient (procedure I) did not provide a well defined separation of NADPH and NADH dependent reductases. Notable, however, was the finding that activity with uracil or thymine as the substrate peaked in the same fraction. The NADH - dependent activity coincided with that of glutamate dehydrogenase whereas the NADPH dependent activity was in the position routinely marked by 5' nucleotidase as reported by Smith and Yamada (30).

Procedure II : Discontinuous Sucrose Gradient

Distribution of Marker Enzymes. Table 1 (B) shows the distribution of the marker enzymes glutamate dehydrogenase and 5' nucleotidase following fractionation of the H.M. fraction on a discontinuous sucrose gradient. The results indicate that 90% of the glutamate dehydrogenase activity was recovered in the M_2 fraction and that the mitochondria were purified an additional 3 - fold by this procedure.

5' Nucleotidase activity was distributed throughout all the mitochondrial fractions with greatest per cent recovery in fraction M_2 while the greatest specific activity was found in fraction M_1.

Distribution of Pyrimidine Reductase Activity

The distribution of NADH - dependent reductase activity was similar with uracil or thymine as substrate (Table 3) and followed the distribution of the mitochondrial marker enzyme glutamate dehydrogenase (Table 2). The M_2 fraction contained the major portion of NADH - dependent activity, with either thymine or uracil as substrate. The specific activity of M_2 with uracil as substrate was usually higher than that found for thymine. The NADH-dependent enzyme was solubilized in 0.1 M Pi buffer (pH 7.4) rather than in glycerol and the apparent K_m values at (pH 7.4) were estimated to be 0.085 mM for uracil, 0.118 mM for thymine and 0.103 mM for NADH with either substrate present. The K_m value for uracil is lower than that reported earlier for the less pure HM fraction (30).

The distribution of the NADPH - dependent reductase activity with either uracil or thymine as substrate corresponded closely with that of the marker enzyme 5' nucleotidase which is associated with plasma and endoplasmic reticulum membranes (50-53). Discontinuous sucrose gradient fractionation resulted in a 2 - fold enrichment of the enzymes and they were now located prinicipally in fraction M_1.

Fraction M_1 was almost as active with uracil as with thymine. The reductase was solubilized as above and its affinity for the two substrates determined; the apparent

K_m values at (pH 7.4) were estimated to be 0.118 mM for uracil, 0.113 mM for thymine and 0.065 mM for NADPH with either substrate present. The K_m value for NADPH is lower than that found earlier for less pure fractions (30).

Reaction Products

Mitochondrial fraction M_2 was incubated in the standard assay mixture containing uracil - 2 - ^{14}C and NADH. Incubation was for 30 minutes at 37^0. The reaction products were separated by paper chromatography as described in "Materials and Methods".

Standards for uracil (R_f= 0.70), dihydrouracil (R_f = 0.53), β-ureidopropionic acid (R_f= 0.02) and B-alanine (R_f= 0.02) were run concurrently with the sample chromatograms. The chromatograms when sprayed with p - dimethyaminobenzaldehyde revealed the presence of uracil (R_f = 0.70), dihydrouracil (R_f = 0.53) and occasionaly a faint spot at R_f = 0.02 appeared implying the possible presence of β - ureidopropionic acid. Strips dipped in ninhydrin revealed a spot near the origin at R_f = 0.02. Whether this spot represented β - alanine or residual protein was uncertain.

Recovery of total radioactivity initially added to the reaction mixture was 85 to 90%. Fifty - two and a half per cent was recovered as uracil while dihydrouracil contained 30% and 7.5% as the ninhydrin - positive spot near the origin (Figure 5).

Formation of CO_2

Purified mitochondria (M_2) were incubated in reaction media containing graded concentrations of uracil - 2 - ^{14}C (at constant specific radioactivity). Figure 6 shows that the formation of radioactive CO_2 increased with increasing uracil concentrations and began to level off at 10 mM. Linearity between CO_2 produced and time was observed for 30 minutes with 6 to 10 mg of mitochondrial protein per flask. The amount of uracil degraded by mitochondria was estimated to be about 10% of that of the homogenate fraction, both with NAD+ as coenzyme.

Disc Gel Electrophoresis

Separation and Detection of Enzyme Bands

Disc gel electrophoresis of the partially purified enzyme preparation in 6.16% acrylamide revealed four bands of enzyme activity when either dihydrouracil or dihydrothymine was used as substrate and NAD+ or NADP+ as the cofactor. In each case two heavily stained bands hereafter referred to as major bands 1 and 2 were accompanied by two very weakly stained bands denoted as minor band 3 and band 4; band 3 usually appeared with both substrates and coenzymes but band 4 was not often discernible. Figures 7 and 10 show developed gels and densitometer tracings. No staining was discernible in controls incubated in the absence of substrate or coenzyme. The weak activity of the minor bands suggest they could possibly be artifacts due to binding to non active proteins. In Figure 7E two very small bands appeared near the tracking dye but were very seldom found with other preparations.

The relative mobilities of the enzyme bands were reduced when the gel concentration was increased to 7-8% and the distance between the bands was also reduced. Other electrophoresis buffers used included Tris - maleate (pH 7.0) and Tris - veronal. In Tris - maleate the two major enzyme bands 1 and 2 were clearly visible but not as well separated as in the Tris glycine (pH 8.3) the electrophoresis buffer used in the standard procedure. The minor bands 3 and 4 were not detected. Staining of enzyme activity was not detectable in Tris - veronal (pH 7.0).

Intensity of Staining and Protein Concentration

Dihydrouracil or Dihydrothymine and NAD+

The intensity of staining of the major bands upon incubation in the presence of dihydrouracil (Figure 7) or dihydrothymine (Figure 9) and NAD+ increased with increasing protein concentration. Color development occurred within 3/4 hour with higher concentrations but took up to 2 hours with the lower concentrations.

From densitometer tracings a linear relationship between intensity of staining and protein concentration up to 200 µg was obtained for both major bands, the intensity being greater for band 1 than for band 2 (Figures 11 and 12) when either dihydrouracil or dihydrothymine was used as the substrate. With NAD+ as coenzyme both major bands showed two times more activity with dihydrouracil as substrate compared to dihydrothymine. No staining was discernible in the control tubes even in those containing the larger aliquots of enzyme protein.

Dihydrouracil or Dihydrothymine and NADP+

The intensity of the staining of the major bands upon incubation in the presence of dihydrouracil (Figure 11) or dihydrothymine (Figure 12) and NADP+ increased with increasing protein concentration.

From densitometer tracings a linear relationship between intensity and protein concentration up to 175 µg

was obtained for both major bands, the intensity being greater for band 1 than band 2 when either dihydrouracil or dihydrothymine was used as the substrate. With NADP+ as coenzyme, both bands showed 2 - 3 times more activity with dihydrothymine as the substrate compared to dihydrouracil.

Protein Staining

Duplicate tubes were stained with Coomassise blue (43) to show the number of protein bands in the enzyme preparations. A typical protein stained gel is shown in Figure 7.

Experiments with 150,000 X g Supernatant

In other experiments enzyme activity was also detected when aliquots of 150,000 X g supernatant (100 - 150 µg protein) were applied to the gels. However, the enzyme bands though visible were faint, particularly band 2 as well as the minor bands 3 and 4 which did not appear routinely on the denistometer tracings. Larger aliquots of protein often retarded the migration of enzyme protein into the separating gel.

Relative Mobilities

The relative mobilities (R_m) of the major enzyme bands calculated from densitometer tracings did not differ significantly when NAD+ was replaced by NADP+ in the staining solution or when the amount of protein added per gel was increased from 110 to 440 µg (Table 5). The standard deviation of the measurement of R_m was in the

same range as that reported by others using a similar method of measurement (45). Furthermore, it was found that the R_m values were also the same with dihydrothymine replacing dihydrouracil with either coenzyme present.

Mobilities and Gel Concentration

The relative mobilities of the two enzyme bands at different gel concentrations were determined (Figure 13). Two nearly parallel lines were obtained indicating that the bands represent isomeric proteins of very similar size but of different charge (46,47). If the bands had represented proteins of different size and charge non parallel lines intersecting at gel concentrations other than 0% gel concentrations would have resulted.

Enzyme Purification Beyond Ammonium Sulfate Fractionation

Further purification of liver extracts beyond ammonium sulfate fractionation (Table 4) included chromatography on Sephadex G-100. An additional two fold purification was achieved but did not significantly improve or alter the separation and detection of the two major enzyme bands upon electrophoresis.

Detection of an Enzyme Band Associated with the Conversion of Pyrimidine to Dihydropyrimidines

A single band with an R_m between 0.5 and 0.6 was detected upon staining of gels for conversion of pyrimidines to dihydropyrimidines. This procedure upon perfection may provide a useful method for detection of the enzyme activity associated with this reaction.

DISCUSSION AND CONCLUSIONS

The pathway for pyrimidine catabolism, shown in Figure 1, has undergone intensive investigation. Early works by Canellakis (1), Rutman et al (6) and Fritzson (15) reported this pathway to be localized primarily in the soluble fraction of rat liver. These early workers concentrated their efforts on investigating the activity of the entire pathway. Canellakis (1) and Rutman et al (6) measured the release of $^{14}CO_2$ from uracil - 2 - ^{14}C or thymine - 2 - ^{14}C in rat liver slices, homogenate and soluble fraction while Fritzson (15) based his investigations on the measurement of β - alanine - ^{14}C released from uracil - 2 - ^{14}C in homogenate and soluble fractions of rat liver. Both Canellakis (1) and Rutman (6) reported however, that their studies contained evidence that indicated that the catabolic pathway may exist in the particulate fraction but was inhibited by endogenous factors.

Early <u>in vivo</u> investigations of pyrimidine catabolism by Fink (2) had indicated that the conversion of dihydrothymine to thymine was not reversible. This work provided the first indication that two pathways might exist; one involved in pyrimidine synthesis and the other involved in pyrimidine catabolism.

