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Studies on Pyrimidine Metabolism in Tumor and Normal Tissues

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Studies on Pyrimidine Metabolism in Tumor and Normal Tissues

Kazuhiro Ikenaka

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PREFACE

When the cells begin to proliferate rapidly, the activities of enzymes involved in pyrimidine nucleotide synthesis increase[3]. The metabolic pathways of pyrimidine nucleotide synthesis are largely devided into two pathways: de novo pathway, which synthesizes pyrimidine nucleotides from small molecules, such as carbon dioxide and ammonia, and the other, salvage pathway, reutilizing bases and nucleosides (Chart 1).

The activities of both pathways are reported to increase during neoplastic development or liver regeneration[3].

The rise of these activities could be the result of

a) inhibition of nucleotide degrading enzymes
b) synthesis of new anabolic enzymes of nucleotide synthesizing enzymes
c) elimination of inhibitors
d) degradation of catabolic enzymes

The reciprocal relationship was found between nucleoside kinase and 5'-nucleotidase during liver regeneration in the previous work from our laboratory[1]. In the first part (Part 1) of this paper, the enzyme levels of pyrimidine nucleotide synthesis in various tissues were measured and the main metabolic pathways of DNA synthesis induced in tumor cells and rapidly growing cells are determined. Among the pyrimidine-metabolising enzymes, the activity of ribonucleoside diphosphate reductase (RR) is very low and clearly increased in parallel with the

growth rate in Morris hepatoma cell line[2]. Moreover the existence of inhibitor of RR in rat liver was suggested[2]. In order to examine the possibility that elimination of inhibitor might activate DNA synthesis, the inhibitor of RR in rat liver was characterized in Part 2. In the last part of the studies (Part 3), the regulation of DNA synthesis by an antimetabolite, 5-fluorouracil, and its metabolism, compared with the natural substrate, uracil, are investigated.

The aim throughout these studies was to clarify the regulatory mechanism of DNA synthesis.

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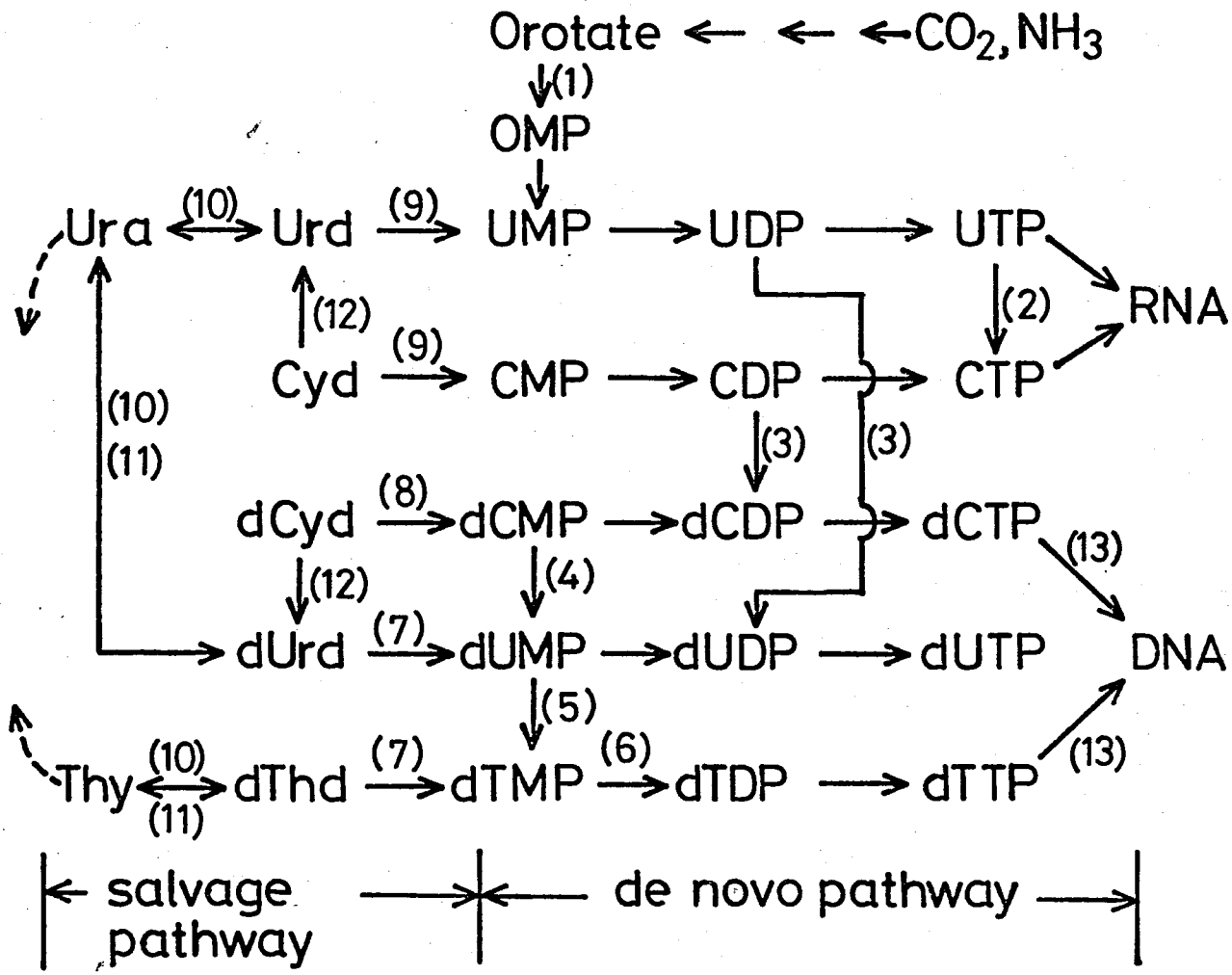

(7) Thymidine kinase

(8) Deoxycytidine kinase

(9) Uridine-cytidine kinase

(10) Uridine phosphorylase

(11) Thymidine phosphorylase

(12) Cytidine deaminase

(1) Orotate phosphoribosyl-transferase

(2) CTP synthetase

(3) Ribonucleoside diphosphate reductase

(4) dCMP deaminase

(5) dTMP synthase

(6) dTMP kinase

(13) DNA polymerase

Chart 1. Metabolic Map of Pyrimidine Nucleotides

Part 1

Metabolism of Pyrimidine Nucleotides in Various Tissues and Tumor Cells from Rodents

SUMMARY

Metabolisms of pyrimidine nucleotide in various tissues and tumor cells of rodents were investigated. Ribonucleoside diphosphate reductase [1.17.4.11], dTMP kinase [2.7.4.9] and DNA polymerase [2.7.7.7] are specifically localized in tumor cells, i.e., activities of these enzymes in tumor cells were at least three times higher than those in normal tissues, including rapidly growing tissues, such as bone marrow, thymus, and spleen. The activity of dCMP deaminase [3.5.4.12], and all the nucleoside kinases was high not only in tumor cells, but also in rapidly growing normal tissues, so that these enzymes are unsuitable as targets for cancer chemotherapy. The tissue distribution of other enzymes, including orotate phosphoribosyltransferases [2.4.2.10], CTP synthetase [6.3.4.2], dTMP synthase [2.1.1.45], nucleoside phosphorylases and Cyd deaminase [3.5.4.5] had no relation with the cell growth rate.

AH-130 tumor cells and thymus showed specific increases in the activities of enzymes involved in de novo DNA synthesis. In contrast, Yoshida sarcoma and bone marrow showed high activities of enzymes in the salvage pathway of DNA synthesis, which suggested that each tumor cell has different patterns of nucleotide metabolism.

Substrate specificity of several enzymes is also discussed, from the results of tissue distribution of enzymes. Urd and Cyd are reconfirmed to be phosphorylated by a single enzyme (Urd-

Cyd kinase), and thymidine and deoxyuridine by dThd kinase. Uridine, deoxyuridine, and thymidine phosphorylase showed similar tissue distribution, which suggests that phosphorylis of dUrd and dThd is due mainly to uridine phosphorylase, rather than thymidine phosphorylase.

INTRODUCTION

During neoplatic development, increases have been observed in the activities of many enzymes involved in pyrimidine nucleotide synthesis, including ribonucleoside diphosphate reductase [1.17.4.1] deoxycytidine monophosphate (dCMP) deaminase [3.5.4.12], cytidine triphosphate (CTP) synthetase [6.3.4.2], thymidine monophosphate (dTMP) synthase [2.1.1.45], uridine (Urd) kinase [2.7.1.21], and DNA polymerase [2.7.7.7] [3,8,11,15]. But the activities of these enzymes are also high in certain rapidly growing normal tissues, such as bone marrow, thymus and spleen, and this limits the use of their antimetabolites as antitumor agents. We became interested in the enzymes that are rate limiting in DNA synthesis and that increase specifically in tumor cells, and also in the main metabolic pathways of pyrimidine nucleotides in normal tissues and tumor cells.

MATERIALS AND METHODS

Animals Donryu and Wister-King strain rats weighing ca. 120 g and ICR male mice weighing 19-25 g were purchased from Kitayama LABES Co., Kyoto, Japan.

Tumor Cells Yoshida sarcoma cells, AH-44 tumor cells, AH-109A tumor cells, and AH-130 tumor cells were maintained by intraperitoneal transfer in Donryu strain rats. They were collected 7 days (10 days in the case of AH-130 tumor cells) after inoculation, and were washed three times with saline before experiments. Sarcoma-180 cells were passaged in ICR mice by intraperitoneal inoculation at weekly intervals and were used for experiments after washing as described above.

Chemicals ^{14}C-labeled or ^{3}H-labeled nucleotides, nucleosides, and bases were purchased from the Japan Radioisotope Association, Tokyo, Japan. All other reagents were purchased from Sigma Chemical Co., U.S.A.

Preparation of Tissues Wistar-King strain rats were decapitated, and their liver was perfused with saline (10 ml) and rapidly removed together with other tissues. These tissues were stored at -20° until use.

Preparation of Orotate Phosphoribosyltransferase, CTP Synthetase, and Urd, dUrd, Cyd, dCyd, dThd, and dTMP kinases All procedures were carried out at 4°. The tissues were homog-

enized with 4 volumes of 50 mM Tris-HCl buffer (pH 8.0) containing 5 mM 2-mercaptoethanol. The homogenates were centrifuged (10,000 x g, 20 min) and the supernatants were used as enzyme solutions.

Preparation of dCMP Deaminase Tissues were homogenized with 4 volumes of 50 mM potassium phosphate buffer (pH 7.0) containing 1.0 mM dithiothreitol (DTT) and 0.5 mM dCTP, and centrifuged at 105,000 x g for 60 min and the supernatants were used as enzyme solutions.

Preparations of Ribonucleoside Diphosphate Reductase and Thymidylate Synthase Tissues were homogenized with 4 volumes of 50 mM potassium phosphate buffer (pH 7.0) containing 25 mM KCl and 5 mM $MgCl_2$ (ribonucleoside diphosphate reductase) or of 50 mM Tris-HCl buffer (pH 8.0) containing 5 mM 2-mercaptoethanol and 1 mM EDTA, (dTMP synthase). The homogenates were centrifuged at 105,000 x g for 60 min, and the supernatants were treated with streptomycin sulfate at 1% final concentration, and centrifuged at 8,000 x g for 20 min. The resulting supernatants were fractionated with 0-35% ammonium sulfate (ribonucleoside diphosphate reductase) or 35-65% ammonium sulfate (dTMP synthase). The pellets obtained by centrifugation at 8,000 x g were dissolved in the homogenizing buffer at one tenth of the volume of the initial homogenates, dialyzed against the same buffer for 12-18 hr, and used for

enzymatic assay.

Preparations of Urd, dUrd, Cyd, dCyd, and dThd Phosphorylases, Cyd and dCyd Deaminases, and DNA Polymerase. Tissues were homogenized with 4 volumes of 0.25 M sucrose containing 5 mM 2-mercaptoethanol and 0.5 mM EDTA, and centrifuged (10,000 x g, 30 min). The supernatants were dialyzed against the homogenizing buffer for 12-18 hr, coagulated protein was removed by centrifugation (10,000 x g, 10 min), and the supernatants were used as enzyme solutions.

Assay of Orotate Phosphoribosyltransferase The activity of orotate phosphoribosyltransferase was measured by the procedure involving PEI-cellulose thin-layer chromatography described by Reyes[12]. The reaction mixture in a final volume of 50 µl consisted of 0.02 M Tris-HCl buffer (pH 8.0), 2 mM magnesium chloride ($MgCl_2$), 1.6 mM 5-phospho-α-D-ribosyl diphosphate (PPRibP), 4 mM sodium fluoride (NaF), 0.05 mM [5-^{3}H] orotic acid (0.31 µCi/tube) and enzyme solution (20 µl). The reaction was started by adding the substrate.

After incubation for 15 (or 5) min at 37°, the reaction mixture was heated in a boiling water bath for 2 min and centrifuged (3,000 rpm, 10 min). The 5 µl of the supernatant was spotted on a 3 x 10 cm PEI-cellulose TLC plate (Merck TLC plates, PEI-cellulose F pre-coated) and promptly developed to the top with methanol. The plate was then dried with cool

air and developed again with a mixture of saturated boric acid (pH 7.0) and 1 M lithium chloride (LiCl) (2:1, v/v). Samples of orotate, UMP, and OMP were applied to the plate before the test sample and spots were located under UV light (254 nm). The area above the spot of orotate was regarded as the location of uridine and uracil. The spots of each substrate were scraped into vials and extracted with 1 M HCl (0.5 ml). Then 10 ml of scintillator [toluene-Triton x-100 system, containing POPOP (0.1 g/liter) and PPO (4.0 g/liter) in toluene] was added and radioactivity was measured in a Beckmann liquid scintillation spectrometer. The activity of orotate phosphoribosyltransferase was calculated from the sum of the amounts of OMP, UMP, uridine and uracil formed.

Assay of CTP Synthetase The assay conditions were essentially as described by Williams et al.[16] The reaction mixture in total volume of 0.20 ml consisted of 70 mM glycylglycine buffer (pH 7.4), 18 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 10 mM L-glutamine, 8 mM ATP, 1 mM GTP, 8 mM phosphoenolpyruvate, 10 mM NaF, 0.2 mM [4-^{14}C] UTP (0.04 μCi/tube) and enzyme solution (0.1 ml). After incubation at 37° for 30 min, the reaction was terminated by heating the mixture in a boiling water bath for 3 min. The amount of CTP formed was determined by converting UTP and CTP to their corresponding nucleosides, i.e., Urd and Cyd, by adding 50 μl of apyrase (20 mg/ml) and

50 µl of alkaline phosphatase (20 mg/ml), and separating the nucleosides on a column of Dowex 50W x 8 (H^+form, 0.5 x 3 cm): ^{14}C-Urd was eluted with 1 mM HCl (5 ml) and ^{14}C-Cyd with 3 M lithium hydroxide (LiOH, 3 ml). Then 1 ml of the eluate with 3 M LiOH was placed in a vial, mixed with the scintillator (10 ml) and 60% perchloric acid (0.3 ml) and kept at room temperature for at least 1 hr, and then the radio activity was counted in a scintillation counter.

Assay of dCMP Deaminase dCMP deaminase was assayed by a slight modification of the method of Maley and Maley[9]. The reaction mixture in a final volume of 0.25 ml consisted of 20 mM potassium phosphate buffer (pH 7.0), 2 mM $MgCl_2$, 20 mM NaF, 0.05 mM dCTP, 2 mM [5-^{3}H]dCMP (1 µCi/tube) and the enzyme solution (0.20 ml). After incubation for 30 min at 37°, the reaction was terminated by heating the mixture in a boiling water bath for 3 min. Then 50 µl of apyrase solution (20 mg/ml) and 50 µl of alkaline phosphatase solution (20 mg/ml) were added and the mixtures were incubated further for 60 min at 37°. Then the reaction was stopped by heating and the mixture was centrifuged at 3,000 rpm for 10 min. The dUrd and Ura in the supernatant (0.2 ml) were separated from dCyd on a column of Dowex 50 x 8 [H^+form] (0.5 x 3 cm) eluted with 0.1 M HCl (2 ml). A sample of 1 ml of the eluate containing dUrd and Ura was mixed with scintillator (10 ml) and

its radioactivity was counted as described above.

