DIHYDROPYRIMIDINE DEHYDROGENASE OF RAT LIVER: SEPARATION INTO REDUCTASE AND DEHYDROGENASE ACTIVITIES.

A THESIS

PRESENTED TO

THE FACULTY OF GRADUATE STUDIES AND RESEARCH

THE UNIVERSITY OF MANITOBA

IN PARTIAL FULFILLMENT

OF THE REQUIREMENTS FOR THE DEGREE

MASTER OF SCIENCE

BY

ASTLEY EUGENE SMITH

DEPARTMENT OF BIOCHEMISTRY, FACULTY OF MEDICINE

JANUARY 1970

ACKNOWLEDGEMENTS

The author wishes to express his sincere appreciation to his supervisor, Dr. Esther V. W. Yamada, for her helpful instruction and valuable criticism.

The author also wishes to thank Mr. Alec D. Leung for his skillful technical assistance and Miss Beverley Smith for her skill and untiring patience in typing this thesis.

ABBREVIATIONS

BAIB	β-aminoisobutyric acid
BUIB	β-ureidoisobutyric acid
BUPA	β-ureidopropionic acid
DHT	dihydrothymine
DHU	dihydrouracil
NADH	nicotinamide adeninedinucleotide - reduced
NADPH	nicotinamide adeninedinucleotide phosphate - reduced
NAD^+	nicotinamide adeninedinucleotide
$NADP^+$	nicotinamide adeninedinucleotide phosphate
AMP	adenosine 5'-monophosphate
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
Mg^{++}	magnesium ions
Pi	inorganic phosphate
ATP--Mg	adenosine 5'-triphosphate magnesium complex
ADP--Mg	adenosine 5'-diphosphate magnesium complex
AMP--Mg	adenosine 5'-monophosphate magnesium complex
TCA	trichloroacetic acid
PCA	perchloric acid
F_5	purified lysosomes
AS_2	ammonium sulphate fraction (55% saturation)

TMP	thymidine 5'-monophosphate
TDP	thymidine 5'-diphosphate
TTP	thymidine 5'-triphosphate
7S	plasma membranes
4g-P	4,000xg-pellet
4g-S	4,000xg-supernatant
S_1	750xg supernatant
F_1	light mitochondria
β-SH	β-mercaptoethanol
EDTA	ethylenediaminetetraacetic acid

TABLE OF CONTENTS

ABSTRACT

From earlier studies of the intracellular localization of the pyrimidine catabolic pathway (9, 8, 21), it is currently believed that the enzyme dihydropyrimidine dehydrogenase is present mainly in the soluble cytoplasmic fraction of rat liver cells, and is responsible for catalysing the hydrogenation of pyrimidine reversibly. However, it was also suggested (9), that this pathway might also be present in the particulate fractions, but the presence of inhibitory factors may be preventing the activity from being expressed.

The purposes of the work to be described in this thesis were two-fold. First to determine the intracellular localization of the enzyme(s) responsible for pyrimidine reduction in rat liver, and to investigate the reversibility of the reaction. Previously, no one had ever determined the intracellular localization of the specific enzyme(s) responsible for pyrimidine reduction in rat liver. By assaying the activity for pyrimidine reduction in the intracellular fractions prepared by differential centrifugation and by following the activity of established marker enzymes for the respective intracellular fractions, it was felt that a proper localization of the specific enzyme(s) responsible for pyrimidine reduction might be obtained. The marker enzymes were chosen as follows: glutamate dehydrogenase for mitochondria, acid phosphatase for lysosomes, glucose-6-phosphatase for microsomes, and 5'-nucleotidase for plasma membranes. Secondly, to investigate further the effect of metabolites, particularly nucleotides, as well as cations

on uracil metabolism. By studying the effect of these metabolites, it should be possible to gain additional information about the regulation of uracil metabolism.

In present studies it was found that this enzyme could be separated into pyrimidine reductase and dihydropyrimidine dehydrogenase activities. The reductase activity was found to be NADH and NADPH dependent, and confined mainly in particulate fractions which were identified by appropriate marker enzymes. The dehydrogenase activity was NAD^+ and $NADP^+$ dependent, but residing mainly in the soluble cytoplasmic fraction. Subsequently, purification of the nuclei, lysosomes and plasma membranes and sucrose density gradient fractionation of the heavy and light mitochondrial fractions showed that most of the NADH-linked pyrimidine reductase activity was associated with the mitochondria, while most of the NADPH-linked pyrimidine reductase activity was associated with the mitochondria and plasma membranes.

Pyrimidine reductase activity was inhibited by Mg^{++}, ATP and ATP-Mg at concentrations above 1mM, whereas, ATP and Mg^{++} were strong activators of dihydropyrimidine dehydrogenase activity.

INTRODUCTION

From what is now known about pyrimidine metabolism, the enzymic pathways for the catabolism of pyrimidines are as represented in Figure 1.

Historical Background

(a) Discovery of pyrimidine catabolic pathway

Evidence for the existence of a pyrimidine catabolic pathway was first introduced by Fink, Henderson and Fink in 1951 (1). These workers in experimenting with rats (1, 2), found that at the dosage levels employed for intraperitoneal administration dihydrothymine (DHT) appeared, on the average, to be over 5 times as effective as thymine in causing urinary excretion of β-aminoisobutyric acid (BAIB). It was suggested that DHT might be a biological intermediate in thymine metabolism. In 1953 these workers (3) extended their study to tissue slice experiments. The tissue slice experiments showed similar results for thymine and DHT and, in addition, the observations were extended to include an analogous formation of β-alanine form dihydrouracil (DHU) a compound which had previously been isolated from animal tissue (4).

In 1954 Fink, Cline, and Kosh produced experimental evidence to show that in bacterial cultures the conversion of thymine and uracil to the corresponding β-amino acids was accompanied by the formation of the corresponding intermediate dihydropyrimidines and β-ureido acids (5). The *in vitro* studies of Fink et al (6) showed that the incubation of the liver slices with thymine gave a slow production of BAIB without detectable accumulation of DHT or β-ureidoisobutyric

acid (BUIB). However, when added as substrates both DHT and BUIB were rapidly and extensively converted to BAIB. In these experiments the conversion of BUIB to BAIB appeared to be irreversible under the conditions used, interconversion of DHT and BUIB was clearly detectable, and thymine appeared in the incubation media when DHT was used as substrate. Results for the analogous compounds uracil, DHU, β-ureidopropionic acid (BUPA), and β-alanine were similar, but the overall conversion of uracil to β-alanine was not clearly demonstrated.

In 1955 the in vivo experiments of Fink (7), produced the following interrelationships: Thymine ← DHT ⇌ BUIB → BAIB. With the related uracil series of compounds only the reaction DHU → BUPA was clearly demonstrated in vivo. At this time it was thought that the absence of detectable urinary β-alanine after administration of uracil, DHU, and BUPA was due, in part, to more rapid metabolism of β-alanine than of BAIB.

(b) Previous work describing intracellular localization of pyrimidine catabolic pathway.

In 1954 Rutman, Cantarow and Paschkis (8) studied the intracellular localization of the degradative pathway in rat liver by measuring the release of $C^{14}O_2$ from uracil-2-C^{14}. After homogenization of the liver slices the rate of $C^{14}O_2$ formation decreased by 95%, and only trace amounts were formed by the subcellular fractions. However, the trace amounts formed by the supernatant fraction greatly exceeded that of a quantity of whole homogenate containing an equal amount of supernatant fraction. It was suggested that the unfractionated homogenate contained inhibitors of the supernatant activity. In 1955 Canellakis studied the degradation of uracil-2-C^{14} and thymine-2-C^{14}

to $C^{14}O_2$ in rat liver slices, whole homogenate, supernatant fluid, and acetone-dried powder extracts of the supernatant fraction (9). The results of these experiments showed that the complete enzyme system responsible for the conversion of both uracil-2-C^{14} and thymine-2-C^{14} to $C^{14}O_2$ was present in rat liver. Similar to the results obtained by other workers (8), the activity of the liver slices was reduced by more than 50% after homogenization in Na-phosphate buffer. However, the activity of the 100,000xg supernatant approximated that of the liver slices. This observation led Canellakis to postulate that the enzyme system which degrades uracil-2-C^{14} to $C^{14}O_2$ resides mainly in the supernatant fraction of rat liver homogenates. Since the addition of microsomes to the supernatant fraction resulted in inhibition of enzymic activity, Canellakis suggested that contrary to the views of other workers (8) the enzymic system may also be present in the various particulate fractions, but the activity could not be measured because of some endogenous inhibitory factor(s). In extracts of acetone-dried powder, Canellakis (9) found that the conversion of uracil-2-C^{14} was both NADH and NADPH dependent. However, after dialysis against 0.05 M Tris buffer for 24 to 40 hours, the NADH-dependent activity was completely lost, and could not be regained even with an NADH generating system. By measuring the rates of degradation of uracil-2-C^{14}, DHU-2-C^{14}, and BUPA-2-C^{14}, Fritzson (14) and Canellakis (10) were able to establish that the initial conversion of uracil to DHU was the rate-limiting reaction in the catabolic pathway.

In 1957 Canellakis did extensive studies on the interaction of the catabolic and anabolic pathways of uracil metabolism in various tissues. From these studies it was shown that small amounts of uracil-2-C^{14} was incorporated into RNA of rat liver *in vitro* when high concentrations of uracil was used (10). This observation clarified the results

of other workers (11, 12), who had previously shown that exogenous preformed pyrimidine at low concentrations was not effectively utilized for RNA biosynthesis by adult mammalian liver. An extention of these studies by Canellakis and other workers showed that in rapidly dividing tissues, e.g. intestinal mucosa (10, 13, 14, 15), various hepatomas (10, 16), and Ehrlich ascites tumor cells (17), an active incorporation of uracil into RNA occurred, whereas in normal resting tissues, rat and mouse liver (10), catabolism of the pyrimidine was the predominant pathway. This indicated a delicate balance between the ability of the tissues to degrade or to utilize the preformed pyrimidine, and Canellakis (27), suggested that this constituted a homeostatic mechanism by which RNA biosynthesis might be regulated. The decreased capacity of regenerating rat liver to degrade uracil was later attributed to a delay in the synthesis of dihydrouracil dehydrogenase shortly after initiation of growth (18).

(c) Previous work about partial purification and properties of dihydrouracil dehydrogenase from different sources.

Between 1956 and 1960 dihydrouracil dehydrogenase was partially purified and some of its properties studied. Campbell was able to purify the enzyme from C. uracilicum, strain M5-2, obtaining a 27-fold purification after ammonium sulphate fractionation and repeated calcium phosphate gel treatment (19). This partially purified enzyme was specific for reducing uracil with the cofactor NADH; it had pH optimum in the range of 7.0-7.8, its apparent K_m value was 1.4×10^{-4}M, and it catalysed the reverse reaction, (i.e. DHU → uracil) in the presence of NAD^+ (19). Grisolia and Cardoso succeeded in purifying dihydrouracil

dehydrogenase from beef liver (20). They were able to obtain a 20-fold purification of the enzyme after repeated ammonium sulphate fractionation and gel treatment. This enzyme catalysed a reversible reaction showing high specificity for uracil and thymine as substrates and NADPH as cofactor. The pH optimum was 7.4, it was activated by sulphosalicylate and salicylate, and its K_m values were calculated to be less than 3×10^{-6}M with pyrimidines and 6×10^{-4}M for dihydropyrimidines. In 1960 Fritzson succeeded in partially purifying dihydrouracil dehydrogenase from the soluble cytoplasmic fraction of rat liver (21). In these studies uracil was used as substrate and NADPH as cofactor. The enzyme catalysed the reaction reversibly, giving a ratio of 0.2 for the rate of DHU oxidation to the rate of uracil reduction at optimal substrate concentrations. The pH optimum was from 7.0 to 7.6 for the partially purified enzyme. The K_m value was estimated to be less than 4×10^{-6}M for uracil and 1.7×10^{-5}M for NADPH. By measuring the formation of β-alanine-C^{14} from uracil-2-C^{14}, Fritzson found that in the presence of ATP and NaF the catabolic activity of the whole homogenate and the supernatant was the same. From this he concluded that the enzyme dihydrouracil dehydrogenase was located exclusively in the soluble cytoplasmic fraction (21).

(d) Previous work describing the inhibition of pyrimidine catabolism.

The inhibition of pyrimidine degradation has been studied in plant seedlings (22), and also in rat and hamster liver (23, 24). Šebesta, Bauerová, and Šormova (22), found that in both plant seedlings and rat liver the production of $C^{14}O_2$ from uracil-2-C^{14} and thymine-2-C^{14} was inhibited competitively by 5-bromouracil and 5-nitrouracil. Since

no labelled compound other than uracil-2-C^{14} or thymine-2-C^{14} was found after incubation, these workers claimed that the two analogues most likely affected the first reaction in each catabolic pathway, i.e. uracil → DHU, or thymine → DHT. Bresnick carried out an extensive study of the effects of various pyrimidine ribo- and deoxyribo-nucleosides and nucleotides on the degradation of uracil in rat liver (23). Using the cytoplasmic supernatant fraction of rat liver as the source of the enzyme system, Bresnick found that the production of $C^{14}O_2$ from uracil-2-C^{14} was inhibited by thymidine, deoxyuridine, uridine, deoxy-TDP and deoxy-TTP. From these results, Bresnick suggested that thymidine, uridine or derivatives thereof may function as regulators of uracil catabolism. Dorsett found that 5-cyanouracil inhibited uracil reduction in rat liver (24).

MATERIALS AND METHODS

Materials

Dihydrothymine (Sigma grade), dihydrouracil (Sigma grade), uracil (Sigma grade), thymine (Sigma grade), β-diphosphopyridine nucleotide, reduced (Sigma grade), triphosphopyridine nucleotide, reduced (Type I), β-diphosphopyridine nucleotide (Sigma grade), triphosphopyridine nucleotide (Sigma grade), adenosine 5'-phosphate (Type III), adenosine 5'-diphosphate (Sigma grade), Sigma 104 phosphatase substrate, disodium salt (Sigma grade), Trizma-HCl (reagent grade), Trizma base (reagent grade), ethylenediaminetetraacetic acid (Sigma grade), and Triton X-100 were purchased from Sigma Chemical Company. Adenosine-5'-triphosphoric acid (ATP) was obtained from General Biochemicals. Glucose-6-phosphate and monosodium glutamate were purchased from Nutritional Biochemicals Corporation. Glycerol (Analar), dipotassium hydrogen orthophosphate (Analar), potassium dihydrogen orthophosphate (Analar), and magnesium chloride (Analar) were obtained from the British Drug Houses Limited. Sucrose (special enzyme grade) and ammonium sulfate (special enzyme grade) were purchased from Mann Research Laboratories.

Preparation of Solutions

Deionized, glass-redistilled water was used to prepare all solutions and was included in the incubation mixtures when necessary. Unless otherwise specified all solutions except salt and sucrose solutions were made up at pH 7.0. The buffers and sucrose solutions were stored in a refrigerator at 2° and all other solutions were stored at -10° until they were required for use.

Animals

Male Holtzman rats weighing 200-350 g were used. The rats were decapitated; then the livers were perfused in situ with ice-cold 0.9% NaCl, removed, blotted and placed in an ice-cold beaker at 0-4°. All additional procedures were done in a cold room at 2-4°.

Preparation of Liver Homogenates

Enzyme-grade reagent sucrose was used for the preparation of all sucrose solutions. For the preparation of intracellular constituents by differential centrifugation, the livers were minced and homogenized in eight volumes of 0.25M sucrose + 3mM magnesium chloride + 5mM β-mercaptoethanol, by means of a Potter-Elvehjem apparatus equipped with a teflon pestle. This homogenate was then filtered through eight layers of cotton gauze.

For the purification of plasma membranes the livers were minced and homogenized in five volumes of 0.02M Tris buffer pH 7.0 + 0.01M EDTA pH 7.0 + 5mM β-mercaptoethanol by means of the same apparatus previously described. This homogenate was also filtered through eight layers of gauze.

Separation of Intracellular Fractions

The intracellular constituents were separated by differential centrifugation as described in Figure 2.

The centrifugation method is that used previously by other workers (25). The homogenate was prepared as described previously for separation of intracellular constituents. A refrigerated Sorvall RC-2 centrifuge with fixed angle SS-34 rotor was used for the preparation of the crude nuclear fraction as well as heavy and light mito-

chondria. A Spinco model L, preparative ultracentrifuge was used to prepare the microsomal and supernatant fractions.

Dialysis of Fractions

A portion of each fraction was dialysed for 16 hours against 100 times its volume of 0.05M phosphate buffer pH 7.0 + 1mM EDTA + 5mM β-mercaptoethanol, in a continuous flow dialysing apparatus (Model B. Oxford Laboratories).

Purification of Nuclei

The nuclei were purified from the crude nuclear pellet according to Figure 3. The method is a slight modification of that used by other workers (26).

Suspension of the crude nuclear pellet in 2.4M sucrose was done by blending in a Virtis at 2,000-3,000 r.p.m. for 2 minutes. The suspension was centrifuged at 64,000xg in a swingout bucket rotor (Spinco SW25) in a Spinco model L preparative ultracentrifuge.

The fraction referred to as "debris" (Figure 3) floated close to the top of the tube in a thick band. The supernatant was removed by means of a syringe, and the "debris" carefully removed with a spatula, before the nuclear pellet was washed. The "debris" contained unbroken cells, subcellular particles and membranes. The purified nuclear fraction contained unbroken nuclei with negligible contamination by other particles.

Purification of Lysosomes

De Duve et al (35) have shown that lysosomes are concentrated in the light mitochondrial fraction of rat liver. Consequently, the lysosomes were purified from the light mitochondrial fraction according to a method used previously by other workers (25).

The purification steps are represented in Figure 4. Acid phosphatase was used as the lysosomal marker enzyme. Total and specific activities of this marker enzyme were measured at each step of fractionation to locate the lysosome-rich fraction.

Purification of Plasma Membranes

Plasma membranes were purified according to the method of Bosmann, Hagopian and Eylar (28). The purification procedure is outlined in Figure 5. 5'-Nucleotidase which has been shown by other workers to be a plasma membrane (28, 29), and microsomal (30) enzyme was used as the plasma membrane marker enzyme. Total and specific activities of this marker enzyme was measured at each step of fractionation to locate the membrane-rich fraction. Glutamate dehydrogenase, acid phosphatase and glucose-6-phosphatase were also assayed at each step of fractionation in order to estimate the amount of other intracellular particles contaminating the plasma membrane-rich fraction.

Sucrose Density Gradient

The heavy and light mitochondrial fractions were separately applied to sucrose density gradients (Figures 6 and 7) in order to separate mitochondria from contaminating intracellular particles. Linear sucrose density gradients were prepared according to the method of Britten and Roberts (31). The gradients made were similar to those used by Nass for further purification of rat liver mitochondrial fractions (32). The gradients were linear from 0.5M sucrose to 1.91M sucrose. A wider separation of the lighter particles and the mitochondria was obtained by using 0.5M instead of 1.03M sucrose (32) as the lowest sucrose concentration. Both sucrose solutions

contained 1mM EDTA and 5mM β-mercaptoethanol. The gradient was always prepared from fresh sucrose solutions by placing with the needle valve connecting the two chambers closed, 12 ml of 1.91M sucrose in the chamber with the outlet to the centrifuge tube and an equal weight of 0.5M sucrose in the other chamber. The combined volume was 27-28 ml.

The needle valve was then opened, a stirrer in the outlet chamber turned on and the gradient started. The solution was allowed to run down the side of the centrifuge tube at approximately 0.5 ml per minute. The gradient was used immediately after preparation. Three milliliters of heavy or light mitochondrial fraction were layered on top of the sucrose density gradient. The top of the solution was then gently stirred with a stirring rod and the tube was centrifuged for 1½ hours at 70,000xg in a swingout bucket rotor (Spinco-SW25) in a Spinco Model L preparative ultracentrifuge. After centrifugation, 3 ml fractions were collected in graduated test tubes beginning at the bottom of the centrifuge tube, through a hypodermic syringe needle, which was inserted in the bottom of the tube.

Partial Purification of Supernatant Enzyme

Liver homogenate was prepared as described previously. The homogenate was centrifuged at 160,000xg for one hour in a Spinco Model L4 preparative ultracentrifuge. The supernatant was carefully withdrawn with a syringe to avoid the lipid layer and loose sediment. After dialysis against 0.05M phosphate buffer, pH 7.0, for 16 hours, ammonium sulphate was added slowly with constant stirring to 25% saturation. The mixture was stirred slowly for 15 minutes and then centrifuged at 12,000xg for 20 minutes. The precipitate (AS_1) was dissolved in a minimum volume of 0.25M sucrose + 5mM β-mercaptoethanol.

To the resulting supernatant, ammonium sulphate salt was added slowly with constant stirring to 55% saturation. The mixture was then centrifuged at 12,000xg for 20 minutes and the precipitate (AS_2) dissolved in minimum volume of 0.25M sucrose + 5mM β-mercaptoethanol. To the resulting supernatant, ammonium sulphate salt was added to 70% saturation. The mixture was centrifuged as before and the precipitate (AS_3) dissolved in 0.25M sucrose + 5mM β-mercaptoethanol. The AS_2 fraction contained 72-77% of the supernatant enzyme activity.

Protein Analysis

Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (33).

Enzyme Assays

Acid Phosphatase

Enzymic activity was measured by following the release of p-nitrophenol from p-nitrophenyl-phosphate (Sigma 104 phosphatase substrate, disodium salt) (34).

The incubation mixture consisted of the following, in μmoles: p-nitrophenyl phosphate, 10.5; acetate buffer, pH 5.0, 50.0. Triton X-100 was added to give a final concentration of 0.1%. Tissue preparation and water was added to give a final volume of 1 ml. The reaction was started by the addition of substrate and incubation was carried out for 10 minutes at 37°. The reaction was stopped by the addition of 1 ml of ice-cold 1 N perchloric acid (PCA). After centrifugation 1 ml of the supernatant was transferred to tubes containing 3 ml of 0.2M NaOH. The optical density of the resulting yellow solution was read against incubated controls at 400 mμ in a Beckman D.U. Spectrophotometer. The hydrolysis of 1.0 μmole

of substrate is equivalent to an increase in optical density of 4.58 units. All assays were done in duplicate.

A unit of enzyme activity is defined as that quantity which catalyses the release of 1 μmole of p-nitrophenol per hour. Specific activity is the number of enzyme units per milligram of protein.

Glucose-6-Phosphatase

Enzymic activity was measured by following the release of inorganic phosphate from glucose-6-phosphate. The method of assay used was a modification of the method of De Duve, Pressman, Gianetto, Wattiaux and Appelmans (35). Unless otherwise stated, the incubation mixture contained the following in μmoles: acetate buffer, pH 6.5 1.0; glucose-6-phosphate, pH 6.5, 40; histidine, pH 6.5, 7; EDTA, pH 6.5, 1.0; tissue preparation and water to give a final volume of 1 ml. The reaction was started by the addition of substrate and incubation was carried out for one hour at 37°, after which the reaction was stopped by the addition of 1 ml 10% TCA. The resulting mixture was centrifuged at 17,500xg for 10 minutes and 0.25 ml of the supernatant was taken for the determination of inorganic phosphate according to the method of Gomori (36). Control tubes were incubated at 37° for 60 minutes without substrate, which was added immediately after the reaction was stopped. Inorganic phosphate was determined as described above and the results of the controls were subtracted from the test values. All assays were done in duplicate.

Correction for unspecific phosphatase activity was done according to a modification of the method of others (37). Identical aliquots of tissue preparation as used in the test samples were adjusted to pH 5.0 by the dropwise addition of 0.1M acetate buffer,

pH 5.0. These aliquots were then heated at 37° for 5 minutes to inactivate glucose-6-phosphatase. The other reagents of the incubation mixture for the test samples were then added, and the reaction was started by the addition of substrate. Incubation was carried out at 37° for one hour and the reaction was stopped with 1 ml 10% TCA. The resulting mixture was centrifuged at 17,500xg in a Sorvall RC-2 for 10 minutes and 0.25 ml of the supernatant was taken for inorganic phosphate determination according to the method of Gomori (36).

Control tubes which were similarly preincubated for 5 minutes were incubated further without substrate for 60 minutes at 37°. Substrate was added immediately after the reaction was stopped, the inorganic phosphate was determined as described above, and the results of the controls were subtracted from the test values. Correction of the values of glucose-6-phosphatase activity was then made for unspecific phosphatase activity.

A unit of enzyme activity is defined as that quantity which catalyses the release of 1 μmole of inorganic phosphate per hour.

Specific activity is the number of enzyme units per milligram of protein.

Glutamate Dehydrogenase

Enzymic activity was measured by the method of Hogeboom and Schneider (1953) as modified by Beaufay, Bendall, Baudhuin and De Duve (38).

The reaction mixture contained the following in μmoles: potassium phosphate buffer, pH 7.7, 20; nicotinamide, 30; potassium cyanide, 0.4; β-mercaptoethanol, 5; potassium glutamate, 13; NAD^+ 1.4; 0.1% Triton X-100; and enzyme in a total volume of 1 ml. The

reaction was started by the addition of glutamate and the increase in optical density was followed at 340 mμ at 25° in a Beckman D.B.G. recording spectrophotometer with a reference cell containing all components of the system except glutamate. Triton X-100 was used to solubilize the enzyme. The extinction coefficient was 6.22×10^6 per mole of NADH.

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

Pyrimidine Reductase

The enzyme activity was assayed spectrophotometrically by the method of Yamada (39), by measuring the decrease in absorption at 290 mμ or 295 mμ 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 as follows: uracil in the presence of NADPH at 290 mμ, 14.94; uracil in the presence of NADH at 290 mμ, 17.74; thymine in the presence of NADPH at 295 mμ, 13.18. Routinely, the incubation mixture contained the following (in μmoles) in a final volume of 1.5 ml: potassium phosphate buffer pH 7.5 (for the assay with NADPH) or pH 6.5 (for the assay with NADH) 250; β-mercaptoethanol, 7.5; enzyme diluted to 0.5 ml with 0.05M potassium phosphate buffer pH 7.0; NADH or NADPH, pH 6.0, 0.25; and uracil or thymine, pH 7.0, 0.2. The final pH of the incubation mixture was optimum (7.45 or 6.5). The reaction was started by the addition of substrate and the tubes were incubated for 10 minutes at 37°. The reaction was stopped by the addition of 0.45 ml 2.12 N PCA in an ice bath. The mixture was centrifuged at 17,500xg at 0° for 10 minutes and 1 ml of the supernatant was added to 0.07 ml

of 10 N NaOH. The readings were then taken on a Beckman D.U. spectrophotometer. The controls were incubated without substrate which was added immediately after the addition of PCA. In some cases the controls were incubated at room temperature since there was no observable difference between the oxidation of NADH or NADPH in control tubes at 37° and at room temperature. All assays were done in duplicate.

One enzyme unit is defined as that quantity which catalyses the formation of 1 μmole of dihydropyrimidine per hour.

Specific activity is the number of enzyme units per milligram of protein.