Further evidence that the enzymes associated with pyrimidine metabolism may be located in the particulate fractions and that two pathways may be involved was supplied by Smith and Yamada (30).

Enzyme localization studies by these workers indicated that the first enzyme of this pathway, dihydropyrimidine dehydrogenase, could be separated into reductase and dehydrogenase activities with most of the reductase activity located in the particulate fractions of the liver cell and most of the dehydrogenase activity located in the cytosol fraction.

The subcelluar distribution studies, as shown in Table 2, indicated that most of the reductase activity was located in the particulate fractions.

The results from sucrose density gradient fractionation of heavy mitochondria (Figure 3, Tables 1 (B) and 3) are in agreement with those reported by Smith and Yamada (30) in terms of distribution of reductase activity. Use of a discontinuous gradient, however, provided a clearer separation of NADPH and NADH dependent reductase activities. Most of the NADPH dependent activity was located in the fraction designated M_1, which also contained the highest specific activity for the plasma membrane marker, 5' nucleotidase. Most NADH dependent

reductase activity as well as the mitochondrial marker enzyme, glutamate dehydrogenase, activity was located in the fraction designated M_2. In addition it was shown that the NADH dependent thymine reductase activity, not previously reported, has a similar distribution pattern to that of NADH dependent uracil reductase. The results also indicated that both the mitochondrial reductase and the reductase activity associated with plasma and endoplasmic reticulum membranes display activities toward uracil and thymine in line with the K_m values found.

Besides coenzyme specificity and intracellular location, the reductases have recently been found to differ most notably in sensititivity to Pi or EGTA and inhibition by calcium ions (54).

That purified mitochondrial fractions catalyze the degradation of uracil to form CO_2 to only a limited extent is shown in the present work. Concentrations of uracil as high as 10 mM were required to obtain maximal formation of CO_2. These results suggest that the degradative enzymes beyond uracil reductase are limiting in amount in the mitochondria so that dihydrouracil (dihydrothymine) formed must be extruded mainly into the cytosol for further degradation. Such an efflux of dihydrouracil (dihydrothymine) may perhaps be the

reason for the virtual non - reversibility of the reaction catalyzed by uracil reductase of these organelles with dihydrouracil (dihydrothymine) as substrate; significant activity in the reverse direction was found only after solubilization of mitochondria by glycerol (30).

It is of interest to note that carbamyl β-alanine hydrolase (β-ureidopropionase) catalyzes the rate - limiting step in the overall degradation of pyrimidines to β-alanine in mouse liver cytosol (18). This finding is in contradiction to early studies of rat liver cytosol by Canellakis (1) and Fritzson (4) in which it was reported that dihydrouracil dehydrogenase catalyzed the rate - limiting step. An additional factor to be considered is the high K_m value of 11.75×10^{-2} M found for dihydrouracil hydrase with dihydrouracil as substrate. This enzyme was purified 200 fold from calf liver (55). This high K_m value is in contrast to those of $1.5 - 20.0 \times 10^{-3}$ M found for dihydrouracil with dihydrouracil dehydrogenase of rat liver cytosol (15,30). However, no conclusions can be drawn as yet from these data since the enzymes were from different sources and only partially purified. Moreover, the activity of the degradative enzymes appears to vary in inverse relationship with growth rate (37,56,57) and as yet no studies have been done to investigate

possible concomitant changes in enzyme properties.

With regard to the substrate specificity of dihydrouracil dehydrogenase, the cytosol enzyme from rat or bovine liver was found to catalyze the reduction of thymine at two thirds to one half the rate of uracil (14,59) with NADPH as coenzyme. Thymine inhibited the catabolism for uracil much more than vice versa (49). For the reverse reaction (14) dihydrothymine appeared to be oxidized at a much more rapid rate than dihydrouracil. In the present work the major and minor bands of dihydrouracil dehydrogenase separated from rat liver by disc gel electrophoresis, did not differ in substrate specificity; both major bands were 2 - 3 times more active towards dihydrothymine as towards dihydrouracil with NADP+ as the cofactor. When NAD+ was used instead of NADP+ the two major bands responded uniformly. The physiological significance of the NAD+ dependent activity cannot, however, be assessed at present mainly because of the high pH optimum of the reaction.

The two distinct minor bands designated band 3 and band 4 appeared inconsistently in the gel electrophoresis studies. The appearance of the minor bands may be attributed to either the retention of enzyme activity by contaminating protein or to the degradation of enzyme protein associated with the major bands. The latter

reason being favored as the possible explanation since the appearance of these bands depended upon the age of the sample used. In all cases densitometer tracings of intensity of staining of bands 3 and 4 indicated they contained less than 1% of the activity of the major bands.

Relative mobility studies of the major bands at varying gel concentrations (Figure 13) revealed two nearly parallel lines indicating that the major bands represented isomeric proteins of very similar size but of different charge (46, 47). The molecular weight of each band was estimated (57) to be about 250,000. Estimations of other physiochemical parameters (45) as well as evidence that the enzyme bands represent isoenzyme forms await the preparation of more highly purified enzyme preparations.

SUMMARY

Present studies confirmed reports by Smith and Yamada (30) that the enzyme dihydrouracil dehydrogenase can be separated into reductase activity located primarily in particulate fractions, and dehydrogenase activity located in the cytosol fraction of mammaliam liver.

Marker enzyme studies and sucrose density gradient fractionation studies have confirmed the previous report (30) that the reductase activity associated with particulate fractions could be separated into NADH dependednt activity located primarily in mitochondrial fractions (sucrose gradient M_2) and NADPH dependant activity located primarily on plasma and endoplasmic reticulum membranes (sucrose gradient (M_1). The apparent K_m values of NADH and NADPH dependant reductase for uracil and thymine at pH 7.4 were determined.

Paper chromatography and radioactive $^{14}CO_2$ studies were done to determine whether or not the complete catabolic pathway for pyrimidines is present in rat liver mitochondria. Paper chromatography studies indicated that dihydrouracil was the prinicipal product produced by reaction mixtures containing purified mitochondria, uracil and cofactors NADH or NADPH. The present work also showed that purified mitochondrial fractions catalyze the degradation of uracil -2-^{14}C to $^{14}CO_2$ to only a limited extent. The results suggested that the degradative enzymes beyond uracil-reductase are in limiting

amounts in the mitochondria so that dihydrouracil (dihydrothymine) formed must be extruded mainly into the cytosol for further degradation.

A rapid and sensitive method for the location of dihydrouracil dehydrogenase after disc gel electrophoresis based on reduction of nitroblue tetrazolium to form an insoluble dye was developed. Two major enzyme bands, differing in charge, were separated giving the first indication that this enzyme may possibly exist in at least 2 isoenzyme forms in rat liver. The major bands did not differ in substrate specificity; both bands were 2 to 3 times more active towards dihydrothymine as towards dihydrouracil with $NADP^+$ as the cofactor while the reverse was true when NAD^+ was used as the cofactor.

TABLE 1

The distribution of marker enzyme activities among subcellular fractions of the rat liver homogenate and fractions obtained in purification of the heavy mitochondria (HM). The fractions were separated by differential centrifugation and by discontinuous sucrose gradient centrifugation according to the procedures outlined in Figures 2 and 4 respectively.

Activity was measured under the standard conditions of assay. The number of enzyme units representing 100% activity in the homogenate is as follows:

Glutamate Dehydrogenase	4066 units
5' Nucleotidase	6885 units

The distribution for each marker enzyme is the average of two experiments.

TABLE 1

A) Distribution of Marker Enzyme Activites in Rat Liver Fractions

Fractions	Glutamate Dehydrogenase		5' Nucleotidase	
	S.A.	%	S.A.	%
Homogenate	1.12	100.0	1.45	100.0
Crude Nuclei	1.93	38.3	2.80	32.7
Heavy Mitochondria	2.86	63.0	2.08	31.7
Light Mitochondria	1.55	7.0	3.21	10.8
Microsomes + Supernatant	0.00	0.0	0.96	33.4
% Recovery		108.3		108.6

B) Distribution of Marker Enzyme Activites in Mitochondrial Subfractions

Fractions	Glutamate Dehydrogenase		5' Nucleotidase	
	S.A.	%	S.A.	%
Homogenate	1.12	100.0	1.45	100.0
Heavy Mitochondria	2.86	63.0	2.08	31.7
M_1	0.00	0.0	2.35	6.0
M_2	6.07	55.8	1.48	16.9
M_3	0.98	4.4	0.93	2.9
% Recovery (M_1 to M_3)		60.2		25.8

S.A. = Specific Activity
% = Percentage of Total Activity

TABLE 2

The distribution of pyrimidine reductase activity among dialysed subcellular fractions of rat liver homogenate. The fractions were separated by differential centrifugation according to the procedure outlined in Figure 2. Activity was measured under standard conditions of assay. The number of enzyme units representing 100% activity in the homogenate is as follows:

NADPH - Linked Uracil Reductase	223 units
NADH - Linked Uracil Reductase	473 units
NADPH - Linked Thymine Reductase	191 units
NADH - Linked Thymine Reductase	410 units

The distribution presented for NADH - Linked Uracil and NADPH - Linked Thymine is the average of three experiments, while the NADPH - Linked Uracil and NADH - Linked Thymine represents the average of two experiments.

Weights of the rats used, 300 - 350 g.