Assay of Ribonucleoside Diphosphate Reductase Assay of ribonucleoside diphosphate reductase activity was based on the principle described by Steeper and Steuart[14]. The reaction mixture, consisting of 10 mM DTT, 4 mM ATP, 1.3 mM $FeCl_3$, 5 mM NaF, 3 mM magnesium acetate, 50 mM potassium phosphate buffer (pH 7.0), 0.125 mM [(U)-^{14}C] CDP (0.4 μCi/tube) and 0.1 ml of enzyme solution in a total volume of 0.3 ml, was incubated at 37° for 60 min. The reaction was terminated by heating the mixture in a boiling water bath for 3 min. Then 50 μl each of 4 mM dCyd, apyrase solution (20 mg/ml) and alkaline phosphatase solution (20 mg/ml) were added successively and incubation was continued for 60 min. The solution was then boiled at 100° for 3 min and centrifuged at 3,000 rpm for 10 min. A sample of 250 μl of the supernatant was loaded onto a 0.5 x 3 cm Dowex 1 x 4 (borate form) column. [^{14}C]dCyd was eluted with 3 ml of water, and the radioactivity of 1 ml of the eluate was counted as described above.

Assay of Thymidylate Synthase The assay method was essentially as described by Dunlap et al.[2]. The assay mixture in a total volume of 0.5 ml consisted of 0.1 M potassium phosphate buffer (pH 7.0), 0.2 mM dl,L-5,10-methylenetetrahydrofolate, 5 mM NaF, 5 mM sodium hydrogen carboxide, 7 mM formaldehyde, 25 mM 2-mercaptoethanol, 0.1 mM [5-^{3}H]dUMP (0.16 μCi/tube) and

enzyme solution. A solution of dl,L-5,10-methylenetetrahydrofolate was prepared under a nitrogen atmosphere just before the assay. After incubation for 15 min at 37° under nitrogen, the reaction was terminated by adding 10% perchloric acid (0.1 ml). The mixture was centrifuged (3,000 rpm, 10 min), 0.2 ml of charcoal suspension in water (80 mg/ml) was added and the mixture was recentrifuged (3,000 rpm, 10 min). Then 0.2 ml of the supernatant containing released tritiated water was mixed with scintillator (10 ml) and its radioactivity was measured as described above.

Assay of dTMP Kinase The reaction mixture in a total volume of 0.25 ml consisted of 50 mM Tris-HCl buffer (pH 8.0), 5 mM ATP, 10 mM NaF, 10 mM $MgCl_2$, 40 μM [2-^{14}C]dTMP (0.05 μCi/tube) and 0.2 ml of enzyme solution. The mixture was incubated at 37° for 30 min, heated in a boiling water bath for 3 min and centrifuged at 3,000 rpm for 10 min. Then 100 μl of the supernatant was spotted on a DEAE-cellulose paper disc and washed successively with 20 mM HCl and ethanol. The paper was dried at 80° and the radioactivity remaining on the disc was counted as described above.

Assay of Urd, dUrd, dThd, Cyd and dCyd Kinase The reaction mixture in a total volume of 0.25 ml consisted of 50 mM Tris-HCl buffer (pH 8.0), 10 mM ATP, 10 mM NaF, 5 mM $MgCl_2$ (or 15 mM $MgCl_2$ for dCyd kinase), 0.6 mM ribonucleoside

([2-^{14}C]Urd and [5-^{3}H]Cyd) or 0.05 mM deoxyribonucleoside ([2-^{14}C]dUrd, [2-^{14}C]dThd, and [5-^{3}H]dCyd; 0.05 μCi/tube) and 0.05 ml of enzyme solution. The mixture was incubated at 37° for 5 or 15 min, heated in a boiling water bath for 3 min, and centrifuged at 3,000 rpm for 10 min. The 50 μl of the supernatant was applied to a PEI-cellulose TLC plate (3 x 10 cm) and developed with water. Phosphorylated compounds at the origin were scraped off, placed in a vial and extracted with 1 M HCl (0.5 ml) and their radioactivity was measured as described above.

Assay of Urd, dUrd, and dThd Phosphorylase The reaction mixture in a final volume of 0.25 ml consisted of 60 mM potassium phosphate buffer (pH 7.6), 0.6 mM [2-^{14}C] nucleosides (0.05 μCi/tube) and 0.1 ml of enzyme solution[17]. The mixture was incubated for 15 min, and then 2 M perchloric acid (50 μl) was added and the mixture was centrifuged (3,000 rpm, 10 min). Then 200 μl of the supernatant was added to 60 μl of 2 M KOH solution. The resulting precipitase ($KClO_4$) was removed by centrifugation (3,000 rpm, 10 min) and 10 μl of the supernatant was applied to a silica gel TLC plate (Merck TLC plates with silica gel 60F_{254} pre-coated; 3 x 10 cm; thickness, 0.25 nm) and developed with a mixture of chloroform, methanol and acetic acid (16:4:1, v/v/v). Samples of the bases and nucleosides were applied to the plate before the test sample and were

located by measuring the UV absorption at 254 nm. The spots of each substance were scraped into vials and extracted with 4 M HCl (50 µl), and this radioactivity was measured as described above.

Cyd and dCyd Deaminase The assay method was similar to that described for phosphorylases, except that the substrates were 5-^{3}H labelled and TLC plates were developed with a mixture of 99% ethanol and 1 M ammonium acetate (5:1, v/v).

Assay of DNA polymerase The reaction mixture in a final volume of 25 µl consisted of 50 mM Tris-HCl (pH 7.9), 6 mM magnesium acetate, 1mM DTT, 16% glycerol, calf serum albumin (10 µg), calf thymus activated DNA (10 µg), 0.1 mM dATP, 0.1 mM dCTP, 0.1 mM dGTP, [Methyl-^{3}H] dTTP (5 µCi/tube) and enzyme solution[10]. After 30 min incubation at 37°, the reaction was stopped by cooling in an ice bath, and the whole reaction mixture was applied to Whatman DEAE-cellulose (DE81) filter paper discs. Free dNTPs were washed off with 5% aqueous dipotassium hydrogen phosphate and the radioactivity remaining on the paper was counted in a toluene scintillator system as described above.

RESULTS

The activities of many enzymes, included those for pyrimidine nucleotide synthesis, are reported to increase in parallel with the cell growth rate[3,8,11,15]. We tried to find an enzyme that increases specifically in tumor cells but does not increase in normal tissues, even in rapid proliferating tissues, such as bone marrow, thymus, and spleen.

Enzymes Concerned in de novo Pyrimidine Biosynthesis

First the activities of various enzymes concerned in de novo pyrimidine nucleotide synthesis were measured. The activity of orotate phosphoribosyltransferase [2.4.2.10], the first enzyme to the de novo synthetic pathway of pyrimidine nucleotides, was similar in tumor cells and normal tissues. All the tumor cells had high activity, but thymus, liver, and testis had as much activity as tumor cells, as shown in Chart 1. CTP synthetase [6.3.4.2] was also not localized specifically in tumor cells, as shown in Chart 2.

The results in Chart 3 show that ribonucleoside diphosphate reductase activity was fairly specifically localized in tumor cells. Even in AH-44 tumor cells, which showed the lowest activity in all the tumor cells tested, the activity was 3 times that in bone marrow, which had the highest activity of the normal tissues examined. dCMP deaminase was not so specifically localized in tumor cells, as shown in Chart 4. But its activity was much higher in rapidly growing cells (tumor

cells, bone marrow and thymus) than in other normal tissues. Thus the activity of this enzyme shows a good correlation with the growth rate of tissues, but was not specific to tumor cells. Chart 5 shows that the activity of thymidylate synthase, which is often used as target in cancer chemotherapy as it is the only enzyme in the de novo synthetic pathway of dTMP, is not correlated with cell growth; perhaps the activity of this enzyme is controlled by the concentration of its substrate, dUMP[4]. In contrast, Chart 6 shows the dTMP kinase is very specific for tumor cells.

Enzymes Concerned in Salvage Pyrimidine Nucleotide Synthesis Next we examined the activities of enzymes involved in the salvage pathway of pyrimidine nucleotides, which utilizes nucleosides and bases. The activity of thymidine kinase, the most important enzyme in the salvage pathway for direct dTMP synthesis, was measured. The activity was high in rapid growing cells, but was not specific to tumor cells; bone marrow had higher activity than all the tumor tested except Yoshida sarcoma cells (Chart 7). The distribution of the activity of dUrd kinase, which is reported to be catalyzed by dThd kinase[1,5], was similar to that of dThd kinase, reconfirming a previous report (Chart 8). dCyd kinase activity had little specificity for tumor cells, as shown in Chart 9. Urd-Cyd kinase, which is reported to catalyze the phosphorylation of both Urd and Cyd[1,7], showed similar tissue distribution with the two substrates, and its activity was high in rapid growing cells,

but not specific to tumor cells (Charts 10, 11).

Next the activities of phosphorylases were measured with Urd, dUrd, and dThd as substrates. Charts 12, 13 and 14 shows that the tissue distributions of Urd, dUrd, and dThd phosphorylases were similar and were not correlated with the cell growth rate. There was no detectable Cyd or dCyd phosphorylase activity in any of rat tissues tested, but Cyd and dCyd were deaminated to Urd and dUrd, respectively, by Cyd deaminase, whose activity was restricted to the intestine, as shown in Charts 15 and 16.

Lastly the activity of DNA polymerase, which catalyzes the final step of DNA-synthesis, was examined. Chart 17 shows that the activity in all tumor cells tested was considerably higher than that in bone marrow, which showed the highest activity of the normal tissues examined.

As summarized in Chart 18, the activities of DNA polymerase, ribonucleoside diphosphate reductase, and dTMP kinase increased specifically in tumor cells; the activity of dCMP deaminase, and all the nucleoside kinase was high not only in tumor cells, but also in rapid growing normal tissues. The activities of the other enzymes tested were not related to cell proliferation. These results show clearly which metabolic pathway of DNA synthesis is increased in tumor cells. AH-130 tumor cells have high activities of the enzymes involved in de novo pyrimidine deoxyribonucleotide synthesis, i.e., CTP synthetase, ribonucleoside diphosphate reductase, and dCMP deaminase (Charts 2, 3, and 4),

but low activity of dThd-dUrd kinase involved in the salvage pathway (Charts 6 and 7). In contrast, Yoshida sarcoma cells have increased activity of dThd-dUrd kinase, but low activities of other enzymes involved in the de novo pathway. Among rapidly growing normal tissues, bone marrow, like Yohida sarcoma cells, has high activity of enzymes involved in the salvage pathway, while thymus, like AH-130 tumor cells, has enhanced activity of enzymes in the de novo pathway.

DISCUSSION

Weber showed that the activities of certain enzymes involved in DNA synthesis increase in parallel with the growth rate of Morris hepatoma cells, and suggested that they could be used as targets in cancer chemotherapy[15]. In this work, we found that the activities of ribonucleoside diphosphate reductase, dTMP kinase, and DNA polymerase were much higher in tumor cells than in normal tissues of rodents. In contrast, the activities of dCMP deaminase, Urd-Cyd kinase, and dThd-dUrd kinase were high in all rapidly growing cells. Although Weber also showed that the activities of these enzymes were related to the growth rate, they were not specific to tumor cells. These findings suggest that use of antimetabolites of these enzymes in chemotherapy would have severe side effects. The tissue distribution of other enzymes, including orotate phosphoribosyltransferase, CTP synthetase, dTMP synthase, phosphorylases and Cyd deaminase had no relation with the cell growth rate. Although Weber[15] or Sköld[13] showed that the activity of uridine phosphorylase was high in rapidly growing cells, we observed liver, intestine, and lung had higher activity than all the tumor cells examined.

Among the rapidly growing cells examined, several, such as AH-130 tumor cells and thymus, showed specific increase in the activities of enzymes involved in de novo DNA synthesis. In contrast, Yoshida sarcoma cells and bone marrow cells showed

high activity of enzymes in the salvage pathway of DNA-synthesis. This observation is important, because tumor cells have been thought to have increased activities of both de novo and salvage pathways. The high activity of the salvage pathway in bone marrow is reasonable, for there are large amounts of nucleosides and bases in the blood (Urd, Ura, Cyd, dCyd), which can serve as substrates (data not shown). These finding showed that each tumor cell have much different patterns of nucleotide metabolism.

Another important finding in this work was on the substrate specificities of several enzymes. Uridine and cytidine have been reported to be phosphorylated by a single enzyme[1,7]. Our study showed similar tissue distributions of Urd and Cyd phosphorylation with 5 times higher activity of Urd kinase than Cyd kinase, although Liacouras et al[7] reported that the V_{max} of the reaction with uridine was about twice that with cytidine. In the same way, dUrd was shown to be phosphorylated by dThd kinase as previously reported[5].

Two phosphorylases of pyrimidine nucleosides are found in rat liver[17]; uridine phosphorylase, which catalyzes the phosphorylis of Urd, dUrd, and dThd, and thymidine phosphorylase, catalyzing phosphorylis of dThd and dUrd. We observed similar tissue distributions of phosphorylase with the three substrates, the ratio of the activities with Urd, dUrd and dThd being 10:7:1. This ratio is consistent with that reported by Kraut and Yamada[6] for highly purified uridine phosphorylase

from rat liver. This suggests that in rat tissues and tumor cells, at least under the experimental condition used in our study, phosphorylis of dUrd and dThd is due mainly to uridine phosphorylase, rather than thymidine phosphorylase.

The present paper described the tissue distribution of enzymes involved in pyrimidine nucleotide synthesis in rodents. Since we measured the enzyme activities in crude extracts of tissues and thus the preparation might include various substances that affect the enzyme activity, the results obtained here do not show the exact enzyme levels. But the activities of enzymes in vivo may be nearer to the activities measured in crude extracts. The activity of ribonucleoside diphosphate reductase was clearly related to the growth rate, but this could be caused by the levels of inhibitors, which were reported by Elford and Cory, besides the level of an enzyme itself. In the next part, the regulation of the activity of ribonucleoside diphosphate reductase will be discussed.

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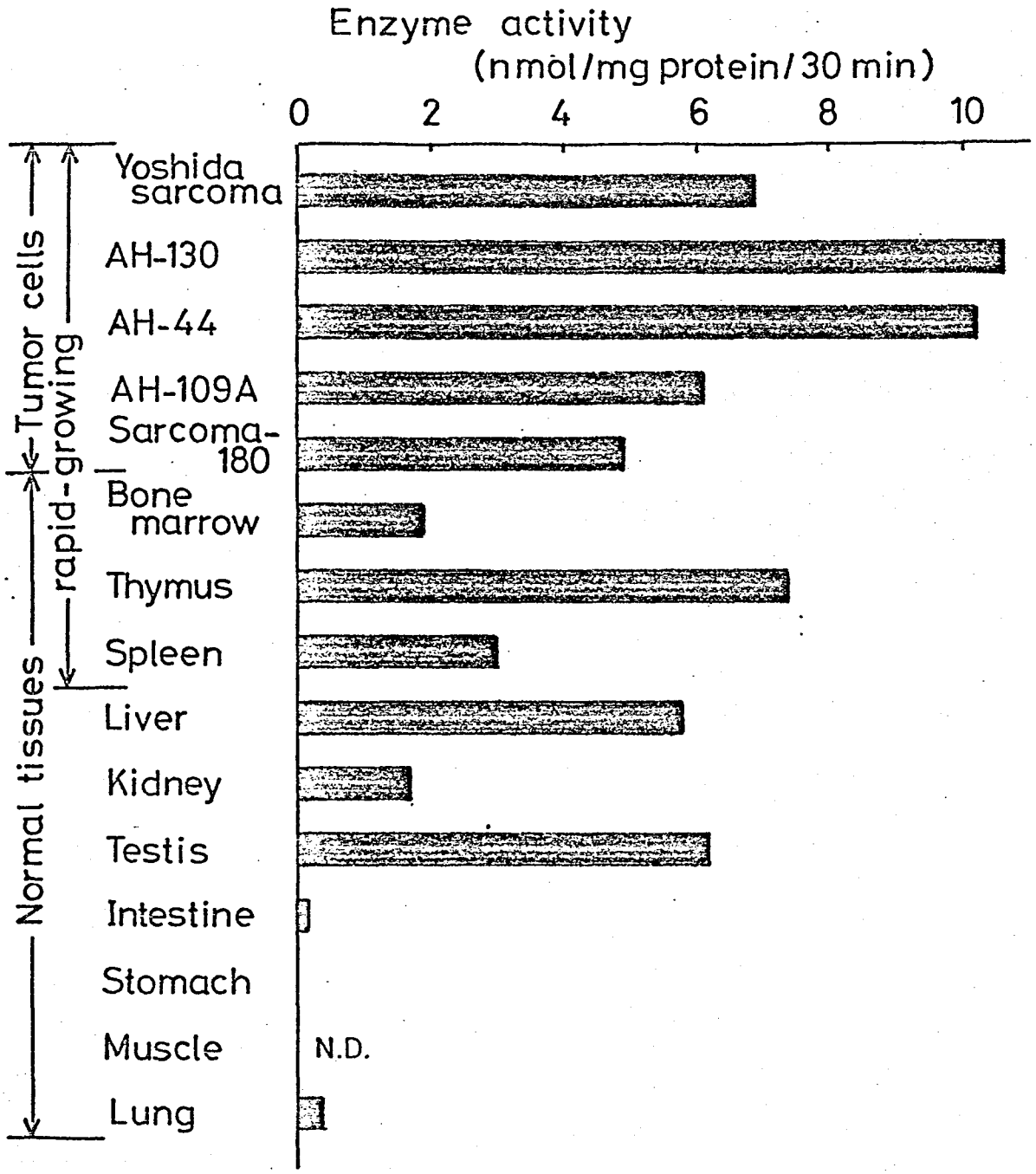

Chart 1.