Dihydropyrimidine Dehydrogenase

The enzymic activity was measured spectrophotometrically by the method of Yamada (39) by measuring the increase in absorption at 290 or 295 mμ resulting from the conversion of dihydropyrimidine to pyrimidine in the presence of NAD^+ or $NADP^+$. The increases in absorption per μmole of product formed are as follows: dihydrouracil in the presence of NAD^+ at 290 mμ, 17.74; dihydrouracil in the presence of $NADP^+$ at 290 mμ, 14.94; dihydrothymine in the presence of $NADP^+$ at 295 mμ, 13.18. Routinely, the incubation mixture contained the following (in μmoles) in a final volume of 1.5 ml: potassium phosphate buffer pH 7.5 (for the assay with $NADP^+$) or Tris buffer pH 9.0 (for the assay with NAD^+) 250; β-mercaptoethanol 7.5; ATP pH 7.0, 15; $MgCl_2$, 15; enzyme diluted to 0.5 ml with 0.05M potassium phosphate buffer, pH 7.0; NAD^+ or $NADP^+$, pH 8.0, 1.5; and dihydrouracil or dihydrothymine pH 7.0, 15. The final pH of the incubation mixture was optimum (7.45 or 8.75). The reaction was started by the addition of substrate

and the tubes were incubated for 10 minutes at 37°. The reaction was stopped by the addition of 0.45 ml of 2.12 N PCA in an ice bath. The mixture was then centrifuged at 17,500xg in a Sorvall RC-2 at 0° for 10 minutes, and 1 ml of the supernatant was added to 0.025 ml of 1 M EDTA, pH 10.8. To this mixture 0.07 ml of 10 N NaOH was added and the readings taken in a Beckman D.U. spectrophotometer.

The controls were incubated without substrate which was added immediately after the addition of PCA. In some cases the controls were incubated at room temperature since there was no observable difference between the reduction of NAD^+ or $NADP^+$ in control tubes at 37° and at room temperature. All assays were done in duplicate.

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

5'-Nucleotidase

Enzyme activity was measured by following the release of inorganic phosphate from 5'-AMP. The assay was done according to the method of other workers (40). Unless otherwise stated, the incubation mixture contained the following, in μmoles: Tris buffer, pH 7.2, 50; KCl, 100; $MgCl_2$, 10; tissue preparation and water to give a final volume of 1 ml. The reaction was started by the addition of substrate and incubation was carried out for 10-15 minutes at 37° after which the reaction was stopped by the addition of 1 ml 10% TCA. The resulting mixture was centrifuged at 17,500xg in a Sorvall RC-2 at 0° for 15 minutes and 1 ml of the supernatant was taken for the determination of inorganic phosphate according to the method of Gomori (36).

Control tubes were incubated without substrate which was

added immediately after the reaction was stopped. Inorganic phosphate was determined as described above and the results of the controls were subtracted from the test values. All assays were done in duplicate.

A unit of enzyme activity is defined as that quantity which catalyses the release of 1 μmole of inorganic phosphate per hour. Specific activity is the number of enzyme units per milligram of protein.

RESULTS

Intracellular Distribution of Enzymes

Marker Enzymes

Table I shows the distribution of the marker enzymes glutamate dehydrogenase, 5'-nucleotidase, acid phosphatase and glucose-6-phosphatase among the subcellular fractions of rat liver homogenate.

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, except that in one case the microsomal fraction was not separated from the supernatant fraction. From Table I it can be seen that 56.13 per cent of the glutamate dehydrogenase activity was present in the heavy mitochondrial fraction which also had the highest specific activity. Most of the acid phosphatase activity was distributed between the debris (21.44 per cent) and the light mitochondrial fraction (24.23 per cent). The highest specific activity for glucose-6-phosphatase (44.48) was present in the microsomes, whereas the highest percentage recovery was in the debris (27.26 per cent). The debris contained the highest percentage of 5'-nucleotidase activity (43.95 per cent), while the rest was spread throughout the other fractions, with the highest specific activity in the light mitochondrial fraction. Purified nuclei contained negligible or no marker enzyme activities, whereas the debris which is usually pelleted in the crude nuclear fraction (Figure 2) contained significant activity for each marker enzyme. These results conform to the distributions obtained by other workers (38)

for glutamate dehydrogenase, acid phosphatase and glucose-6-phosphatase of rat liver, in which however the crude nuclear fraction (N) (38) contained at least half the activity of the debris fraction. Although the highest specific activity for glucose-6-phosphatase was present in the microsomes, the percentage recovery in this fraction was low when compared to the results of other workers (38, 41). The distribution of 5'-nucleotidase (Table I) is similar to the distribution obtained by Wattiaux-De Coninck and Wattiaux (41) in rat liver, except that their percentage recovery in the microsomes and soluble fraction is higher. Dialysis of the fractions did not have any significant effect on the marker enzyme activities.

Pyrimidine Reductases

Table II shows the distribution of NADPH-linked uracil reductase, NADH-linked uracil reductase and NADPH-linked thymine reductase among the subcellular fractions of rat liver homogenate. The homogenate was prepared as described under "Materials and Methods" and the intracellular constituents were separated by differential centrifugation according to the procedures outlined in Figure 2. From Table II it is seen that over 57 per cent of the NADPH-linked uracil reductase activity is distributed between the heavy and light mitochondrial fractions with the highest specific activity in the light mitochondrial fraction. On the other hand 54.34 per cent of the NADH-linked uracil reductase activity was found in the heavy mitochondrial fraction which also had the highest specific activity. The distribution of NADPH-linked thymine reductase activity is very similar to the distribution of NADPH-linked uracil reductase activity, except that 27.54 per cent of NADPH-linked thymine reductase activity was found in the soluble fraction. Purified nuclei contained no activity, and again the debris fraction contained significant activity.

Table III shows the effect of dialysis on the activity of the reductases. The distribution of each reductase activity remains almost unchanged, but slight decreases in specific activity were noticed. The activity distribution pattern for NADPH-linked uracil reductase does not support the results of others who suggested that the localization of uracil catabolic pathway was mainly in the supernatant fraction (9, 8).

Dihydropyrimidine Dehydrogenases

Table IV shows the distribution of $NADP^+$-linked dihydrouracil dehydrogenase, NAD^+-linked dihydrouracil dehydrogenase and $NADP^+$-linked dihydrothymine dehydrogenase among the subcellular fractions of rat liver homogenate. 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 IV it is seen that over 80 per cent of each dehydrogenase activity was found in the soluble fraction with only trace activity in the particulate fractions. In these experiments 5.4M glycerol was added to the subcellular fractions for both solubilization and stabilization of the activity. In the absence of glycerol the particulate fractions had little or no dehydrogenase activity and almost 100 per cent was recovered in the supernatant fraction.

Purification of Nuclei

Marker Enzymes

Table V shows the distribution of the marker enzymes in the fractions obtained during the purification of nuclei. The homogenate was prepared as described under "Materials and Methods". The homogenate was divided into a crude nuclear fraction and supernatant (S_1).

The crude nuclear fraction was then separated into "pure nuclei" and debris according to the procedure outlined in Figure 3. From Table V-A it is seen that there is a very low percentage and a very low specific activity for each marker enzyme in the nuclear fraction. This indicates that the nuclear fraction was only negligibly contaminated by the particulate fractions.

Pyrimidine Reductases

Table V-B shows the distribution of the pyrimidine reductases in the fractions obtained during the purification of nuclei. The homogenate was prepared as described under "Materials and Methods" and the fractions were prepared according to the procedure outlined in Figure 3. It can be seen from Table V-B that the activities of the pyrimidine reductases were not found in the purified nuclei.

Purification of Lysosomes

Marker Enzymes

The distribution of marker enzymes in the fractions obtained during the purification of lysosomes is shown in Table VI. The homogenate was prepared as described under "Materials and Methods", and the fractions were prepared according to the procedure outlined in Figure 4. The light mitochondria (F_1) (Figure 4) was used as the source of lysosomes. F_5 represents the purified lysosomes. From Table VI it is seen that the absence of glutamate dehydrogenase from F_5 is an indication that this fraction is free of mitochondria. The very low recovery of glucose-6-phosphatase and 5'-nucleotidase in F_5 indicates that this fraction is slightly contaminated with smooth membranes of microsomes (30, 62). The fact that acid phosphatase had a 42 fold purification in F_5 is an indication that the lysosomal particles were significantly purified. Other workers (25) have reported a 70 fold purification of acid phosphatase activity and a 10% recovery of lysosomes from rat liver.

Pyrimidine Reductases

The activity distribution of the pyrimidine reductases in the fractions obtained during the purification of lysosomes is shown in Table VII. The homogenate was prepared as described under "Materials and Methods" and the fractions were prepared by differential centrifugation according to the procedure outlined in Figure 4.

From Table VII it is seen that in the purified lysosomal fraction only 0.001 per cent of NADPH-linked uracil reductase activity was present having very low specific activity, and no activity was found for the other reductases. Since glucose-6-phosphatase and 5'-nucleotidase were purified in F_5 (Table VI), this is an indication that uracil reductase is not a part of the smooth membranes.

Purification of Plasma Membranes

Marker Enzymes

Table VIII shows the distribution of the marker enzymes in the fractions obtained during the purification of plasma membranes. The homogenate was prepared as described under "Materials and Methods" and the fractions were prepared by discontinuous sucrose density gradient according to the procedure outlined in Figure 5. 1S-2S represents smooth internal membranes and vesicular smooth membranes, 3S-6S represents ribosomal and particulate material and 7S represents plasma membranes (28).

Glutamate Dehydrogenase

As seen from Table VIII, 91.70 per cent of the glutamate dehydrogenase activity in the homogenate was found in the 4,000xg pellet (4g-P). Of the 5.70 per cent found in the 4,000xg supernatnat fluid (4g-S) almost all (5.10 per cent) was found in the 70,000xg

supernatant (70S-Supernatant). This indicates that 5.10 per cent of the glutamate dehydrogenase activity was released from the mitochondria contaminating the 4g-S supernatant into the supernatant fluid. These results indicate that almost all the mitochondrial material is sedimented in the 4g-P pellet.

Acid Phosphatase

The acid phosphatase activity in the homogenate was partitioned almost equally between 4g-P and 4g-S (Table VIII). The 46.13 per cent present in 4g-S was distributed between 1S-6S (9.80 per cent), the plasma membranes (10.40 per cent), and the 70S-Supernatant (24.70 per cent). The presence of 24.70 per cent in the 70S-Supernatant is an indication that this amount of acid phosphatase was released under the conditions used for preparation of the fractions. Other workers (50) have also found acid phosphatase associated with purified plasma membranes of rat liver.

Glucose-6-Phosphatase

The activity of glucose-6-phosphatase present in the homogenate was partitioned between 4g-P and 4g-S (Table VIII). The plasma membrane fraction contained 18.17 per cent with a four-fold purification by comparing the specific activities of the homogenate (5.17), and the plasma membranes (20.70). Other workers (42, 43) have also found glucose-6-phosphatase present in purified plasma membranes of rat liver. The plasma membrane fraction is probably contaminated by microsomes.

5'-Nucleotidase

Table VIII shows that 35.90 per cent of the 5'-nucleotidase activity was present in 4g-P and 61.80 per cent in 4g-S. Most of the activity present in 4g-S was recovered in the plasma membrane fraction.

which had 45.40 per cent. The fractions 1S-6S had 11.9 per cent. These results strongly suggest that 5'-nucleotidase is an integral part of the plasma membranes.

By comparing the specific activity of the homogenate (2.00) with the specific activity of 7S the plasma membrane fraction (15.60) the degree of plasma membrane purity is 7.8 fold. These results are similar to results obtained by other workers for the distribution of 5'-nucleotidase in the membrane fractions of Hela cells (28), although, in our case the purification is lower.

Dialysis had no significant effect on the marker enzyme activities in these fractions.

Pyrimidine Reductases

The distribution of the pyrimidine reductases in the fractions obtained during the purification of plasma membranes is shown in Table IX. The homogenate was prepared as described under "Materials and Methods" and the fractions were prepared by discontinuous sucrose density gradient according to the procedure outlined in Figure 5.

NADPH-linked Uracil Reductase

From Table IX it is seen that the activity of NADPH-linked uracil reductase in the homogenate was almost equally divided between 4g-P and 4g-S. A large portion of the 4g-S activity was found in the 70S-Supernatant indicating that 37.90 per cent of this enzyme was released into the 70S-Supernatant fraction because of lysis of particulate matter during homogenization. This was not the case when homogenization was done in sucrose (Tables II and III). The presence of 8 per cent in 1S-6S and 14.20 per cent in the plasma membrane fraction strongly suggests that a significant percentage of the NADPH-linked uracil reduc-

tase, is membrane bound.

NADH-linked Uracil Reductase

Table IX shows that 76.90 per cent of the NADH-linked uracil reductase activity was found in 4g-P with 27.20 per cent in 4g-S. Of the latter only 6.30 per cent was found in the plasma membrane fraction but 18.80 per cent in the 70S-Supernatant which indicates that 18.80 per cent was extracted under the conditions used for preparation of the fractions. These results suggest that most of the NADH-linked uracil reductase activity is associated with the material which sedimented in the 4g-P pellet in which the activity of the mitochondrial enzyme glutamate dehydrogenase was almost completely recovered (91.70 per cent) (Table VIII).

NADPH-linked Thymine Reductase

Table IX shows that 66.30 per cent of NADPH-linked thymine reductase activity was found in 4g-S with 31.50 per cent in 4g-P. The activity of 4g-S was distributed between the membrane fractions and the 70S-Supernatant. The plasma membrane fraction had 11.90 per cent and the presence of 48.90 per cent in the 70S-Supernatant indicates that a significant amount of this activity was released into the 70S-Supernatant due to lysis of particulate matter during homogenization. These results suggest that some of the NADPH-linked thymine reductase is also associated with the plasma membranes.

Dihydropyrimidine Dehydrogenases

The distribution of the dihydropyrimidine dehydrogenases in the fractions obtained during the purification of plasma membranes is shown in Table X. The homogenate was prepared as described under "Materials and Methods" and the fractions were prepared by discontinuous

sucrose density gradient according to the procedure outlined in Figure 5. Table X shows that 4g-S contains 108 per cent of $NADP^+$-linked dihydrouracil dehydrogenase activity, 87.50 per cent of NAD^+-linked dihydrouracil dehydrogenase activity and 100 per cent $NADP^+$-linked dihydrothymine dehydrogenase activity, with only trace amounts for each dehydrogenase in 4g-P. The relatively low percentage and low specific activity of each dehydrogenase in 1S-6S and the plasma membrane fraction (7S), and the presence in the 70S-Supernatant of almost all the activity for each dehydrogenase is an indication that most of the dihydropyrimidine dehydrogenase activity is in the soluble matrix.

Sucrose Density Gradient Fractionation

Heavy Mitochondrial Fraction

Figure 6 shows the activity of NADPH-linked uracil reductase, NADH-linked uracil reductase, glutamate dehydrogenase and 5'-nucleotidase in the fractions collected after centrifugation of the heavy mitochondrial fraction on a linear sucrose density gradient. The gradient was prepared as described under "Materials and Methods". Glutamate dehydrogenase and NADH-linked uracil reductase have similar activity profiles and both of these enzymes have their activity peaks in the same fraction. The activity peak for NADPH-linked uracil reductase lies between the respective peaks for glutamate dehydrogenase and 5'-nucleotidase. In tube number two the NADH-linked uracil reductase activity is greater than the NADPH-linked uracil reductase activity, while in tubes number three to ten the NADPH-linked uracil reductase activity switches over to become slightly greater than the NADH-linked uracil reductase activity. From these results (Figure 6) it appears as if most of the NADH-linked uracil reductase activity is associated with the mitochondria, while the NADPH-linked uracil reductase activity

seems to be divided between the mitochondria and the plasma membranes.

Light Mitochondrial Fraction

Figure 7 shows the activities of NADPH-linked uracil reductase, NADH-linked uracil reductase, glutamate dehydrogenase and 5'-nucleotidase in the fractions collected after centrifugation of the light mitochondrial fraction on a linear sucrose density gradient. The gradient was prepared as described under "Materials and Methods". In these fractions the NADPH-linked uracil reductase activity is higher than the NADH-linked uracil reductase activity. The activity profile for all four enzymes show that glutamate dehydrogenase and NADH-linked uracil reductase have their activity peaks in the same fraction (similar to results in Figure 6), while NADPH-linked uracil reductase and 5'-nucleotidase also have their activity peaks in a common fraction. There is no significant separation of the two uracil reductases, but the NADH-linked uracil reductase activity does seem to follow the activity profile of glutamate dehydrogenase, while the NADPH-linked uracil reductase activity more closely follows the activity profile of 5'-nucleotidase.

Properties of Enzymes

Marker Enzymes

Under the conditions of assay used, linearity with protein and with time was obtained for all marker enzymes in the enzyme fractions.

NADH-linked Uracil Reductase

pH Optimum

From Figure 8A it is seen that the pH optimum of NADH-linked uracil reductase activity of the heavy mitochondrial fraction is 6.5 while it is 6.7 in the light mitochondrial fraction (Figure 8B). Figure 9 shows that the NADH-linked uracil reductase activity of the

soluble cytoplasmic fraction is 6.5. These results indicate that there is very little variation between the optimum pH values obtained for NADH-linked uracil reductase activity in the heavy mitochondria, light mitochondria or soluble fraction.

Activity and Protein Concentration

The relationship between protein concentration and NADH-linked uracil reductase activity of the heavy mitochondrial fraction is shown in Figure 10. It is apparent that there is a linear relationship between activity and protein concentration up to 0.80 mg of protein. The heavy mitochondrial fraction was used for this study since it had the highest specific activity and largest percentage of NADH-linked uracil reductase activity.

Time Relationship

Figure 11 shows that NADH-linked uracil reductase activity of the heavy mitochondrial fraction is linear with time for 16 minutes. Linearity was observed up to a change in optical density of 0.40.

Cofactor Concentration

The heavy mitochondrial fraction was used as the source of NADH-linked uracil reductase activity. Figure 12 shows the relationship between uracil reduction and the concentration of the cofactor NADH. The Michaelis-Menten constant (Km) was found to be 3.3×10^{-5}M, and the optimal NADH concentration was above 2.5×10^{-4}M. The finding that uracil reduction is NADH-linked is in agreement with the results of Canellakis (9), for rat liver and Campbell (19) for bacteria. Canellakis also found that the NADH-linked uracil reductase activity disappeared after extensive dialysis against Tris buffer for 24-40 hours (9). No further studies of the NADH-linked uracil reductase activity of rat liver have been reported

to date and hence it is impossible to make any further comparisons.

Inhibition Study

In these studies the heavy mitochondrial fraction was used as the source of NADH-linked uracil reductase activity. Table XI shows that 5mM and 10mM ATP concentrations inhibited the NADH-linked uracil reductase activity by 20.1 per cent and 29.6 per cent respectively. Lower inhibition was also produced by 5mM and 10mM Mg^{++} (Table XI). When ATP and Mg^{++} were added in equimolar amounts 10mM concentration of the ATP-Mg complex produced 47.2 per cent inhibition (Table XI). No inhibition was observed in the presence of ADP or AMP up to a concentration of 10mM. ADP-Mg and AMP-Mg had very low inhibitory effects at 5mM and 10mM concentrations, while inorganic phosphate showed no inhibition at concentrations up to 50 and 100mM (Table XI).

NADPH-linked Uracil Reductase

pH Optimum

The relationship between pH and NADPH-linked uracil reductase activity is shown in Figure 8. With the heavy mitochondrial fraction the pH optimum is 7.45 (Figure 8-A), while it is 7.7 with the light mitochondrial fraction (Figure 8-B).

From Figure 9 it is apparent that the NADPH-linked uracil reductase activity of the soluble cytoplasmic fraction has its pH optimum at 7.45. It is therefore very clear that there is only a very slight variation between the pH optimum obtained for the NADPH-linked uracil reductase activity of the heavy mitochondrial fraction, light mitochondrial fraction or soluble fraction. These optimum pH values are in relatively close agreement with those obtained by other workers for NADPH-linked uracil reductase activity from rat liver (21)

~~and beef liver (20).~~

Activity and Protein Concentration

The relationship between NADPH-linked uracil reductase activity and protein concentration in the light mitochondrial fraction is shown in Figure 13. A linear relationship between uracil reduction and protein concentration was observed up to 0.4 mg of protein.

Time Relationship

Figure 14 shows the relationship between time and NADPH-linked uracil reductase activity of the light mitochondrial fraction. The reduction of uracil was linear with time for 14 minutes. Linearity was observed up to a change in optical density of 0.40. The relationship between time and NADPH-linked uracil reductase activity of an ammonium sulphate fraction (AS_2) prepared from the soluble cytoplasmic fraction is shown in Figure 15. Using 6.35 mg of AS_2 protein, the reduction of uracil was linear with time for 9 minutes. Linearity was observed up to a change in optical density of 0.38. In all subsequent studies done with the AS_2 fraction the amount of protein used was considerably less than 6.35 mg so as to ensure linearity for at least 10 minutes.

Substrate Concentration

The relationship between uracil concentration and NADPH-linked uracil reductase activity of the light mitochondrial fraction is shown in Figure 16. The Michaelis-Menten constant (Km) was found to be 3.6×10^{-6}M, and the optimal uracil concentration was 2.75×10^{-5}M. In an ammonium sulphate fraction (AS_2) prepared from the soluble cytoplasmic fraction, the Km for uracil was determined to be 6.7×10^{-6}M, and the optimal uracil concentration was 2.75×10^{-5}M (Figure 17). These Km values obtained in the light mitochondrial fraction and in the ammonium sulphate fraction (AS_2) are in close agreement

with Km values obtained by other workers for NADPH-linked uracil reductase in an ammonium sulphate fraction prepared from the soluble fraction of rat liver (21), and in an enzyme fraction partially purified from acetone dried powder extracts of beef liver (20).

Cofactor Concentration

The relationship between uracil reduction and NADPH concentration in the light mitochondrial fraction is shown in Figure 18-A. The shape of the curve obtained indicates positive cooperativity (44). From this sigmoid curve, the NADPH concentration required to give 50 per cent saturation is estimated to be 1.1×10^{-4}M. Figure 18-B shows the relationship between NADPH concentration and uracil reduction in an ammonium sulphate fraction (AS_2) prepared from the soluble fraction. The curve obtained indicates positive cooperativity (similar to the results of Figure 18-A), and the NADPH concentration required to give 50 per cent saturation is estimated to be 7.5×10^{-5}M.

Inhibition Study

The inhibition of NADPH-linked uracil reductase activity of the light mitochondrial fraction is shown in Table XI. It can be seen that 5 and 10mM concentrations of ATP gave inhibitions of 17.4 per cent and 31.2 per cent respectively. ADP and AMP had no inhibitory effect at concentrations of 5 and 10mM. Mg^{++} showed low inhibitory effects at 5 and 10mM concentrations. When ATP and Mg^{++} were added in equimolar amounts, 10mM concentration of ATP-Mg complex gave 47.6 per cent inhibition (Table XI). ADP-Mg and AMP-Mg each at 5 and 10mM concentrations showed very low inhibition, while 50 and 100mM concentrations of inorganic phosphate had no inhibitory effects (Table XI). In an undialysed light mitochondrial fraction, ATP, Mg^{++} and ATP-Mg also in-

bited NADPH-linked uracil reductase activity (Figure 19). In this case ATP-Mg complex gave 49 per cent inhibition at 10mM concentration. The inhibitory effects of ATP and Mg^{++} in the light mitochondrial fraction is almost additive when compared to the inhibition produced by ATP-Mg (Table XI), (Figure 19). Figure 20 shows the inhibition of NADPH-linked uracil reductase activity in the ammonium sulphate fraction (AS_2). Similar to results obtained for the light mitochondrial fraction (Table XI), various concentrations of ATP-Mg complex were inhibitory. The concentration of ATP-Mg complex required to give 50 per cent inhibition was about 10.5mM (Figure 20).

Solubilization of NADPH-linked Uracil Reductase

Table XII shows the effect of glycerol on the activities of NADPH-linked uracil reductase as compared to the effect on glutamate dehydrogenase in various liver fractions. The fractions were prepared as described under "Materials and Methods". The ratio of the specific activity after addition of glycerol and the specific activity before the addition of glycerol shows that in mitochondrial fractions both glutamate dehydrogenase and NADPH-linked uracil reductase have been solubilized. For glutamate dehydrogenase which is a mitochondrial enzyme, the highest ratio $\frac{(\text{S.A. After})}{(\text{S.A. Before})}$ was found in the heavy mitochondrial fraction. In the case of NADPH-linked uracil reductase the highest ratio was found in the light mitochondrial fraction (Table XII). Glycerol has been used by other workers for the solubilization and stabilization of mitochondrial enzymes (45). The NADPH-linked uracil reductase activity increased by more than two-fold with glycerol in the debris and plasma membranes, whereas this was not the case for glutamate dehydrogenase (Table XII). This increase in specific activity for

glutamate dehydrogenase and NADPH-linked uracil reductase can also be obtained by repeated freezing and thawing. However, for fractions stored in sucrose, the NADPH-linked uracil reductase activity starts to disappear quickly after freezing and thawing 3-4 times. If these fractions are dialysed the NADPH-linked uracil reductase activity and the other reductase activities disappear at a more rapid rate after freezing and thawing.

Triton X-100 was much more effective than glycerol in solubilizing glutamate dehydrogenase but not NADPH-linked uracil reductase, particularly in the homogenate, where it had inhibitory effects. The supernatant activity is probably inhibited by Triton X-100. The solubilization of NADPH-linked uracil reductase shows that glycerol can be used to make the enzyme become more accessible to the substrate in the particulate fractions.

NAD^+-linked Dihydrouracil Dehydrogenase

Partial Purification

Partial purification of NAD^+-linked dihydrouracil dehydrogenase was achieved by ammonium sulphate fractionation of the soluble supernatant. The purification steps were carried out as described under "Materials and Methods". The ammonium sulphate fraction (AS_2) which was prepared by a 55 per cent saturation of the supernatant fraction with ammonium sulphate salt, showed 72 per cent recovery of the enzyme activity with approximately a four-fold purification (Table XIII). For all subsequent studies done on NAD^+-linked dihydrouracil dehydrogenase the ammonium sulphate fraction (AS_2) was used.

pH Optimum

Figure 21 shows the relationship between activity and pH.

It is apparent from Figure 21 that the pH optimum is approximately 8.75 when measured in Tris-HCl or glycine-NaOH buffers. Activity measured in glycine-NaOH buffers is slightly higher than that observed in Tris-HCl buffers at high pH values.

Activity and Protein Concentration

The relationship between protein concentration and NAD^+-linked dihydrouracil dehydrogenase activity is shown in Figure 22. It is seen that dihydrouracil dehydrogenation is linear with protein concentration up to 1.64 mg of protein.

Time Relationship

Figure 23 shows the relationship between time and NAD^+-linked dihydrouracil dehydrogenase activity. From Figure 23, it is apparent that the dehydrogenation of dihydrouracil is linear with time for approximately 14 minutes. Linearity was observed up to a change in optical density of 0.60.

$NADP^+$-linked Dihydrouracil Dehydrogenase

Partial Purification

$NADP^+$-linked dihydrouracil dehydrogenase was partially purified by ammonium sulphate treatment of the soluble cytoplasmic fraction. The purification steps were carried out as described under "Materials and Methods". In the ammonium sulphate fraction (AS_2), 77 per cent recovery and approximately a four-fold purification was obtained (Table XIII). In all subsequent studies done on $NADP^+$-linked dihydrouracil dehydrogenase the ammonium sulphate fraction (AS_2) was used.

pH Optimum

From Figure 24 it can be seen that the pH optimum for $NADP^+$-linked dihydrouracil dehydrogenase activity is 7.45. This result is in very close agreement with the pH optimum obtained by other workers

for partially purified $NADP^+$-linked dihydrouracil dehydrogenase of beef liver (20).