TABLE 2

Distribution of Pyrimidine Reductase in Dialysed Rat Liver Fractions

Fractions	NADPH + URACIL		NADH + URACIL		NADPH + THYMINE		NADH + THYMINE	
	S.A.	%	S.A.	%	S.A.	%	S.A.	%
Homogenate	0.042	100.0	0.067	100.0	0.045	100.0	0.088	100.0
Crude Nuclei	0.095	30.4	0.103	26.5	0.089	29.9	0.123	24.1
Heavy Mitochondria	0.055	35.8	0.286	47.7	0.064	33.2	0.214	51.0
Light Mitochondria	0.111	16.1	0.086	5.9	0.140	23.2	0.107	6.0
Microsomes + Supernatant Fraction	0.38	23.2	0.020	16.0	0.016	17.9	0.014	12.4
% Recovery		105.5		96.1		103.2		93.5

S.A. = Specific Activity

% = Percentage of Total Activity

TABLE 3

The distribution of pyrimidine reductase activity in different fractions obtained during the purification of Heavy Mitochondria. The fractions were prepared by discontinuous sucrose density gradient according to the procedure outlined in Figure 4. Activity was measured under the standard conditions of assay. The number of enzyme units representing 100% activity in the homogenate is as follows:

NADPH - Linked Uracil Reductase	223 units
NADH - Linked Uracil Reductase	473 units
NADPH - Linked Thymine Reductase	191 units
NADH - Linked Thymine Reductase	410 units

The distribution presented for the NADH - Linked Uracil and NADPH - Linked Thymine is the average of three experiments, while the NADPH - Linked Uracil and NADH - Linked Thymine represents the average of two experiments.

Weights of the rats used, 300 - 350 g.

TABLE 3

Purification of Mitochondria

Distribution of Pyrimidine Reductase

Fractions	NADPH + Uracil		NADH + Uracil		NADPH + Thymine		NADH + Thymine	
	S.A.	%	S.A.	%	S.A.	%	S.A.	%
Homogenate	0.070	100.0	0.067	100.0	0.045	100.0	0.088	100.0
Heavy Mitochondria	0.055	35.8	0.286	47.7	0.064	33.2	0.214	51.0
M_1	0.243	28.4	0.052	3.8	0.265	21.4	0.064	3.1
M_2	0.013	7.0	0.535	41.0	0.078	9.9	0.482	37.0
M_3	0.031	2.4	0.047	2.4	0.033	3.3	0.144	4.8
% Recovery (M_1 - M_3)		37.8		47.2		34.6		44.9

S.A. = Specific Activity

% = Percentage of Total Recovery

TABLE 4

A summary of the purification of dihydropyrimidine dehydrogenase activity. The fractions were separated by differential centrifugation according to the procedures outlined in Figures 2 and 4. After separation each fraction was dialyzed for 16 hours. Activity was measured under the standard conditions of assay except that fractions were made 5.4M with glycerol immediately prior to assaying for activity. The number of enzyme units representing 100% activity in the homogenate is as follows:

NADP+	- Linked Dihydrouracil Dehydrogenase	129 units
NAD+	- Linked Dihydrouracil Dehydrogenase	99 units
NADP+	- Linked Dihydrothymine Dehydrogenase	132 units
NAD+	- Linked Dihydrothymine Dehydrogenase	109 units

Purification of NAD+ linked enzymes in Sephadex G - 100 was assayed in a different preparation.

TABLE 4

Summary of Purification of Dihydrouracil Dehydrogenase

Step and Treatment	Volume (ml)	Specific Activity units/mg protein			
		DHU		DHT	
		NAD+	NADP+	NAD+	NADP+
1. Homogenate	146	0.079	0.061	0.070	0.082
2. Supernatant	91	0.134	0.123	0.134	0.144
3. Ammonium Sulfate	63	0.283	0.267	0.291	0.342
4. Sephadex G - 100*	20	0.236	-----	0.205	-----

* A different preparation was used for Sephadex G - 100 purifications, the homogenate used had specific activities of 0.028 (DHU + NAD+) and 0.026 (DHT + NAD+)

TABLE 5

Relative mobilities of enzyme bands. The means of 4 experiments in which 110, 176, 220, and 330 µg protein were applied to each gel are given for each coenzyme. For NADP+, 176 µg protein was replaced by 440 µg.

TABLE 5

Relative Mobilities of Enzyme Bands

Coenzyme	Band 1 (mean ± SD)	Band 2 (mean ± SD)
NAD+	0.178 ± 0.006	0.623 ± 0.015
NADP+	0.184 ± 0.011	0.606 ± 0.026

FIGURE 1

Representation of the

pathways for pyrimidine catabolism.

PYRIMIDINE CATABOLIC PATHWAYS

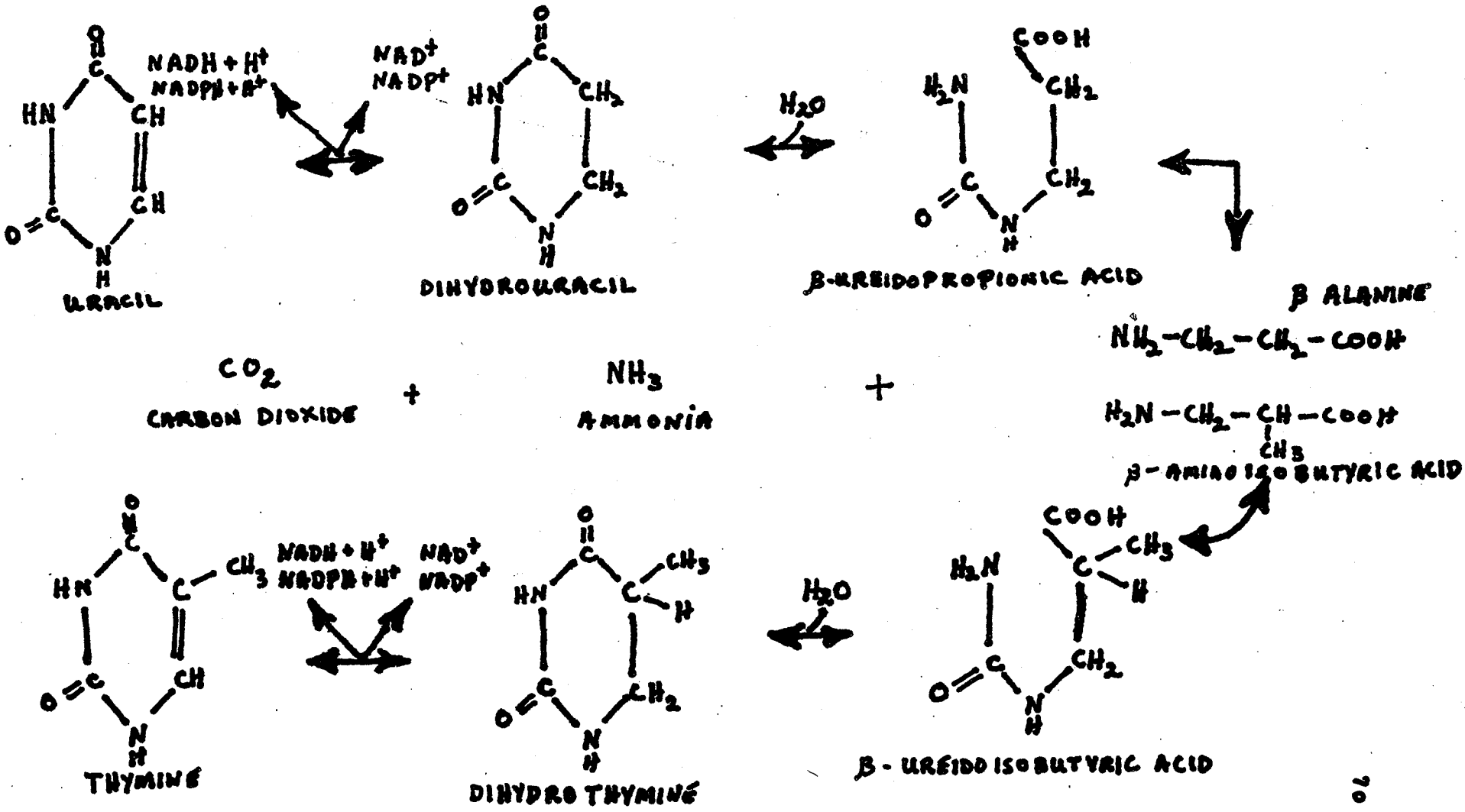


FIGURE 2

Procedure for the separation of subcellular fractions of rat liver by differential centrifugation. The tissue was homogenized as described under "Materials and Methods".