Activity of orotate phosphoribosyltransferase in rat tissues and tumor cells from rodents.

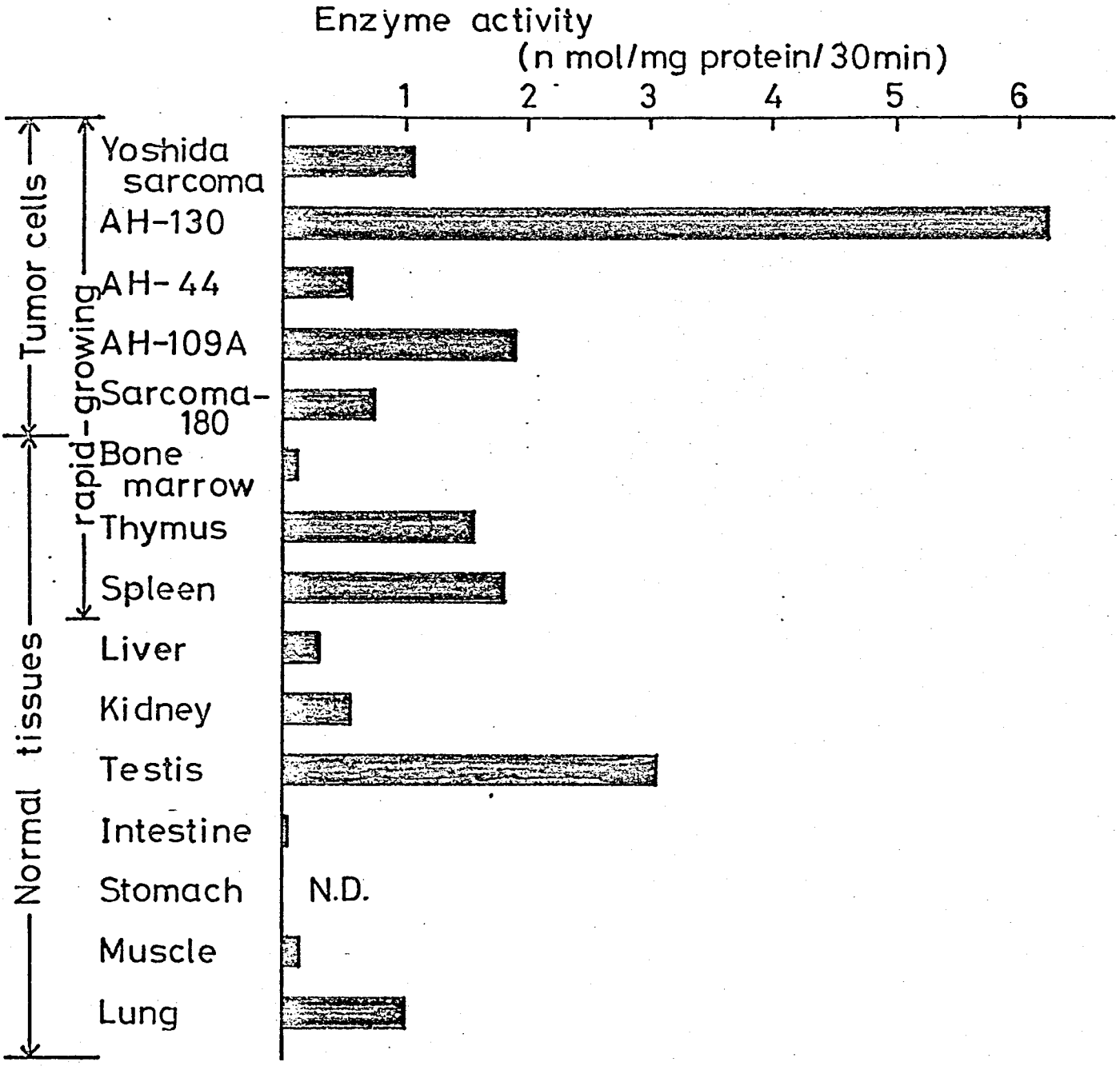

Chart 2.

Activity of CTP synthetase in rat tissues and tumor cells from rodents.

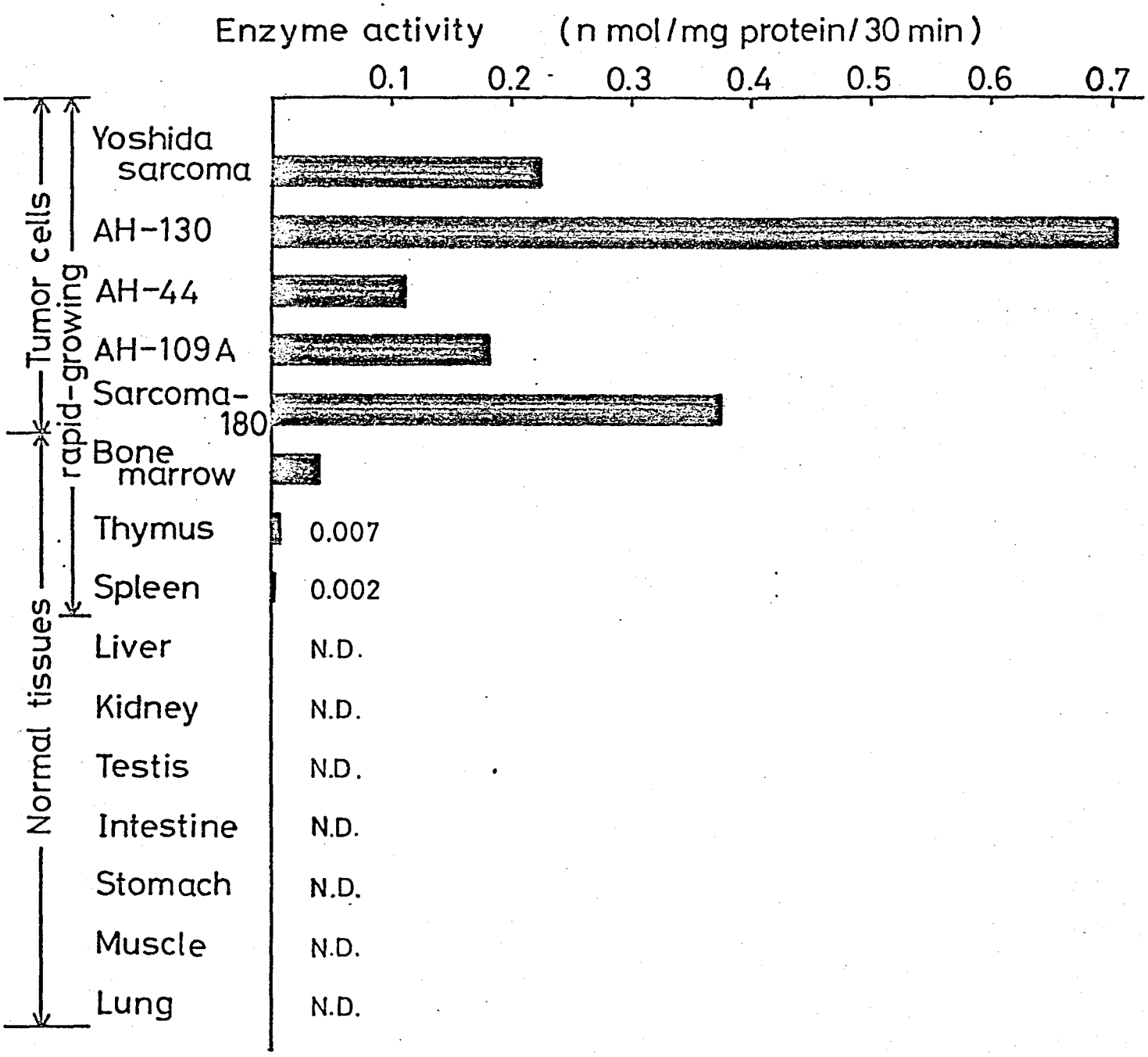

Chart 3.

Activity of ribonucleoside diphosphate reductase in rat tissues and tumor cells from rodents.

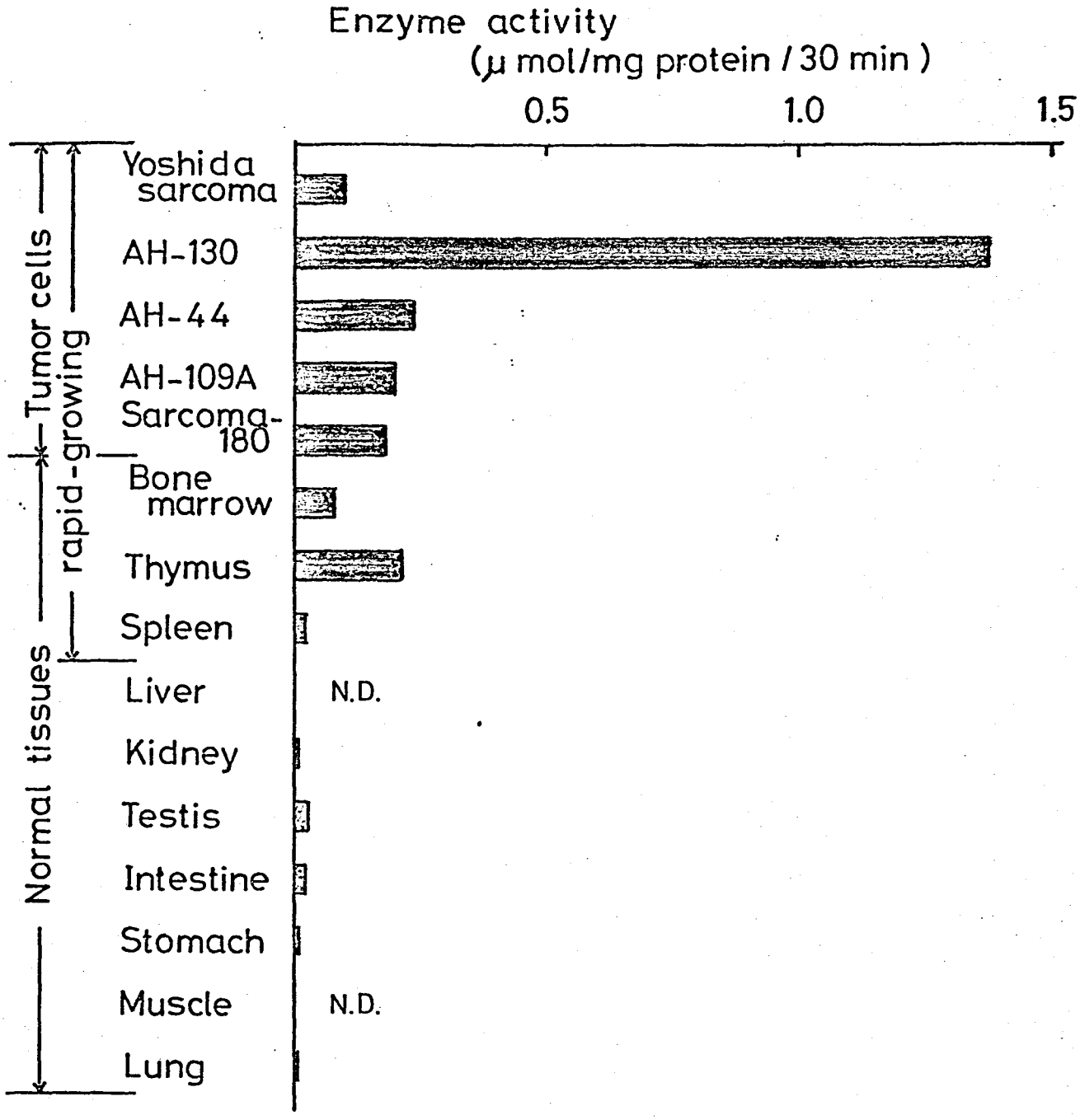

Chart 4.

Activity of dCMP deaminase in rat tissues and tumor cells from rodents.

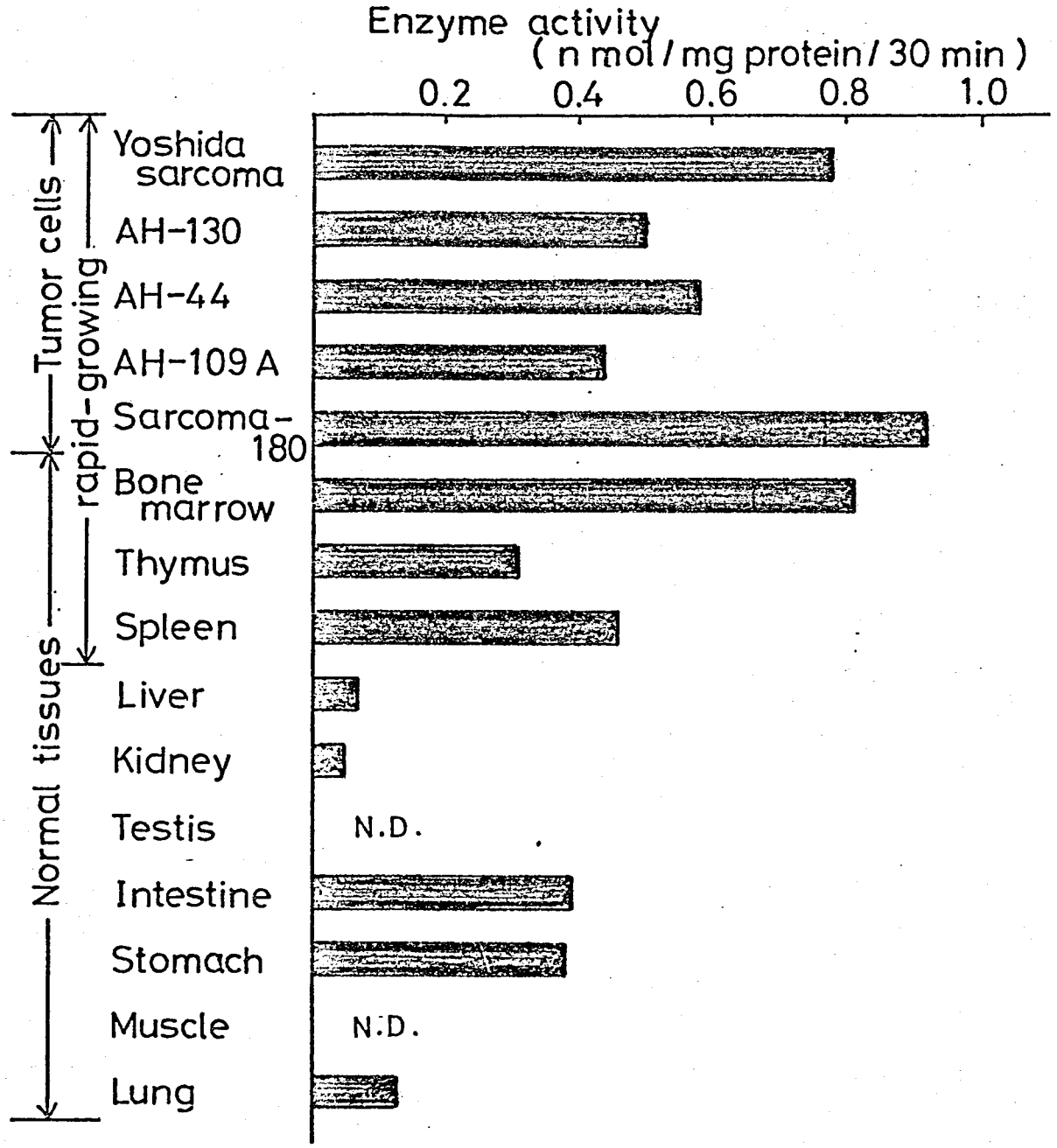

Chart 5.

Activity of dTMP synthase in rat tissues and tumor cells from rodents.