Activity and Protein Concentration

Figure 25 shows the relationship between $NADP^+$-linked dihydrouracil dehydrogenase activity and protein concentration. A linear relationship existed up to a protein concentration of 1.64 mg of protein.

Time Relationship

The relationship between time and $NADP^+$-linked dihydrouracil dehydrogenase is shown in Figure 26. A linear relationship between dihydrouracil dehydrogenation and time was observed for at least 16 minutes up to a change in optical density of 0.50.

Substrate Concentration

Figure 27 shows the relationship between dihydrouracil concentration and $NADP^+$-linked dihydrouracil dehydrogenase activity. It can be seen from Figure 27, that the shape of the plot of initial rate against DHU concentration is slightly sigmoid. The shape of this curve cannot be compared with the shape obtained by Fritzson for the same enzyme (21), since he did not investigate dihydrouracil concentrations below 2.5×10^{-3}M. The Lineweaver-Burke plot does not give a straight line but curves upwards. This is expected for a sigmoid curve which indicates activation by substrate (46). The concentration of dihydrouracil required to give 50 per cent saturation is estimated to be 1.5×10^{-3}M. This is in close agreement with the Km obtained by Fritzson for the same enzyme partially purified from the soluble fraction of rat liver (21).

Activation

The activation of $NADP^+$-linked dihydrouracil dehydrogenase by ATP, Mg^{++}, and ATP-Mg complex is shown in Figure 28. In the presence

of 5-10mM Mg^{++}, 19 per cent activation was observed (Figure 28-A).

Figure 28-B shows that in the presence of 6.6-10mM ATP 34 per cent activation was obtained. When ATP was varied in the presence of 10mM Mg^{++}, approximately 200 per cent activation was observed at a concentration of 6.6-10mM ATP-Mg (Figure 28-C). The ATP-Mg complex seems to be the most powerful activator. ATP-Mg showed activation of the homogenate and soluble cytoplasmic fraction similar to that of AS_2 (Figure 28-C).

Tissue Distribution of Pyrimidine Reductase and Dihydropyrimidine Dehydrogenase Activities

Table XIV shows that NADH-linked and NADPH-linked uracil reductase activities are present in the liver, kidney and testis of the rat, while NADPH-linked thymine reductase is present in the liver and kidney only.

All three dehydrogenase activities were found in the liver and kidney. It is interesting to note that all three dihydropyrimidine dehydrogenase activities were present in the brain where there was no reductase activity, and $NADP^{+}$-linked dihydrothymine dehydrogenase activity was found in heart and spleen, where again there was no reductase activity. No reductase or dehydrogenase activity was found in the blood or muscle.

In the absence of ATP and Mg^{++}, dihydropyrimidine dehydrogenase activity could not be measured in the tissues except for the liver where the activity was also very low.

DISCUSSION AND CONCLUSIONS

Our results indicate that in rat liver, the enzyme commonly known as dihydropyrimidine dehydrogenase can be separated into reductase and dehydrogenase activities with most of the reductase activity located in the particulate fractions of the cell and with most of the dehydrogenase activity located in the soluble cytoplasmic fraction. The pathway for the catabolism of pyrimidines (Figure 1) has been reported by Canellakis (9), Rutman et al (8) and Fritzson (21) to be localized mainly in the soluble cytoplasmic fraction of rat liver cells. The studies of Canellakis (9) and Rutman et al (8) were based upon measuring the release of $C^{14}O_2$ from uracil-2-C^{14} or thymine-2-C^{14} in rat liver slices, homogenate and soluble fraction. The studies of Fritzson (21) were based mainly on measuring the release of β-alanine-C^{14} from uracil-2-C^{14} in rat liver homogenate and soluble fraction. These workers were measuring the activity of the entire pyrimidine catabolic pathway and although they claim that this pathway was mainly in the soluble fraction, Canellakis had obtained evidence (9) which led him to suggest that this pathway might also be present in the particulate fractions. The studies of Rutman et al (8) produced evidence to show that the catabolic pathway for pyrimidines was present in the particulate fractions but in relatively low amounts although 95 per cent of the activity of liver slices was lost upon homogenization of the tissue. Fritzson (21) did not investigate the particulate fractions for activity.

Previously no one has described intracellular localization of the specific enzyme or enzymes responsible for pyrimidine reduction or dihydropyrmidine oxidation. However, from the findings of the above mentioned workers (9, 8 , 21) one would expect to find these specific enzymes mainly in the soluble cytoplasmic fraction of rat liver cells.

In present studies, the findings did not support this view. Our results for the intracellular localization of pyrimidine reductase, showed that the activity in the homogenate could be almost completely recovered, with most of it distributed between the heavy and light mitochondrial fractions, and a lesser amount in the soluble cytoplasmic fraction. The reductase activity present in the 150,000xg supernatant (soluble fraction) varied from 3-10 per cent with uracil as substrate and 25-27 per cent with thymine as substrate.

The debris contained a reasonable percentage of reductase activity, but since the marker enzyme study (Table I) and microscopic observation indicated that this fraction was almost equally contaminated with the other particulate fractions and it contained whole cells, this fraction is not homogenous and the reductase activity in it can be regarded as belonging to constituents which are mixed together in this fraction of rat liver cell.

The differences that were found between our localization studies and those of other workers (9, 8 , 21) are not surprising, in view of the number of differences in procedure which were employed for our work, and these factors are probably responsible for the variations obtained. Some of the differences and similarities are discussed below.

In present studies homogenization of the liver tissue was done

in isotonic sucrose (0.25M) containing 3mM $MgCl_2$ and 5mM β-mercaptoethanol using a Potter-Elvehjem homogenizer equipped with a teflon pestle. This was quite different from the equipment and hypotonic Tris and phosphate buffer media used by Rutman et al (8) and Canellakis (9) for their homogenization of liver slices. We do not know how much, if any, reductase activity was lost from the tissue after our homogenization, but probably the activity which was measured in the homogenate and was found distributed in the subcellular fractions was protected and stabilized by β-mercaptoethanol, since, on one occasion when β-mercaptoethanol was not stirred into the dialysing solution reductase activity was completely lost from all fractions which were assayed for activity immediately after dialysis. In these fractions the activity of uridine phosphorylase which is protected (39) and can be reactivated (47) by β-mercaptoethanol, was also completely lost.

We do not claim to have dialysed out the reductase inhibitors which Canellakis (9) and Rutman et al (8) suggested might be in the particulate fractions, since our undialysed fractions showed slightly higher reductase activity when compared to the dialysed fractions (Tables II and III). The very rapid loss of reductase activity, particularly from the particulate fractions, which we observed after the fractions were dialysed against hypotonic Tris or phosphate buffer, could be one of the reasons why little or no reductase activity was found in the particulate fractions by the above mentioned workers (8, 9). This instability of the reductase activity after dialysis, even in the presence of β-mercaptoethanol, could also be the reason why the NADH-linked uracil reductase activity of rat liver was completely lost after dialysis against hypotonic Tris buffer without a protecting agent as reported by Canellakis (9).

The results of Table IX show that when the liver tissue was homogenized in hypotonic Tris buffer with EDTA more than one third of NADPH-linked uracil reductase activity and almost one half of NADPH-linked thymine reductase activity were present in the soluble fraction. The percentage of NADH-linked uracil reductase activity in the soluble fraction also increased considerably (Table IX). This is a clear indication that the amount of reductase activity present in the soluble fraction is very dependent upon the medium in which the tissue is homogenized. The extent of homogenization might also be of importance. In the studies done by Fritzson (21), the tissue was homogenized in isotonic sucrose (o.25M) but containing EDTA at a lower concentration than in our medium. It is possible that the presence of EDTA, particularly at a high concentration, could have influenced the amount of reductase activity in the soluble fraction, but since Fritzson did not investigate the particulate fractions for reductase activity it is impossible to make further comparisons.

A comparison of the specific activities and recoveries of glutamate dehydrogenase and acid phosphatase in the 70S-Supernatant (Table VIII) and the supernatant fraction (Table I) shows that some of these particulate enzymes were extracted and released into the soluble cytoplasmic fraction when the tissue was homogenized in a hypotonic medium. The presence of a large percentage of reductase activity in the 70S-Supernatant (Table IX) is an indication that some of the reductase might be loosely bound to the particulate fractions and is released during homogenization This conforms to the suggestion of Bosmann et al (28), that enzymes that are loosely bound or adsorbed to membranes will probably be removed as part of the

soluble protein found in the supernatant fluid upon homogenization in 0.02M Tris + 0.01M EDTA and centrifugation at 70,000xg of the gradient fractions.

The purification of the nuclei, lysosomes and plasma membranes has shown that pyrimidine reductase activity is absent in the nuclei (Table V-B) and lysosomes (Table VII) but present in the plasma membranes (Table IX). Since the percentage of pyrimidine reductase activity present in the purified plasma membrane fraction (Table IX) was obtained from 61.80 per cent of the plasma membranes as judged by 5'-nucleotidase activity, it is estimated that the plasma membranes of rat liver contain about 23 per cent of NADPH-linked uracil reductase activity, 10.2 per cent NADH-linked uracil reductase activity and 19.3 per cent NADPH-linked thymine reductase activity.

The heavy and light mitochondrial fractions together contain 34.69 per cent of 5'-nucleotidase activity (Table I). Since the respective percentages of pyrimidine reductase activities in 7S (Table IX) are obtained from 61.80 per cent of plasma membranes as judged by 5'-nucleotidase activity, the reductase activities in the two mitochondrial fractions due to contamination by plasma membranes are estimated by multiplying the respective percentages of reductase activities in 7S by the factor $\frac{34.69}{61.80}$. When the respective corrections are made for reductase activities due to plasma membrane contamination, it is estimated that the two types of mitochondria together contain 49.2 per cent NADPH-linked uracil reductase activity, 58.8 per cent NADH-linked uracil reductase activity and 42.5 per cent NADPH-linked thymine reductase activity.

Although glycerol did solubilize NADPH-linked uracil reductase in the particulate fractions before freezing and thawing (Table XII), it was less effective in solubilizing the dihydropyrimidine dehydrogenase activity of the particulate fractions (Table IV). Most of the activity for dihydropyrimidine dehydrogenase was found in the soluble cytoplasmic fraction when the tissue was homogenized in an isotonic medium (Table IV)

or in a hypotonic medium (Table X). The presence of very low dehydrogenase activity in the particulate fractions even in the presence of ten times the protein used to assay for reductase activity is puzzling, but two possible reasons might be responsible for these results. Firstly the pyrimidine reductase reactions seem to have their chemical equilibrium far to the right. This is quite different from other $NADP^+$-linked and NAD^+-linked dehydrogenases which catalyse freely reversible reactions. It is possible that the inhibitor ATP "polarizes" the reductase reaction. In this regard, it is of interest to note that an amphidirectional ornithine δ-transaminase has been described by other workers (48). This transaminase, however, is "polarized" by substrate repression. Secondly the catabolic enzymes may form a multi-enzyme-complex in which the intermediates are enzyme-bound. If this is the case, free dihydropyrimidine would not bind to this enzyme-complex, unless the complex was dissociated in the presence of an agent such as glycerol as used in present studies. The dihydropyrimidine dehydrogenase catalysed reaction of the soluble supernatant fraction may also be irreversible for reasons similar to those mentioned for the reductase except that ATP activates the reaction.

The localization of most of the pyrimidine reductase activity in the particulate fractions, and most of the dihydropyrimidine dehydrogenase activity in the soluble fraction, is a clear indication that the enzyme commonly known as dihydropyrimidine dehydrogenase can be separated into reductase and dehydrogenase activities in rat liver cells. This indicates that there are two separate pathways; one for the degradation of pyrimidines, the other for the synthesis of pyrimidines.

A number of workers (49, 51, 52, 53, 54, 55, 56, 57, 58) have reported that both RNA and DNA are present in the mitochondria and it has been indicated that mitochondrial DNA is a part of the machinery responsible for the production of the mitochondrial RNA (52, 53, 57). The presence

of RNA and DNA in the mitochondria makes it reasonable to assume that the pathway for the degradation of the pyrimidine nucleotides to pyrimidine bases, and the pathway for the catabolism of pyrimidines should also be present in the mitochondria. We do not have evidence to show that these pathways are present in the mitochondria, but since our results show that some of the pyrimidine reductase activity is present in the mitochondria (Tables II and III), and pyrimidine nucleoside phosphorylase activity has been detected in the mitochondria (59), it is strongly suspected that the pathway for the degradation of pyrimidine nucleotides and the pathway for pyrimidine catabolism might also be present in the mitochondria.

The presence of pyrimidine reductase activity in the plasma membranes may be of significance. According to our results (Table XIV) and the findings of other workers (10), pyrimidine reductase activity is absent from most tissues, except liver and kidney. Since the pathway for the degradation of pyrimidine nucleotides to pyrimidine bases is present in most tissues, the pyrimidine bases could be either recycled within the tissues to form DNA and RNA or transported to the liver for degradation. If the latter is the case, then, the pyrimidine bases could go through their first step of degradation in the plasma membranes upon entering the liver cells. In this way the plasma membranes could serve as a point for regulating the concentration of pyrimidine bases in the liver cells and in the blood. It is also of interest to note that pyrimidine nucleoside phosphorylase has also been found to be present in purified plasma membranes (59). This might be an indication that the plasma membranes may also possess the enzymic pathway for the degradation of pyrimidine nucleotides to the pyrimidine bases.

The question as to whether the pyrimidine reductase activities found in rat liver are being expressed by the same enzyme is an important one. The subcellular distributions have shown that most of the reductase activity is located in the particulate fractions (Tables II and III), but, the sucrose density gradient fractionation of the heavy and light mitochondrial fractions (Figures 6 and 7), and the purification of plasma membranes (Table IX), show that most of the NADH-linked uracil reductase, like glutamate dehydrogenase, is associated with the mitochondria, while most of the NADPH-linked uracil and thymine reductases seem to be divided between the mitochondria and plasma membranes to which they seem to be more loosely bound than the NADH-linked uracil reductase. NADPH-linked thymine reductase has a similar activity distribution pattern to that of NADPH-linked uracil reductase, except in the supernatant fraction where there is a higher percentage (Tables II and III). This difference remains whether the tissue was homogenized in isotonic sucrose (Tables II and III) or in hypotonic Tris buffer (Table IX). The fact that the dependence of uracil reduction on NADH concentration produced a hyperbolic curve (Figure 12), while the dependence on NADPH concentration gave a sigmoid curve (Figure 18) is a major difference between the two reductase activities. In the light of these differences and similarities, it is concluded that the NADH-linked and the NADPH-linked activities are likely expressed by different enzymes, but the NADPH-linked thymine and NADPH-linked uracil reductase activities might be expressed by the same enzyme. More detailed investigation on more purified fractions containing reductase activity will have to be done before further differentiation can be made between the proteins which are expressing the reductase activities.

NADH-linked uracil reductase activity has approximately the same pH optimum in the heavy and light mitochondrial fractions (Figure 8) and in the soluble fraction (Figure 9). The same also applies to the pH optimum for NADPH-linked uracil reductase activity (Figures 8 and 9). Kinetic studies on NADPH-linked uracil reductase have shown, that the dependence of uracil reduction on NADPH concentration produces a sigmoid curve in both the light mitochondrial fraction and in the ammonium sulphate fraction (AS_2) (Figure 18); and, the respective concentrations of NADPH required to give 50 per cent saturation in both cases show only a small difference. These results and the fact that the Km values obtained for uracil in both the light mitochondria and ammonium sulphate fraction (AS_2) are relatively close (Figure 16 and 17), lead us to conclude that the proteins which are expressing NADPH-linked uracil reductase activity in the particulate fractions and in the soluble fraction are very similar.

The fact that most of the pyrimidine reductase activity has been localized in the particulate fractions (Tables II and III), whereas almost all of the dehydrogenase activity has been located in the soluble fraction (Tables IV and X) suggests very strongly, that different proteins may be responsible for the expression of the reductase and the dehydrogenase activities.

The increase in the specific activities of NAD^+-linked and $NADP^+$-linked dihydrouracil dehydrogenase activities show that these activities were partially purified in AS_2. The fact that partially purified NAD^+-linked and $NADP^+$-linked dihydrouracil dehydrogenase activities have different pH optimums (Figures 21 and 24) suggests that the NAD^+-linked dehydrogenase activity and the $NADP^+$-linked

dehydrogenase activities might be expressed by different proteins. However, more detailed studies will have to be done before further differentiation can be made between the dehydrogenases.

Our results have shown that NADH-linked and NADPH-linked uracil reductase activities are inhibited to approximately the same degree by Mg^{++}, ATP and ATP-Mg complex in the particulate fractions (Table XI), and also in the soluble fraction (Figure 20) in the case of NADPH-linked uracil reductase. On the other hand, all three dehydrogenase activities are activated by Mg^{++}, ATP and ATP-Mg complex in the homogenate, soluble cytoplasmic fraction, and in the ammonium sulphate fraction (AS_2). Rabinowitz and Wilhite (60) have recently reported that in normal and leukemic leukocytes a certain optimum concentration of ATP was required for maximal production of TTP from TMP, while, the same concentration of ATP strongly inhibited the breakdown to thymine. In this case the concentration of ATP appears to be the deciding factor as to whether the anabolic or the catabolic function will predominate. These findings seem to fit in with our findings that ATP and ATP-Mg will inhibit pyrimidine reduction but activate pyrimidine formation from dihydropyrimidine. In our studies the concentration of ATP and Mg^{++} seems to be the deciding factor as to whether pyrimidine anabolism or catabolism will predominate. The optimum concentration of ATP used to activate the formation of TTP is approximately the same as the concentration of ATP and ATP-Mg used to give maximal formation of pyrimidine from dihydropyrimidine. In the light of these findings it is concluded that changes in concentrations of ATP and Mg^{++} in the cell may serve to regulate pyrimidine metabolism in rat liver.

The finding that the dependence of dihydrouracil dehydrogenation on DHU concentration gives a sigmoid curve (Figure 27) is of major significance since ATP and ATP-Mg complex are strong activators (Figure 28). It should be interesting to investigate the effect of Mg^{++}, ATP and ATP-Mg on the other enzymes in the pathway for pyrimidine metabolism.

The tissue distribution studies of pyrimidine reductase and dihydropyrimidine dehydrogenase activities have shown that while some tissues (liver, kidney, testis) contain both the pyrimidine catabolic and anabolic enzymes, other tissues seem to contain the anabolic enzymes only (Table XIV). It does appear as if in the heart and spleen of the rat, thymine could be synthesized from dihydrothymine, whereas uracil cannot be synthesized from dihydrouracil in these two tissues. The true significance of these observations are unknown, and no further conclusions can be drawn from the data in Table XIV until more detailed investigations are conducted.

SUMMARY

The intracellular distribution of the enzyme commonly known as dihydropyrimidine dehydrogenase has been studied in rat liver. The intracellular constituents of the liver homogenate were separated by differential centrifugation according to procedures which have been successfully used by others for this purpose. Each fraction was further identified by marker enzymes for the respective subcellular particles.

The intracellular distribution studies have shown that dihydropyrimidine dehydrogenase can be separated into reductase activity, which resides mainly in the particulate fractions and to a lesser degree in the soluble cytoplasmic fraction, and dehydrogenase activity which resides mainly in the soluble cytoplasmic fraction. Subsequent purification of the nuclei, lysosomes, plasma membranes, and, the fractionation of the heavy and light mitochondrial fractions on sucrose density gradient have revealed, that the NADH-linked uracil reductase activity is mainly associated with the mitochondria, while the NADPH-linked uracil and thymine reductase activities are divided between the mitochondria, plasma membranes and soluble fraction. The presence of glycerol in the incubation mixture produced solubilization of NADPH-linked uracil reductase activity in the particulate fractions before freezing and thawing, but it was less effective in solubilizing the dehydrogenase activity. Some of the possible reasons for low dehydrogenase activity in the particulate

fractions have been discussed.

NADH-linked and NADPH-linked uracil reductase activities had different pH optimums in the particulate fractions and in the soluble fraction. This and other differences existing between NADH-linked uracil reductase activity and NADPH-linked uracil and thymine reductase activities is probably an indication that these activities might be expressed by different proteins. Since a sigmoid curve was obtained when the initial rate of uracil reduction was plotted against NADPH concentration for the light mitochondrial fraction and for the ammonium sulphate fraction (AS_2), NADPH-linked uracil reductase seems to have allosteric properties. Sufficient studies were not done on NADH-linked uracil reductase and NADPH-linked thymine reductase to determine whether or not any allosteric properties were present.

NAD^+-linked dihydrouracil dehydrogenase activity had a pH optimum of 8.75, while the $NADP^+$-linked dihydrouracil dehydrogenase activity had a pH optimum of 7.45. $NADP^+$-linked dihydrouracil dehydrogenase seems to have allosteric properties since a sigmoid curve was obtained when the initial rate of dihydrouracil oxidation was plotted against dihydrouracil concentration. All the dihydropyrimidine dehydrogenase activities are activated by Mg^{++}, ATP and ATP-Mg, but sufficient information is not available to decide whether or not the dehydrogenase activities are being expressed by one or more proteins.

The fact that Mg^{++}, ATP and ATP-Mg inhibits the reductase activity while activating the dehydrogenase activity, seems to indicate that the concentration of Mg^{++} and ATP in the cell could be the deciding factor as to whether pyrimidine anobolism or catabolism will

predominate *in vivo*. Localization of the other enzymes in the pyrimidine metabolic pathway will probably attach greater meaning and significance to the separation of dihydropyrimidine dehydrogenase into reductase and dehydrogenase activities.

BIBLIOGRAPHY

1. Fink, K., Henderson, R.B., and Fink, R.M., Proc. Soc. Exp. Biol. and Med., 78, 135 (1951)

2. Fink, K., Henderson, R.R., and Fink, R.M., J. Biol. Chem., 197, 441 (1952)

3. Fink, R.M., Fink, K., and Henderson, R.B., J. Biol. Chem., 201, 349 (1953)

4. Funk, C., Merritt,A.J., and Ehrlich, A., Arch. Biochem. Biophys., 35, 468 (1952)

5. Fink, R.M., Cline, R.E., and Koch, H.M.G., Fed. Proc., 13, 207 (1954)

6. Fink, R.M., McGaughey, C., Cline, R.E., and Fink, K., J. Biol. Chem., 218, 1 (1956)

7. Fink, K., J. Biol. Chem., 218, 9 (1956)

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

9. Canellakis, E.S., J. Biol. Chem., 221, 315 (1956)

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

11. Plentl, A., and Schoenheimer, J., J. Biol. Chem., 153, 203 (1944)

12. Rutman, R.J., Cantarow, A., and Paschkis, K.E., Cancer Res., 14, 119 (1954)

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

14. Fritzson, P., J. Biol. Chem., 226, 223 (1957)

15. Skold, O., Biochim. Biophys. Acta, 44, 1 (1960)

16. Potter, V.R., Pitot, H.C., Ono, T., and Morris, H.P., Cancer Res., 21, 1255 (1960)

17. Reichard, P., and Skold, O., Biochim. Biophys. Acta, 28, 376 (1958)

18. Fritzson, P., Biochim. Biophys. Acta, 91, 374 (1964)

19. Campbell, L. Leon, Jr., J. Biol. Chem., 227, 693 (1957)

20. Grisolia, S., Cardoso, S.S., Biochim. Biophys. Acta, 25, 430 (1957)

21. Fritzson, P., J. Biol. Chem., 235, 719 (1960)

22. Šebesta, K., Bauerová, J., and Šormova, Z., Biochim. Biophys. Acta, 50, 393 (1961)

23. Bresnick, E., Texas Reports on Biology and Medicine, 22, 431 (1964)

24. Dorsett, M.T., Cancer Res., 29, 79 (1969)

25. Sawant, P.L., Shibko, S., Kumta, U.S., and Tappel, A.L., Biochim. Biophys. Acta, 85, (1964) 82-92

26. Pogo, A.O., Allfrey, V.G., and Mirsky, A.E., Proc. Nat. Acad. Sci. U.S., 56, 550 (1966)

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

28. Bosmann, H.B., Hagopian, A., and Eylar, E.H., Arch. Biochem. Biophys., 128, 51-69 (1968)

29. Weaver, R.A., and William, B., Biochim. Biophys. Acta, 173, 377-388 (1969)

30. Song, C.S., and Bodansky, O., J. Biol. Chem., 242, 694 (1967)

31. Britten, R.J., and Roberts, R.B., Science, 131, 32 (1960)

32. Nass, M.M.K., Proc. Nat. Acad. Sci., 56, 1215 (1966)

33. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., J. Biol. Chem., 193, 265 (1951)

34. Schmidt, G., in Methods in Enzymology, Vol. II Edited by S.P. Colowick and N.O. Kaplan, Academic Press Inc., New York (1955) p. 523

35. De Duve, C., Pressman, B.C., Gianetto, R., Wattiaux, R., and Appelmans, F., Biochem. J., 60, 604 (1955)

36. Gomori, G., J. Lab. Clin. Med., 27, 955 (1941-42)

37. Hers, H.G., and Van Hoff, F., in Methods in Enzymology, Vol. VIII, Edited by S.P. Colowick and N.O. Kaplan, Academic Press Inc., New York (1966) p. 530

38. Beaufay, H., Bendall, D.S., Baudhuin, P., and De Duve, C., Biochem. J., 73, 623 (1959)

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

40. Emmelot, P., Bos, C.J., Benedetti, E.L., and Rümke, P.H., Biochim. Biophys. Acta, 90, 126 (1964)

41. Wattiaux-De Coninck, S., and Wattiaux, R., Biochim. Biophys. Acta, 183, 118-128 (1969)

42. Berman, H.M., Gram, W., and Spirtes, M.A., Biochim. Biophys. Acta, 183, 10-18 (1969)

43. Song, C.S., Rubin, W., Rifkind, A.B., and Kappas, A., J. Cell Biol., 41, 124-132 (1969)

44. Koshland, D.E., Jr., Nemethy, G., Filmer, D., Biochemistry, 5, 355 (1966)

45. Swick, R.W., Stange, J.L., Nance, S.L., and Thomson, J.F., Biochemistry, 6, 737 (1967)

46. Dixon, M., Webb, E.C., Enzymes, Second Edition, Longmans, Green and Co. Ltd., London (1964) p. 81-84

47. Kraut, A., Ph.D. Thesis, University of Manitoba

48. Volpe, P., and Strecker, H.J., Biochem. Biophys. Res. Commun., 32, 240 (1968)

49. Vesco, C., Penman, S., Nature 224, 1021 (1969)

50. Lansing, A.L., Belkhode, M.L., Lynch, W.E., and Lieberman, I., J. Biol. Chem., 242, 1772 (1967)

51. Nass, M.M.K., Science, 165, 25 (1969)

52. Handi, G., and Attardi, B., Proc. Nat. Acad. Sci. U.S., 58, 1051 (1967)

53. Dawid, I., Fed. Proc., 28, 349 (1969)

54. Smith, A.E., and Marcker, K.A., J. Mol. Biol., 38, 241 (1968)

55. Nass, M.M.K., and Buck, C.A., Proc. Nat. Acad. Sci. U.S., 62, 506 (1968)

56. Galper, J.B., and Darnell, J.E., Biochem. Biophys. Res. Commun., 34, 205 (1969)

57. Zylber, E., Vesco, C., Penman, S., J. Mol. Biol., 44, 195 (1969)

58. Gross, N.J., Getz, G.S., Rabinowitz, M., J. Biol. Chem., 244, 1552 (1969)

59. Bose, R., and Yamada, E.W., (Unpublished results)

60. Rabinowitz, Yale, and Wilhite, Betty A., Blood, 33, 759 (1969)

61. Hogeboom, G.H., and Schneider, W.C., J. Biol. Chem., 194, 513 (1953)

62. Decloitre,F. and Chauveau, J., FEBS Letters, 2, 227 (1969)

FIGURES

Figure 1 Representation of the pathways for pyrimidine catabolism.