SEPARATION OF SUBCELLULAR FRACTIONS

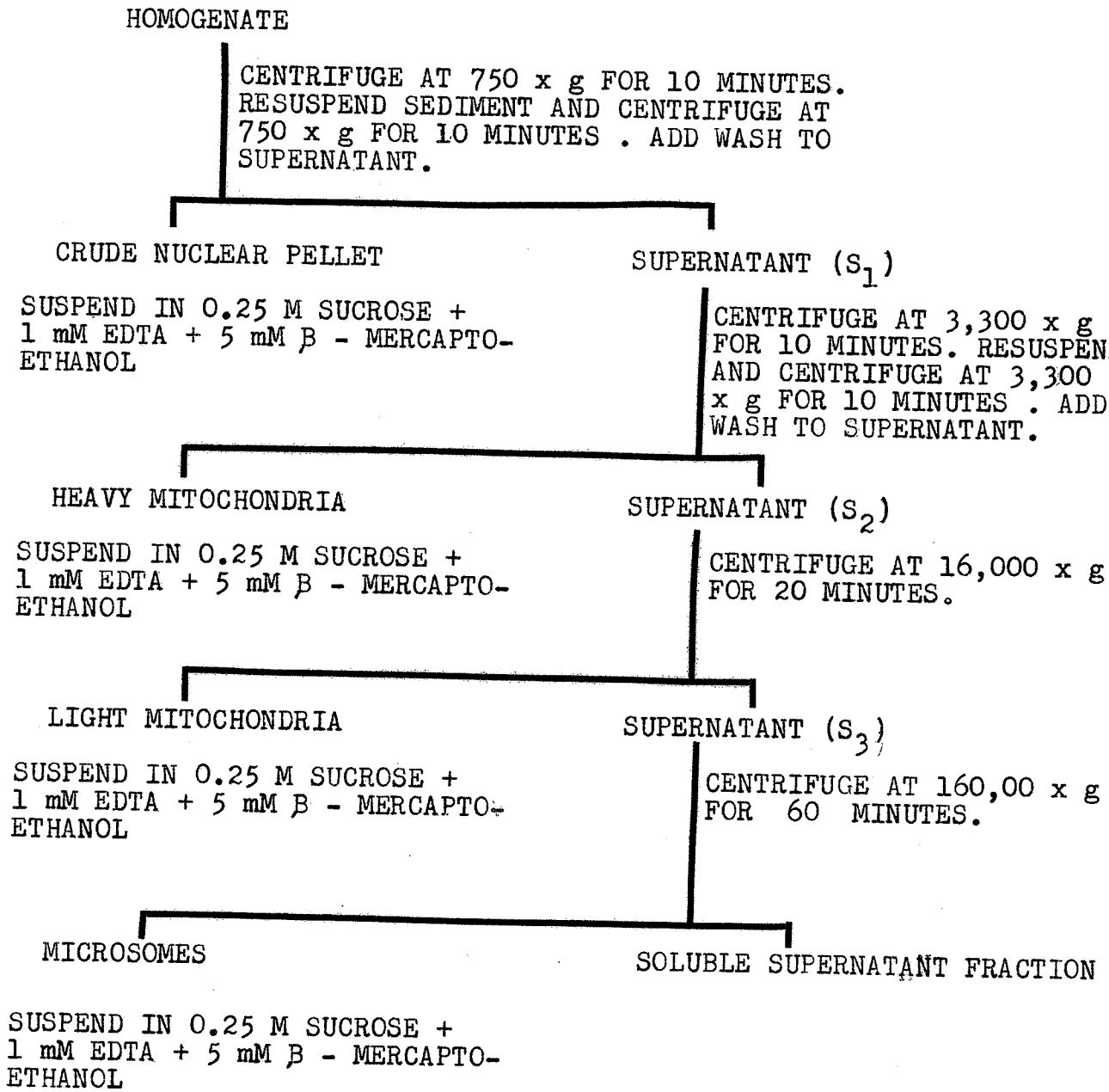


FIGURE 3

Enzyme distribution in HM fractions (heavy mitochondria) centrifuged in linear sucrose density gradients (procedure 1). Activity is expressed as the μmoles of dihydrouracil (DHU) or dihydrothymine (DHT) formed per hour per tube with uracil (U) or thymine (T) as substrates; 1.5 ml fractions were collected. A unit of glutamate dehydrogenase activity is expressed as the μmoles of NADH formed per hour per tube; a 3 ml aliquot containing 75.6 mg protein of the HM fraction was used.

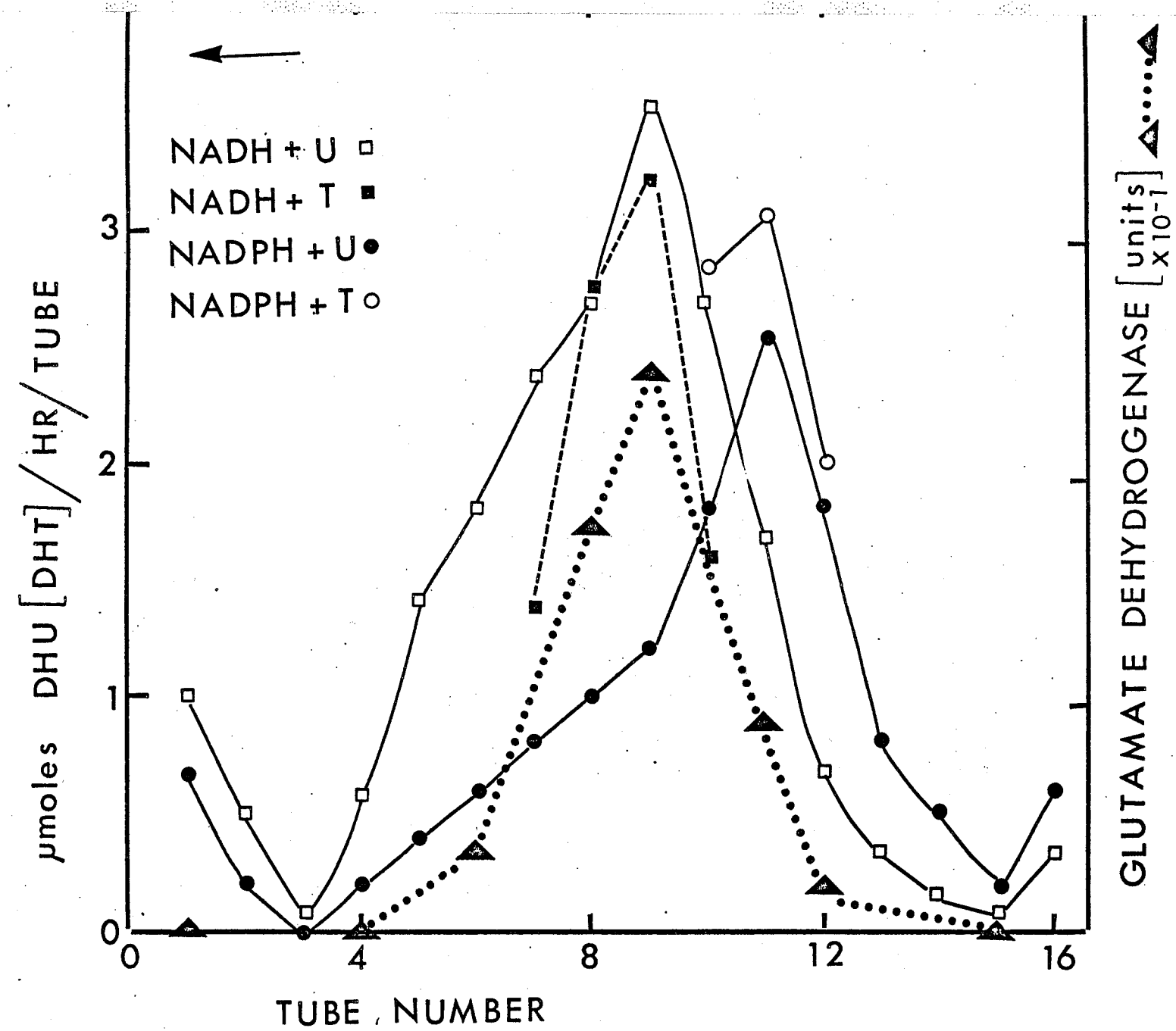

NADH + U
NADH + T
NADPH + U
NADPH + T
μmoles DHU [DHT] / HR / TUBE
GLUTAMATE DEHYDROGENASE [units X 10⁻¹]
TUBE NUMBER
0
1
2
3
0
4
8
12
16

FIGURE 4

Procedure for the purification of Heavy Mitochondria by discontinuous sucrose density gradient centrifugation (procedure 2). Layering was done in a cold room in the volumes specified. Centrifugation was at 4^0 in an International model B-60 ultracentrifuge.

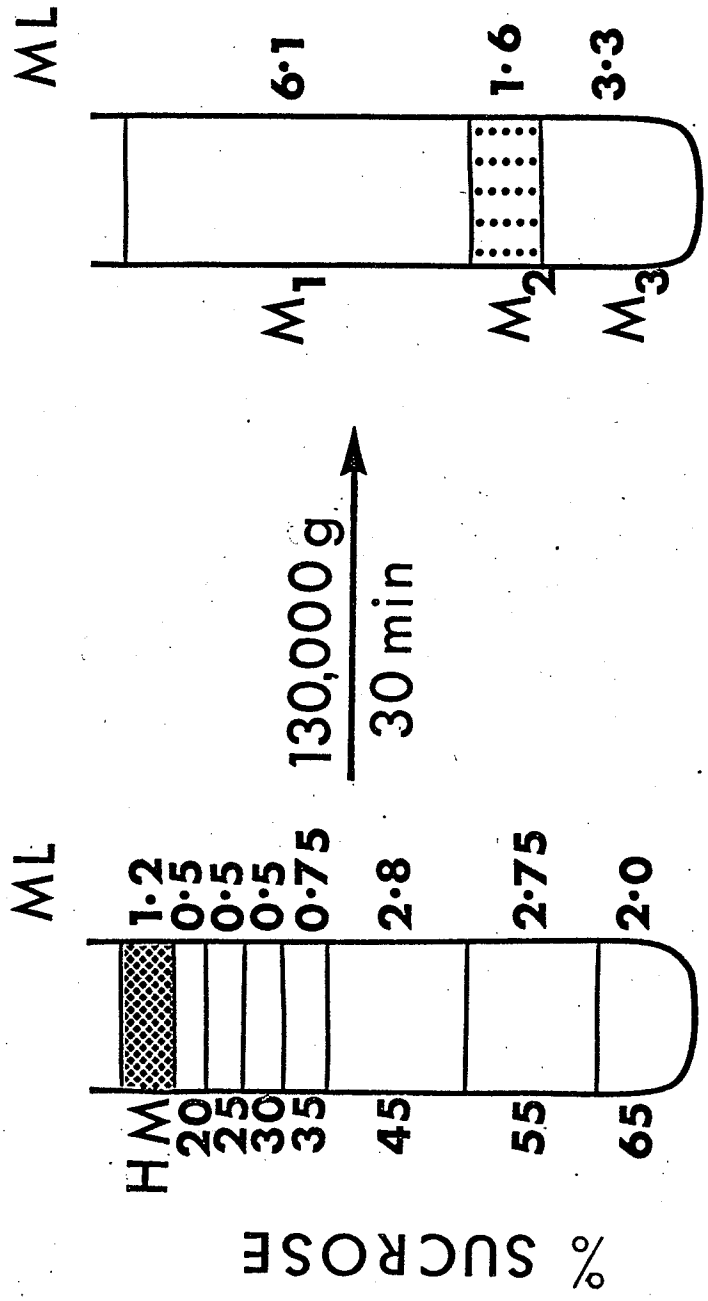
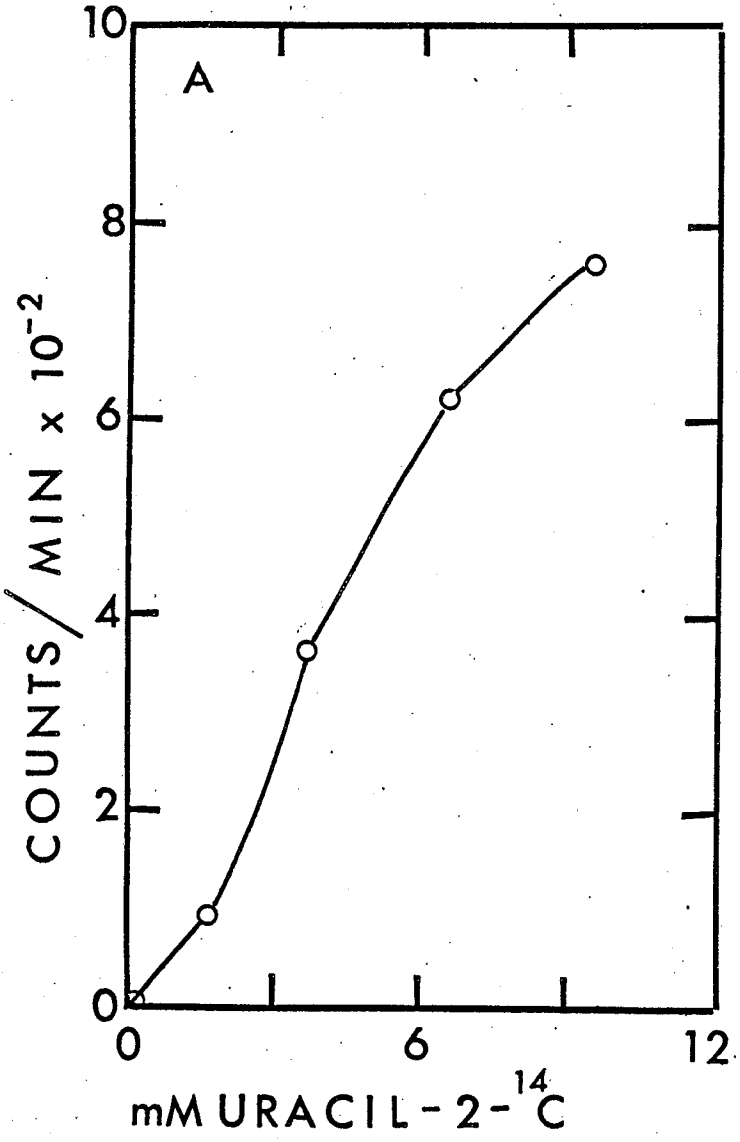