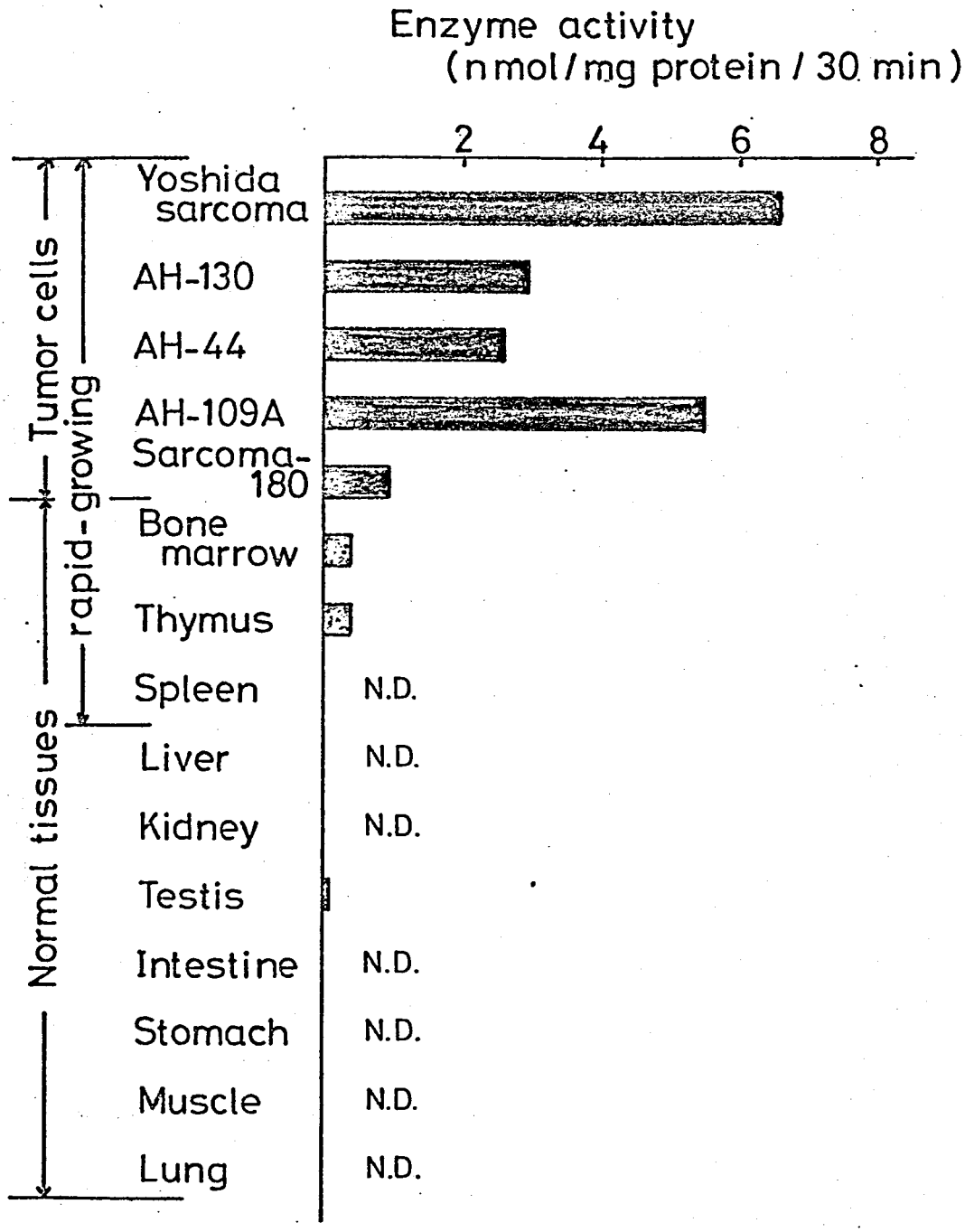

Chart 6.

Activity of dTMP kinase in rat tissues and tumor cells from rodents.

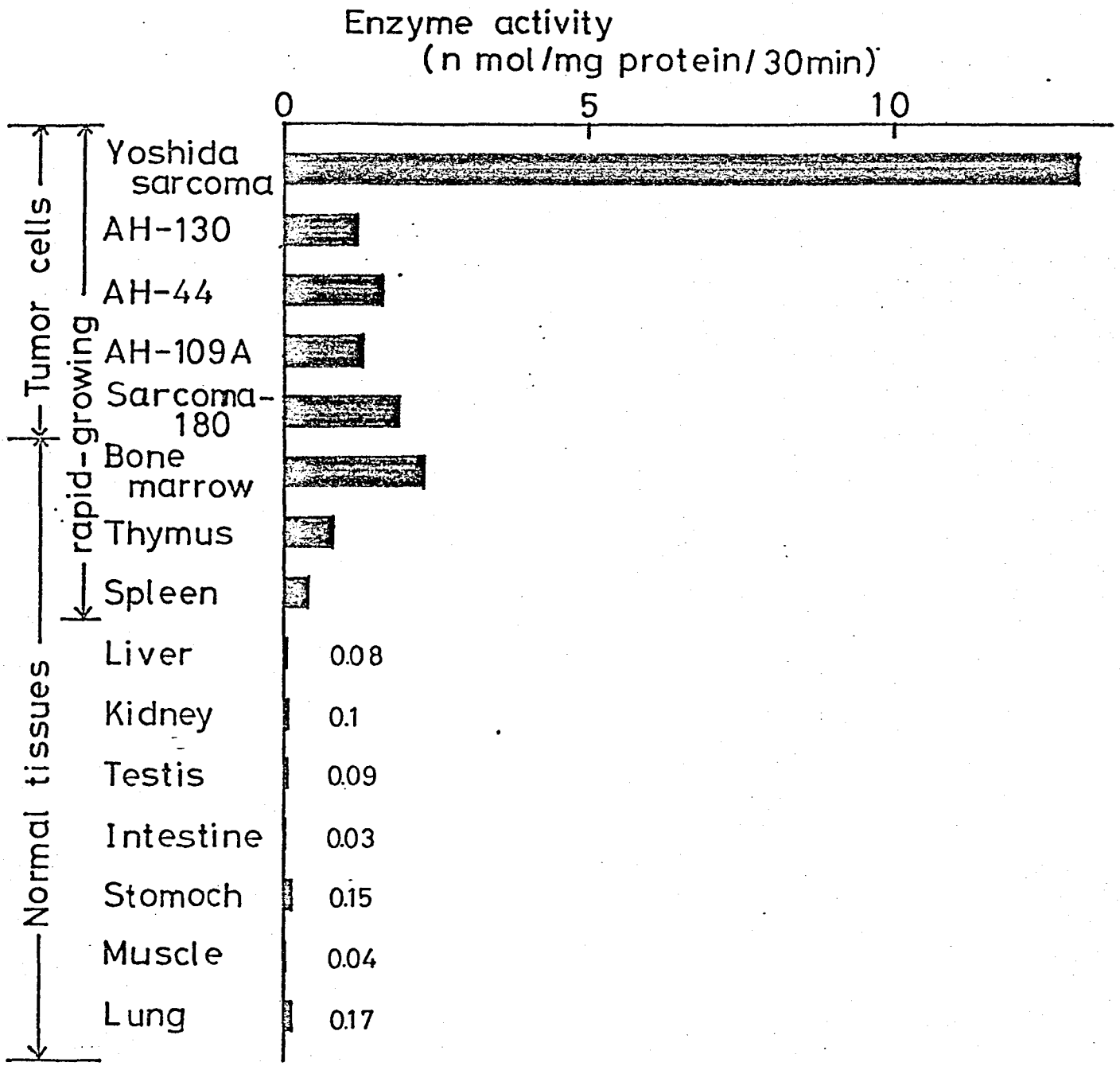

Chart 7.

Activity of dThd kinase in rat tissues and tumor cells from rodents.

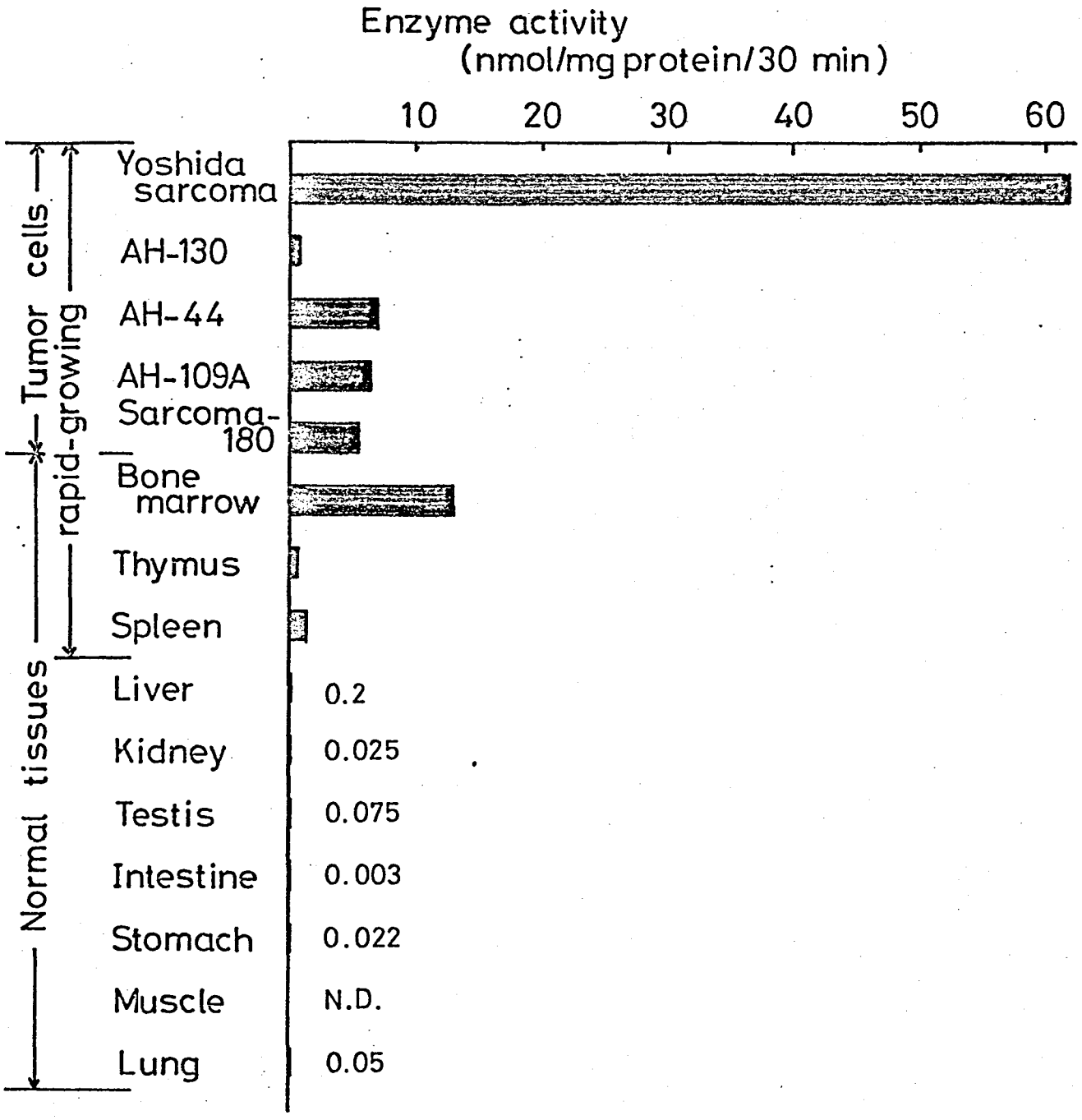

Chart 8.

Activity of dUrd kinase in rat tissues and tumor cells from rodents.

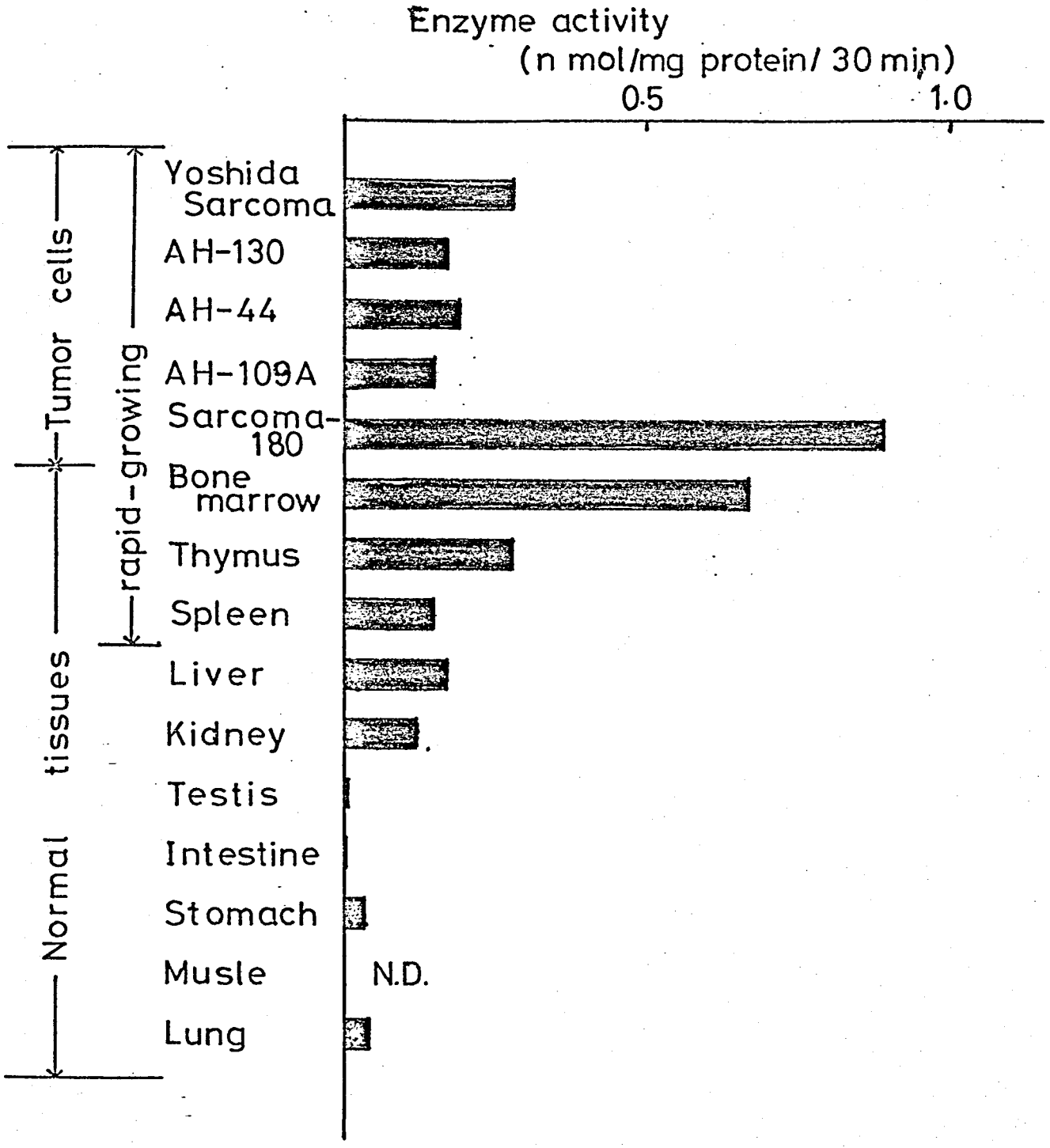

Chart 9.

Activity of dCyd kinase in rat tissues and tumor cells from rodents.