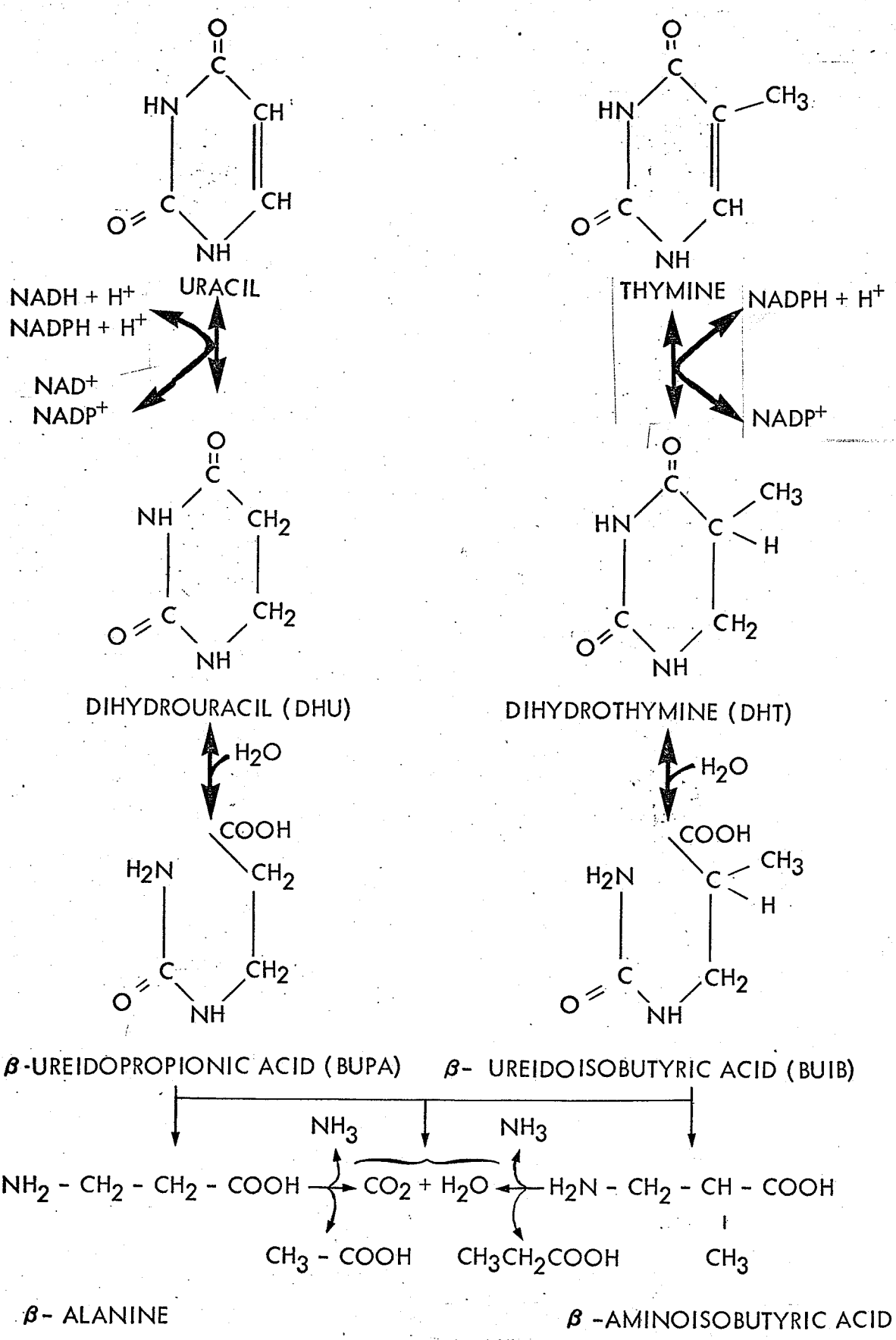

URACIL
NADH + H+
NADPH + H+
NAD+
NADP+
THYMINE
NADPH + H+
NADP+
DIHYDROURACIL (DHU)
DIHYDROTHYMINE (DHT)
H2O
COOH
β-UREIDOPROPIONIC ACID (BUPA)
β- UREIDOISOBUTYRIC ACID (BUIB)
NH3
NH3
NH2 - CH2 - CH2 - COOH
CO2 + H2O
H2N - CH2 - CH - COOH
CH3 - COOH
CH3CH2COOH
β- ALANINE
β -AMINOISOBUTYRIC ACID (BAIB

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 INTRACELLULAR FRACTIONS

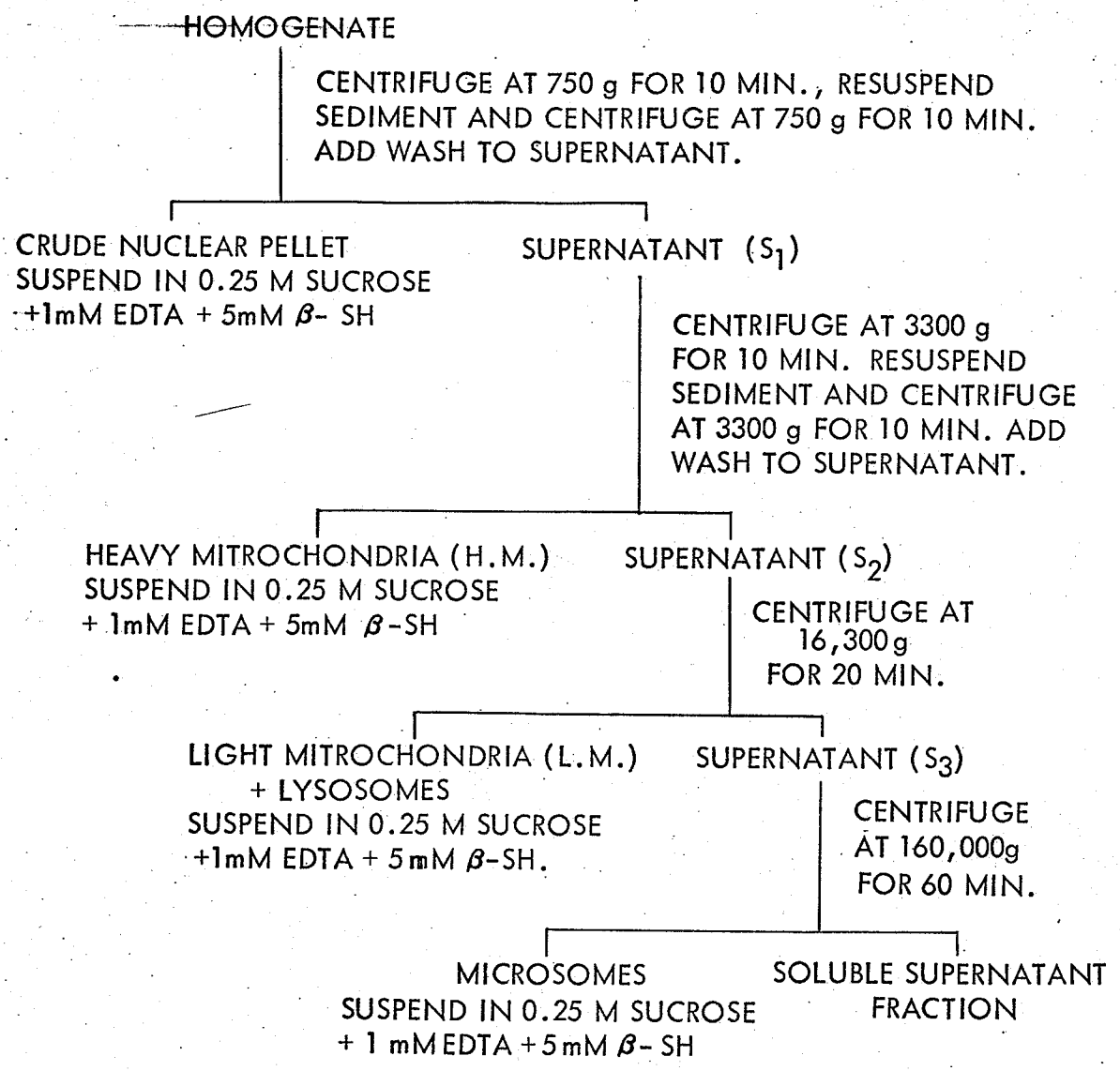


Figure 3 Procedure for the purification of nuclei by differential centrifugation. The tissue was homogenized as described under "Materials and Methods".

PURIFICATION OF NUCLEI

HOMOGENATE

CENTRIFUGE AT 750 g FOR 10 MIN.
RESUSPEND SEDIMENT AND CENTRIFUGE
AT 750 g FOR 10 MIN.

CRUDE NUCLEAR PELLET — SUPERNATANT (S_1)

RESUSPEND IN 10 VOLUMES OF 2.4 M SUCROSE
+1mM $MgCl_2$
CENTRIFUGE AT 64,000 g FOR 60 MIN.

NUCLEAR PELLET — SUPERNATANT (DISCARD) — DEBRIS

SUSPEND IN 0.25 M SUCROSE
+ 1mMEDTA + 5mM β- SH

WASH WITH 20 VOLUMES
0.25 M SUCROSE - 4mM $MgCl_2$-
10mM TRIS BUFFER pH 8.3
CENTRIFUGE AT 1,000 g FOR 6 MIN.
REPEAT WASHING TWICE AND FINALLY
SUSPEND THE NUCLEAR PELLET IN
0.25M SUCROSE +5mM β-SH.

PURE NUCLEI

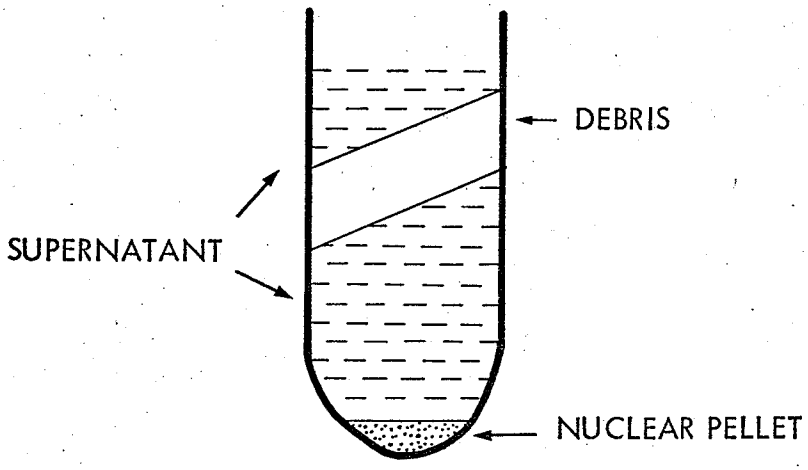


Figure 4 Procedure for the purification of lysosomes by differential centrifugation. The tissue was homogenized as described under "Materials and Methods".

PURIFICATION OF LYSOSOMES

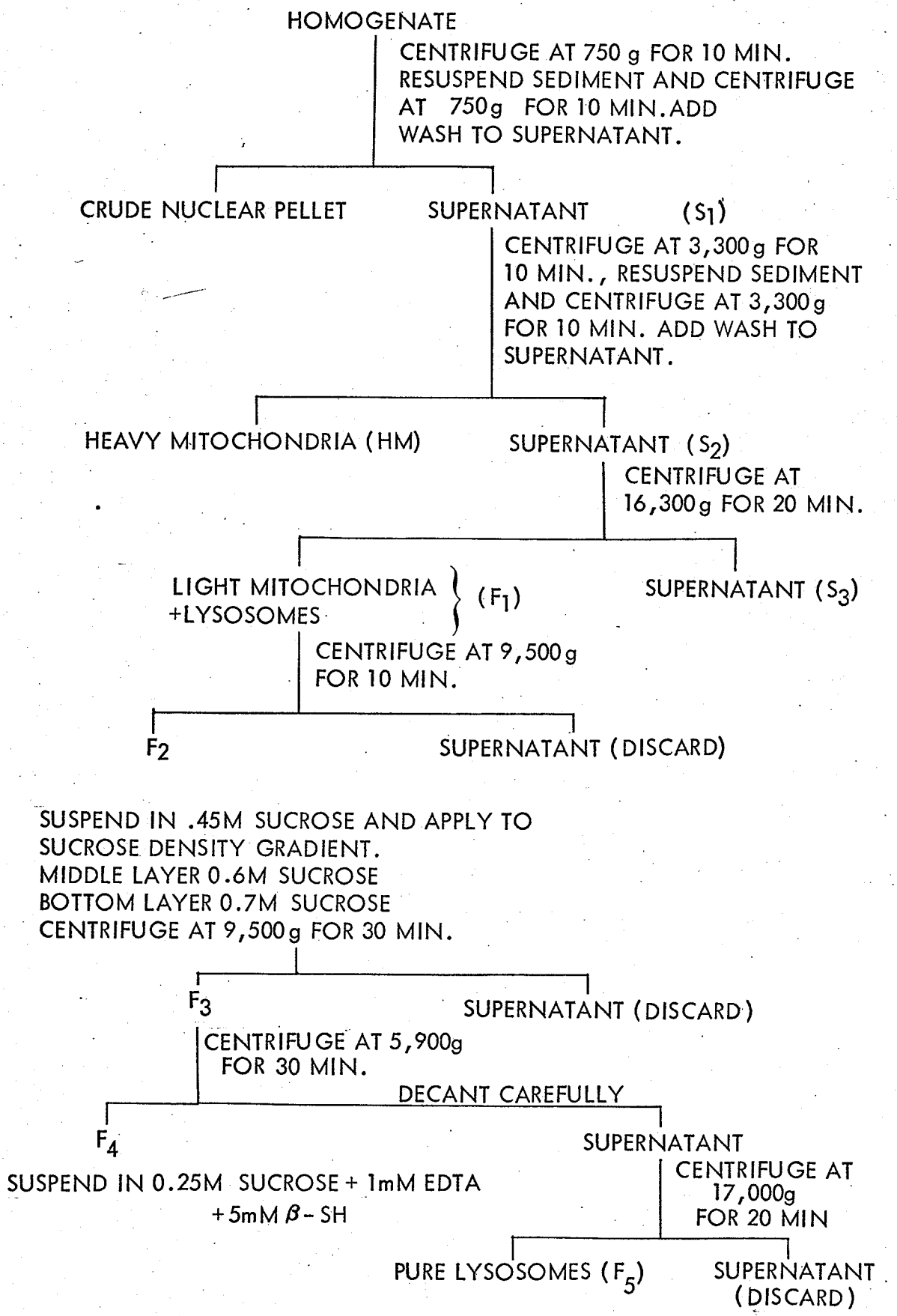


Figure 5 Procedure for the purification of plasma membranes by discontinuous sucrose density gradient. The tissue was homogenized as described under "Materials and Methods".

PURIFICATION OF PLASMA MEMBRANES

HOMOGENATE

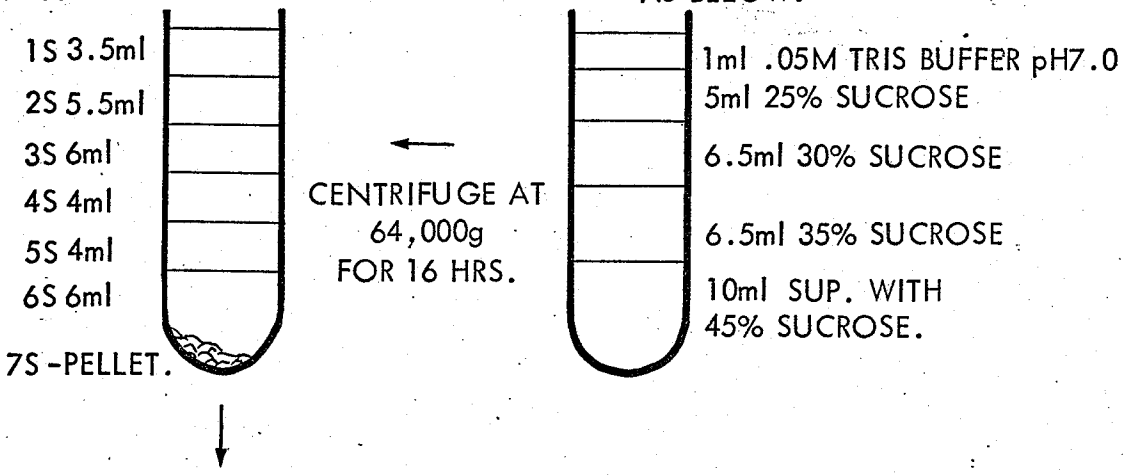
CENTRIFUGE AT 4000g FOR 15 MIN.

4000g PELLET (4g-P).

WASH ONCE WITH 0.02M TRIS BUFFER pH 7.0 + .01M EDTA pH 7.0, AND ADD WASHING TO SUPERNATANT. FINALLY SUSPEND PELLET IN 0.05M TRIS BUFFER pH 7.0.

4000g-SUP. (4g-S)

ADD SUCROSE TO MAKE SUPERNATANT 45% WITH RESPECT TO SUCROSE. MAKE DICONTINUOUS SUCROSE DENSITY GRADIENT AS BELOW.



DILUTE EACH FRACTION TO 3 TIMES ITS VOLUME WITH 0.05M TRIS,BUFFER pH 7.0, AND CENTRIFUGE AT 70,000g FOR 1 HOUR. SUSPEND EACH PELLET IN 20 VOLUMES OF 0.05M TRIS BUFFER pH 7.0 + 5mM β-SH.

THE 70,000 X g SUPERNATANTS FROM ALL THE FRACTIONS WERE COMBINED TO GIVE THE 70S - SUPERNATANT

7S - PELLET FINALLY GIVES PURE PLASMA MEMBRANES.

Figure 6 Sucrose density gradient (0.5M-1.91M) fractionation of heavy mitochondrial fraction. The gradient was prepared as described under "Materials and Methods" and 3 ml of heavy mitochondrial fraction containing 60 mg protein was applied to the gradient. Three milliliter fractions were collected and stored at 0-4° overnight along with a sample of unfractionated heavy mitochondria and then assayed on the next day. Activity was measured under the standard conditions of assay for glutamate dehydrogenase (open squares), 5'-nucleotidase (closed circles), NADPH-linked uracil reductase (open circles), NADH-linked uracil reductase (open triangles).

 direction of sedimentation.

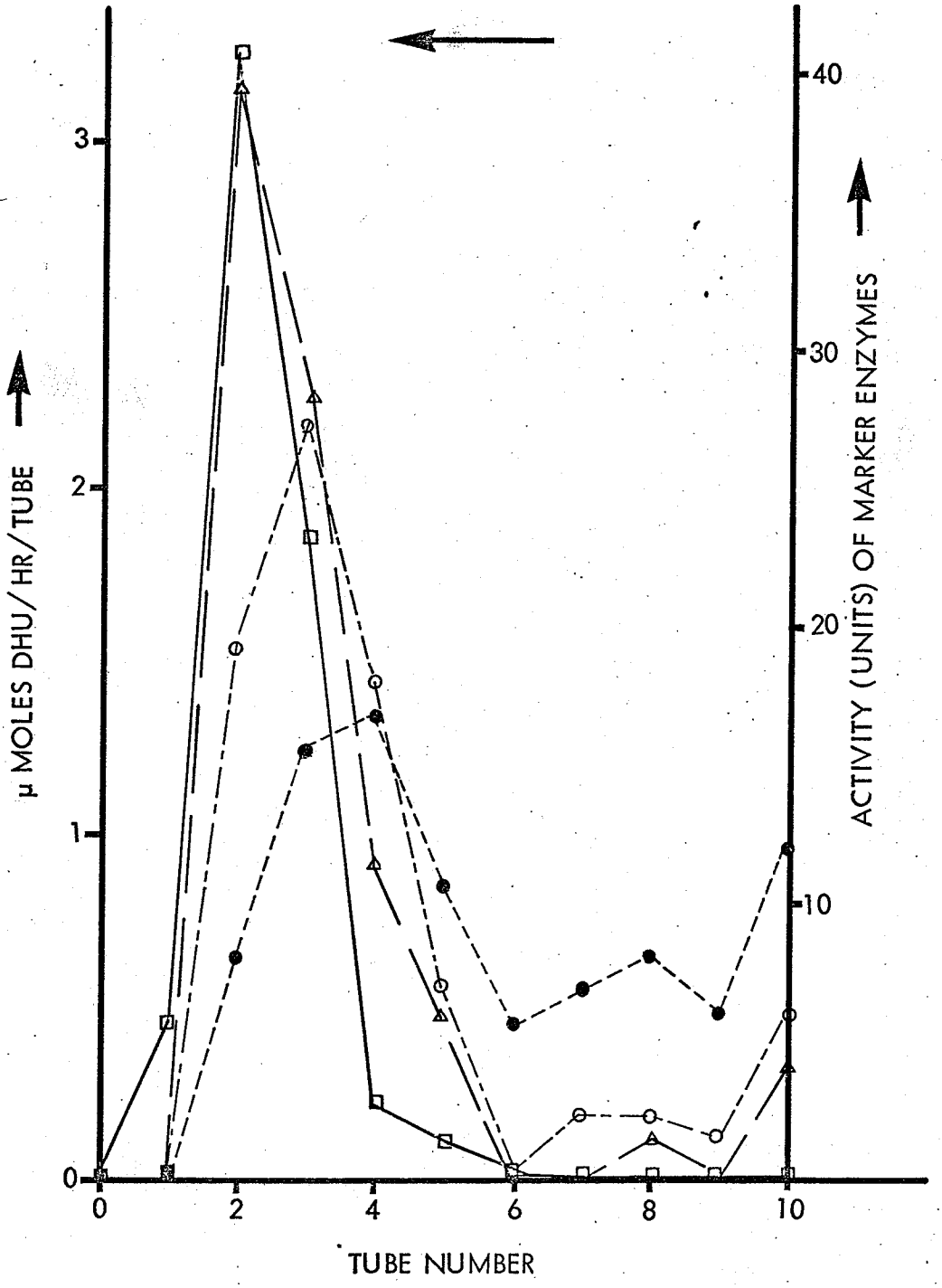

μ MOLES DHU/HR/TUBE
ACTIVITY (UNITS) OF MARKER ENZYMES
TUBE NUMBER
0
1
2
3
10
20
30
40
0
2
4
6
8
10

Figure 7 Sucrose density gradient (0.5M-1.91M) fractionation of light mitochondrial fraction. The gradient was prepared as described under "Materials and Methods" and 3 ml of light mitochondrial fraction containing 38 mg protein was applied to the gradient. Three milliliter fractions were collected and stored at 0-4° overnight along with a sample of unfractionated light mitochondria and then assayed on the next day. Activity was measured under the standard conditions of assay for glutamate dehydrogenase (open squares), 5'-nucleotidase (closed circles), NADPH-linked uracil reductase (open circles), NADH-linked uracil reductase (open triangles). ← direction of sedimentation.

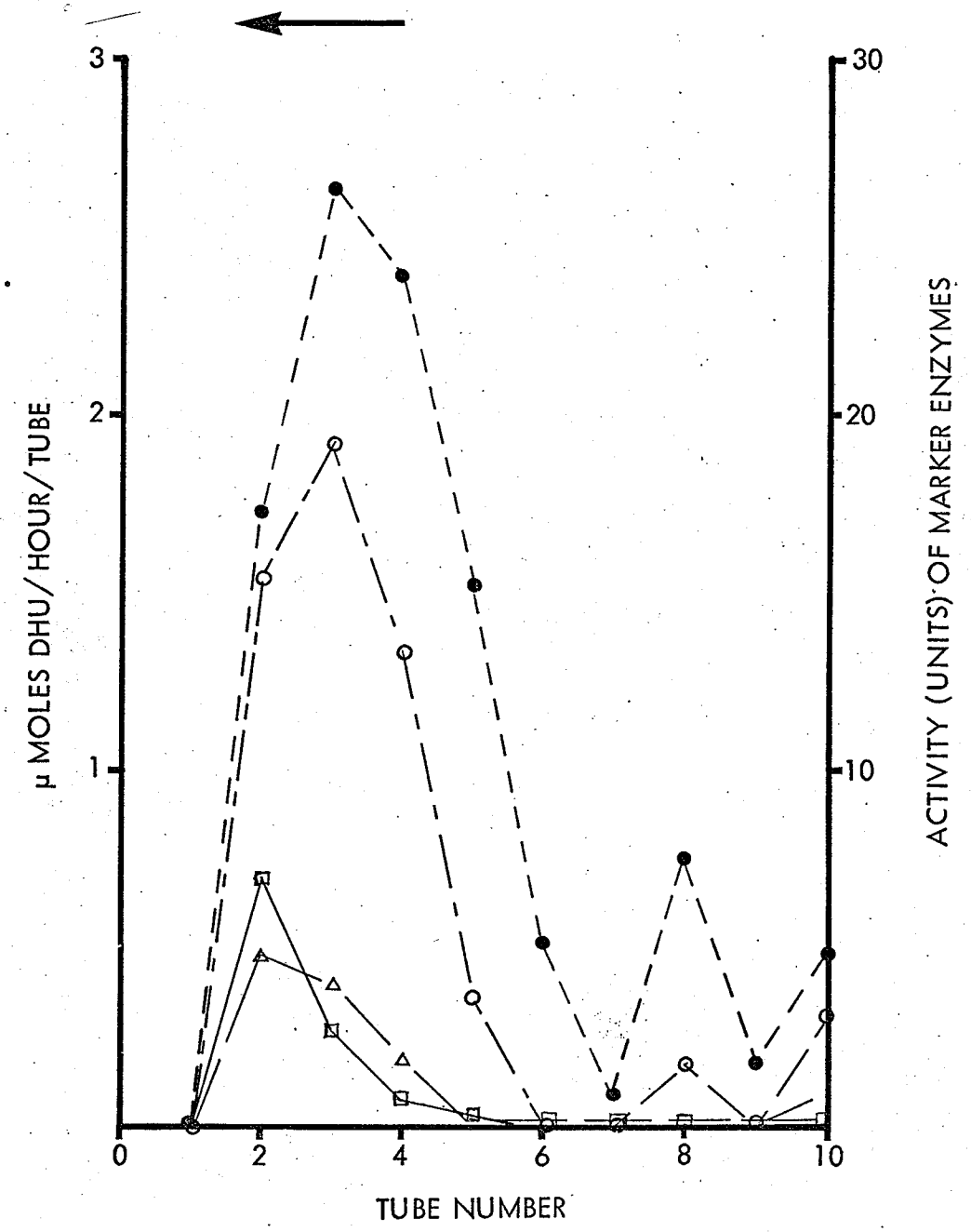

3
2
1
0
2
4
6
8
10
30
20
10
μ MOLES DHU/HOUR/TUBE
ACTIVITY (UNITS) OF MARKER ENZYMES
TUBE NUMBER

Figure 8 A: The relationship between pH and uracil reductase activity of heavy mitochondria. Activity was measured under the standard conditions of assay but at differing pH's for NADPH-linked uracil reductase (closed squares) and NADH-linked uracil reductase (closed triangles).