ML
6·1
1·6
3·3
M1
M2
M3
130,000 g
30 min
ML
1·2
0·5
0·5
0·5
0·75
2·8
2·75
2·0
H M
20
25
30
35
45
55
65
% SUCROSE

FIGURE 5 (to the left)

Distribution of radioactivity along the strips after chromatography in collidine saturated with water of the supernatant fluid from the incubation mixture containing 20 µmoles uracil -2-^{14}C and M_2 fraction as described in "Materials and Methods".
The solvent traveled 35 cm beyond the point of application of the sample. The activity of a compound, represented by a peak in the diagram is expressed in per cent of the total activity on the strip.

FIGURE 6 (to the right)

Relationship between radioactive CO_2 formed (counts/minute) and uracil -2-^{14}C concentration; fraction M_2 (10 mg protein) was incubated for 30 minutes at 37^0.


A
10
8
6
4
2
0
0
6
12
COUNTS / MIN x 10^{-2}
mM URACIL - 2 - ^{14}C

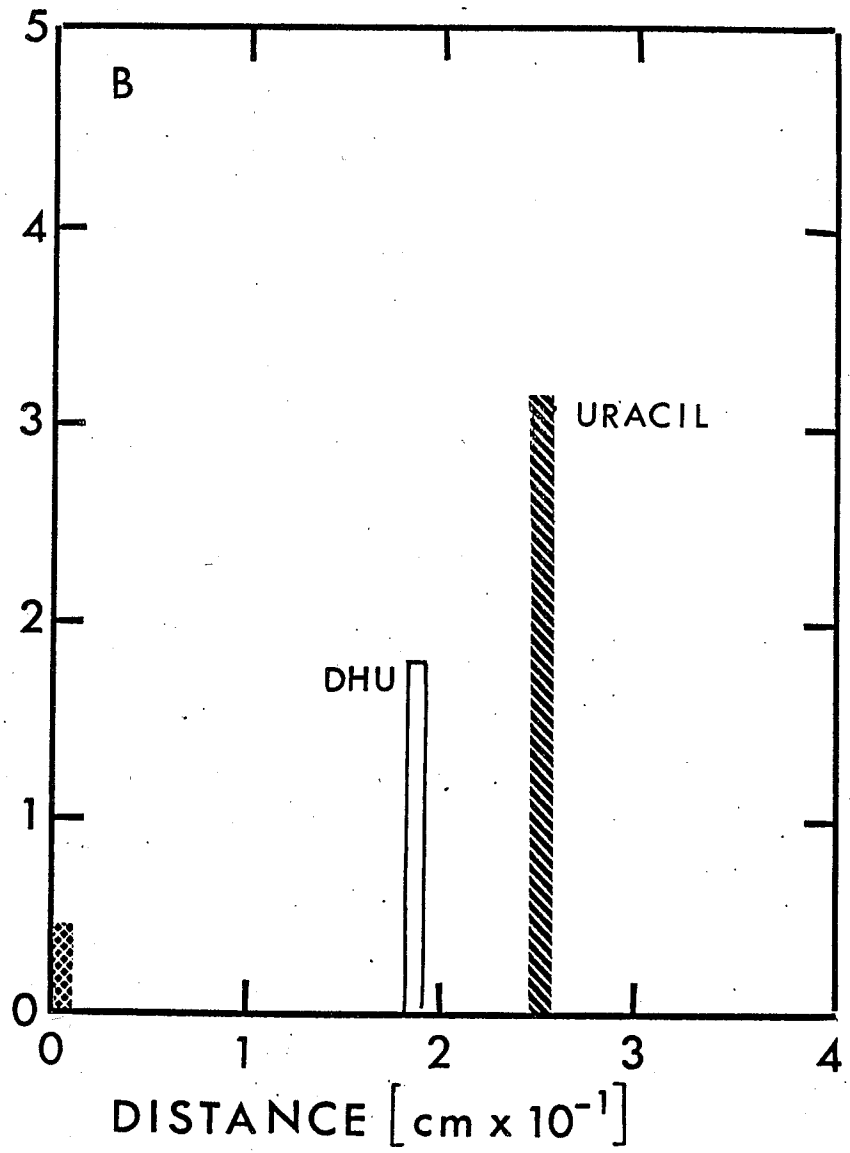

B
5
4
3
2
1
0
0
1
2
3
4
URACIL
DHU
DISTANCE [cm x 10^{-1}]

FIGURE 7

NAD+ - linked DHU dehydrogenase activity and protein concentration (A-E). Gels (6.16%) were stained, after gel electrophoresis, for 2 hours as described under "Materials and Methods". Control tubes (gel A) were stained in the absence of coenzyme. In gels (B-E), 110, 176, 220 and 330 µg of protein were applied in order from left to right. The specific activity of the partially purified enzyme sample was 0.140. The arrows show the position of the origin (0), enzyme bands (1,2,3, and 4) and tracking dye(TD). Gels F and H were stained for NADP+ - linked enzyme activity; gel G was stained for protein with Coomassie blue. In gel F, 220 µg of protein were applied, and in gels G and H, 330 µg of protein corresponding to 0.029 and 0.043 units of enzyme activity,respectively.
The location of minor bands 3 and 4 is not noted, however, due to faintness they are barely visible in photographs, but are clearly visible in densitometer tracings (see figure 8).