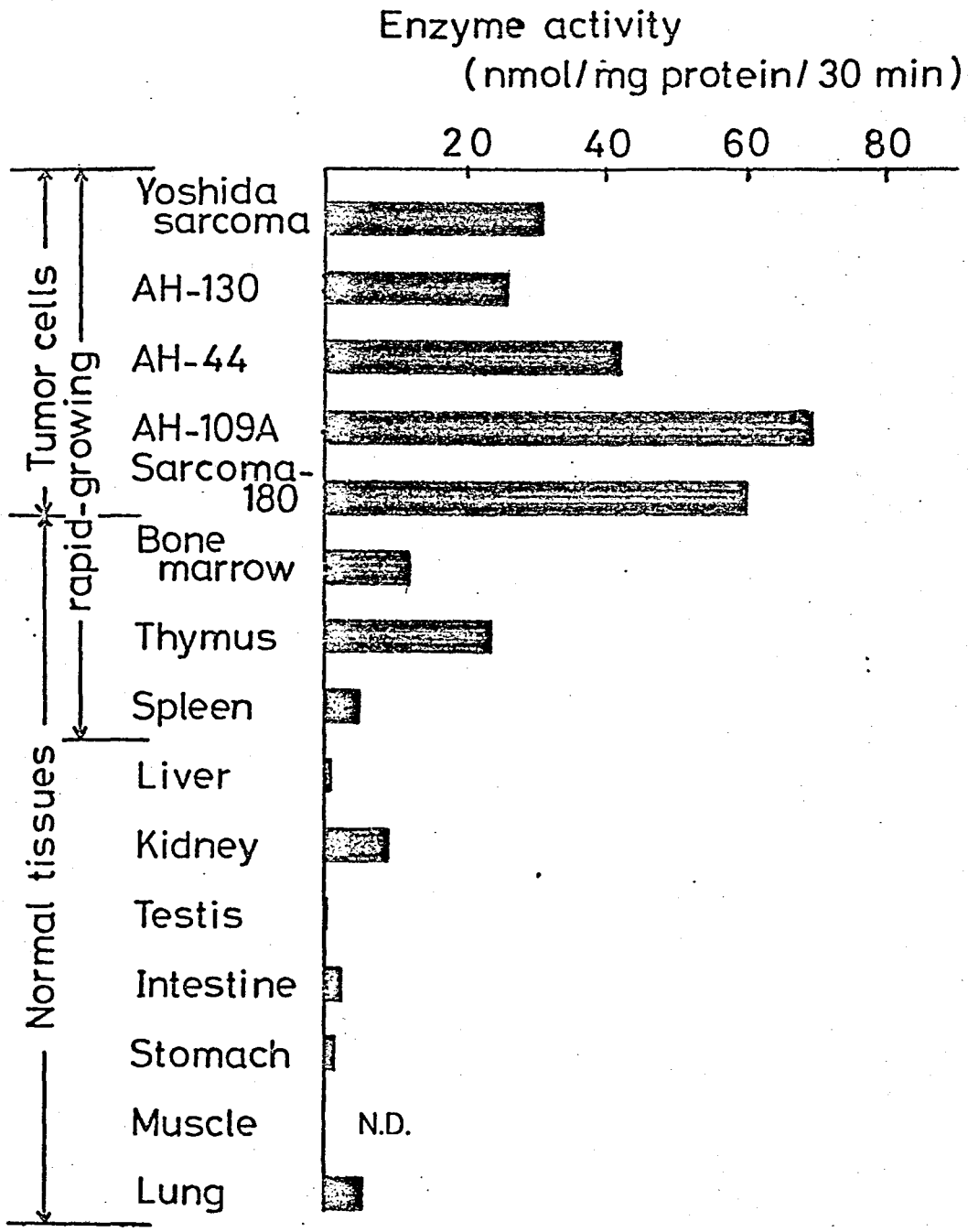

Chart 10.

Activity of Urd kinase in rat tissues and tumor cells from rodents.

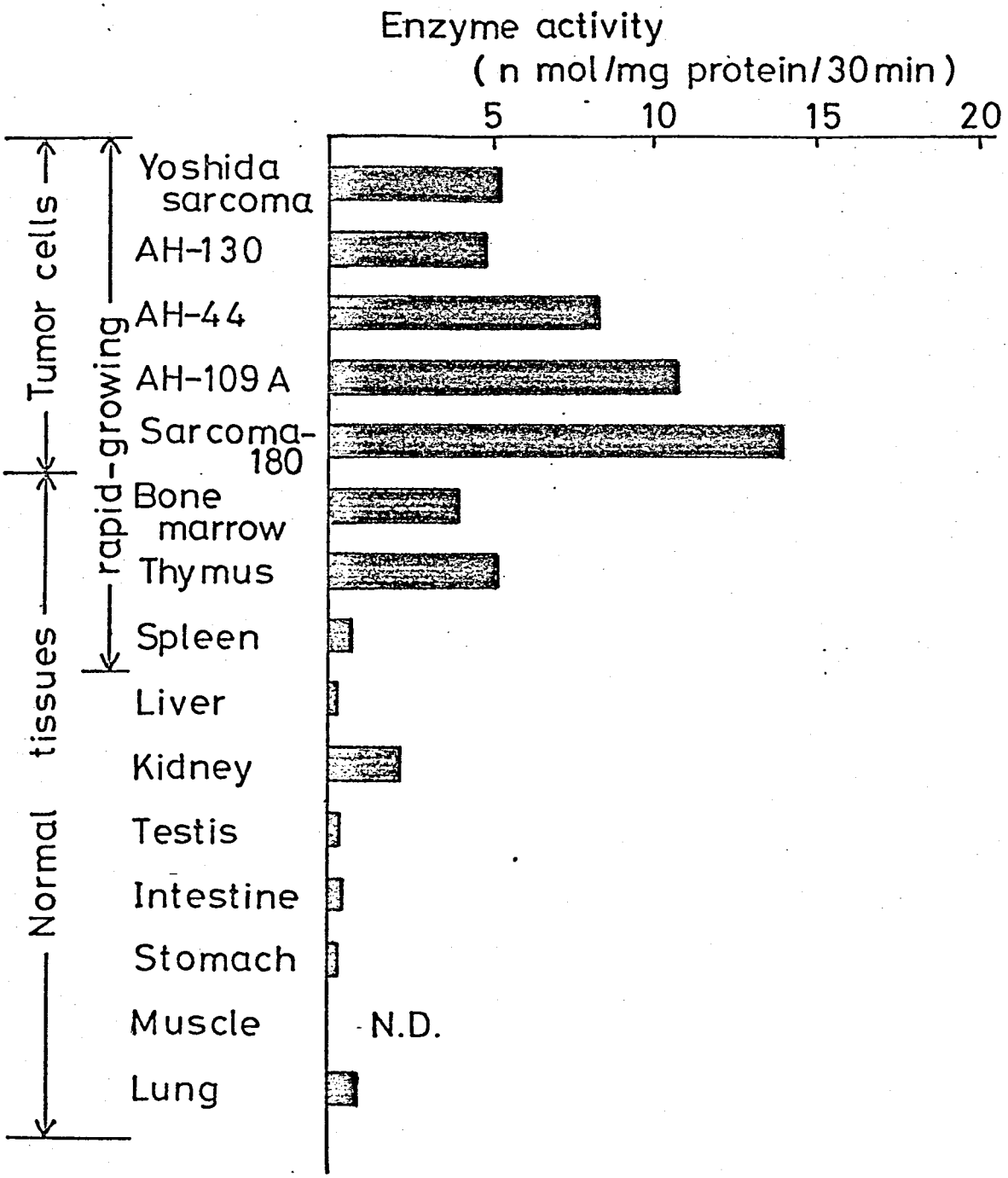

Chart 11.

Activity of Cyd kinase in rat tissues and tumor cells from rodents.

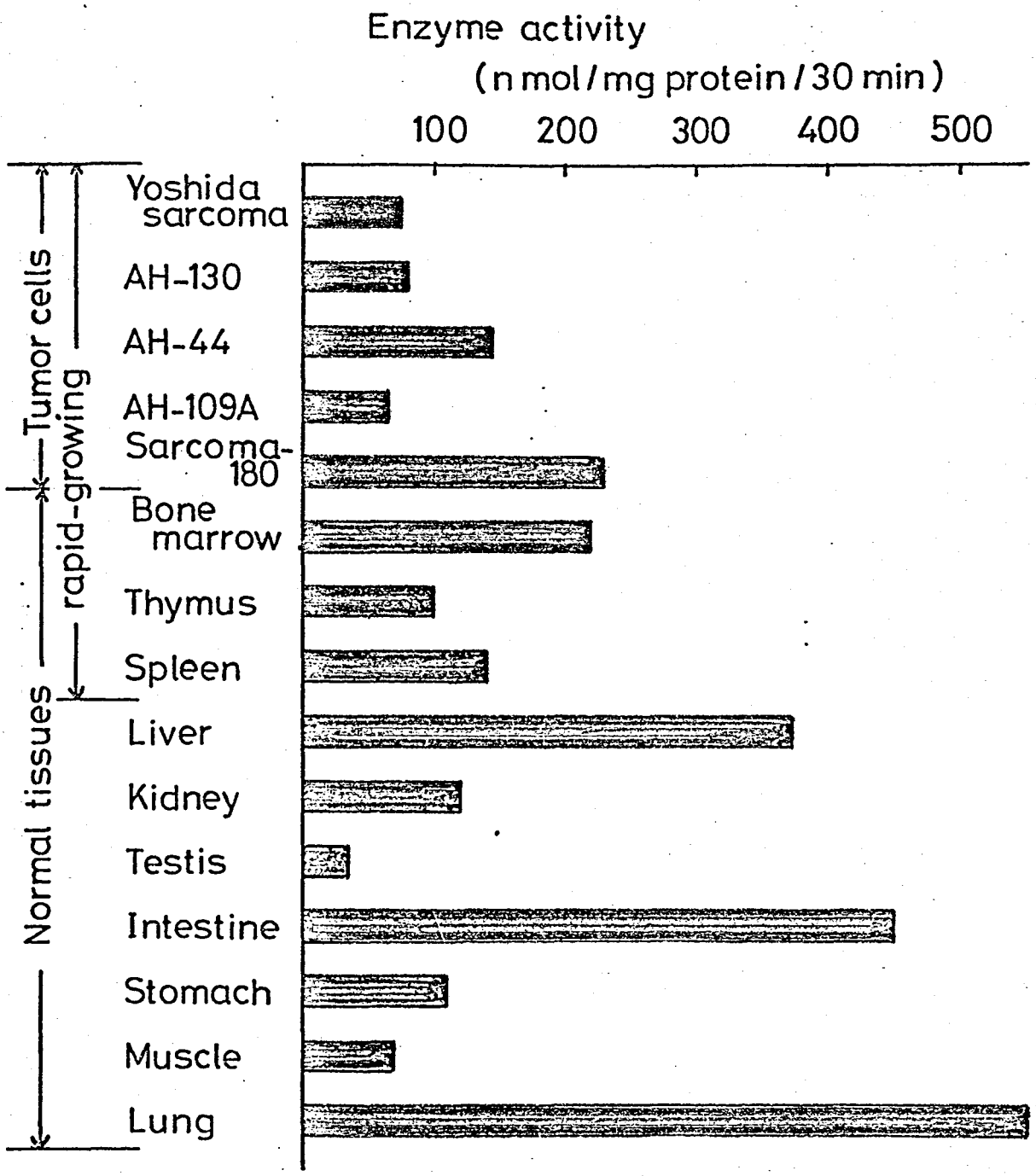

Chart 12.

Activity of Urd phosphorylase in rat tissues and tumor cells from rodents.

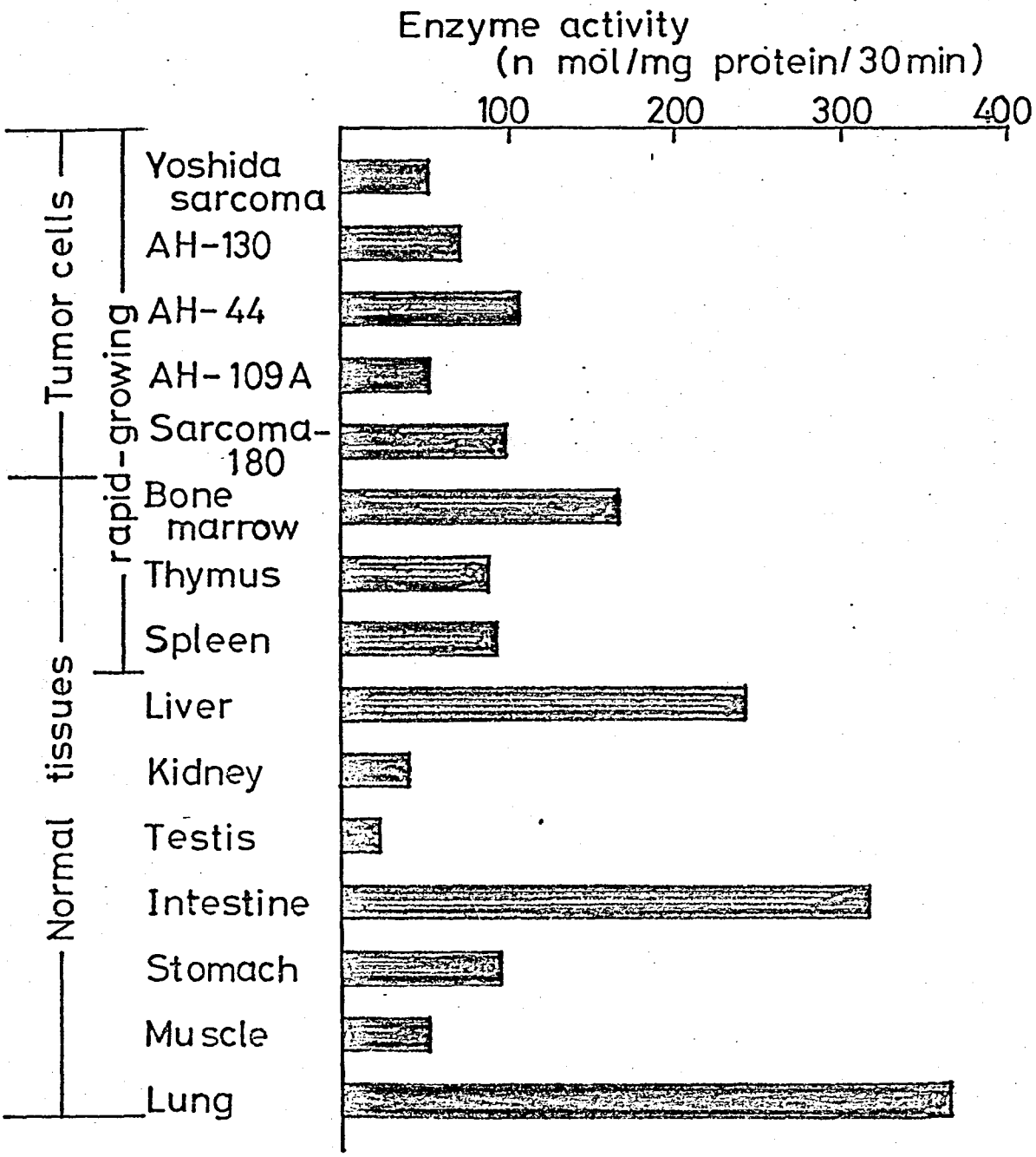

Chart 13.

Activity of dUrd phosphorylase in rat tissues and tumor cells from rodents.

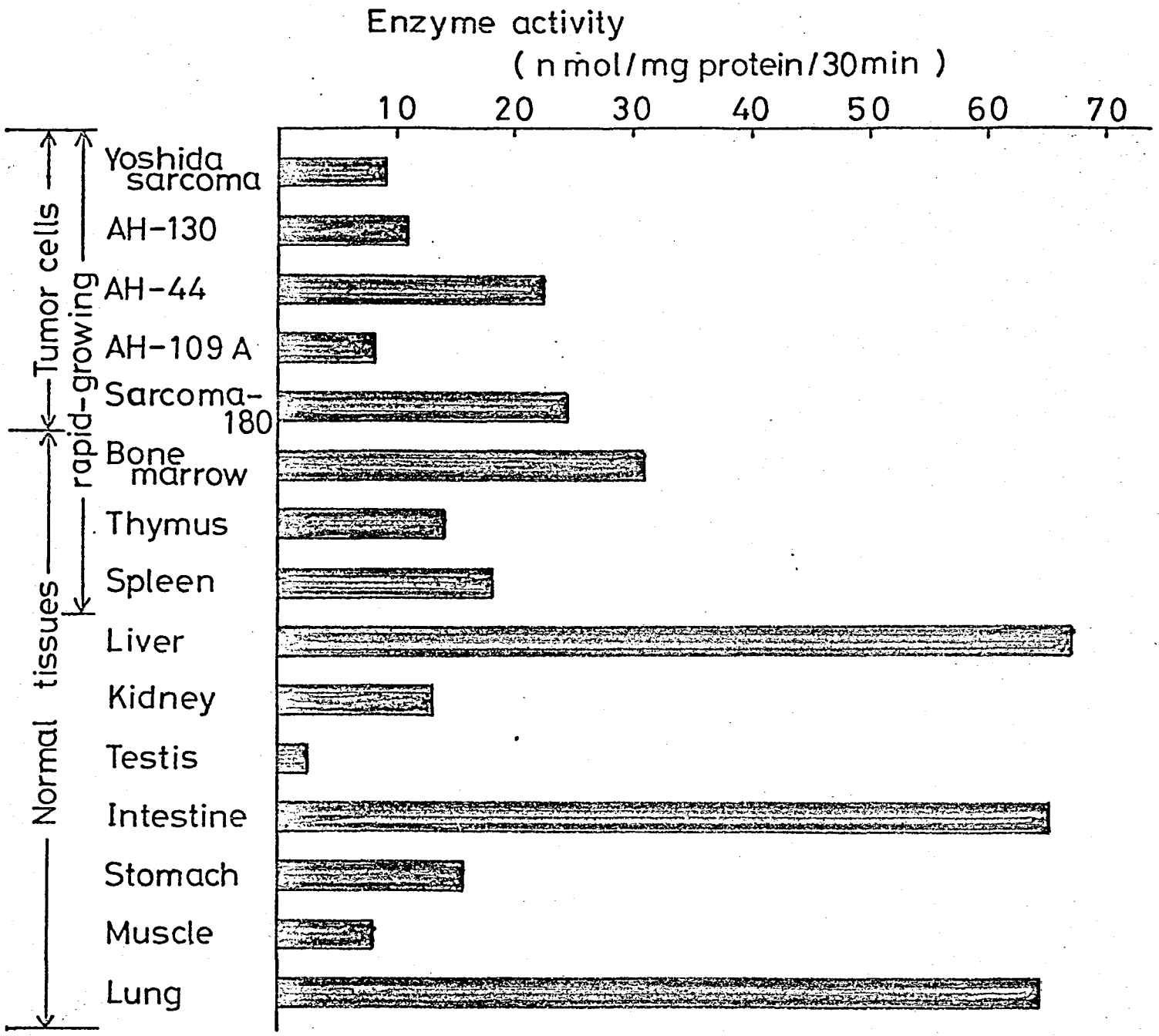

Chart 14.