B: The relationship between pH and uracil reductase activity of light mitochondria. Activity was measured under the standard conditions of assay but at differing pH's for NADPH-linked uracil reductase (closed squares) and NADH-linked uracil reductase (closed triangles).

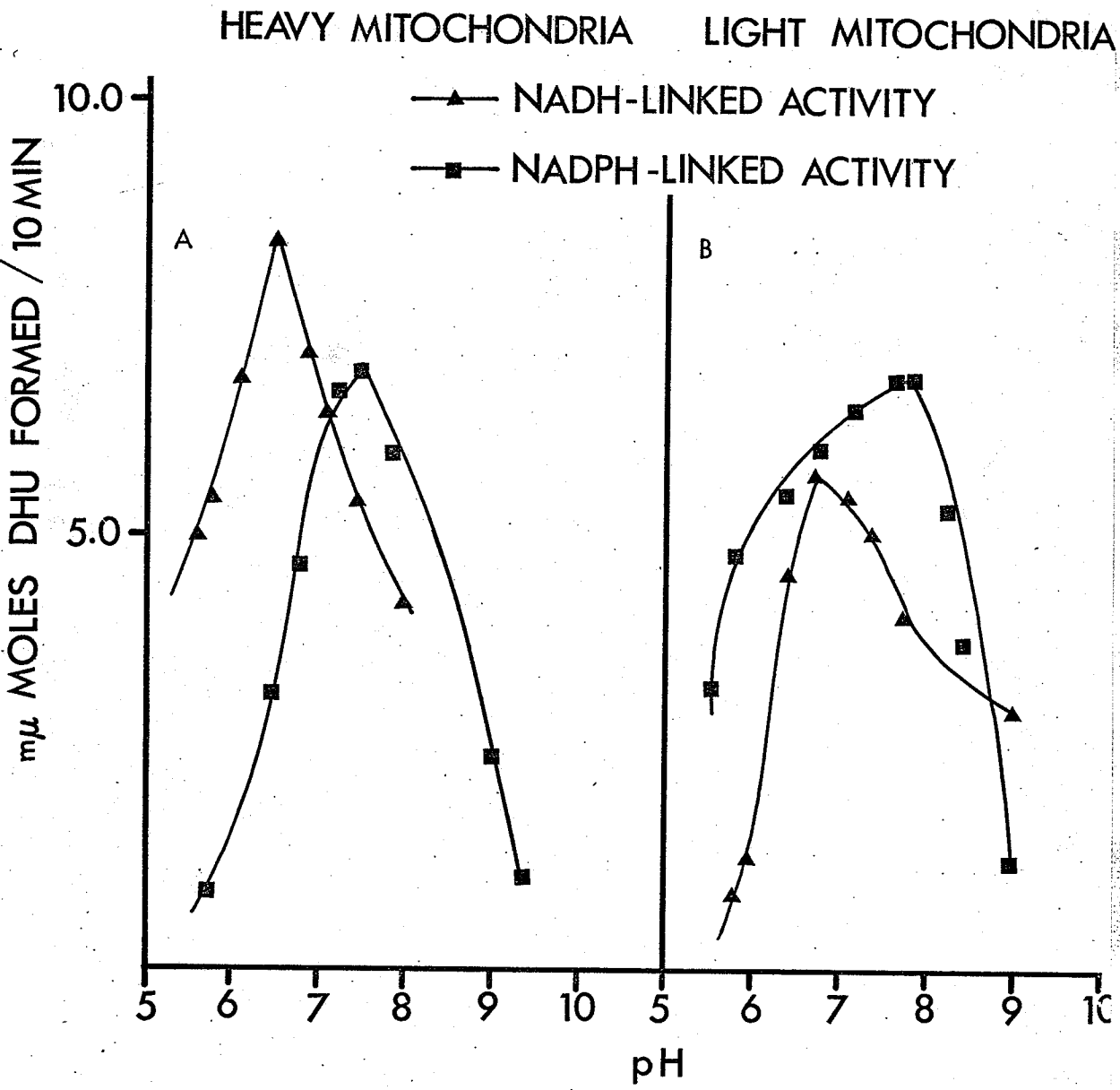

HEAVY MITOCHONDRIA
LIGHT MITOCHONDRIA
NADH-LINKED ACTIVITY
NADPH-LINKED ACTIVITY
10.0
5.0
mμ MOLES DHU FORMED / 10 MIN
A
B
5
6
7
8
9
10
pH

Figure 9 The relationship between pH and uracil reductase activity of soluble fraction. Activity was measured under the standard conditions of assay but at differing pH's for NADPH-linked uracil reductase (closed squares) and NADH-linked uracil reductase (closed circles).

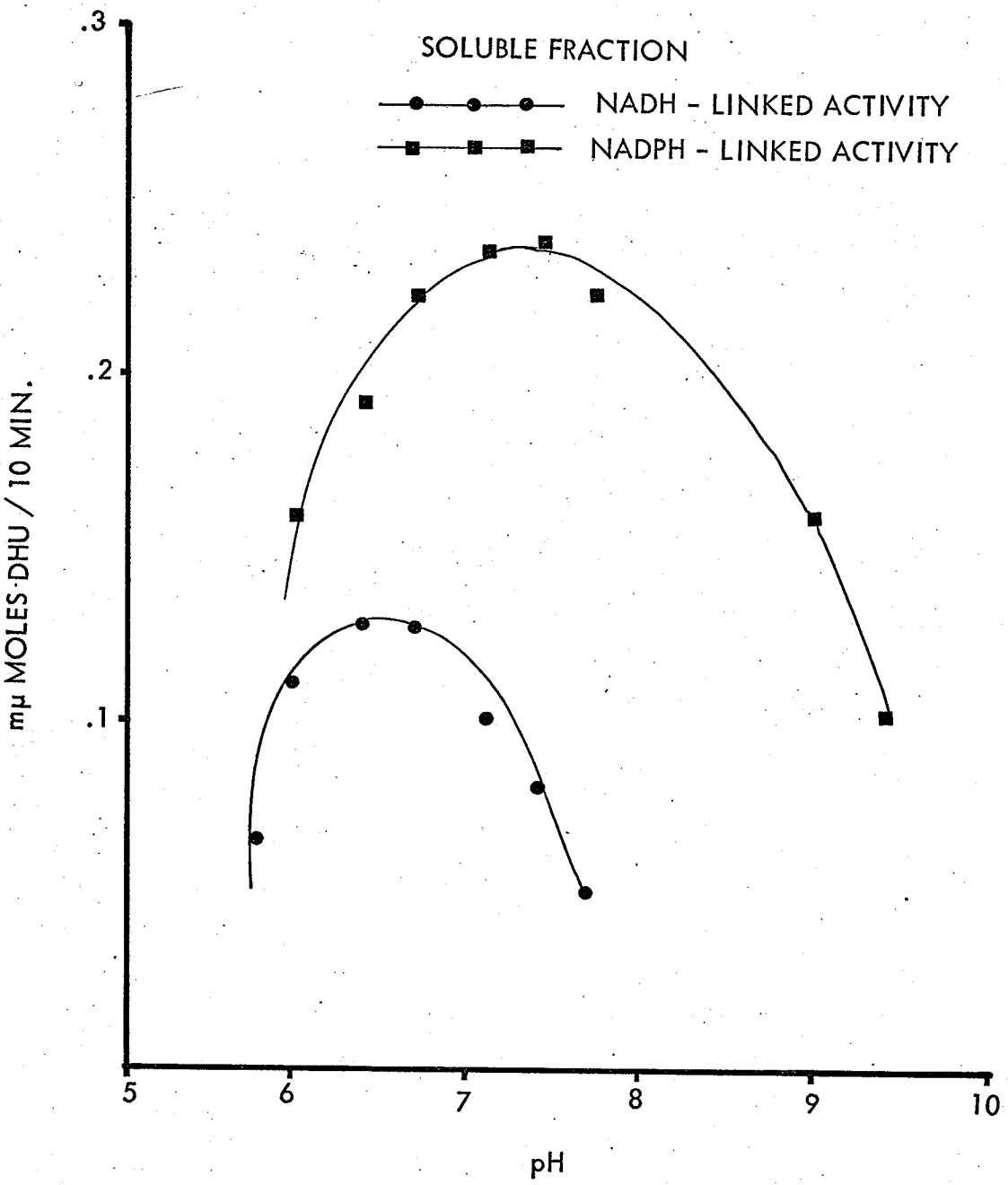

SOLUBLE FRACTION
NADH - LINKED ACTIVITY
NADPH - LINKED ACTIVITY
.3
.2
.1
mμ MOLES·DHU / 10 MIN.
5
6
7
8
9
10
pH

Figure 10 Relationship between protein concentration and NADH-linked uracil reductase activity of heavy mitochondria. Activity was measured under the standard conditions of assay but with varying amounts of heavy mitochondrial protein.

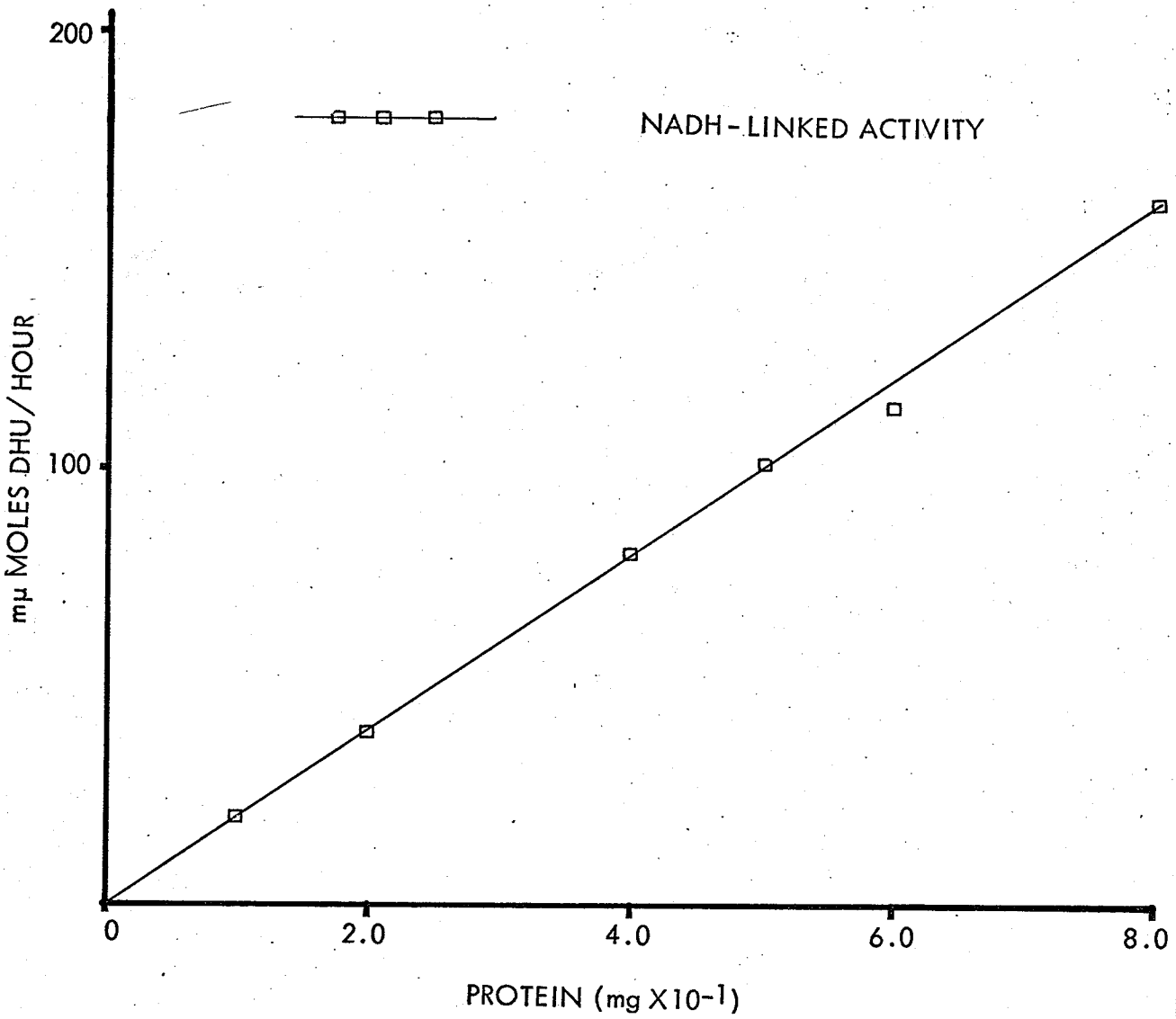

200
100
NADH-LINKED ACTIVITY
mμ MOLES DHU/HOUR
0
2.0
4.0
6.0
8.0
PROTEIN (mg X10-1)

Figure 11 The relationship between time and NADH-linked uracil reductase activity of heavy mitochondria. Activity was measured under the standard conditions of assay but with varying incubation times. The incubation mixture contained 0.50 mg heavy mitochondrial protein.

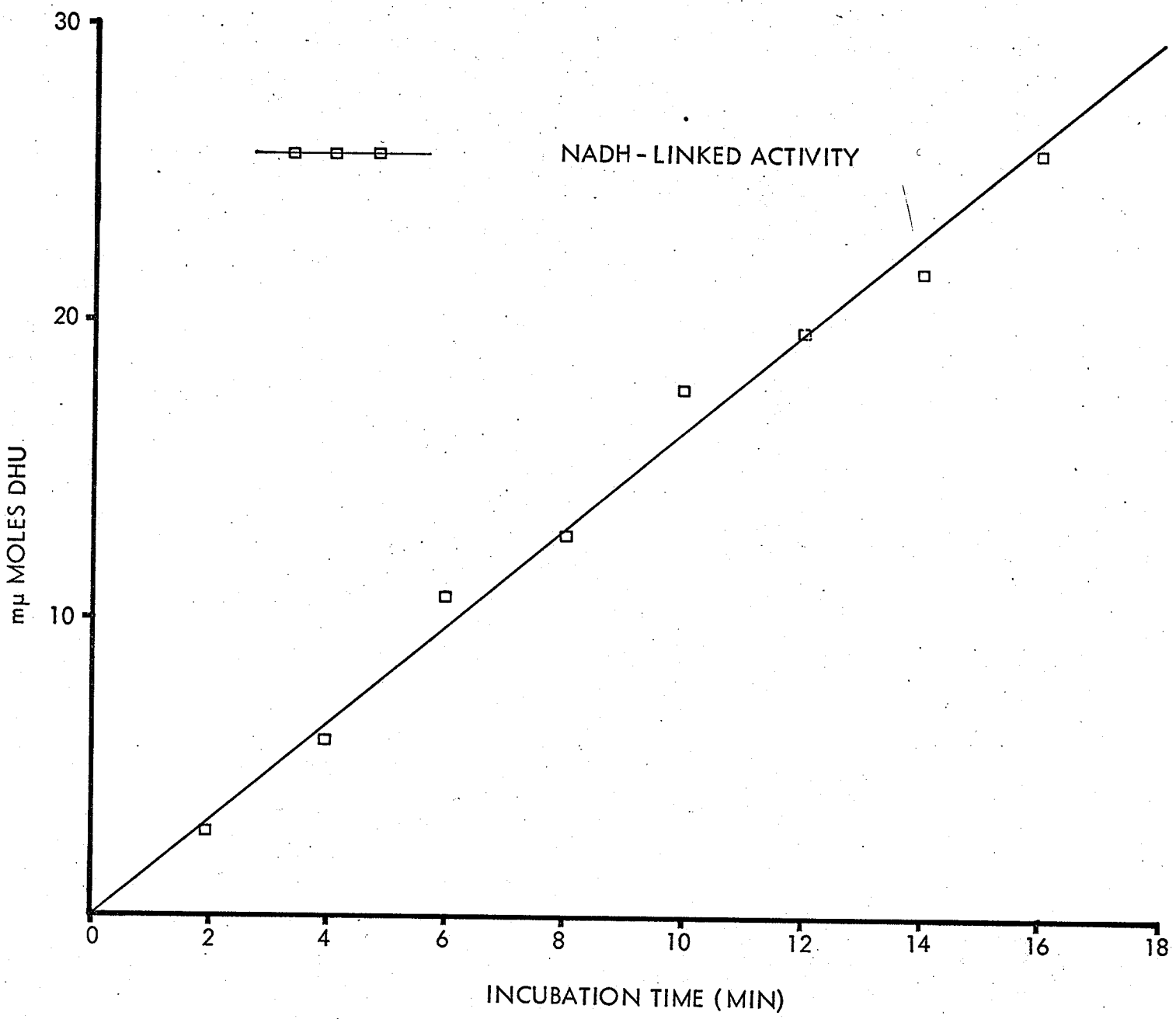

NADH - LINKED ACTIVITY
mμ MOLES DHU
30
20
10
0
2
4
6
8
10
12
14
16
18
INCUBATION TIME (MIN)

Figure 12 The relationship between NADH concentration and the initial rate of uracil reduction in heavy mitochondria. Activity was measured under the standard conditions of assay but with varying concentrations of NADH. 0.28 mg heavy mitochondrial protein was used in the incubation mixture.

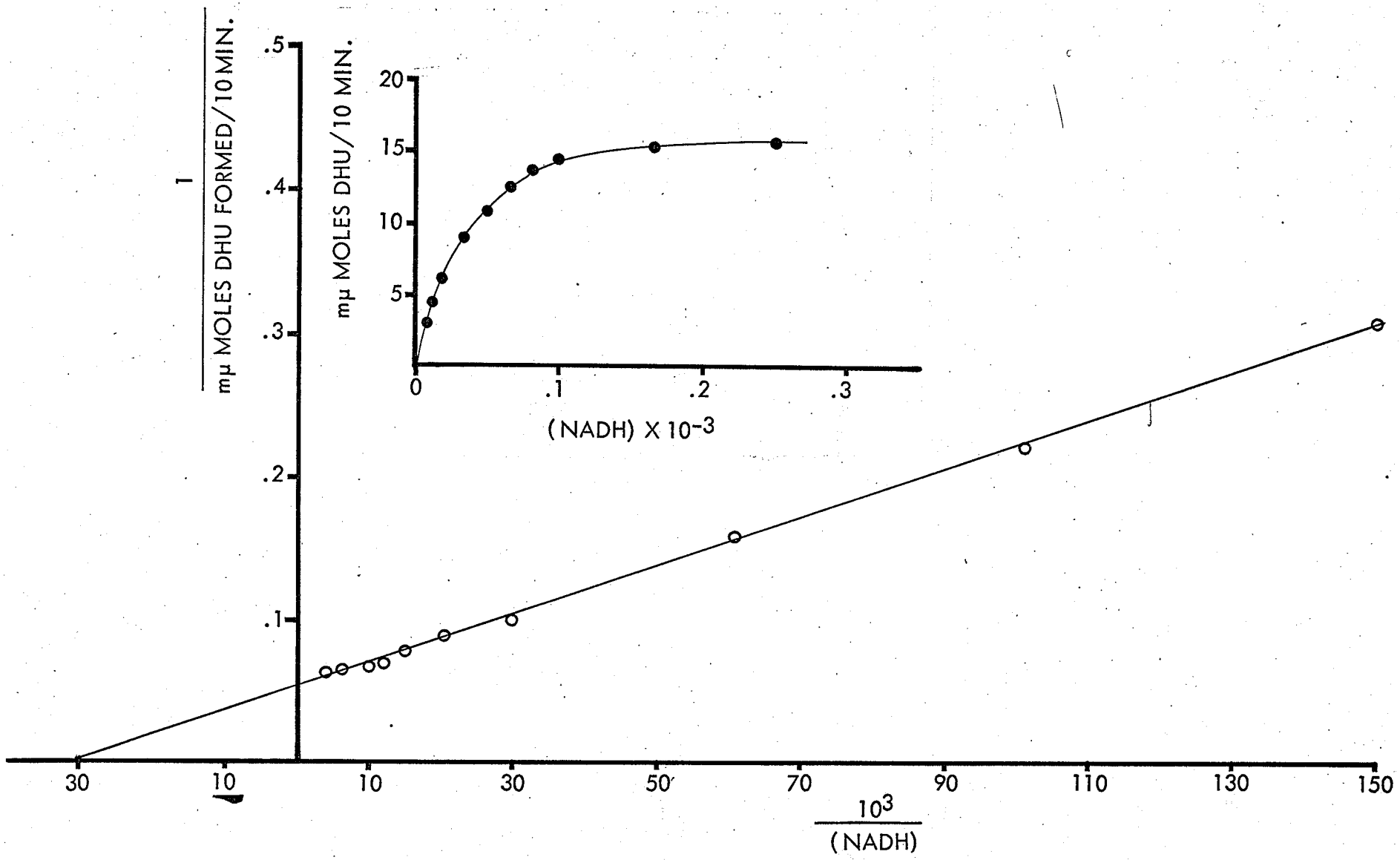

1
mμ MOLES DHU FORMED/10 MIN.
.5
.4
.3
.2
.1
mμ MOLES DHU/10 MIN.
20
15
10
5
0
.1
.2
.3
(NADH) X 10-3
30
10
10
30
50
70
90
110
130
150
10³
(NADH)

Figure 13 Relationship between protein concentration and NADPH-linked uracil reductase activity of light mitochondria. Activity was measured under the standard conditions of assay but with varying amounts of light mitochondrial protein.

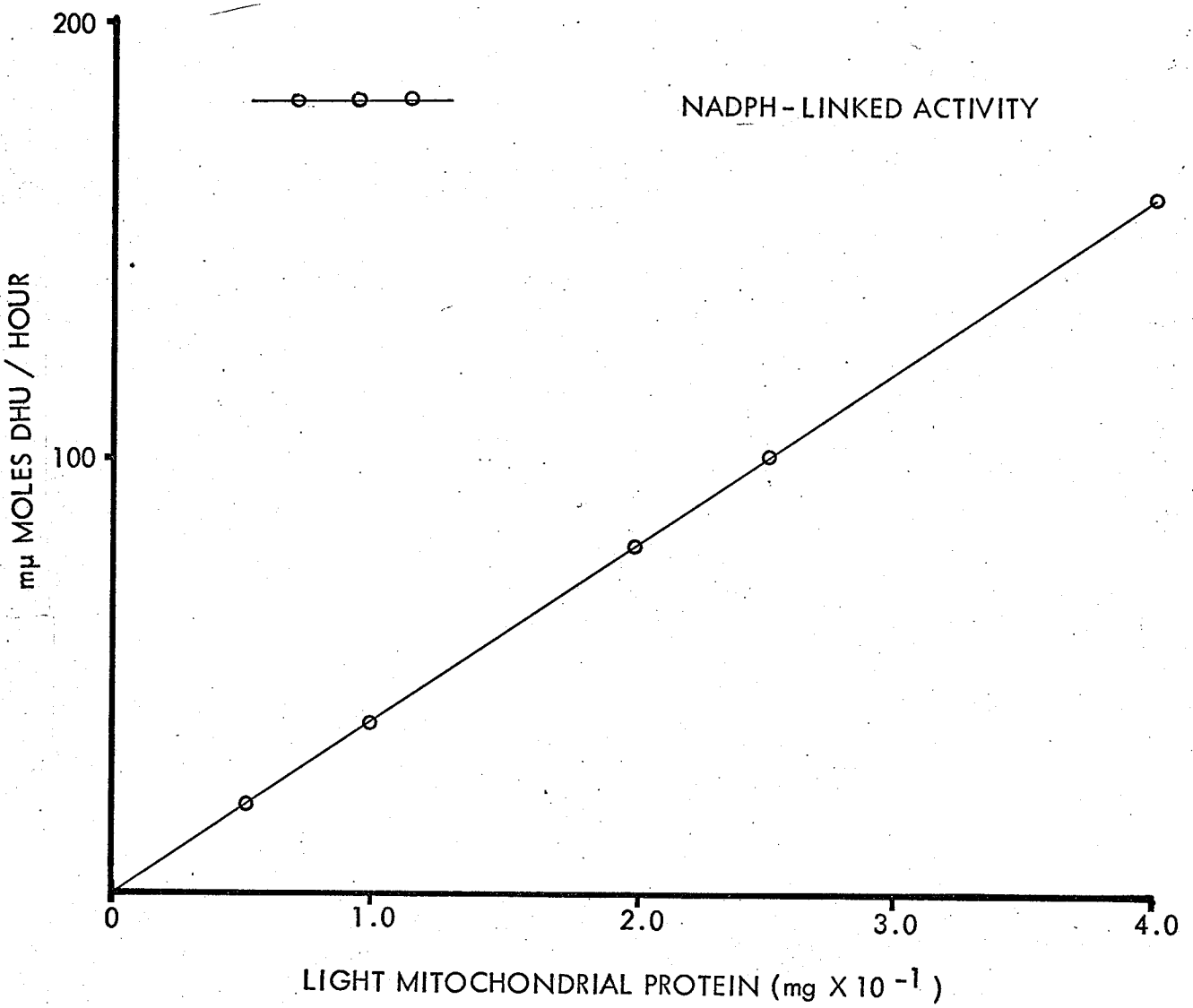

200
100
mμ MOLES DHU / HOUR
NADPH-LINKED ACTIVITY
0
1.0
2.0
3.0
4.0
LIGHT MITOCHONDRIAL PROTEIN (mg X 10 -1)

Figure 14 The relationship between time and NADPH-linked uracil reductase activity of light mitochondria. Activity was measured under the standard conditions of assay, but with varying incubation times. 0.25 mg light mitochondrial protein was used in the incubation mixture.