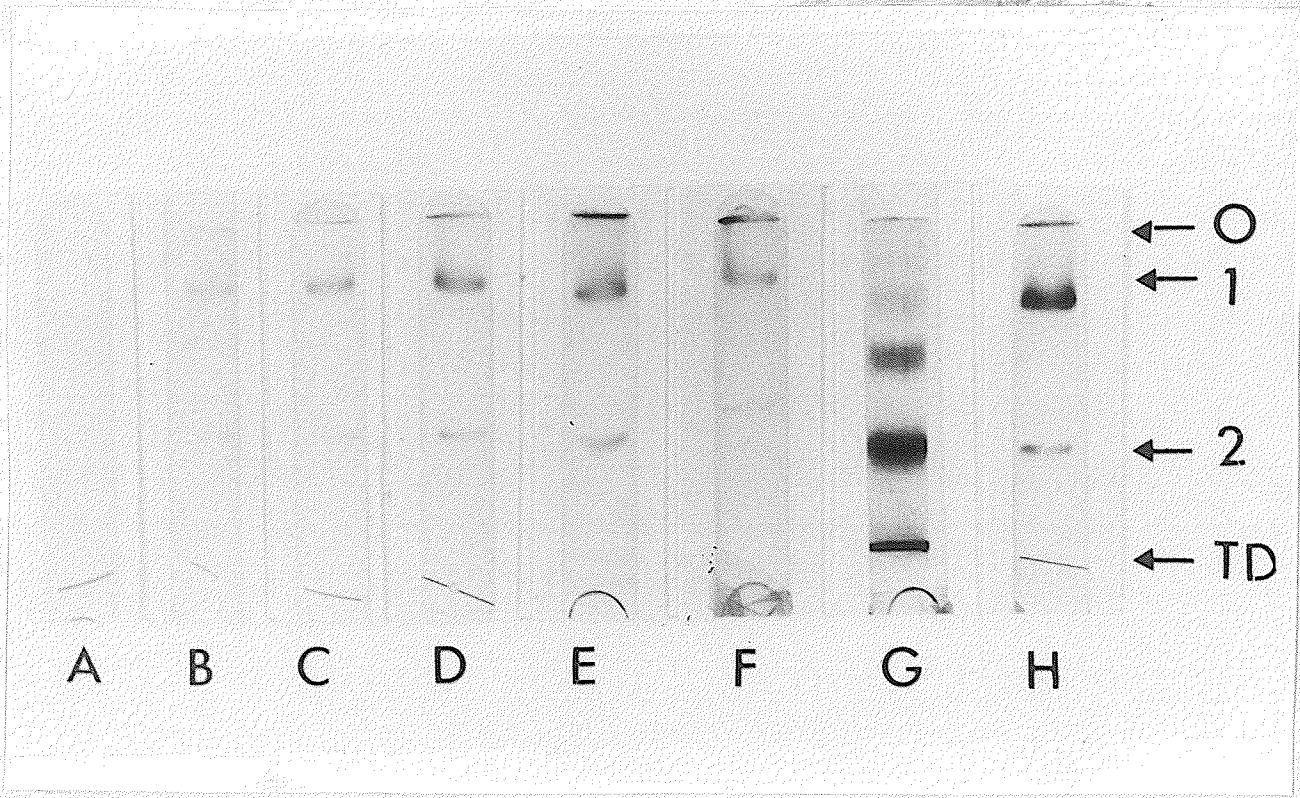

O
1
2
TD
A
B
C
D
E
F
G
H

FIGURE 8

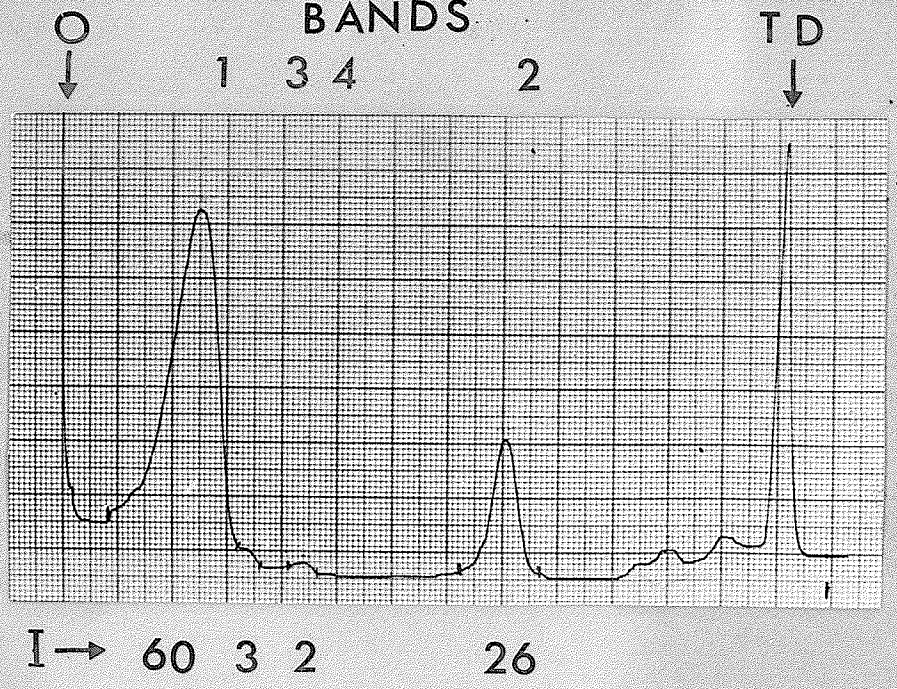
Representative densitometer tracings showing the separation and detection of dihydrouracil dehydrogenase activity after electrophoresis in 6.16% gel. The gels were stained for enzyme activity for 2 hours by the standard procedure outlined in "Methods and Materials".

A: Gel (see tube B, Figure 7) stained for NAD+ - linked dihydrouracil dehydrogenase activity contained 110 µg of protein (0.018 units)

B: Gel (see tube H, Figure 7) stained for NADP+ - linked dihydrouracil dehydrogenase activity contained 330 µg of protein (0.043 units).

The vertical arrows (↓) show the positions of the origin (0) major enzyme bands 1 and 2 and tracking dye (TD). Location of minor bands 3 and 4 are also indicated although they did not appear routinely. The area under the peaks (Intensity (I)) were estimated by disc integrator.

A)



B)

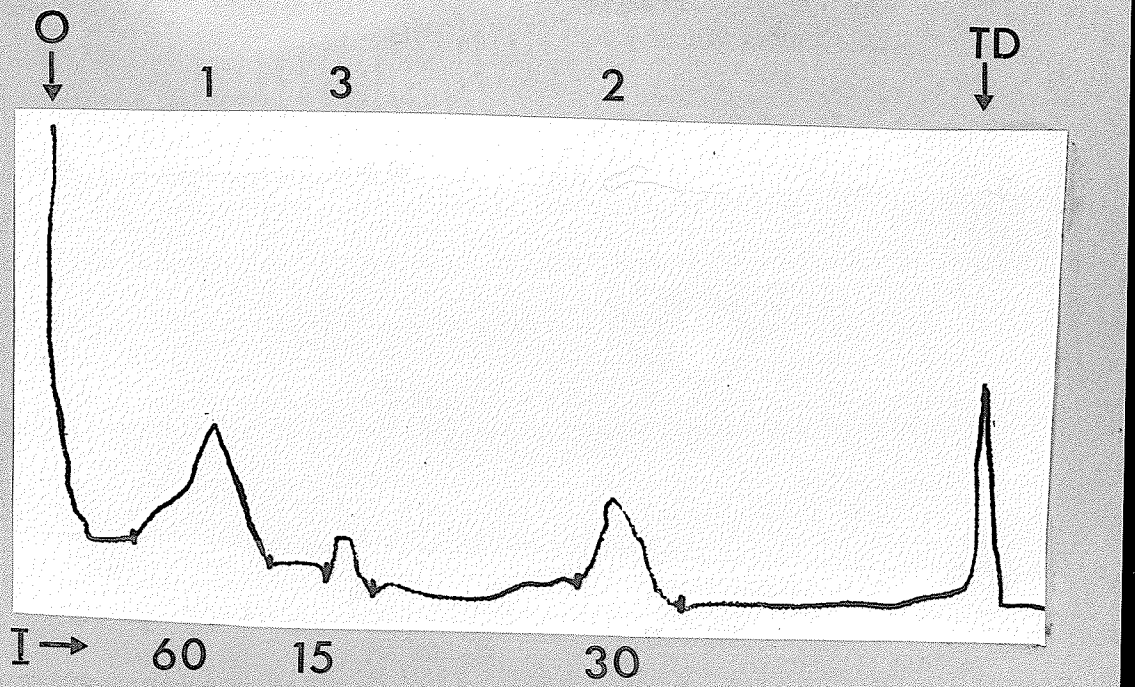


FIGURE 9

NAD+ - linked DHT dehydrogenase activity and protein concentration (A-E). Gels (6.16%) were stained, after gel electrophoresis, for 2 hours as described under "Materials and Methods". Control tubes (gel A) were stained in the absence of DHT or in the absence of coenzyme. In gels B-E, 110, 176, 220, and 330 ug of protein were applied in order from left to right. The specific activity of the partially purified sample was 0.150. The arrows show the position of the origin (0), enzyme bands (1,2,3 and 4) and tracking dye (TD). Gels F and H were stained for NADP+ - linked enzyme activity; gel G was stained for protein with Coomassie blue. In gel F, 220 µg of protein were applied, and in gels G and H, 330 µg of protein corresponding to 0.030 and 0.045 units of enzyme activity,respectively.
The location of minor bands 3 and 4 is not noted however, due to faintness they are barely visible in photographs,but are clearly visible in densitometer tracings (see Figure 10).