Activity of dThd phosphorylase in rat tissues and tumor cells from rodents.

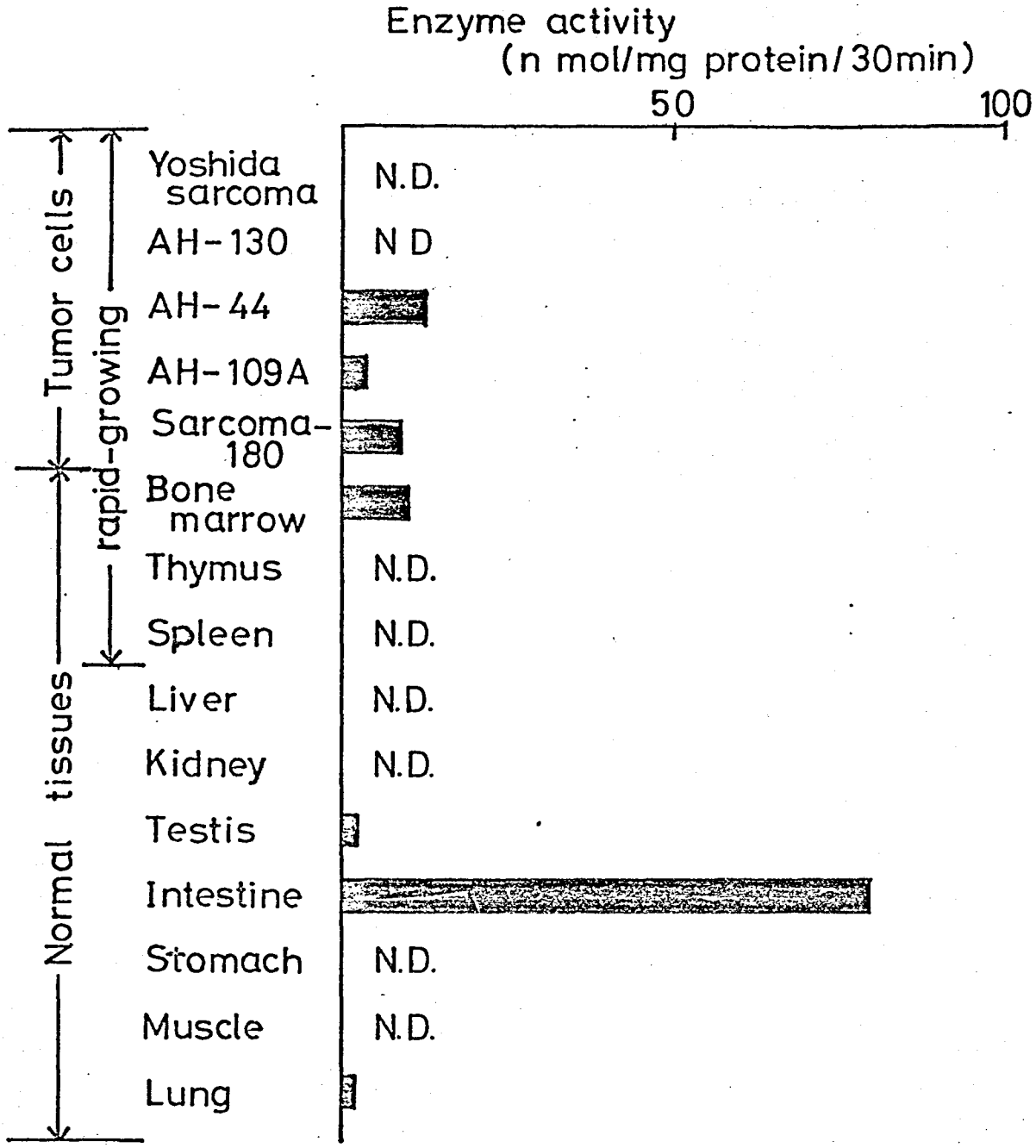

Chart 15.

Activity of Cyd deaminase in rat tissues and tumor cells from rodents.

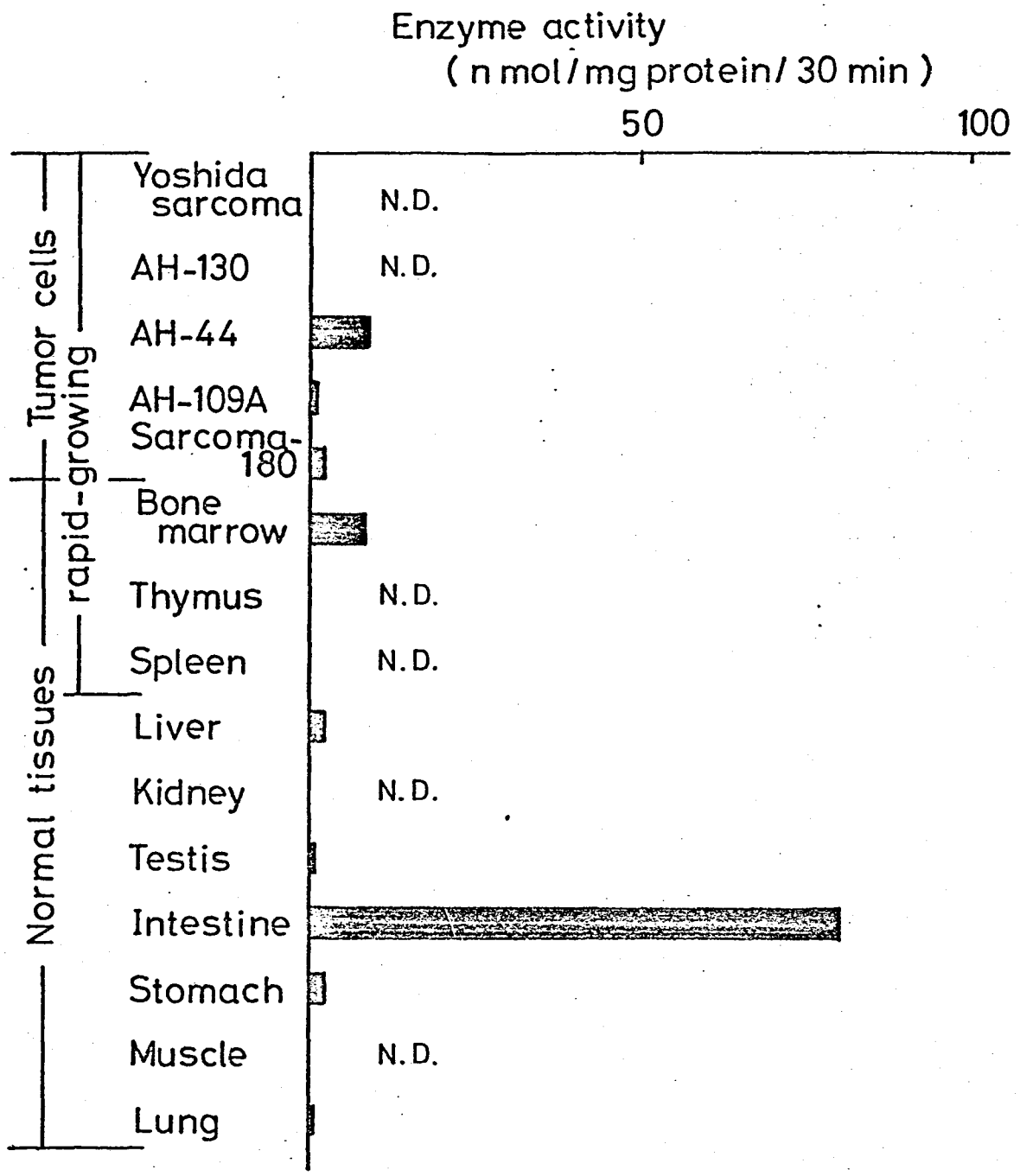

Chart 16.

Activity of dCyd deaminase in rat tissues and tumor cells from rodents.

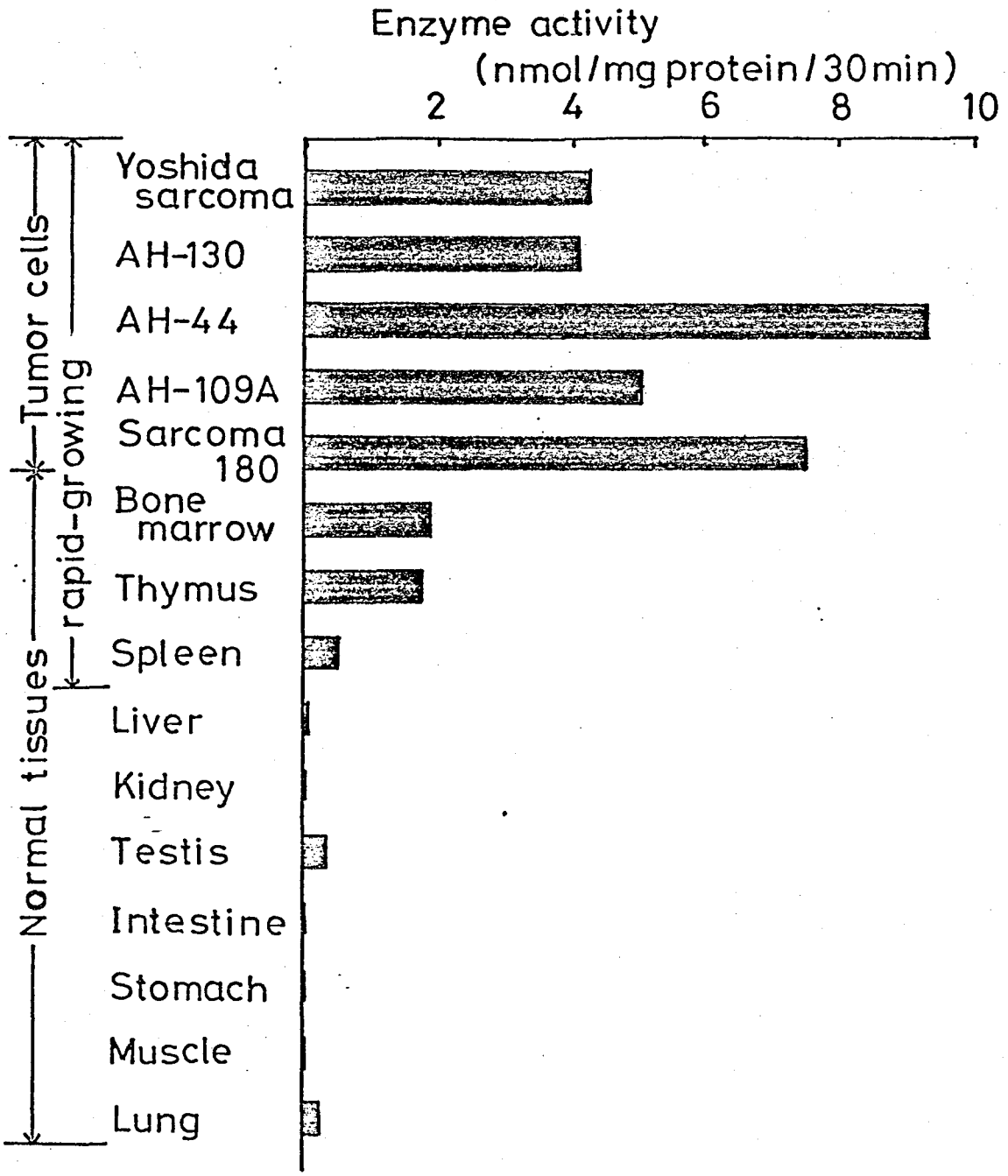

Chart 17.

Activity of DNA polymerase in rat tissues and tumor cells from rodents.

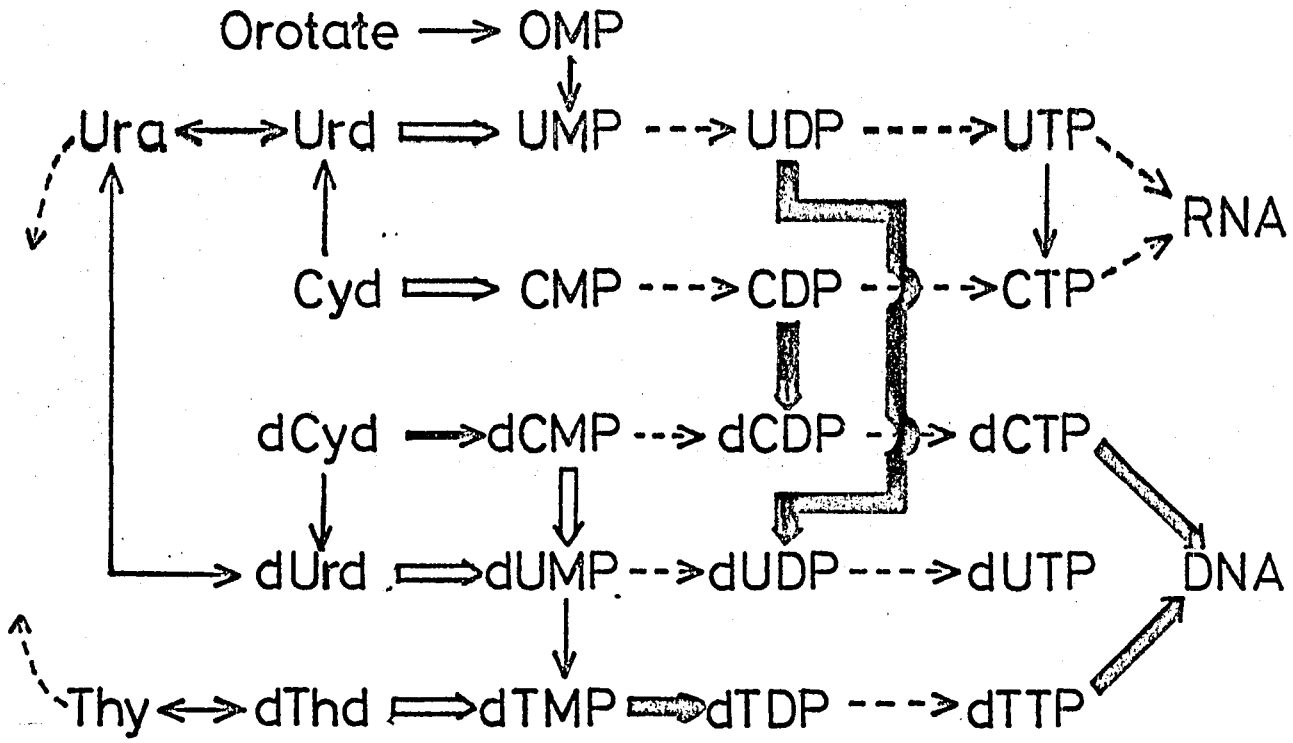

Chart 18.

Metabolic map of pyrimidine nucleotides. The activities of enzymes which have specificity to tumor cells are shown by wide black arrows (⟹); those which are propotional to the growth rate, but have no specificity to tumor cells are shown by open wide arrows (⟹); those which have no correlation with the growth rate, are shown by thin arrows (⟶). The activities which were not determined are shown by broken arrows (⇢).

Part 2

Possible Regulation of Ribonucleoside Diphosphate Reductase *in vivo* and *in vitro*

SUMMARY

Two inhibitors of ribonucleoside diphosphate reductase (RR) [1.17.4.1] *in vitro* were isolated from normal rat liver: a nondialysable and heat-labile, high molecular weight ribonucleoside diphosphate reductase inhibitor (HRRI), and a dialysable, heat-stable, low molecular weight ribonucleoside diphosphate reductase inhibitor (LRRI). The activities of both inhibitors varied inversely with the cell growth rate. HRRI from the cytosol fraction of rat liver was partially purified by ammonium sulfate fractionation (0-50%), and gel filtration on a Sepharose 6B column. It was eluted in the void volume from this column, with ATP hydrolyzing activity. The HRRI fraction also contained CDP kinase and CDPase activities, suggesting that HRRI is a complex of several enzymes that reduce the substrate of RR, CDP, and the allosteric activator, ATP.