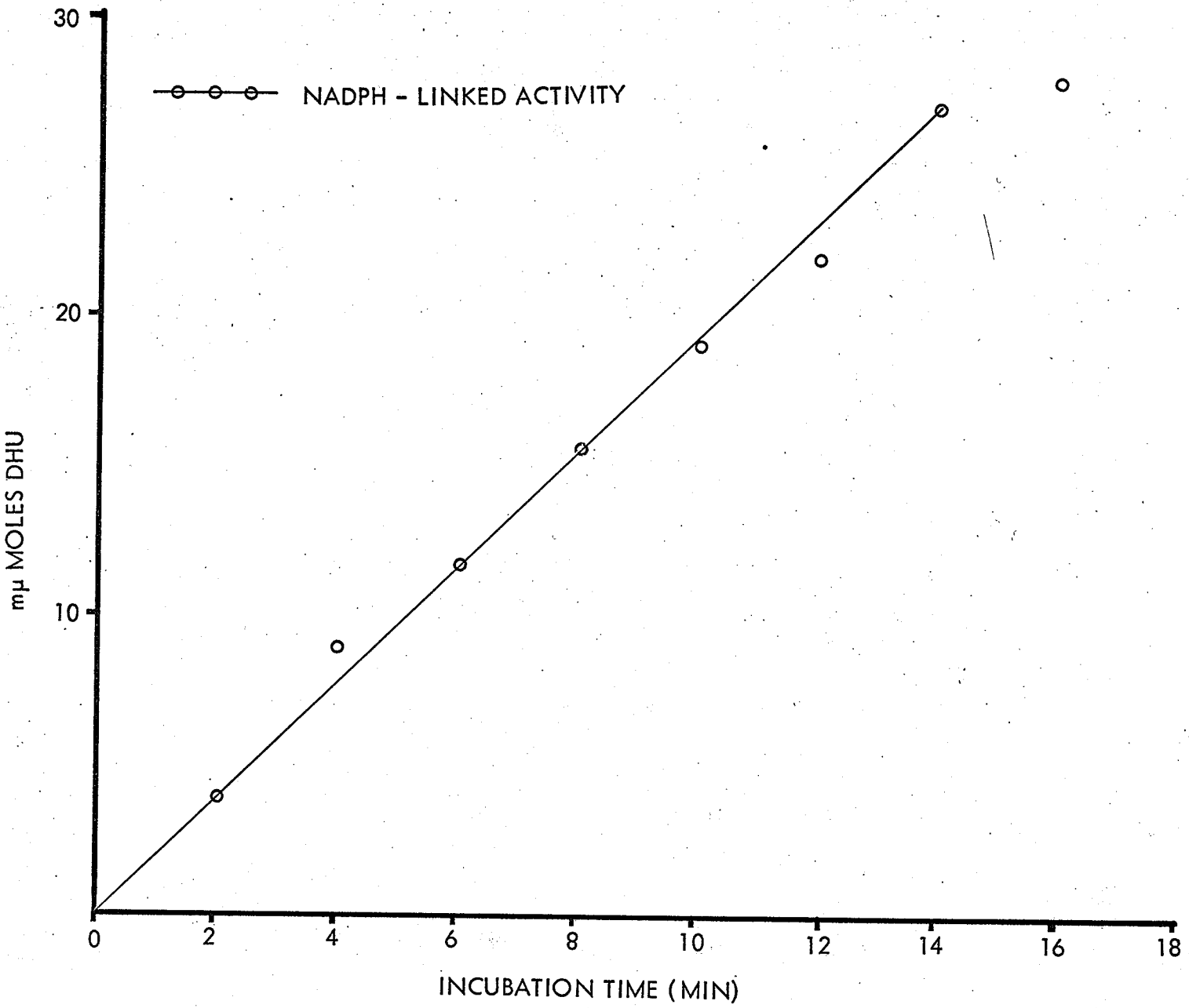

NADPH - LINKED ACTIVITY
mμ MOLES DHU
30
20
10
0
2
4
6
8
10
12
14
16
18
INCUBATION TIME (MIN)

Figure 15 The relationship between time and NADPH-linked uracil reductase activity in an ammonium sulphate fraction (AS_2) prepared from dialysed soluble fraction. Activity was measured under the standard conditions of assay but with varying incubation time. 6.35 mg of AS_2 protein was used in the incubation mixture.

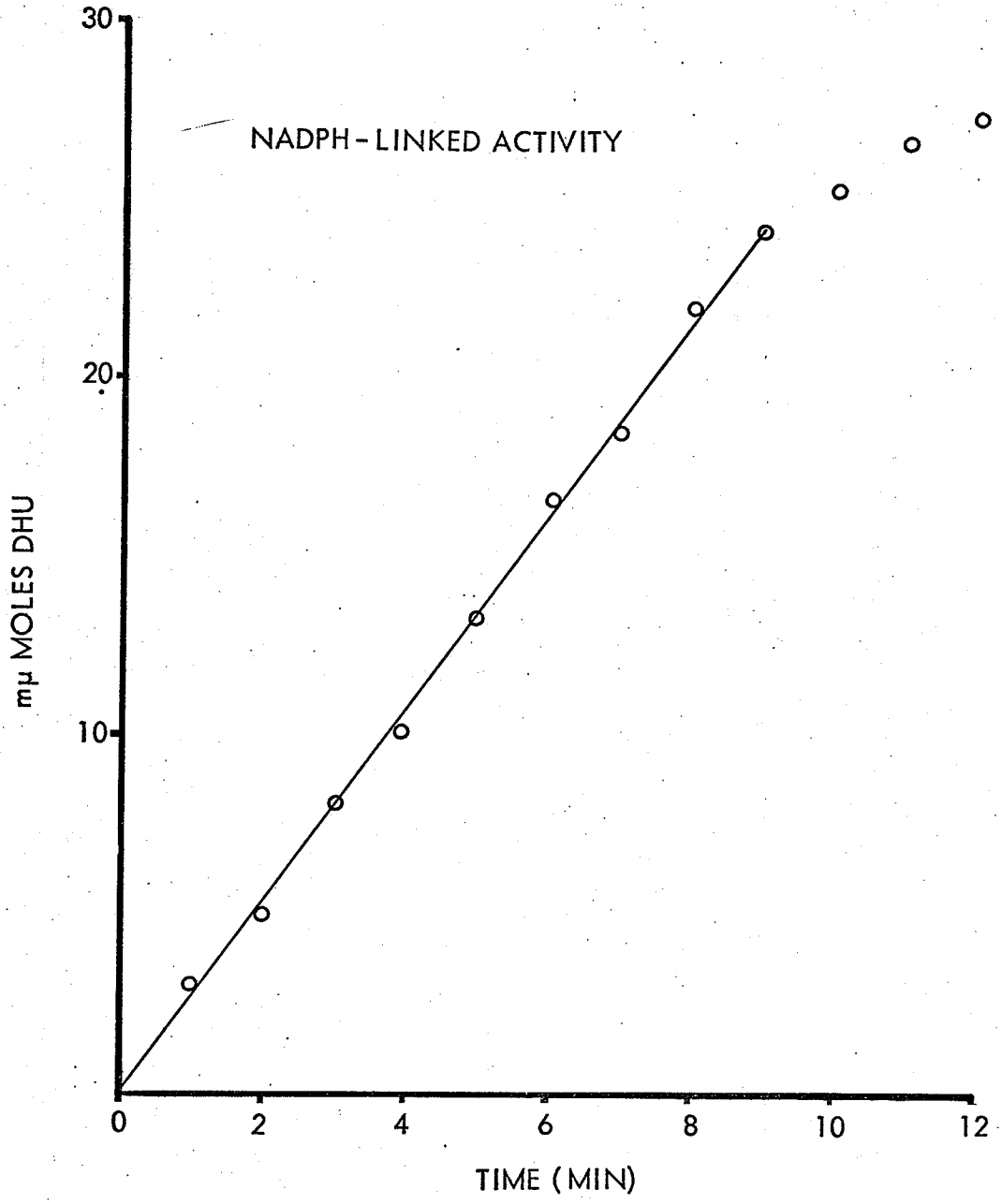

NADPH - LINKED ACTIVITY
mμ MOLES DHU
30
20
10
0
2
4
6
8
10
12
TIME (MIN)

Figure 16 The relationship between uracil concentration and NADPH-linked uracil reductase activity in light mitochondria. Activity was measured under the standard conditions of assay but with varying concentrations of uracil. 0.114 mg of dialysed light mitochondrial protein was used in the incubation mixture.

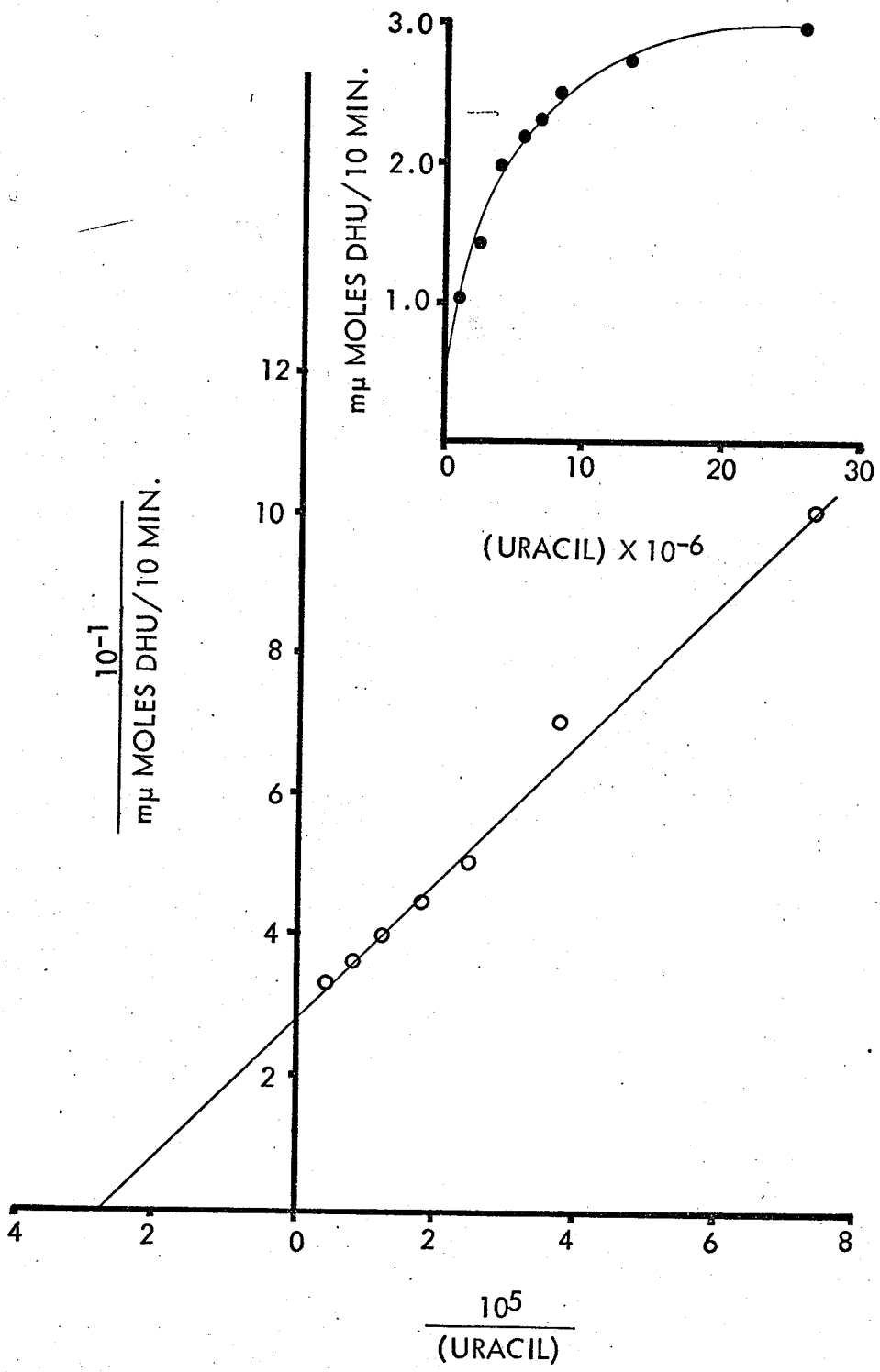

3.0
2.0
1.0
mμ MOLES DHU/10 MIN.
0
10
20
30
(URACIL) X 10⁻⁶
12
10
8
6
4
2
10⁻¹ / mμ MOLES DHU/10 MIN.
4
2
0
2
4
6
8
10⁵ / (URACIL)

Figure 17 The relationship between uracil concentration and NADPH-linked uracil reductase activity in an ammonium sulphate fraction (AS_2) prepared from dialysed soluble fraction. Activity was measured under the standard conditions of assay but with varying concentrations of uracil. 2.55 mg AS_2 protein was used in the incubation mixture.

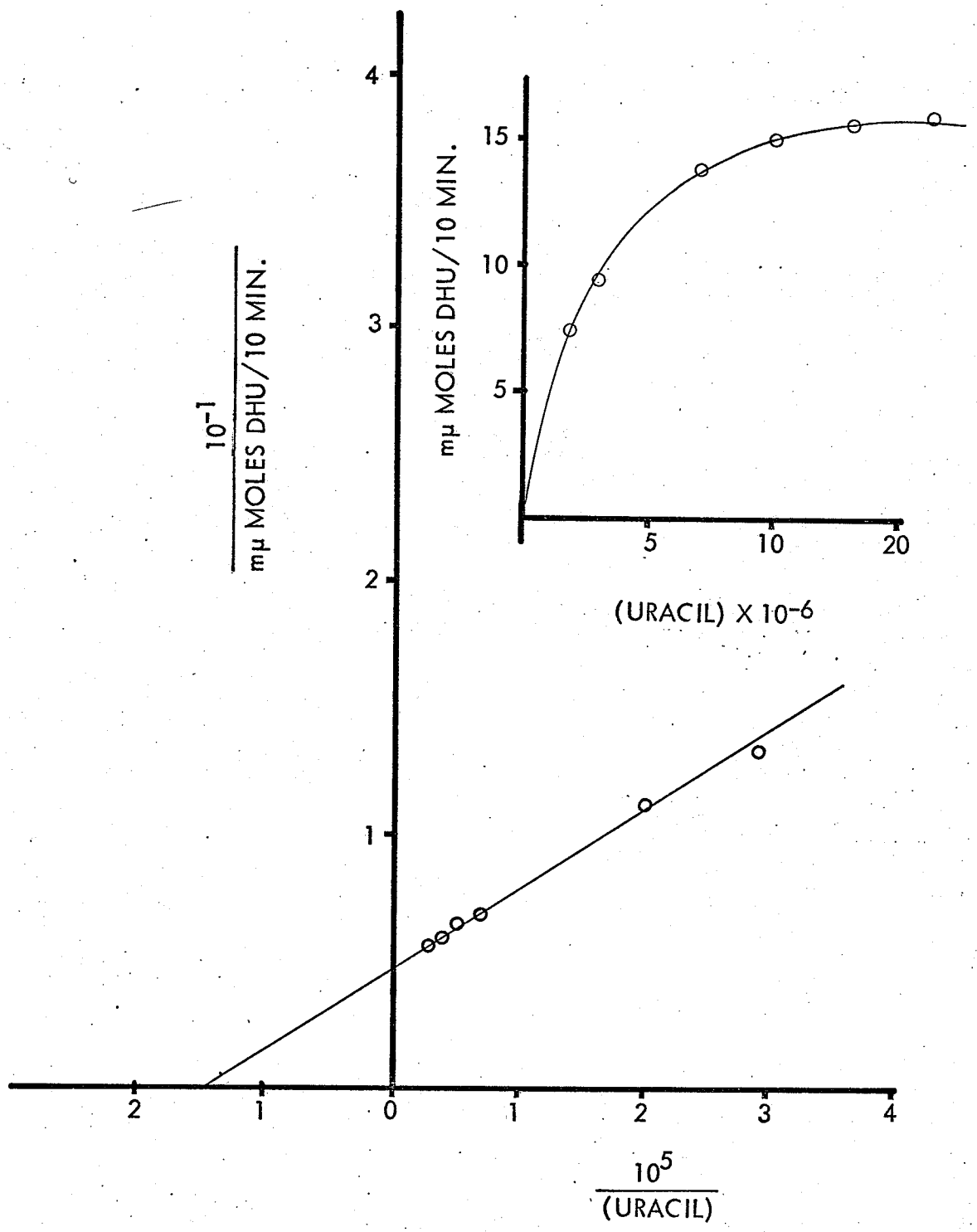

4
3
2
1
0
1
2
3
4
2
1
$\frac{10^{-1}}{\text{mμ MOLES DHU/10 MIN.}}$
$\frac{10^{5}}{\text{(URACIL)}}$
15
10
5
5
10
20
mμ MOLES DHU/10 MIN.
(URACIL) X 10^{-6}

Figure 18 A: Relationship between NADPH concentration and NADPH-linked uracil reductase activity in light mitochondria. Activity was measured under the standard conditions of assay but with varying concentrations of NADPH. 0.25 mg light mitochondrial protein was used in the incubation mixture.

B: Relationship between NADPH concentration and NADPH-linked uracil reductase in an ammonium sulphate fraction (AS_2) prepared from dialysed soluble fraction. Activity was measured under the standard conditions of assay but with varying concentrations of NADPH. 2.55 mg AS_2 protein was used in the incubation mixture.

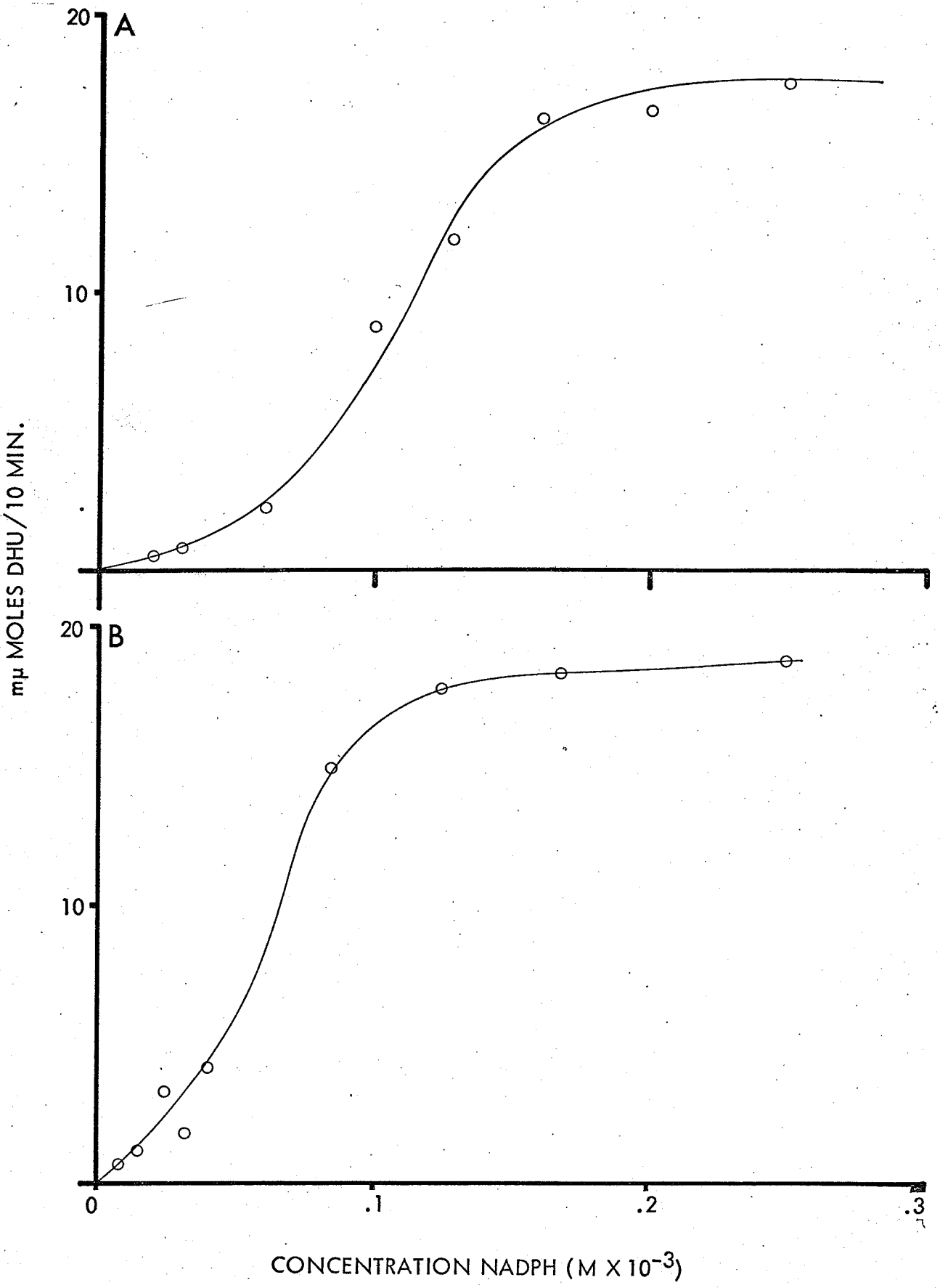

20
A
10
20
B
10
0
.1
.2
.3
mμ MOLES DHU/10 MIN.
CONCENTRATION NADPH (M X 10⁻³)

Figure 19 Inhibition of NADPH-linked uracil reductase in light mitochondrial fraction by $MgCl_2$ (open circles) ATP (closed squares) and ATP-Mg (closed triangles). Activity was measured under the standard conditions of assay but with varying concentrations of $MgCl_2$, ATP, and ATP-Mg.

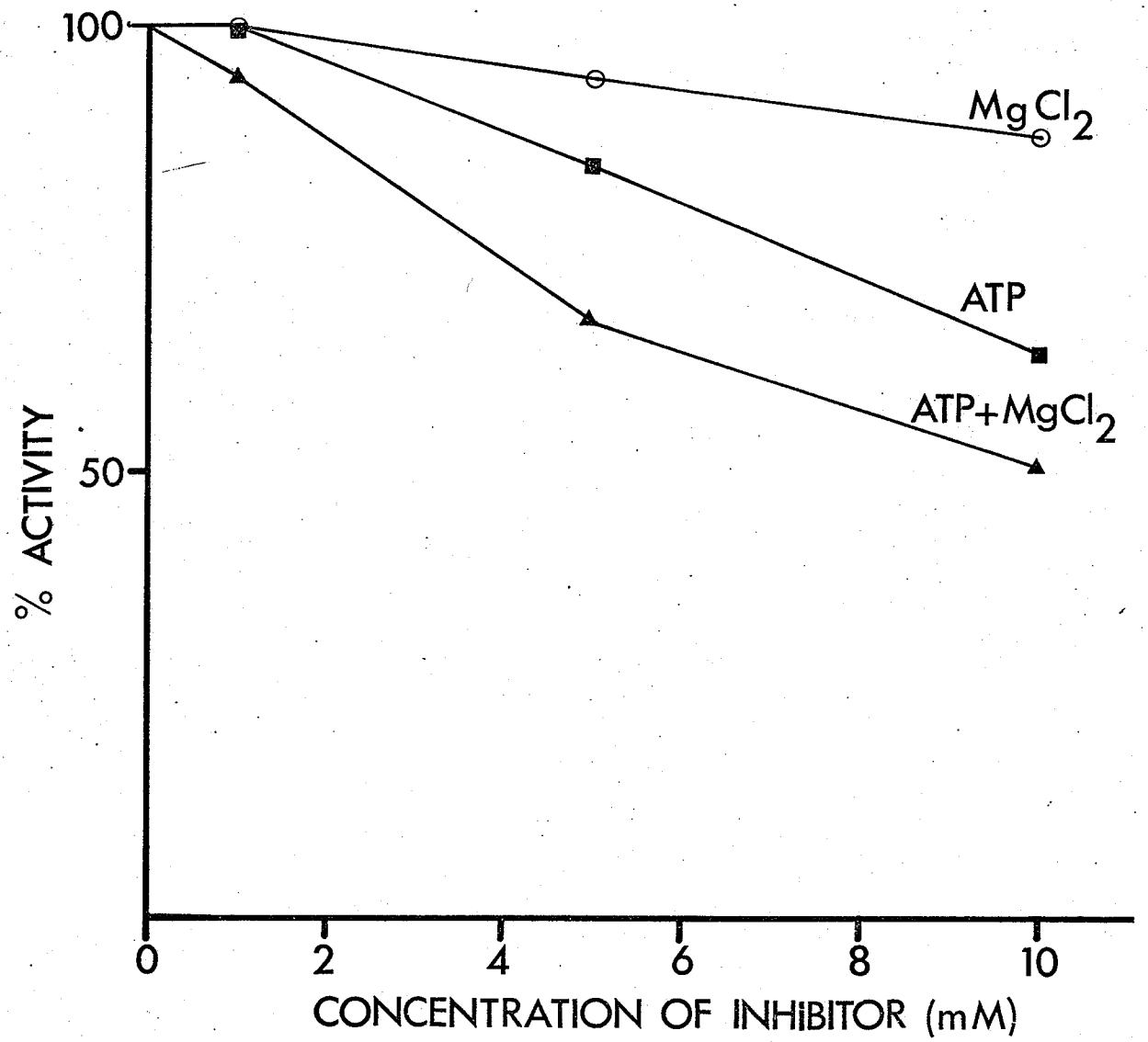

100
50
% ACTIVITY
$MgCl_2$
ATP
$ATP+MgCl_2$
0
2
4
6
8
10
CONCENTRATION OF INHIBITOR (mM)

Figure 20 Inhibition of NADPH-linked uracil reductase in an ammonium sulphate fraction (AS_2) prepared from dialysed soluble fraction. Activity was measured under the standard conditions of assay but with varying concentrations of ATP-Mg complex. 2.55 mg AS_2 protein was used in the incubation mixture.

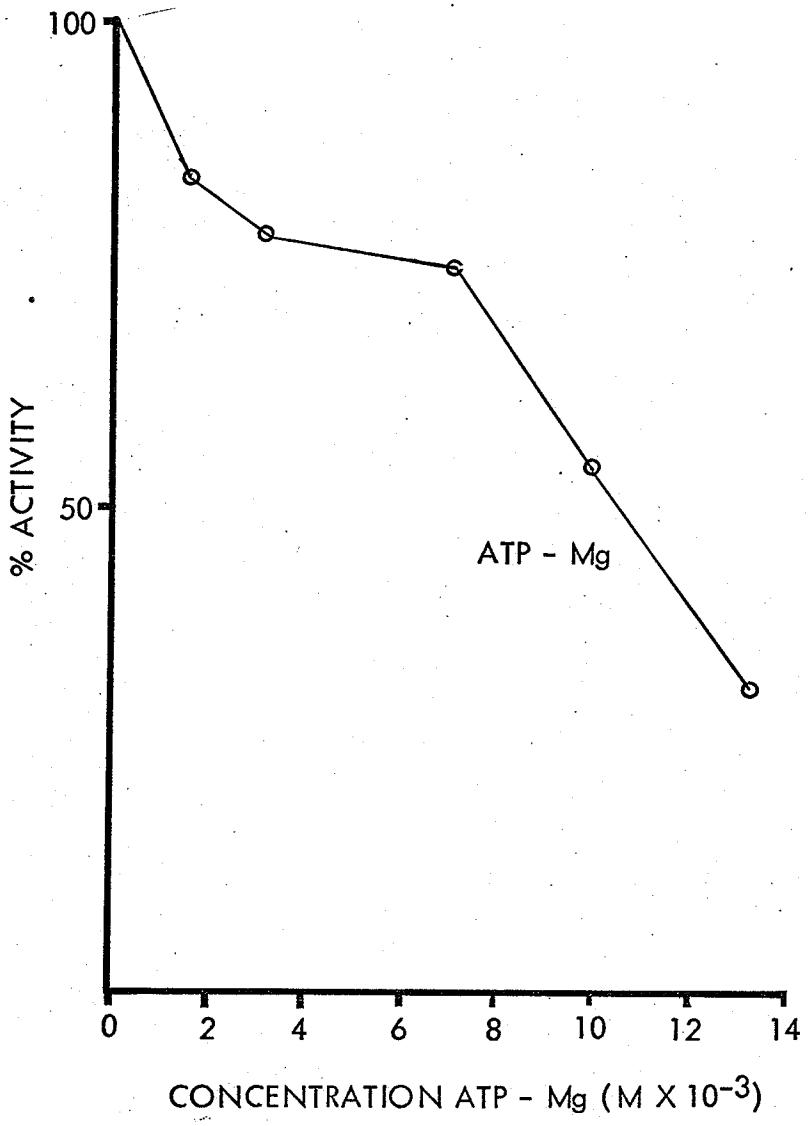

100
50
% ACTIVITY
ATP - Mg
0
2
4
6
8
10
12
14
CONCENTRATION ATP - Mg (M X 10-3)

Figure 21 The relationship between pH and NAD^+-linked dihydrouracil dehydrogenase activity in an ammonium sulphate fraction (AS_2) prepared from dialysed soluble fraction. Activity was measured under the standard conditions of assay but with differing pH's using Tris buffer (open circles), and glycine-NaOH buffer (open squares). 0.65 mg AS_2 protein was used in the incubation mixture.