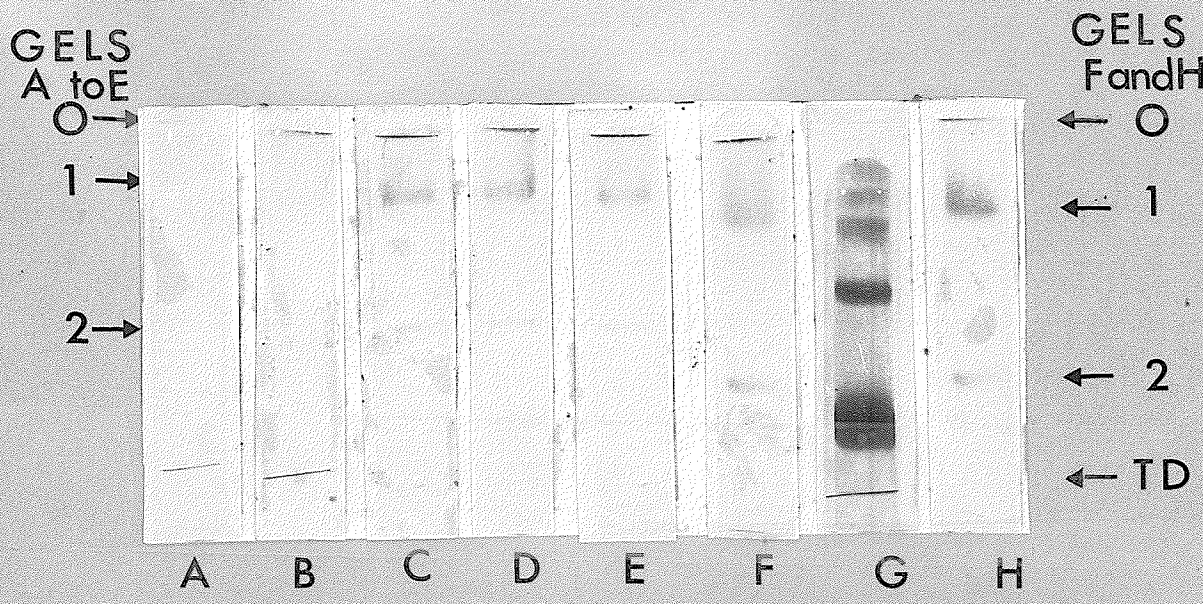

GELS
A toE
O
1
2
GELS
FandH
O
1
2
TD
A
B
C
D
E
F
G
H

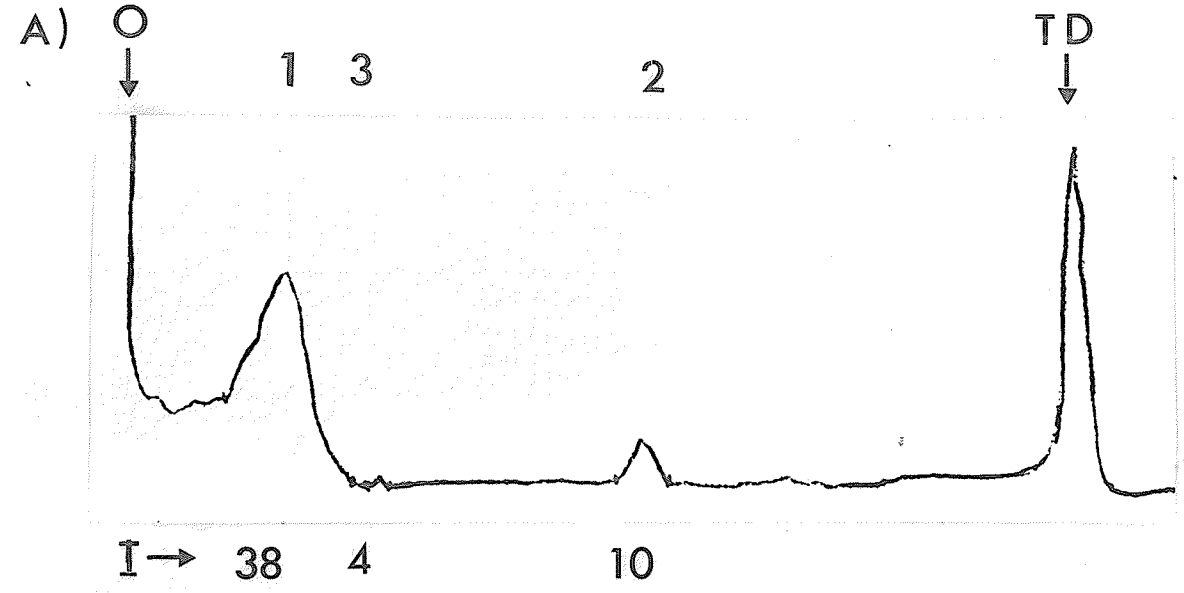
FIGURE 10

Representative densitometer tracing showing the separation and detection of dihydrothymine dehydrogenase activity after electrophoresis in 6.16% gel. The gels were stained for enzyme activity for 2 hours by the standard procedure outlined in "Methods and Materials".

A: Gel (see tube B, Figure 9) stained for NAD+ - linked dihydrothymine activity contained 176 µg of protein (0.015 units).

B: Gel (see tube 11, Figure 9) stained for NADP+ - linked dihydrothymine contained 330 µg of protein (0.45 units).

The vertical arrows (↓) show the positions of the origin (O) major enzyme bands 1 and 2 and tracking dye (TD). Location of minor bands 3 and 4 are also indicated although they did not appear routinely. The area under the peaks (Intensity (I)) were estimated by disc integrator.


A)
O
1
3
2
TD
I→ 38
4
10

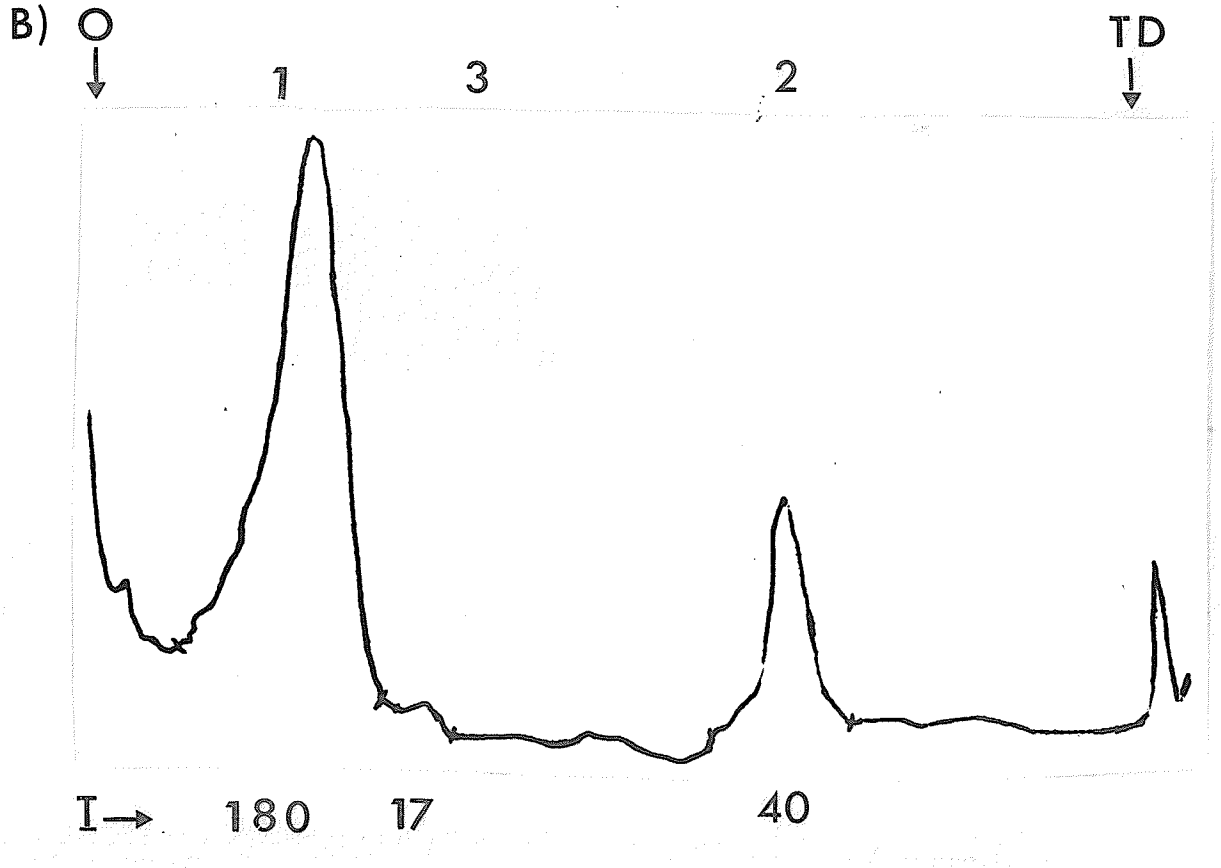

B)
O
1
3
2
TD
I→ 180
17
40

FIGURE 11

A: The relationship between staining intensity and protein concentration for NAD+ - linked DHU dehydrogenase. Major bands 1 (●) and 2 (■) and minor bands 3 (▲) and 4 (▼)

B: The relationship between staining intensity and protein concentration for NADP+ - linked DHU dehydrogenase. Major bands 1 (○) and 2 (□) and minor bands 3 (△).

The area under each peak in densitometer tracings of gels was determined by disc integrator to give the intensity of staining in arbitrary units.

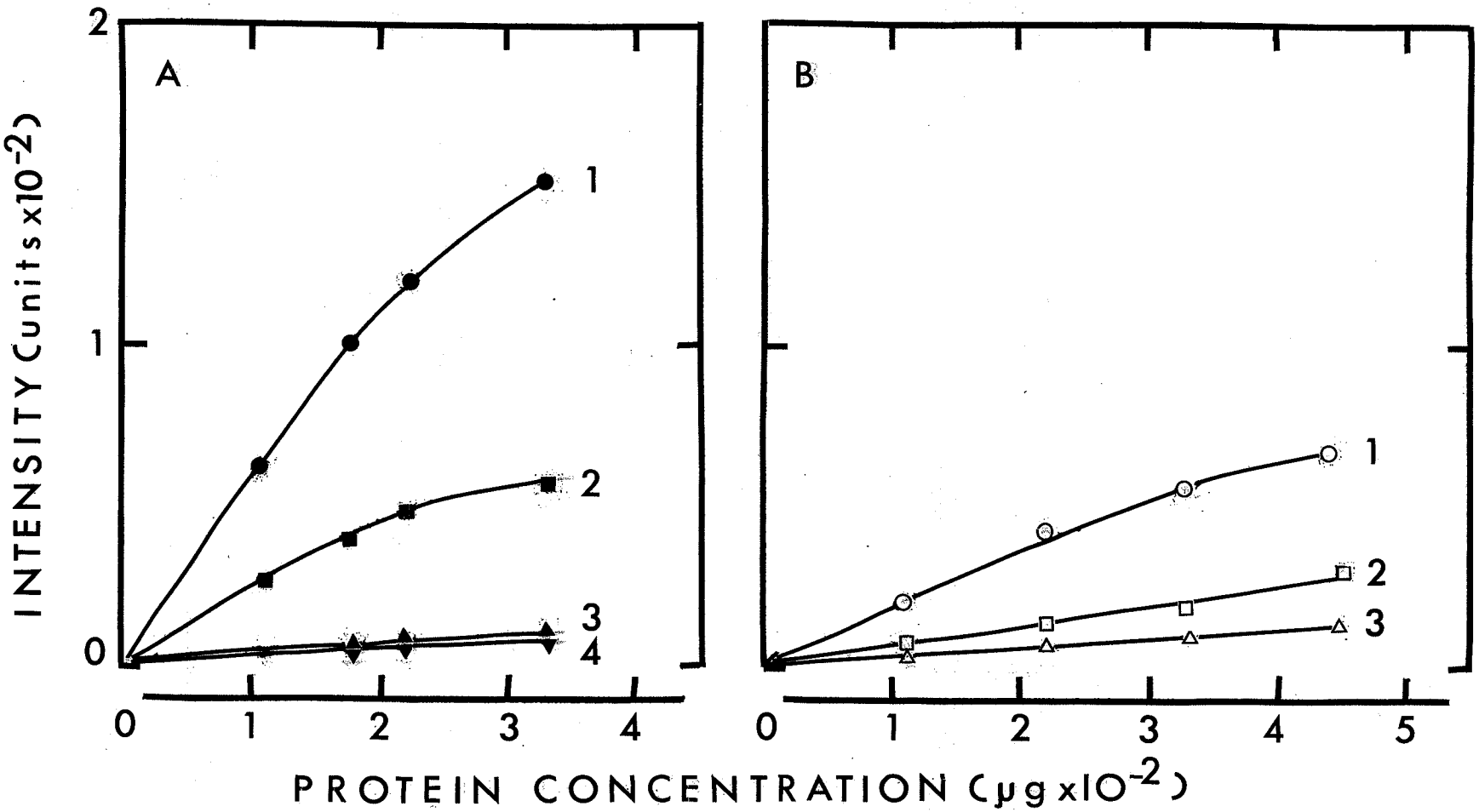

A
B
1
2
3
4
INTENSITY (units x10^{-2})
PROTEIN CONCENTRATION (µg x10^{-2})
0
1
2
3
4
5