LRRI was extracted from the cytosol of rat liver with ethanol (80% final concentration) and purified further by washing with organic solvent, and by chromatographies on Amberlite IR-45 and Dowex 50. Finally, it was shown to be glucose, which was phosphorylated to glucose-6-phosphate by hexokinase present in the RR enzyme solution (0-35% ammonium sulfate fraction of AH-130 cytosol), thus causing ATP depletion.

Thus neither inhibitor reacted directly with the RR enzyme, but both may regulate the enzyme activity *in vivo* by reducing the intracellular levels of substrates or cofactors.

INTRODUCTION

During neoplastic development and liver regeneration the activities of several enzymes involved in pyrimidine nucleotide synthesis increase in parallel with the cell growth rate[5,12,16,20]. This phenomenon could be the result of synthesis of new enzymes or elimination of inhibitors. In support of the latter possibility, the following reports indicate the existence of inhibitors in the microsomal fraction of normal liver: Fausto found that microsomal fractions from normal liver inhibit the conversion of orotic acid to UMP by the cytosol fraction of regenerating liver[6]; Fiala and Fiala reported the presence of an inhibitor of dCMP deaminase and dTMP kinase in the microsomal fraction of normal rat liver[7,8]; we found that the thymidine kinase and uridine kinase activities of regenerating liver were inhibited by the microsomal fraction of normal liver[1].

Ribonucleoside diphosphate reductase may be very important in regulation of DNA synthesis, since its activity is much lower than other DNA synthesising enzymes in mammalian cells[10] even in tumor cells and rapidly proliferating cells, and thus it is believed to be a ratelimiting step in DNA synthesis[5]. The activity of this enzyme is regulated by various nucleotide triphosphates, and the balance of the four deoxyribonucleotide triphosphate pools is thought to be maintained mainly by this

allosteric effect[2,13,14,17].

Elford[5] showed that the activity of RR varies in parallel with the growth rate in Morris hepatoma cell lines, and also suggested[4] that the cytosol fraction of normal rat liver contains a natural inhibitor of RR, which is nondialysable and heat-labile. The inhibitory activity was low in fetal liver and in regenerating liver, in which DNA synthesis is active, and thus it may regulate cell proliferation by inhibiting RR activity. Recently, Cory recomfirmed the existence of this inhibitor[3].

We[10] found that RR activiy is much higher in tumor cells than in normal tissues, including rapidly growing tissues, such as bone marrow cells, thymus and spleen. These findings led us to investigate the characters of this natural inhibitor, described by Elford, and to clarify the mechanism of its effect.

As reported in this paper, we obtained two fractions that inhibited ribonucleoside diphosphate reductase in vitro: a nondialysable, heat-labile protein-like fraction, similar to Elford's inhibitor, and a dialysable, heat-stable fraction. The mechanisms of inhibition by these two fractions are discussed.

MATERIALS AND METHODS

Chemicals [(U)-^{14}C]CDP (420 mCi/mmol) was purchased from New England Nuclear Corp., Boston, Mass. All other chemicals used were obtained from Sigma Chemical Co., St. Louis, U.S.A.

Preparation of Ribonucleoside Diphosphate Reductase from AH-130 tumor cells AH-130 tumor cells were collected from male Dontyu rat ascites, washed with cold saline, and frozen at -20° until use. The frozen packed cells (20 g) were homogenized with 4 volumes of 50 mM potassium phosphate buffer (pH 7.0) containing 5 mM $MgCl_2$ and 25 mM KCl and centrifuged at 105,000 x g for 50 min. The supernatant was treated with streptomycin sulfate at 1% final concentration, and centrifuged at 10,000 x g for 20 min, and the resulting supernatant was fractionated with ammonium sulfate (0-35% saturation). The pellet obtained by centrifugation at 10,000 x g for 20 min was dissolved in 5 ml of the homogenizing buffer and dialyzed against the same buffer. This enzyme solution contained 20-30 mg of protein per ml.

Assay of Ribonucleoside Diphosphate Reductase The assay of ribonucleoside diphosphate reductase activity was based on the principle described by Steeper and Steuart[18]. The reaction mixture, consisted of 10 mM DTT, 4 mM ATP, 1.3 mM $FeCl_3$, 5 mM

NaF, 3 mM magnesium acetate, 50 mM potassium phosphate (pH 7.0), 0.125 mM [(U)-^{14}C]CDP (0.2 μCi/tube) and 0.1 ml of enzyme solution, with or without an inhibitor fraction in a total volume of 0.3 ml. The mixture was incubated at 37° for 60 min, and then the reaction was stopped by heating the mixture in a boiling water bath for 3 min. Then 50 μl each of 4 mM dCyd, apyrase solution (20 mg/ml) and alkaline phosphate solution (20 mg/ml) were added successively and the mixture was incubated further for 60 min. This solution was boiled at 100° for 3 min and centrifuged (3,000 rpm, 10 min). An aliquot of the supernatant was loaded onto a 0.5 x 5 cm Dowex 1 x 4 (borate form) column. [^{14}C]dCyd was eluted first with 3 ml of water, and 1 ml of the eluate was mixed with 10 ml of scintillator (toluene-Triton X-100 system, containing 100 mg/liter of 1,4-bis[2-(5-phenyloxazolyl)]-benzene and 4 g/liter of 2,5-diphenyloxazole in toluene). Radioactivity was counted in a Beckman liquid scintillation spectrometer.

Assay of Hexokinase Hexokinase activity, coupled with glucose-6-phosphate (G-6-P) dehydrogenase, in a solution of ribonucleoside diphosphate reductase was assayed by measuring increase in UV-absorption at 340 nm (formation of NADPH). The reaction mixture in a final volume of 3.0 ml consisted of 10 mM DTT, 3.3 mM ATP, 1.3 mM $FeCl_3$, 10 mM NaF, 3 mM magnesium acetate, 3.3 mM potassium phosphate (pH 7.0) (all

these reagents are present in the R.R. assay mixture), 1.1 mM NADP, G-6-P dehydrogenase (5 unit), 1 mM glucose, and 0.5 ml of enzyme solution. The reference cell contained all these reagents except G-6-P dehydrogenase and ATP. The cells were incubated at 37° in a Hitachi 124 spectrophotometer attached to a Kinshiwick circulator, and the reaction was started by adding ATP solution. The change in absorbance at 340 nm was then measured.

Effect of High Molecular Weight Ribonucleoside Diphosphate Reductase Inhibitor (HRRI) on CDP HRRI was incubated at 37° for 30 min in the reaction mixture for RR assay but without RR enzyme solution. Then the mixture was boiled for 3 min, and 5 µl of the supernatant obtained by centrifugation (3,000 rpm, 10 min) was applied to a polyethyleneimino cellulose TLC plate (Merck TLC plates, PEI-cellulose F pre-coated, 3 x 10 cm). The plate was developed first with methanol and then after drying in cool air, with 0.75 M LiCl, 25 mM formic acid. After development the plates were dried and the radioactivity was scanned with a Packard Radiochromatogram Scanner, model 7201.

Detection of ATP Protein was removed with perchloric acid (final 5%) and then the solution was neutralized with 2 M KOH and subjected to high pressure liquid chromatography, (Hitachi 633 liquid chromatogram; column, Nucleosil 10 NH_2 (Macherey-Nagel Co.) 4 x 250 mm; eluant, a linear gradient of

5 mM KH_2PO_4, 6 mM H_3PO_4 to 400 mM KH_2PO_4, 6 mM H_3PO_4; detection, U.V. 254 nm)

Protein Determination Protein was measured by the method of Lowry et al.[17], using bovine serum albumin as a standard.

Purification of HRRI All procedures were conducted at 0-4°. Rats (Wistar-King strain, weighing 150-200 g) were decapitated and their livers were perfused with cold 0.9% NaCl. The livers (60 g) were homogenized with 4 volumes of cold 50 mM potassium phosphate buffer (pH 7.0) and centrifuged at 10,000 x g for 30 min. The supernatant was recentrifuged at 105,000 x g for 60 min. The microsome-free supernatant (cytosol fraction) was treated with streptomycin sulfate (1% final concentration) for 2 hr, and then centrifuged at 10,000 x g for 10 min. The supernatant was treated with diisopropylsulfonylfluoride (DFP) (10 mM final concentration) for 1 hr and fractionated with ammonium sulfate (0-50%). This fraction was dissolved in 50 mM potassium phosphate buffer (pH 7.0), dialyzed against the same buffer, and applied to a Sephadex G-200 column (3.1 x 90 cm) previously equilibrated with 50 mM potassium phosphate buffer (pH 7.0). The column was eluted with the same buffer at a flow rate of 0.3 ml/min, and 10-ml fractions were collected. In another experiment, since the inhibitory activity was eluted in the void volume

from a Sephadex G-200 column, the ammonium sulfate fraction was loaded onto a Sepharose 6B column (3.1 x 90 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.0), and eluted with the same buffer and 7-ml fractions were collected.

Purification of LRRI Rat livers (10 g), obtained as described for Purification of HRRI, were homogenized with an equal volume of water, and centrifuged at 10,000 x g for 30 min. Protein in the supernatant fluid was precipitated with 80% ethanol and the mixture was centrifuged (10,000 x g, 10 min). The supernatant was condensed in vacuo at 35% (rotary evaporator) and the residue was dissolved in water (5.0 ml) (Ethanol fraction). This solution was washed twice with 10 ml of ethyl acetate and then twice with 10 ml of water saturated n-butanol in a separating funnel. The aqueous layer was applied to an Amberlite IR-45 (OH-form) column (2.7 x 10 cm). Material was eluted with water, and 6 ml-fractions were collected. The non-adsorbed fractions (Fraction number 4-7) were collected, lyophilized and dissolved in water (1.0 ml). This solution was then loaded onto a column of Dowex 50 x 4 (H^+form) (1.0 x 5.0 cm), the column was eluted with water and 1 ml-fractions were collected. The inhibitory activity was eluted between Frac. 3 and 9.

Detection of Glucose Silica gel TLC plates (Merck TLC plates with silica gel 60, without fluorescence, 3 x 10 cm, thickness 0.25 mm) were soaked in 0.5 M NaH_2PO_4 and dried at 105° for 1 hr. Samples were spotted on the plates and developed with a mixture of 2-propanol, acetone, 0.1 M lactic acid (4:4:2 v/v), and glucose was located with diphenylamine-aniline-phosphoric acid reagent.

RESULTS

Ribonucleoside Diphosphate Reductase Inhibitory Activity in the Cytosol Fraction of Rat Liver The cytosol fraction of rat liver, obtained by centrifugation of a liver homogenate at 105,000 g for 60 min, was incubated with ribonucleoside diphosphate reductase (RR) enzyme solution (0-35% ammonium sulfate fraction of AH-130 cytosol), and RR activity was measured. The liver cytosol was strongly inhibitory (79% inhibition relative to the control), as shown in Table I. This cytosol fraction was further fractionated with either ammonium sulfate (0-50%) or ethanol. When the cytosol was fractionated with ammonium sulfate and assayed after dialysis, the inhibitory activity was found in material precipitated at 0-50% saturation with none in the supernatant. This inhibitor was heat-labile and was inactivated by ethanol treatment (60% saturation). In addition, the cytosol contained inhibitory activity that was soluble in 60% ethanol and was heat-stable (100°, 2 min) (Table I). These findings suggested that there are two different inhibitors in rat liver cytosol; a nondialysable and heat-labile, high molecular weight ribonucleoside diphosphate reductase inhibitor (HRRI), and a dialysable, heat-stable, low molecular weight reductase inhibitor (LRRI). HRRI seemed to be the same as the natural inhibitor of ribonucleotide reductase firstly described by Elford[4].

Tissue Distribution, Purification and Characterization of HRRI The amounts of HRRI in ammonium sulfate (0-50%) fractions

of the cytosol of various tissues were compared. The fraction from liver, at a protein concentration of 2.7 mg/ml, inhibited the RR activity 50%. The relative activities of fractions from other tissues, calculated from the following formula, are shown in Table II.

$$\text{Relative activity} = \frac{2.7}{\text{Protein concentration needed to reduce RR activity to 50\% (mg/ml)}} \times 100$$

Slowly proliferating tissues, such as liver, kidney, lung, muscle, and testis had high HRRI activity; rapid proliferating tissues, such as spleen, thymus, bone marrow had little or no activity. The fractions from bone marrow cells and Yoshida sarcoma cells appeared to stimulate RR activity, because they contained RR activity. In regenerating liver obtained 48 and 72 hr after 66% partial hepatectomy, in which DNA synthesis was very active, the HRRI activities were only 50% and 55% of normal. These results suggest that the HRRI activity in tissues is inversely related to the growth rate and thus may regulate DNA synthesis by inhibiting RR activity. This finding is consistent with Elford's data[4].

Further purification was necessary for characterization of HRRI. For this purpose we applied the ammonium sulfate fraction to a Sephadex G-200 column. Chart 1. shows that the inhibitory activity was eluted in the void volume, indicating that HRRI had a molecular weight of more than 200,000. The material in

the void volume was collected, concentrated by lyophilization, and applied to a DEAE-cellulose column or a CM-cellulose column. Sometimes the inhibitory activity was recovered in poor yield from the nonadsorbed fraction (less than 10%), but the results were not reproducible. Thus ion-exchange column chromatography seemed unsuitable for purifying HRRI. Next the ammonium sulfate fraction was subjected to Sepharose 6B gel filtration. Again the inhibitory activity was found in the void volume (sepharose 6B fraction, molecular weight; 2,000,000) (Chart 2).

The effects of this HRRI on a substrate, CDP, and on co-factors were examined. The Sepharose 6B fraction (void volume) was incubated at 37° for 30 min with CDP and with all the co-factors needed for RR assay, but without RR enzyme solution. The radioactivities in CTP, CDP, CMP, and Cyd, separated on PEI-cellulose TLC, were measured with a radiochromatogram scanner. As shown in Chart 3A, almost 45% of the activity was found in CTP and 15% in CMP, while the radioactivity in CDP was reduced to 40%. When the mixture became depleted of ATP almost all the substrate CDP was degraded to CMP (Chart 3B), suggesting that the high molecular weight fraction contained CDP kinase (nucleoside diphosphate kinase) and CDPase (nucleosidediphosphatase). But this finding alone was not sufficient to explain why HRRI was inhibitory, because HRRI inhibited RR activity more than 70%. Therefore, we examined the effects of HRRI on cofactors.

HRRI seemed to have no effect on Mg^{2+}, Fe^{3+}, or DTT, because when these cofactors were added to the reaction mixture at twice their usual concentrations, no reversal of the inhibition was observed (data not shown). The effect of HRRI on ATP, the allosteric activator, was examined by measuring ATP, ADP and AMP by high pressure liquid chromatogtaphy. As shown in Chart 2, ATP degrading (ATPase) activity was eluted with HRRI activity from a Sepharose 6B column; fraction 29 caused 55% inhibition of RR activity and 85% degradation of ATP when incubated for 30 min, which is half the period used for assay of RR activity.

These results show that this very high molecular weight HRRI is a complex of several enzymes, including CDP kinase, CDPase, and ATPase, and that it is inhibitory because it destroys the substrate, CDP and allosteric activator, ATP.