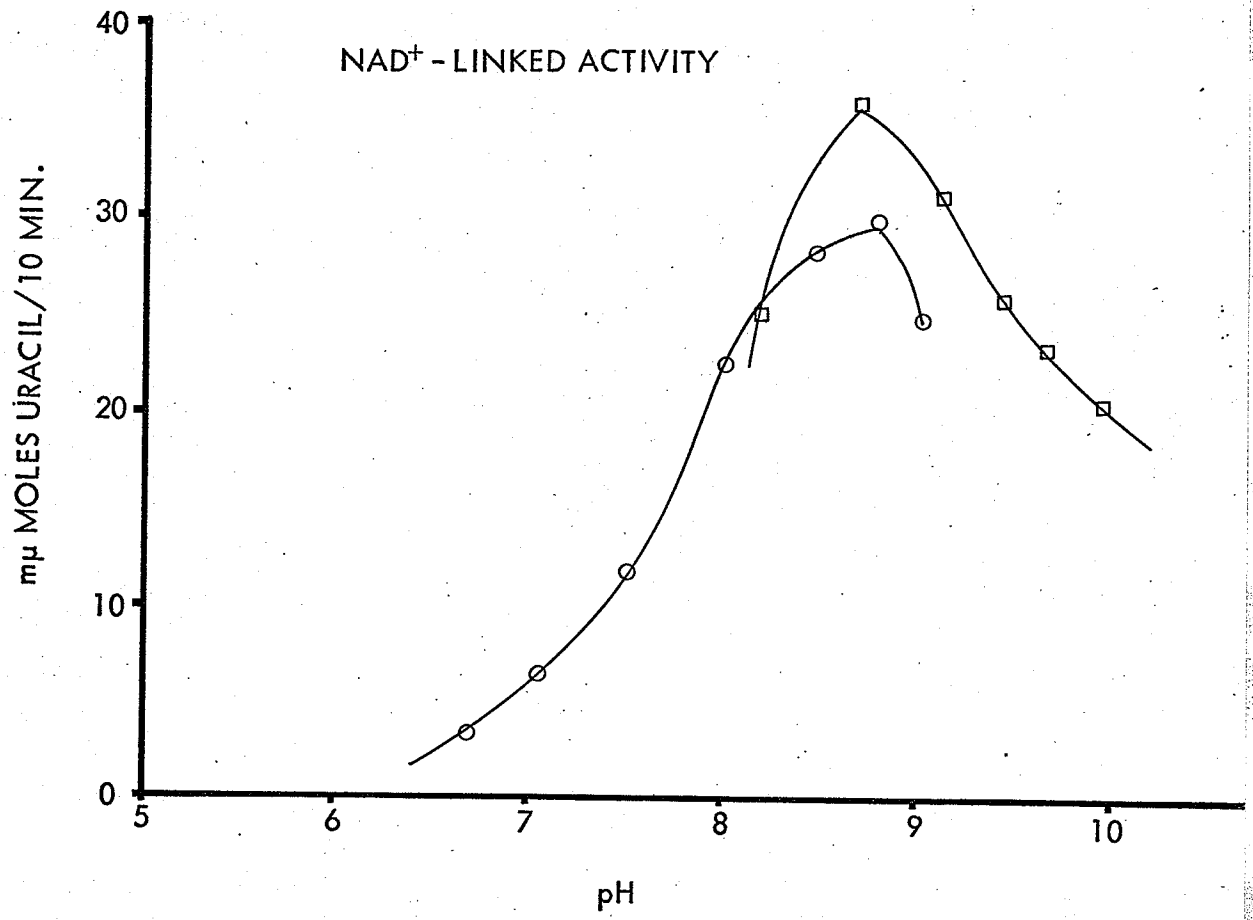

NAD+ - LINKED ACTIVITY
mμ MOLES URACIL/10 MIN.
40
30
20
10
0
5
6
7
8
9
10
pH

Figure 22 The relationship between protein concentration and NAD^+-linked dihydrouracil dehydrogenase activity in an ammonium sulphate fraction (AS_2) prepared from dialysed soluble fraction. Activity was measured under the standard conditions of assay but with varying concentrations of protein.

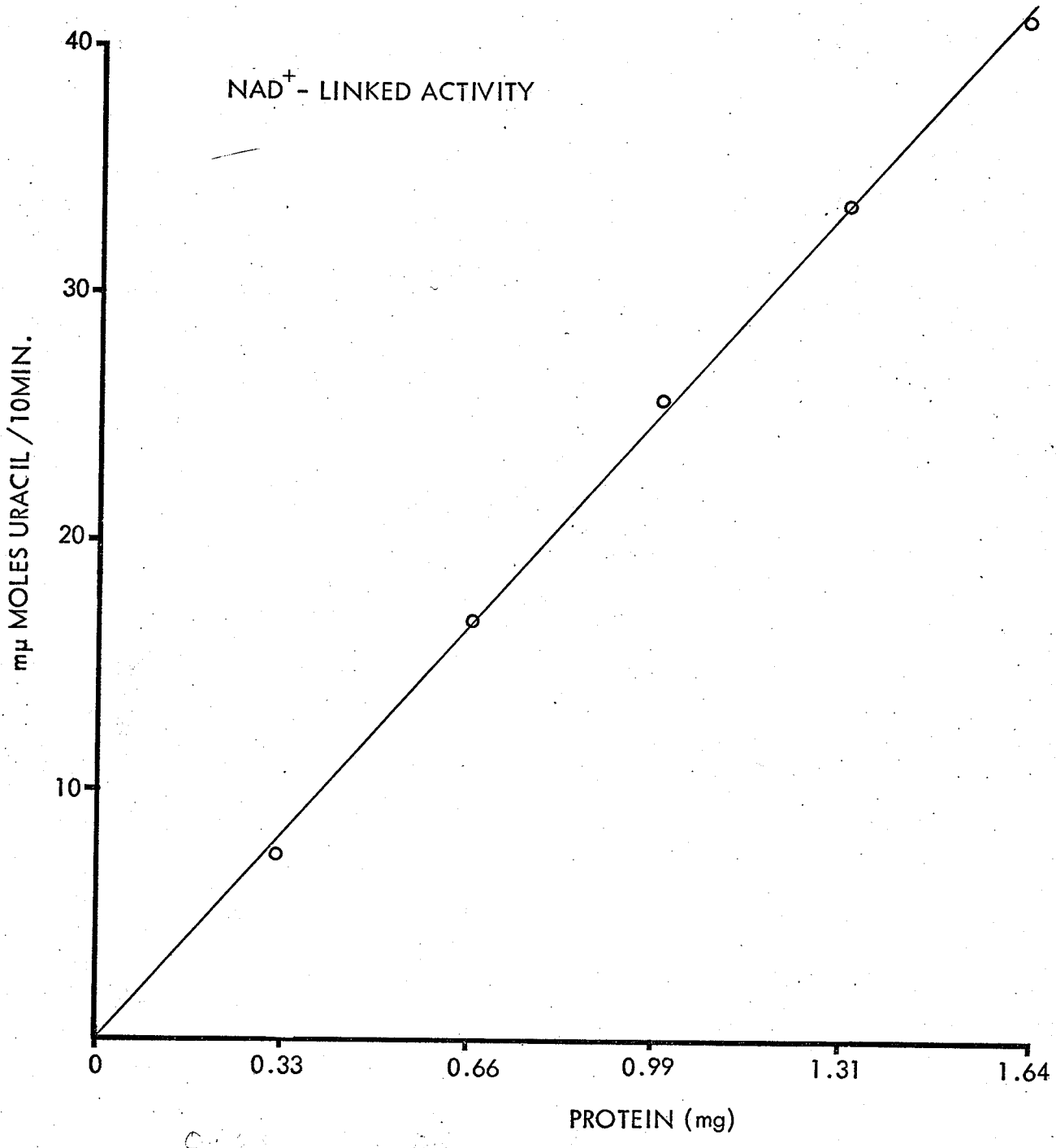

NAD+- LINKED ACTIVITY
mμ MOLES URACIL /10MIN.
40
30
20
10
0
0.33
0.66
0.99
1.31
1.64
PROTEIN (mg)

Figure 23 The relationship between time and NAD^+-linked dihydrouracil dehydrogenase activity in an ammonium sulphate fraction (AS_2) prepared from dialysed soluble fraction. Activity was measured under the standard conditions of assay but with varying incubation times. 1.63 mg AS_2 protein was used in the incubation mixture.

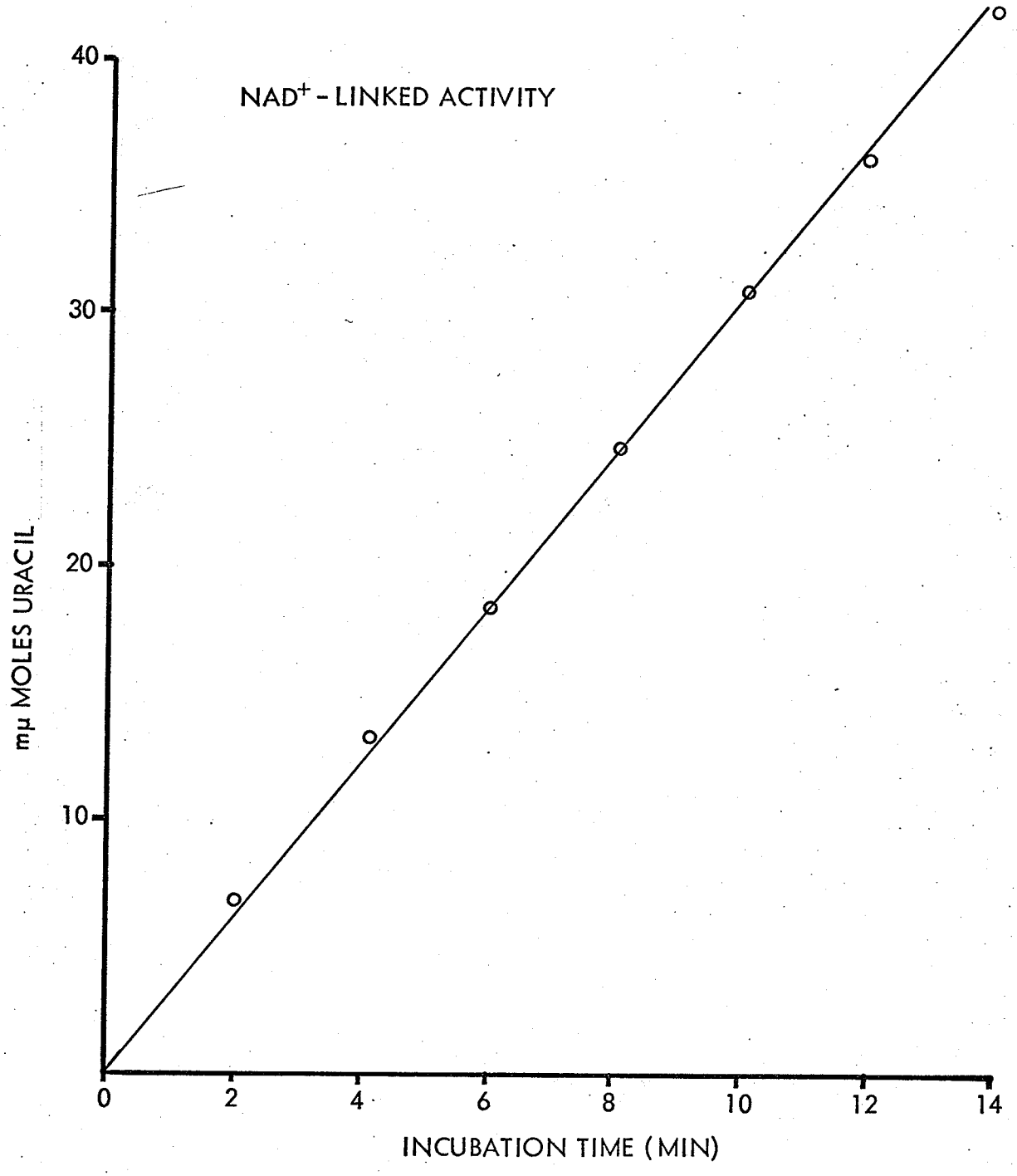

NAD+ - LINKED ACTIVITY
mμ MOLES URACIL
40
30
20
10
0
2
4
6
8
10
12
14
INCUBATION TIME (MIN)

Figure 24 The relationship between pH and $NADP^+$-linked dihydrouracil dehydrogenase activity in an ammonium sulphate fraction (AS_2) prepared from dialysed soluble fraction. Activity was measured under the standard conditions of assay using acetate buffer (open triangles) and phosphate buffer (open circles). 0.65 mg AS_2 protein was used in the incubation mixture.

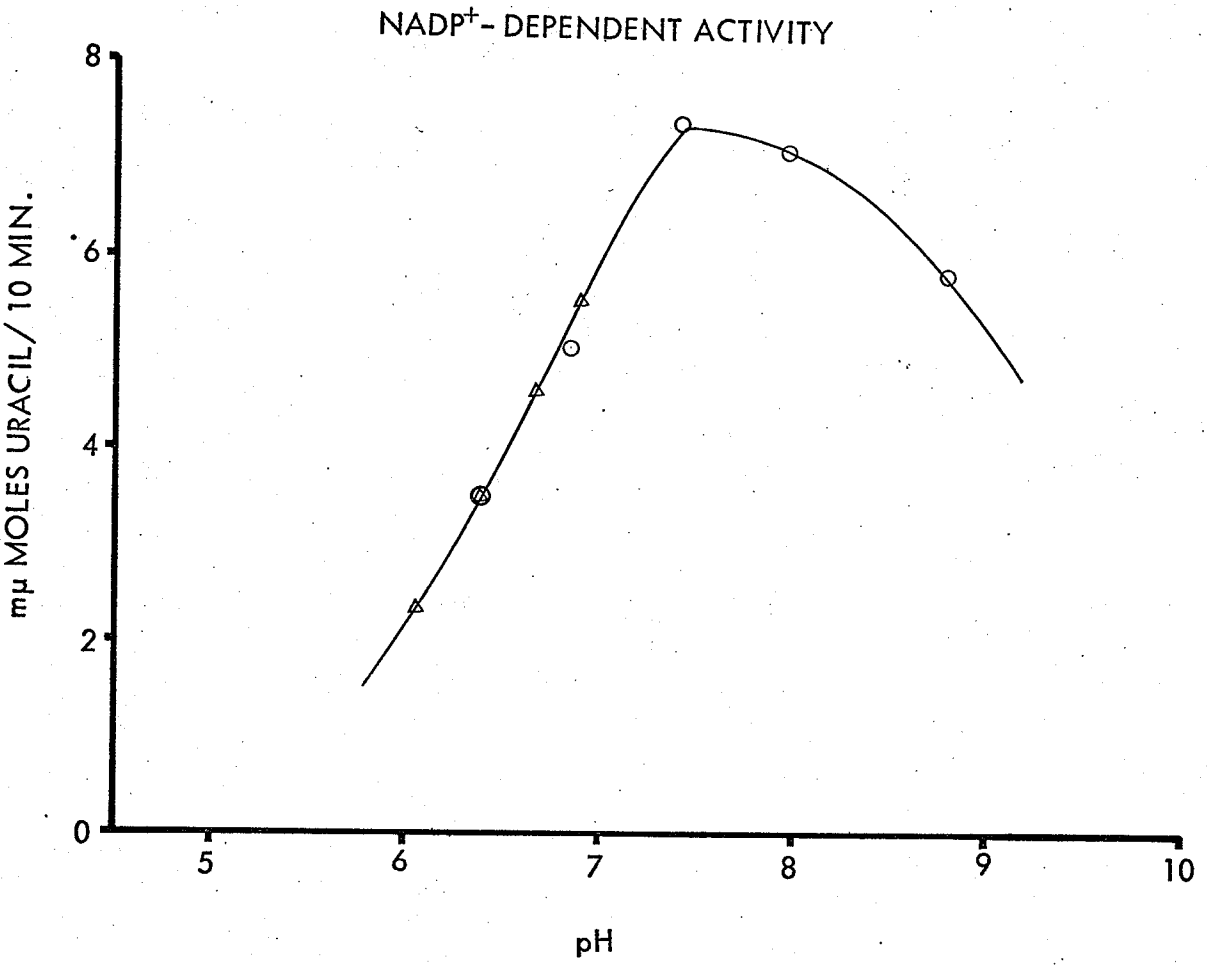

NADP+- DEPENDENT ACTIVITY
mμ MOLES URACIL/ 10 MIN.
0
2
4
6
8
5
6
7
8
9
10
pH

Figure 25 The relationship between protein concentration and $NADP^+$-linked dihydrouracil dehydrogenase activity of an ammonium sulphate fraction (AS_2) prepared from dialysed soluble fraction. Activity was measured under the standard conditions of assay but with varying protein concentrations.

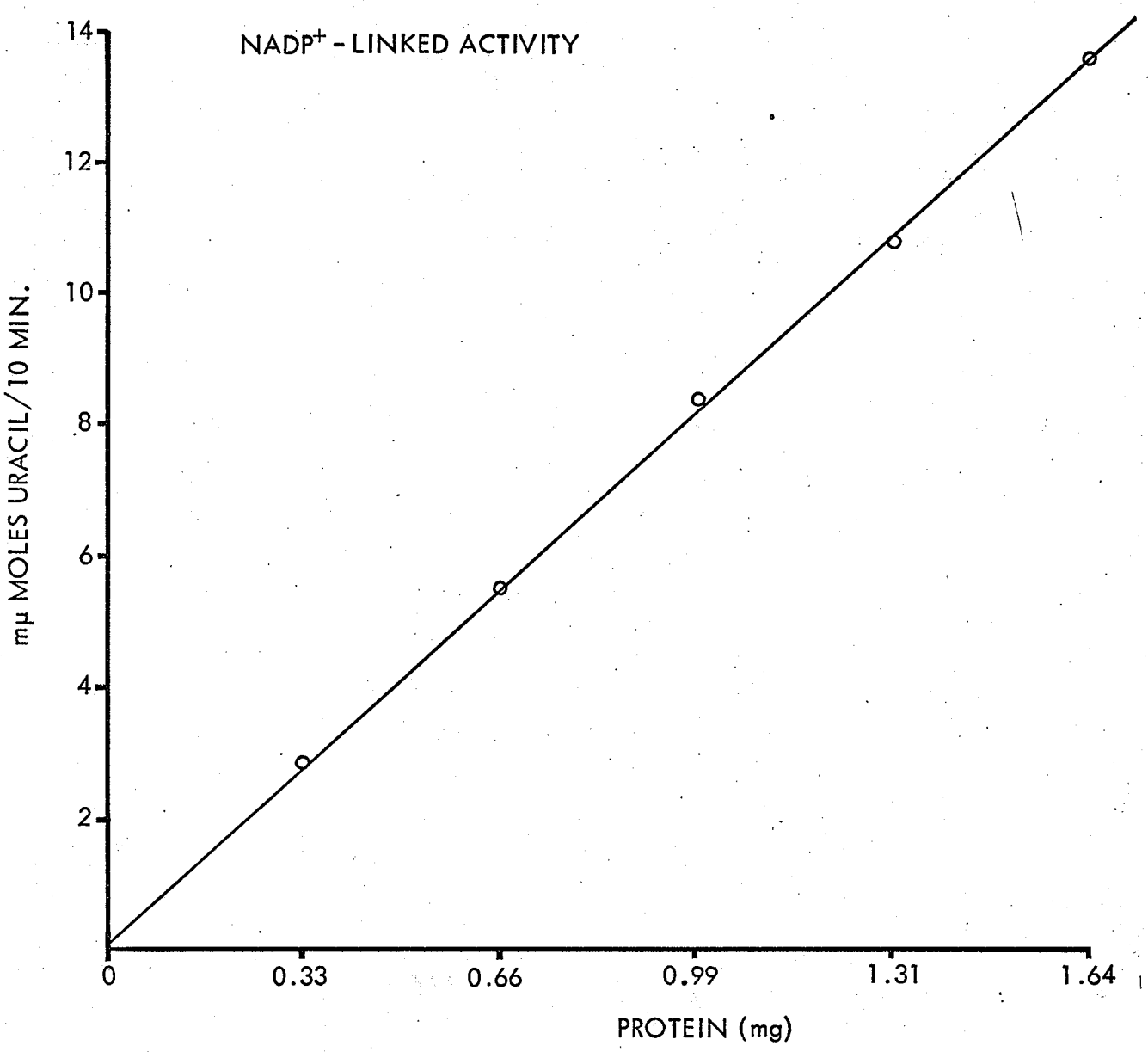

NADP+ - LINKED ACTIVITY
mμ MOLES URACIL/10 MIN.
14
12
10
8
6
4
2
0
0.33
0.66
0.99
1.31
1.64
PROTEIN (mg)

Figure 26 The relationship between time and $NADP^+$-linked dihydrouracil dehydrogenase activity in an ammonium sulphate fraction (AS_2) prepared from dialysed soluble fraction. Activity was measured under the standard conditions of assay but with varying incubation times. 3.25 mg AS_2 protein was used in the incubation mixture.

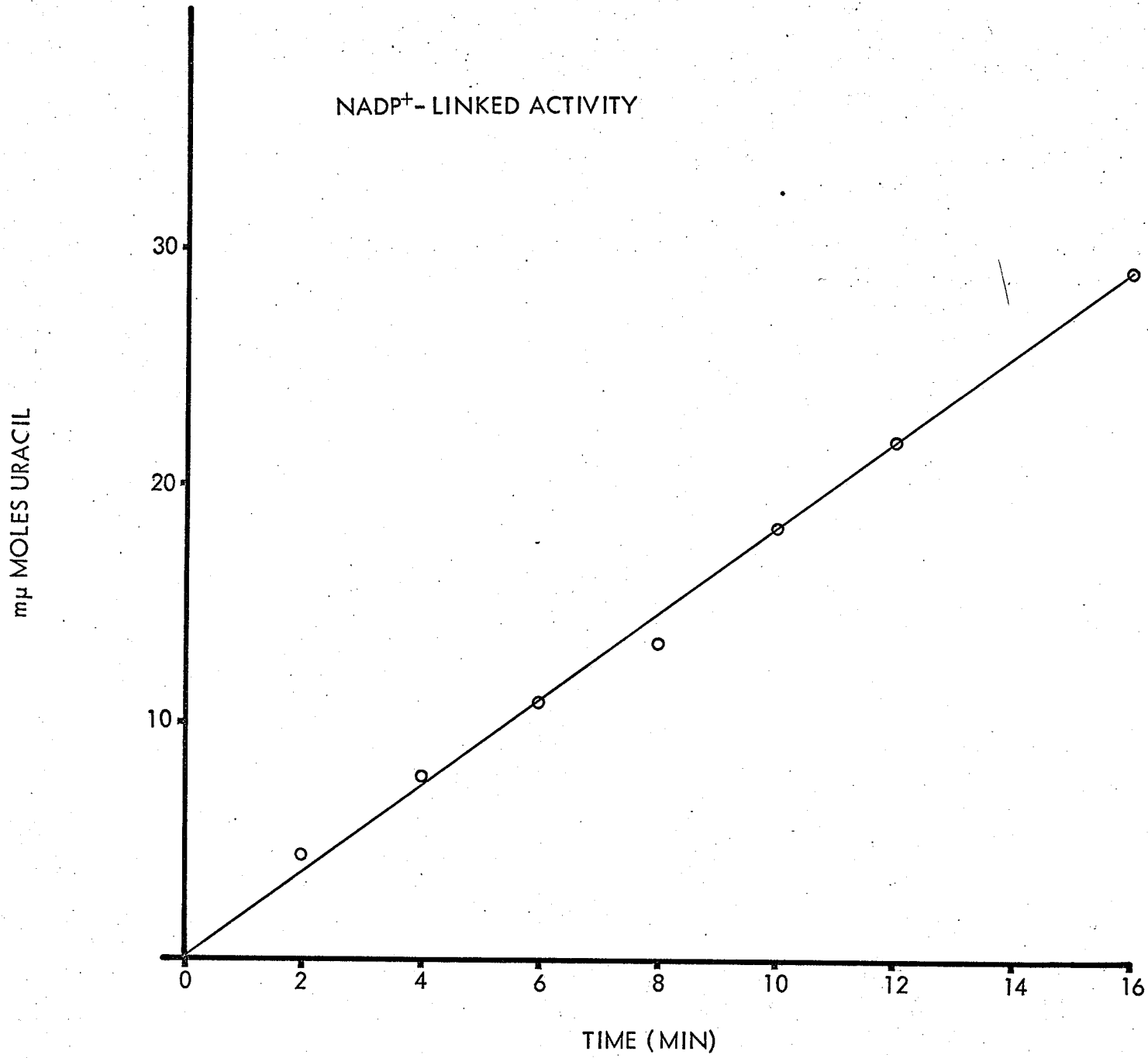

NADP+- LINKED ACTIVITY
mμ MOLES URACIL
30
20
10
0
2
4
6
8
10
12
14
16
TIME (MIN)

Figure 27 The relationship between dihydrouracil concentration and $NADP^+$-linked dihydrouracil dehydrogenase activity in an ammonium sulphate fraction (AS_2) prepared from dialysed soluble fraction. Activity was measured under the standard conditions of assay but with varying concentrations of dihydrouracil in the absence of ATP and $MgCl_2$. 1.64 mg AS_2 protein was used in the incubation mixture.