FIGURE 12

A: The relationship between staining intensity and protein concentration for NAD+ - linked DHT dehydrogenase. Major bands 1 (●) and 2 (■) and minor band 3 (▲).

B: The relationship between staining intensity and protein concentration for NADP+ - linked DHT dehydrogenase. Major bands 1 (○) and 2 (□) and minor bands 3 (△).

The area under the peak in densitometer tracings of gels was determined by disc integrator to give the intensity of staining in arbitrary units.

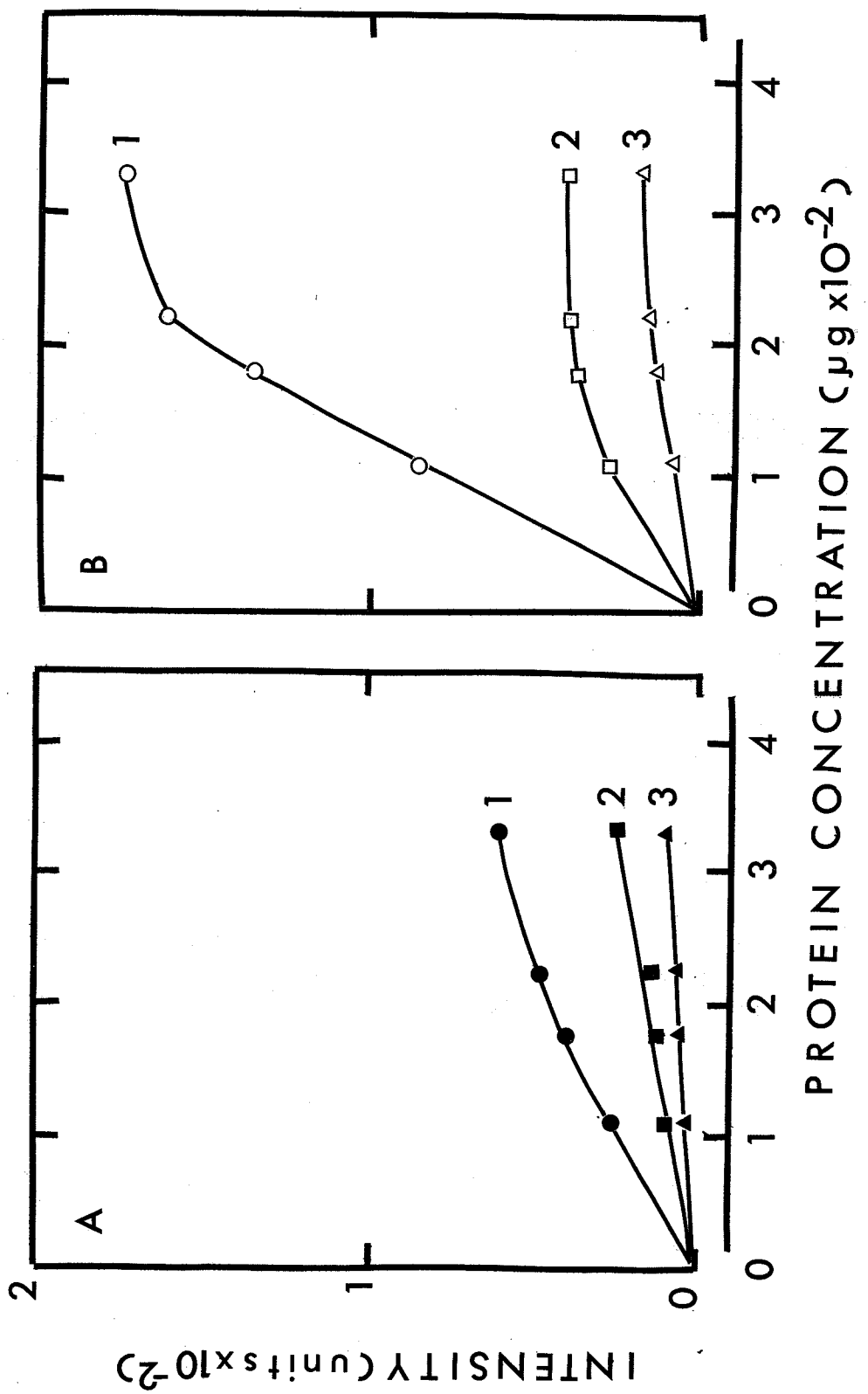
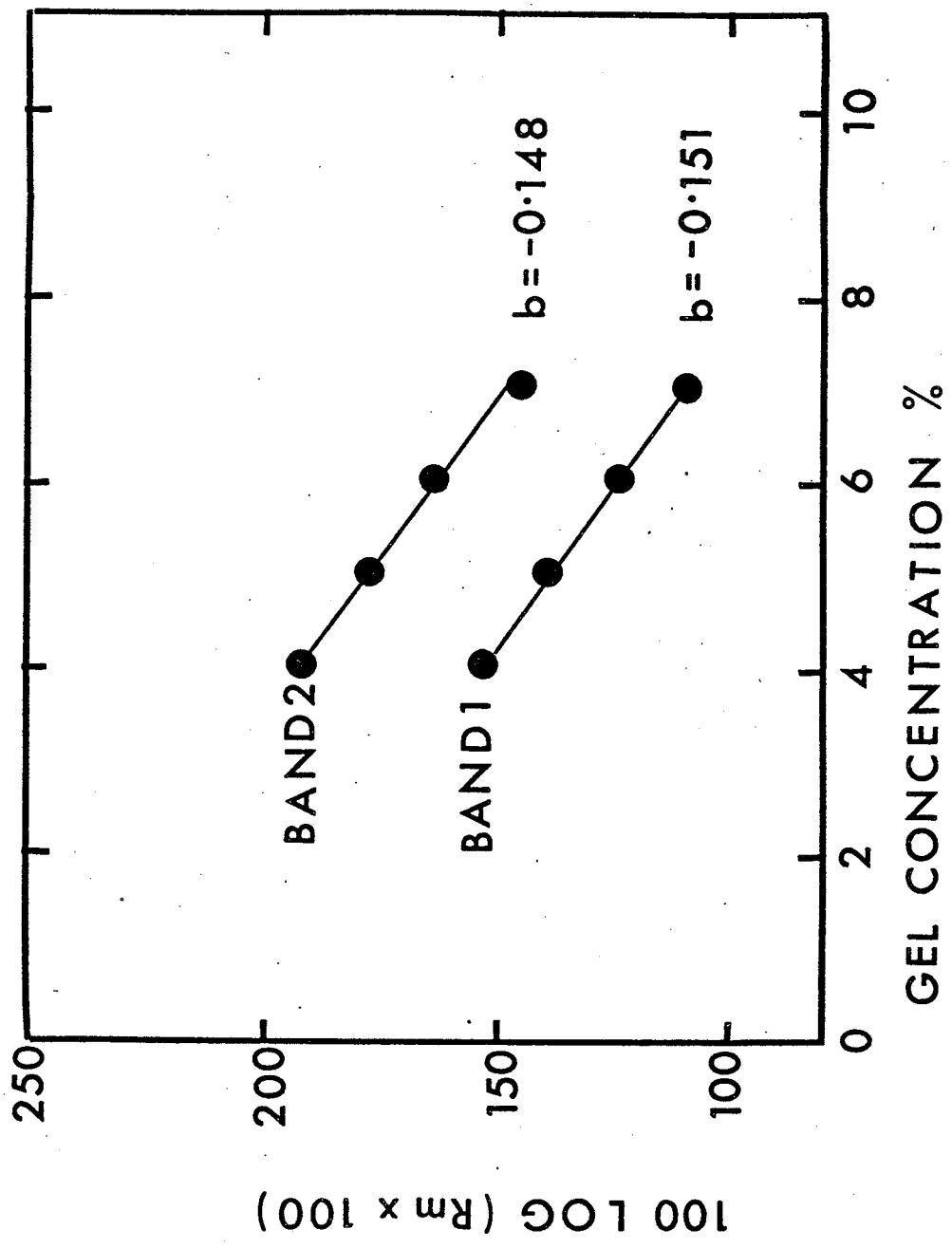

INTENSITY (units x10-2)
PROTEIN CONCENTRATION (µg x10-2)
A
B
1
2
3
0
1
2
3
4

FIGURE 13

Relationship between log R_m and total gel concentration; % C was kept constant at 2.6. The lines of best fit were computed by unweighted least squares regression analysis (45). The slopes of the lines (b) (X 10^{-2}) are noted on the figure.


100 LOG (Rm x 100)
250
200
150
100
0
2
4
6
8
10
GEL CONCENTRATION %
BAND2
BAND1
b=-0·148
b=-0·151

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