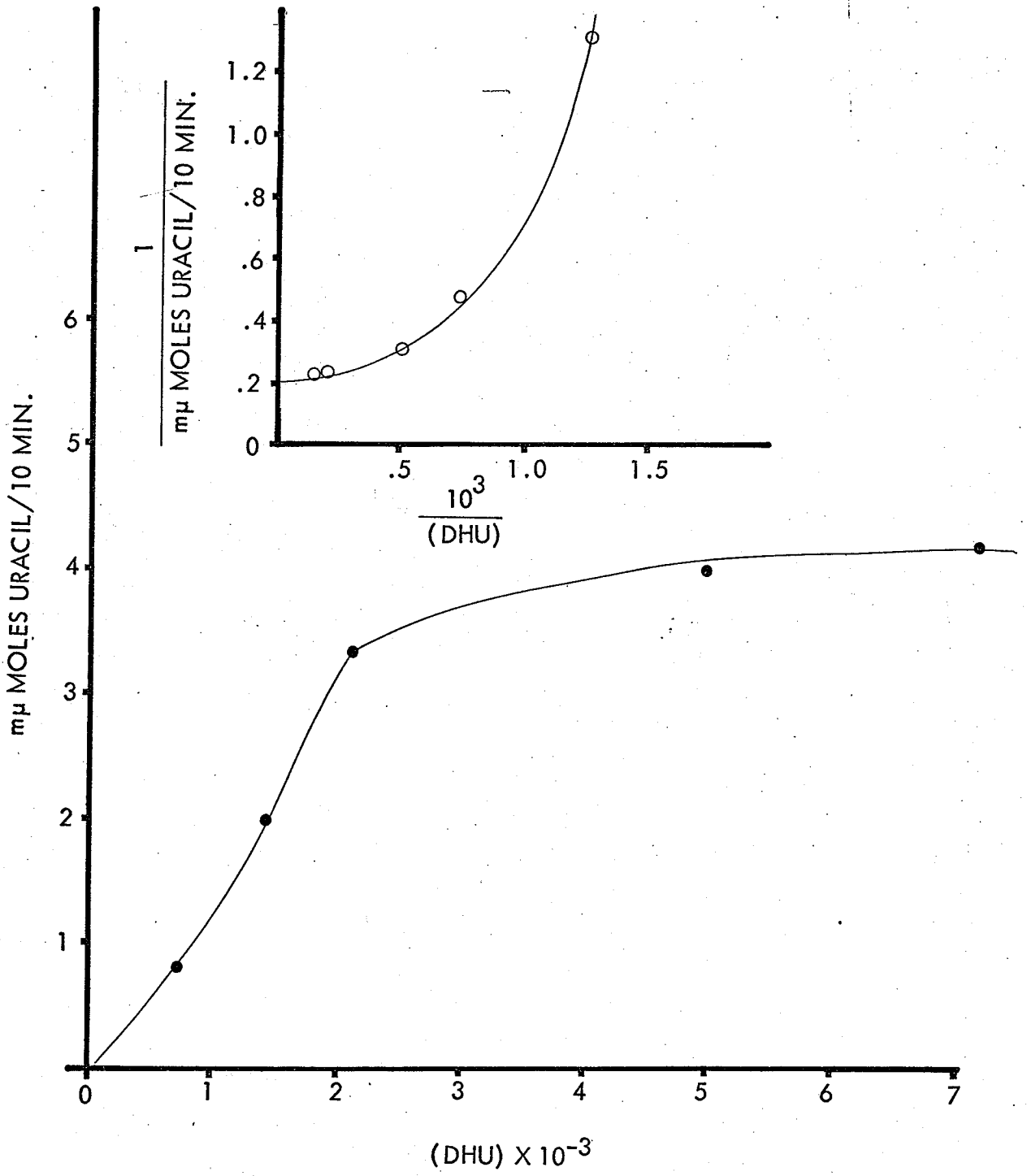
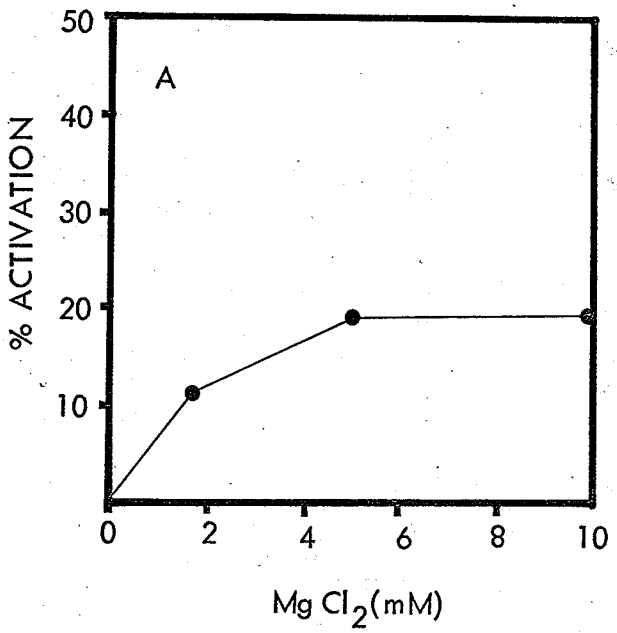
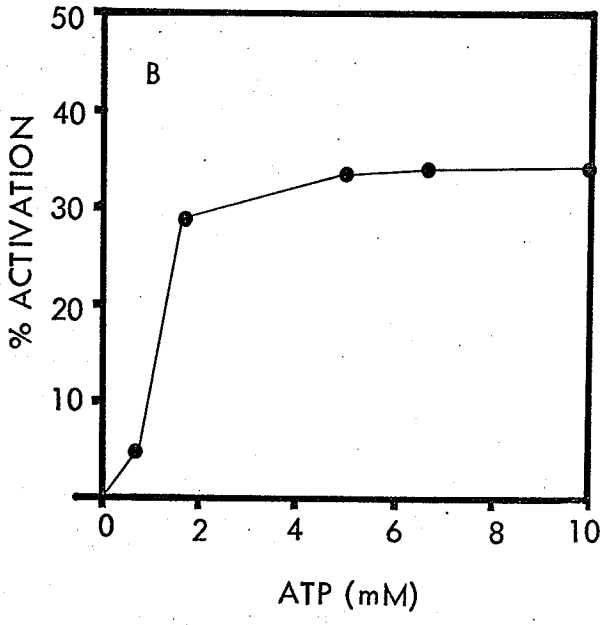
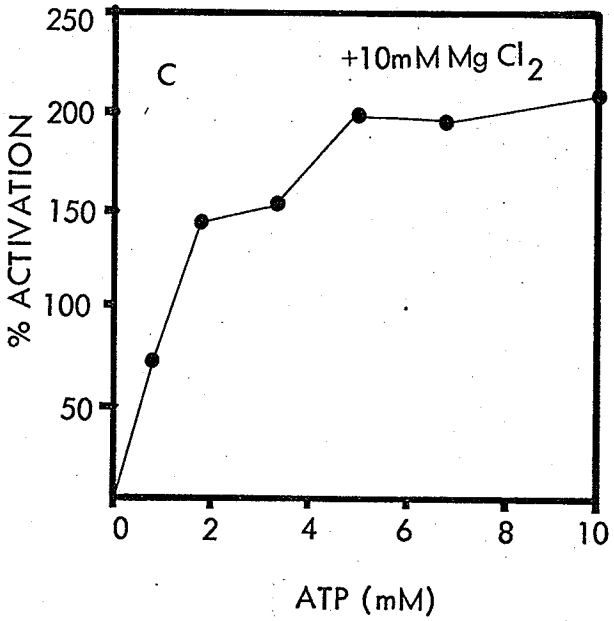

mμ MOLES URACIL/10 MIN.
(DHU) X 10^{-3}
0
1
2
3
4
5
6
7
$\frac{1}{\text{mμ MOLES URACIL/10 MIN.}}$
1.2
1.0
.8
.6
.4
.2
0
.5
1.0
1.5
$\frac{10^3}{(DHU)}$

Figure 28 The relationship between activators and $NADP^+$-linked dihydrouracil dehydrogenase activity in an ammonium sulphate fraction AS_2 prepared from dialysed soluble fraction. Activity was measured under the standard conditions of assay but with varying concentrations of $MgCl_2$ (A), ATP (B) and ATP + $MgCl_2$ (C). 1.64 mg AS_2 protein was used in the incubation mixture.


A
% ACTIVATION
50
40
30
20
10
0
2
4
6
8
10
Mg Cl2(mM)


B
% ACTIVATION
50
40
30
20
10
0
2
4
6
8
10
ATP (mM)


C
+10mM Mg Cl2
% ACTIVATION
250
200
150
100
50
0
2
4
6
8
10
ATP (mM)

TABLES

Unless otherwise stated the results in Tables I-X represent typical distributions of the enzymes in the respective fractions.

Table I Distribution of marker enzyme activities among subcellular fractions of rat liver homogenate. The fractions were separated by differential centrifugation according to the procedures outlined in Figures 2 and 3. 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	8,709 units
5'-Nucleotidase	7,648 units
Acid Phosphatase	1,987 units
Glucose-6-Phosphatase	8,833 units

The distribution for each marker enzyme is the average of two experiments. Acid Phosphatase and Glucose-6-Phosphatase were assayed on one preparation while Glutamate Dehydrogenase and 5'-Nucleotidase were assayed on another preparation.

TABLE I

Distribution of Marker Enzyme Activities in Rat Liver Fractions

Fractions	Glutamate Dehydrogenase		Acid Phosphatase		Glucose-6-phosphatase		5'-Nucleotidase	
	S.A.	%	S.A.	%	S.A.	%	S.A.	%
Homogenate	2.16	100	2.04	100	4.24	100	1.98	100
Debris	2.67	27.73	0.73	21.44	3.99	27.26	3.52	43.95
Purified Nuclei	0.09	0.07	0.00	0.00	0.00	0.00	0.00	0.00
Heavy Mitochondria	21.68	56.13	1.23	17.38	6.62	14.62	6.27	19.98
Light Mitochondria	4.15	8.06	3.31	24.23	12.82	16.28	8.47	14.71
Microsomes	0.011	0.44	1.65	7.68	44.48	24.99	1.86	32.50
Supernatant Fraction	0.004	0.22	0.88	15.07	0.00	0.00		
% Recovery		92.65		85.80		83.15		111.14

S.A. = Specific Activity

% = Percentage of Total Activity

Table II Distribution of pyrimidine reductase activity among subcellular fractions of rat liver homogenate. The fractions were separated by differential centrifugation according to the procedures outlined in Figures 2 and 3. 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	384 units
NADH-linked Uracil Reductase	276 units
NADPH-linked Thymine Reductase	236 units

NADPH-linked Uracil Reductase and NADH-linked Uracil Reductase were assayed on one preparation while NADPH-linked Thymine Reductase was assayed on a different preparation.

TABLE II

Distribution of Pyrimidine Reductase in Subcellular Fractions of Rat Liver

Fractions	NADPH + Uracil		NADH + Uracil		NADPH + Thymine	
	S.A.	%	S.A.	%	S.A.	%
Homogenate	0.131	100	0.091	100	0.047	100
Debris	0.355	34.89	0.203	24.62	0.135	23.30
Purified Nuclei	0.000	0.00	0.000	0.00	0.000	0.00
Heavy Mitochondria	0.324	32.03	0.401	54.34	0.095	29.23
Light Mitochondria	0.629	25.10	0.144	7.97	0.374	19.91
Microsomes	0.222	3.12	0.204	3.98	0.168	2.96
Supernatant Fraction	0.024	8.33	0.017	6.15	0.028	27.54
% Recovery		103.38		97.06		102.94

S.A. = Specific Activity

% = Percentage of Total Activity

Table III Distribution of pyrimidine reductase activity among dialysed sub-cellular fractions of rat liver homogenate. The fractions were separated by differential centrifugation according to the procedures outlined in Figures 2 and 3. After separation each fraction was dialysed for 16 hours. 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	326 units
NADH-linked Uracil Reductase	223 units
NADPH-linked Thymine Reductase	215 units

NADPH-linked Uracil Reductase and NADH-linked Uracil Reductase were assayed on one preparation while NADPH-linked Thymine Reductase was assayed on a different preparation.

TABLE III

Distribution of Pyrimidine Reductase in Dialysed Rat Liver Fractions

Fractions	NADPH + Uracil		NADH + Uracil		NADPH + Thymine	
	S.A.	%	S.A.	%	S.A.	%
Homogenate	0.100	100.00	0.086	100.00	0.042	100.00
Debris	0.300	30.15	0.184	26.00	0.092	17.67
Purified Nuclei	0.000	0.00	0.000	0.00	0.000	0.00
Heavy Mitochondria	0.307	30.46	0.353	49.32	0.081	27.90
Light Mitochondria	0.608	24.00	0.112	7.60	0.332	19.53
Microsomes	0.140	2.50	0.134	3.50	0.120	2.32
Supernatant Fraction	0.021	7.00	0.011	4.90	0.026	26.51
% Recovery		94.11		91.32		93.93

S.A. = Specific Activity

% = Percentage of Total Activity

Table IV Distribution of dihydropyrimidine dehydrogenase activity among dialysed subcellular fractions of rat liver homogenate. The fractions were separated by differential centrifugation according to the procedures outlined in Figures 2 and 3. After separation each fraction was dialysed for 16 hours. Activity was measured under the standard conditions of assay except that the fractions were made 5.4M with glycerol immediately before assay. The number of enzyme units representing 100% activity in the homogenate is as follows:

$NADP^+$-linked Dihydrouracil Dehydrogenase	225 units
NAD^+-linked Dihydrouracil Dehydrogenase	267 units
$NADP^+$-linked Dihydrothymine Dehydrogenase	170 units

TABLE IV

Distribution of Dihydropyrimidine Dehydrogenase in Rat Liver Fractions

Fractions	$NADP^+$ + DHU		NAD^+ + DHU		$NADP^+$ + DHT	
	S.A.	%	S.A.	%	S.A.	%
Homogenate	0.038	100.00	0.045	100.00	0.028	100.00
Debris	0.004	0.88	0.008	1.50	0.003	0.86
Purified Nuclei	0.000	0.00	0.000	0.00	0.000	0.00
Heavy Mitochondria	0.005	5.33	0.012	11.20	0.003	4.11
Light Mitochondria	0.016	2.66	0.004	0.74	0.011	2.35
Microsomes	0.003	0.16	0.005	0.26	0.088	0.20
Supernatant Fraction	0.101	82.70	0.124	85.39	0.085	88.23
% Recovery		91.73		99.09		95.75

S.A. = Specific Activity

% = Percentage of Total Activity

Table V Distribution of marker enzymes (A) and pyrimidine reductase (B) activities in different fractions obtained during purification of nuclei. The fractions were prepared by differential centrifugation according to the procedures outlined in Figure 3. Activity was measured under the standard conditions of assay. The number of enzyme units representing 100% activity is as follows:

Glutamate Dehydrogenase	8,709 units
Acid Phosphatase	7,762 units
Glucose-6-Phosphatase	15,840 units
5'-Nucleotidase	7,648 units
NADPH-linked Uracil Reductase	348 units
NADH-linked Uracil Reductase	271 units
NADPH-linked Thymine Reductase	180 units

TABLE V

Purification of Nuclei

(A) Distribution of Marker Enzymes

Fractions	Glutamate Dehydrogenase		Acid Phosphatase		Glucose-6-phosphatase		5'-Nucleotidase	
	S.A.	%	S.A.	%	S.A.	%	S.A.	%
Homogenate	1.83	100.00	1.63	100.00	4.15	100.00	2.43	100.00
S_1	2.65	57.71	3.45	83.53	5.42	64.77	3.23	60.30
Debris	3.14	27.73	1.08	7.47 *	5.08	16.99 *	5.04	58.65
Pure Nuclei	0.05	0.07	1.33	0.20	1.45	1.16	0.00	0.00
% Recovery		85.51		91.20		82.92		118.95

(B) Distribution of Pyrimidine Reductase

Fractions	NADPH + Uracil		NADH + Uracil		NADPH + Thymine	
	S.A.	%	S.A.	%	S.A.	%
Homogenate	0.138	100.00	0.104	100.00	0.078	100.00
S_1	0.234	63.50	0.201	70.47	0.147	77.22
Debris	0.223	29.31	0.148	25.10	0.081	20.55
Pure Nuclei	0.000	0.00	0.000	0.00	0.000	0.00
% Recovery		92.81		95.57		97.77

S.A. = Specific Activity

% = Percentage of Total Activity

* A different preparation was used for the assay of these activities and the debris of these fractions was less contaminated by particulate matter.

Table VI Distribution of marker enzyme activities in different fractions obtained during purification of lysosomes. The fractions were prepared by differential centrifugation 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 is as follows:

Glutamate Dehydrogenase	5,384 units
Acid Phosphatase	5,343 units
Glucose-6-Phosphatase	16,692 units
5'-Nucleotidase	11,608 units

(F_5 = Purified Lysosomes)

TABLE VI

Purification of Lysosomes

Distribution of Marker Enzymes

Fractions	Glutamate Dehydrogenase		Acid Phosphatase		Glucose-6-phosphatase		5'-Nucleotidase	
	S.A.	%	S.A.	%	S.A.	%	S.A.	%
Homogenate	1.53	100.00	1.38	100.00	3.82	100.00	2.48	100.00
F_1	1.36	6.60	6.84	33.40	12.18	18.69	5.76	13.06
F_2	1.36	2.70	4.81	9.35	11.81	7.47	5.04	4.71
F_3	1.42	1.40	6.67	5.98	12.52	4.07	3.92	1.85
F_4	1.21	0.04	8.33	3.74	16.69	2.04	6.96	1.23
F_5	0.00	0.00	54.63	2.61	16.25	0.24	4.92	1.05

S.A. = Specific Activity

% = Percentage of Total Activity

F_1 = Light Mitochondria

F_5 = Purified Lysosomes

Table VII Distribution of pyrimidine reductase activity in different fractions obtained during purification of lysosomes. The fractions were prepared by differential centrifugation 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 is as follows:

NADPH-linked Uracil Reductase	599 units
NADH-linked Uracil Reductase	402 units
NADPH-linked Thymine Reductase	311 units

(F_5 = Purified Lysosomes)

TABLE VII

Purification of Lysosomes

Distribution of Pyrimidine Reductase in Fractions

Fractions	NADPH + Uracil		NADH + Uracil		NADPH + Thymine	
	S.A.	%	S.A.	%	S.A.	%
Homogenate	0.137	100.00	0.092	100.00	0.071	100.00
F_1	0.446	18.10	0.120	7.70	0.085	7.39
F_2	0.460	8.10	0.122	3.40	0.088	2.89
F_3	0.641	5.80	0.123	1.70	0.120	2.25
F_4	0.737	2.50	0.100	0.40	0.184	0.96
F_5	0.050	0.001	0.000	0.00	0.000	0.00

S.A. = Specific Activity

% = Percentage of Total Activity

F_1 = Light Mitochondria

F_5 = Purified Lysosomes

Table VIII Distribution of marker enzyme activities in different fractions obtained during purification of plasma membranes. The fractions were prepared by discontinuous sucrose density gradient according to the procedure outlined in Figure 5. 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	2,787 units
Acid Phosphatase	3,026 units
Glucose-6-Phosphatase	6,528 units
5'-Nucleotidase	2,544 units

TABLE VIII

Purification of Plasma Membranes

Distribution of Marker Enzymes in Fractions

Fractions	Glutamate Dehydrogenase		Acid Phosphatase		Glucose-6-phosphatase		5'-Nucleotidase	
	S.A.	%	S.A.	%	S.A.	%	S.A.	%
Homogenate	2.19	100.00	2.37	100.00	5.17	100.00	2.00	100.00
4g-P	5.38	91.70	2.69	42.40	3.47	52.27	1.92	35.90
4g-S	0.34	5.70	1.64	46.13	2.61	40.44	1.84	61.80
1S-2S	0.00	0.00	3.30	4.80	18.40	5.60	3.20	3.10
3S	0.00	0.00	0.00	0.00	12.00	0.76	5.71	1.10
4S-5S	2.20	0.10	9.81	2.00	15.60	8.07	2.54	1.50
6S	0.00	0.00	4.58	3.00	11.89	9.29	8.13	6.20
7S	0.63	1.10	3.85	10.40	20.70	18.17	15.60	45.40
70S-Supernatant	0.45	5.10	1.39	24.70	0.14	1.06	3.02	6.40

S.A. = Specific Activity

% = Percentage of Total Activity

7S = Plasma Membranes

70S-Supernatant = A combination of the 70,000xg supernatants obtained after the fractions from the discontinuous sucrose density gradient were centrifuged.

Table IX Distribution of pyrimidine reductase activity in different fractions obtained during purification of plasma membranes. The fractions were prepared by discontinuous sucrose density gradient according to the procedure outlined in Figure 5, except that 1S-6S were pooled. The fractions were dialysed for 16 hours. Activity was measured under the standard conditions of assay. The number of enzyme units representing 100% activity is as follows:

NADPH-linked Uracil Reductase	211 units
NADH-linked Uracil Reductase	143 units
NADPH-linked Thymine Reductase	92 units

TABLE IX

Purification of Plasma Membranes

Distribution of Pyrimidine Reductase in Fractions

Fractions	NADPH + Uracil		NADH + Uracil		NADPH + Thymine	
	S.A.	%	S.A.	%	S.A.	%
Homogenate	0.129	100.00	0.088	100.00	0.052	100.00
4g-P	0.183	51.60	0.177	76.90	0.048	31.50
4g-S	0.179	53.50	0.062	27.20	0.097	66.30
1S-6S	0.393	8.00	0.066	2.10	0.136	6.50
7S	0.404	14.20	0.119	6.30	0.142	11.90
70S-Supernatant	0.103	37.90	0.035	18.80	0.058	48.90

S.A. = Specific Activity

% = Percentage of Total Activity

7S = Plasma Membranes

70S-Supernatant = A combination of the 70,000xg supernatants obtained after the fractions from the discontinuous sucrose density gradient were centrifuged.

Table X Distribution of dihydropyrimidine dehydrogenase activity in different fractions obtained during purification of plasma membranes. The fractions were prepared by discontinuous sucrose density gradient according to the procedure outlined in Figure 5, except that the fractions 1S-6S were pooled. The fractions were dialysed for 16 hours. Activity was measured under the standard conditions of assay. The number of enzyme units representing 100% activity is as follows:

$NADP^+$-linked Dihydrouracil Dehydrogenase	24 units
NAD^+-linked Dihydrouracil Dehydrogenase	40 units
$NADP^+$-linked Dihydrothymine Dehydrogenase	28 units

TABLE X

Purification of Plasma Membranes

Distribution of Dihydropyrimidine Dehydrogenase in Fractions

Fractions	$NADP^+$ + DHU		NAD^+ + DHU		$NADP^+$ + DHT	
	S.A.	%	S.A.	%	S.A.	%
Homogenate	0.013	100.00	0.024	100.00	0.016	100.00
4g-P	0.0008	2.08	0.0012	1.88	0.0008	1.85
4g-S	0.038	108.00	0.056	87.50	0.041	100.00
1S-6S	0.023	3.75	0.040	4.42	0.029	5.17
7S	0.019	5.83	0.042	7.50	0.019	5.00
70S-Supernatant	0.032	91.66	0.038	85.00	0.036	92.85

S.A. = Specific Activity

% = Percentage of Total Activity

7S = Plasma Membranes

70S-Supernatant = A combination of the 70,000xg supernatants obtained after the fractions from the dis-continuous sucrose density gradient were centrifuged.

Table XI Inhibition of NADPH-linked and NADH-linked uracil reductase by nucleotides and cations. Dialysed light mitochondrial fraction was used to assay the activity of NADPH-linked uracil reductase, and dialysed heavy mitochondrial fraction was used to assay the activity of NADH-linked uracil reductase. The enzyme fractions used when Pi was included in the incubation mixture were dialysed as described under "Materials and Methods" except that Tris buffer pH 7.0 was substituted for phosphate buffer pH 7.0. Activity was measured under the standard conditions of assay, but in the presence of various concentrations of the nucleotides, cations or complexes.

TABLE XI

Inhibition of Uracil Reductase by Nucleotides and Cations

Inhibitor	Conc.	NADPH-linked Uracil Reductase % Inhibition	NADH-linked Uracil Reductase % Inhibition
ATP	1mM	0.0	0.0
	5mM	17.4	20.1
	10mM	31.2	29.6
ADP	1mM	0.0	0.0
	5mM	0.0	0.0
	10mM	0.0	0.0
AMP	1mM	0.0	0.0
	5mM	0.0	0.0
	10mM	0.0	0.0
Mg^{++}	1mM	0.0	0.0
	5mM	6.6	8.1
	10mM	12.2	9.2
ATP-Mg	1mM	5.6	0.0
	5mM	27.5	29.6
	10mM	47.6	47.2
ADP-Mg	1mM	0.0	0.0
	5mM	3.4	2.9
	10mM	5.6	5.2
AMP-Mg	1mM	0.0	0.0
	5mM	2.6	2.2
	10mM	6.5	5.6
Pi	50mM	0.0	0.0
	100mM	0.0	0.0

Table XII Solubilization of NADPH-linked uracil reductase and glutamate dehydrogenase. The respective fractions were prepared according to the procedures outlined in Figures 2, 3 and 5. Activity was measured under the standard conditions of assay, except that a portion of each fraction was made 5.4M with glycerol to test for solubilization, and all assays were done before the fractions were frozen.

TABLE XII

Solubilization of NADPH-linked Uracil Reductase and Glutamate Dehydrogenase in Rat Liver Fractions

Fractions	Glutamate Dehydrogenase			NADPH-linked Uracil Reductase		
	S.A. Before	S.A. After	Ratio $\frac{\text{S.A. After}}{\text{S.A. Before}}$	S.A. Before	S.A. After	Ratio $\frac{\text{S.A. After}}{\text{S.A. Before}}$
Homogenate	0.921	1.694	1.84	0.088	0.155	1.76
Debris	2.372	2.765	1.17	0.057	0.117	2.05
Purified Nuclei	0.846	0.846	1.00	0.000	0.000	0.00
Heavy Mitochondria	1.806	5.637	3.12	0.043	0.161	3.74
Light Mitochondria	0.423	0.853	2.02	0.137	0.645	4.71
Plasma Membrane	0.016	0.016	1.00	0.156	0.425	2.72

S.A. = Specific Activity

Table XIII Partial purification of $NADP^+$-linked and NAD^+-linked dihydrouracil dehydrogenase. The purification steps were carried out as described under "Materials and Methods". Activity was measured under the standard conditions of assay, except that phosphate buffer pH 7.5 was used as the buffering medium in both cases.

TABLE XIII

Partial Purification of $NADP^+$-linked and NAD^+-linked Dihydrouracil Dehydrogenase

Fraction	$NADP^+$-linked Dihydrouracil Dehydrogenase			NAD^+-linked Dihydrouracil Dehydrogenase		
	Total Activity	S.A.	% Recovery	Total Activity	S.A.	% Recovery
Homogenate	133.00	0.041	100.00	152.00	0.046	100.00
Supernatant	121.00	0.094	90.00	126.00	0.097	83.00
AS_1	1.00	0.023	0.70	1.40	0.033	0.90
AS_2	103.00	0.150	77.00%	109.00	0.160	72.00
AS_3	3.70	0.040	2.70%	7.30	0.079	4.80

S.A. = Specific Activity

Table XIV Tissue distribution of pyrimidine reductase and dihydropyrimidine dehydrogenase activities. Each tissue was homogenized in 0.25M sucrose + 5mM β-mercaptoethanol, then dialysed for 16 hours. Activity was measured under the standard conditions of assay.

TABLE XIV

Tissue Distribution of Pyrimidine Reductase and Dihydropyrimidine Dehydrogenase

Enzyme	Specific Activity							
	Heart	Liver	Spleen	Whole Blood	Kidney	Testis	Brain	Muscle
NADPH-linked Uracil Reductase	0.0	0.101	0.0	0.0	0.0035	0.0042	0.0	0.0
NADH-linked Uracil Reductase	0.0	0.086	0.0	0.0	0.0032	0.0090	0.0	0.0
NADPH-linked Thymine Reductase	0.0	0.042	0.0	0.0	0.0050	0.0	0.0	0.0
$NADP^+$-linked Dihydrouracil Dehydrogenase	0.0	0.038	0.0	0.0	0.0034	0.0	0.0029	0.0
NAD^+-linked Dihydrouracil Dehydrogenase	0.0	0.045	0.0	0.0	0.0066	0.0058	0.0015	0.0
$NADP^+$-linked Dihydrothymine Dehydrogenase	0.0065	0.028	0.0056	0.0	0.0048	0.0035	0.0133	0.0