Tssue Distribution, Purification, and Characterization of LRRI The 60% ethanol soluble inhibitor was apparently different from the inhibitor described by Elford. It was purified further by ethanol fractionation. Potassium phosphate was precipitated with 80% ethanol from solution. To avoid this, we used water instead of buffer for homogenization. The supernatant obtained by centrifuging a 50% homogenate of rat liver at 10,000 x g for 30 min was 80% saturated with ethanol and centrifuged (10,000 x g, 10 min). The supernatant was then evaporated and the residure was redissolved in water (one quarter of the volume of the ini-

tial homogenate) (80% ethanol fraction). This 80% ethanol soluble fraction strongly inhibited RR activity: 12 µl of this fraction per tube caused 50% inhibition of RR activity. The amounts of LRRI in the 80% ethanol fractions of various tissues were compared and the relative activity are shown in Table III, calculated from following fomula:

$$\text{Relative activity} = \frac{12}{\mu\text{l of 80\% ethanol fraction causing 50\% inhibition of RR activity}} \times 100$$

Liver showed the highest LRRI activity, and this decreased during liver regeneration (48 hr, 41%; 72 hr, 17%). Tumor cells (Yoshida sarcoma) showed low activity (19%) (Table III). But there was not such a clear reciprocal relation between LRRI activity and the cell growth rate as in the case of HRRI. Next the 80% ethanol fraction was extracted with ethylacetate and then with water saturated n-butanol. Each fraction was evaporated under reduced pressure (20-30Torr) and the residue was dissolved in water (an equal volume to the 80% ethanol fraction) and tested for inhibitory activity. Table IV shows that most of the activity remained in the aqueous layer (n-butanol-treated fraction). When this fraction was applied to a column of Amberlite IR-45 (free form), the LRRI activity was not adsorbed. The active fractions were collected, lyophilized, dissolved in water and applied to a column of Dowex 50 x 4 (H^+form). Again LRRI was not retained on the column. The yield in the

n-butanol treated fraction was 57%. These results suggest that LRRI was a small, nonionic molecule, possibly sugar. Therefore we examined the sugars in this fraction by Silica gel TLC before and after hydrolysis (2 M sulfuric acid, 100°, 4 hr). The samples, before and after hydrolysis both gave only one spot with the same Rf value as glucose (data not shown). This led us to investigate the effect of glucose on RR activity. Chart 4 shows that glucose caused concentration-dependent inhibition up to a concentration of 4 mM, and that 2 mM glucose caused 50% inhibition, confirming that LRRI was glucose. The effects of the various other sugars listed in Table V were also tested. Fructose, mannose, and 2-deoxyglucose, which are all substrates of hexokinase, inhibited RR activity. The hexokinase activity in the RR enzyme solution, measured using glucose (1 mM or 5 mM) as substrate, is shown in Table VI. This activity broke down the ATP in the RR assay mixture in 8.3 min. The presence of hexokinase was confirmed by the fact that the reaction was inhibited by N-acetylglucosamine. Thus LRRI was proved to be glucose and its inhibitory effect was concluded to be due to depletion of ATP as a result of the hexokinase activity present in the RR enzyme solution.

DISCUSSION

There are many reports of the existence of natural, protein-like inhibitors of DNA synthesis[1,3,4,6,7,8], but the mechanisms of their inhibition have not been clarified. In this study, we found two inhibitors of ribonucleoside diphosphate reductase (RR); one with a high molecular weight (HRRI) and the other with a low molecular weight (LRRI). HRRI was apparently the same as the inhibitor of RR described by Elford[4] of by Cory[3], since its activity varied inversely with the growth rate, and it was heat-labile and nondialysable. HRRI activity was eluted in the void bolume from a Sepharose 6B column, with ATP hydrolysing activity (Chart 2). Moreover, when HRRI was incubated with the substrate, CDP, and the co-factors needed for RR assay., CDP was phosphorylated to CTP and degraded to CMP (Chart 3). These results show that HRRI was a very large molecular weight (>2,000,000) complex of CDP kinase (nucleoside diphosphate kinase), CDPase (nucleoside diphosphatase), and ATPase. If so, HRRI should also inhibit kinase, which require ATP as a phosphate donor. Indeed, when, HRRI was added to mixtures for assay of thymidine kinase and thymidylate kinase, it was inhibitory (data not shown). CDP hydrolyzing activiy in liver and Yoshida sarcoma cells was 1.05 and 0.10 (μ mol/mg protein/30 min), respectively, and

high HRRI activity in liver was again agreeable from this results. Consequently, HRRI is not a direct inhibitor of RR. But its activity clearly varied reciprocally with the growth rate, and thus it may be important in regulating DNA synthesis *in vivo*, by reducing the levels of the substrate (CDP) and allosteric activator (ATP) of RR. Further studies on the characters and cellular localization of HRRI are now in progress, since HRRI seems to be a membrane fragment with several enzyme activities.

The other inhibitor, LRRI, was shown to be glucose. This was phosphorylated to glucose-6-phosphate by hexokinase present in the RR enzyme solution (0-35% ammonium sulfate fraction of cytosol of AH-130 tumor cells), and thus caused depletion of ATP. This phenomenon may have no biological importance, but the activities of G-6-P dehydrogenase and transaldolase, which are involved in pentose phosphate biosynthesis from glucose, are increased in all hepatomas irrespective of their growth rate[9,12]. Therefore, if excess glucose was added to tumor cells, it may decrease the ATP pool and thus inhibiting tumor growth.

Nakahara et al., in studies on a factor from normal rat liver that inhibits tumor growth[15,19], found that glucose inhibits the tumor growth *in vivo*, but they did not examine mechanism of this effect. Our results suggest the possible mechanism.

Neither of the inhibitors separated in this work had a direct effect on RR enzyme, like several inhibitors of proteases. Both HRRI and LRRI seemed to regulate enzyme activity by reducing the levels of the substrate or cofactors. Our results suggest that the balance of the activities of catabolic and anabolic enzymes seems to be one of important regulatory mechanism in nucleotide metabolism.

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Table I Evidence for two Ribonucleoside Diphosphate Reductase Inhibitors in Rat Liver Cytosol

Addition	RR activity (nmol/tube/hr)	Percent of Control
None	1.52	100
105,000 x g supernatant	0.32	21
0-50% ammonium sulfate fraction	0.26	17
" heated[a)]	0.17	112
" 60% ethanol supernatant	1.34	88
60% ethanol pellet	2.19	144
60% ethanol supernatant	0.24	16
" dialyzed[b)]	0.88	58
" heated[a)]	0.14	9

Rat liver cytosol or fractionated solution was added to the RR assay mixture.

a) heated in boiling water for 2 min

b) dialyzed against an equal volume of homogenizing buffer

Table II Levels of High Molecular Weight Ribonucleoside Diphosphate Reductase Inhibitor (HRRI) in Various Tissues of Rats

Tissue	Relative activity (%)
Liver	100
Kidney	82
Lung	81
Muscle	87
Testis	78
Spleen	43
Thymus	0
Bone Marrow	0*
Regenerating Liver (48 hr)	50
" (72 hr)	55
Yoshida Sarcoma	0

The HRRI activities in 0-50% ammonium sulfate fractions of various tissues, prepared as described in the Materials and Methods, are shown as relative activity, calculated as described in the text.

* Stimulation of RR activity was observed.

Table III Levels of Low Molecular Weight Ribonucleoside Diphosphate Reductase Inhibitor (LRRI) in Various Tissues of Rats

Tissue	Relative activity (%)
Liver	100
Kidney	45
Lung	24
Muscle	26
Testis	43
Spleen	48
Thymus	59
Regenerating Liver (48 hr)	41
" (72 hr)	17
Yoshida Sarcoma	19

The LRRI activities in 80% ethanol fractions of various tissues, prepared as described in the Materials and Methods, are shown as relative activity, calculated as described in the text.

Table IV Extraction of Low Molecular Weight Ribonucleoside Diphosphate Reductase Inhibitor (LRRI) with Organic Solvents

Fractions of LRRI	Volume of Sample added per tube (ml)	Percent of Control
80% Ethanol fraction	0.10	12
	0.02	14
	0.01	62
Ethylacetate layer	0.10	102
	0.02	117
	0.01	109
Water saturated n-butanol layer	0.10	51
	0.02	94
	0.01	100
Aqueous layer	0.10	9
	0.02	17
	0.01	71

The 80% ethanol fraction of rat liver (1 ml) was washed with ethylacetate (2 ml, twice) followed by water saturated n-butanol (2 ml, twice). The layers were each condensed *in vacuo*. The residues were dissolved in water (1 ml) and their effects on RR activity were examined.

Table V Effects of Various Sugars on Ribonucleoside Diphosphate Reductase Activity in vitro

Sugar	Concentration (mM)	Percent of Control
α-Cyclodextrin	2	98
	10	82
α,α-Trehalose	2	101
	10	105
Maltose	2	90
	10	95
N-Acetyl-D-glucosamine	2	88
	10	97
L-Rhamnose	2	101
	10	98
D-Galactose	2	95
	10	97
D-Fructose	2	65
	10	11
D-Mannose	2	68
	10	8
2-Deoxy-D-glucose	2	84
	10	8
L-Fucose	2	94
	10	95
D-Ribose	2	101
	10	92
D-Xylose	2	104
	10	97
2-Deocy-D-ribose	2	91
	10	89
L-Arabinose	2	89
	10	91

Various sugars at concentrations of 2 and 10 mM, were incubated with the RR assay mixture to examine their effects on RR activity.

Table VI Hexokinase Activity in RR Enzyme Solution

(0-35% Ammonium Sulfate Fraction of AH-130 Cytosol)

Substrate and Inhibitor	Rate of ATP Degradation (μmol/min/1 ml enzyme)
Glucose (1 mM)	1.44
Glucose (5 mM)	3.50
Glucose (5 mM) + N-acetylglucosamine (50 mM)	0.77 (78% Inhibition)

Hexokinase activity in the RR enzyme solution was measured using glucose as a substrate. The reaction was coupled with glucose-6-phosphate dehydrogenase and the amount of NADPH produced was determined by measuring the increase in U.V. absorbance at 340 nm.

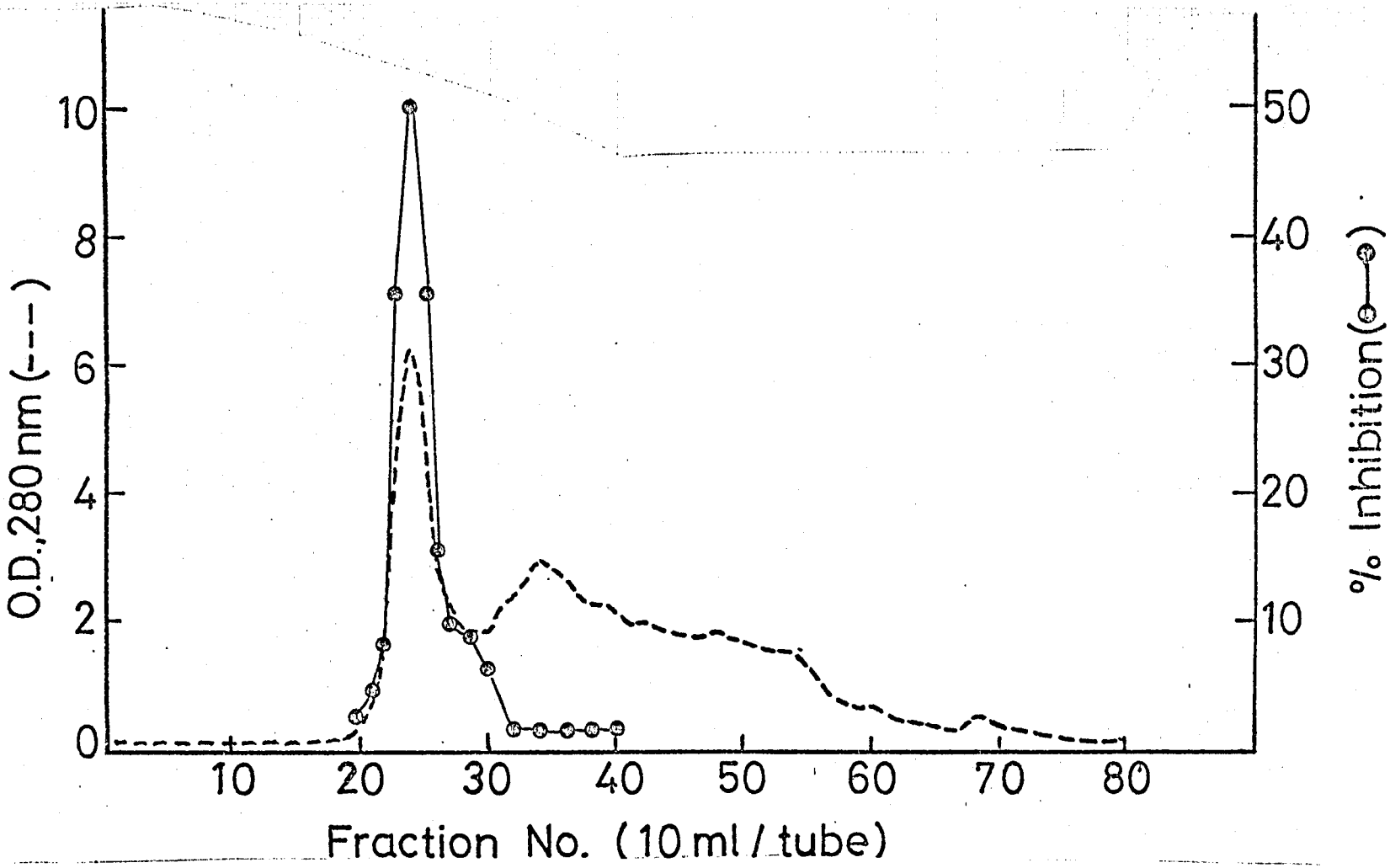

Chart 1 Gel filtration of high molecular weight ribonucleoside diphosphate reductase inhibitor from rat liver on a Sephadex G-200 column. The 0-50% ammonium sulfate fraction of rat liver cytosol was loaded on a column of Sephadex G-200, and the inhibitory activity of RR was measured.

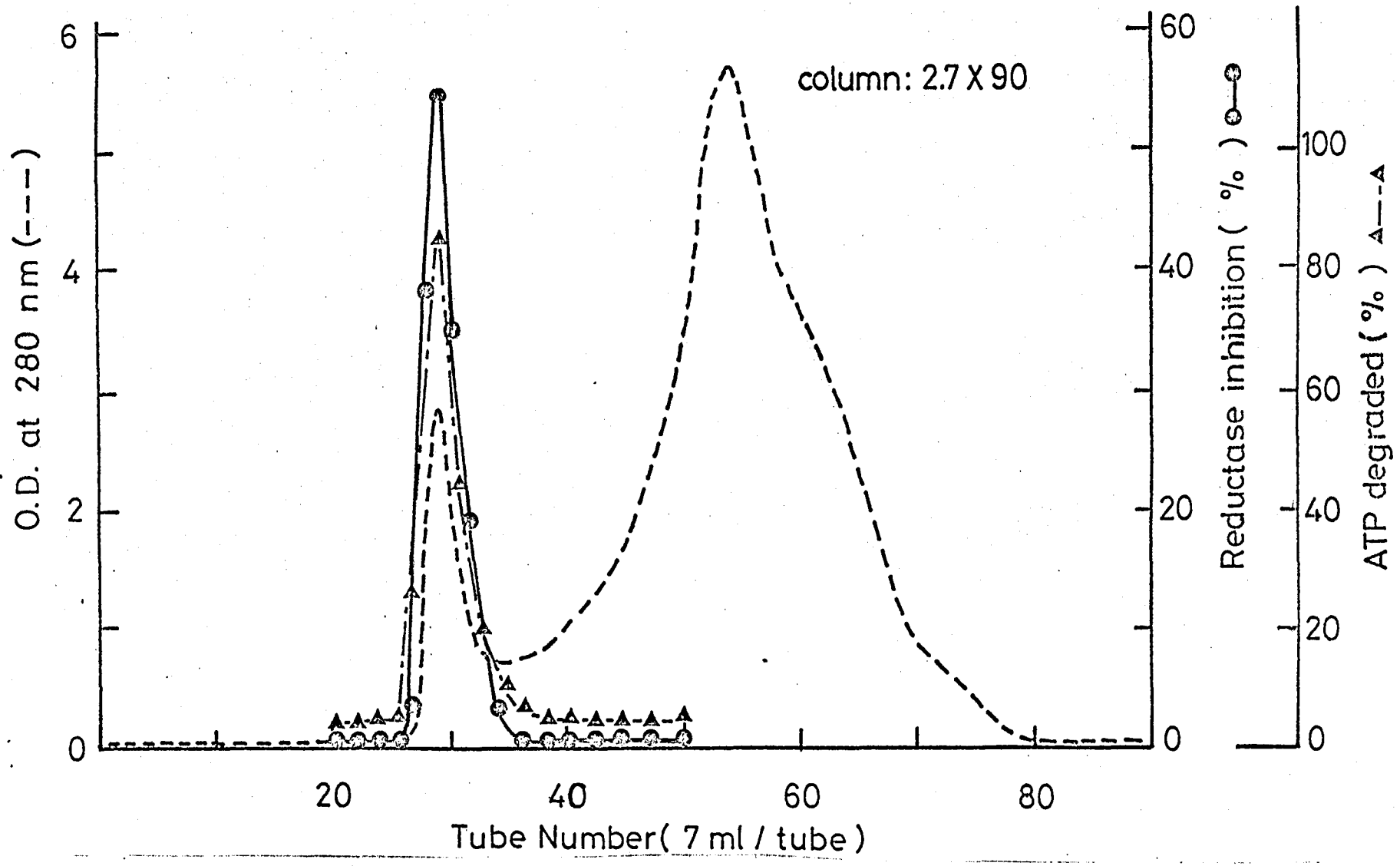

Chart 2 Gel filtration of high molecular weight ribonucleoside diphosphate reductase inhibitor from rat liver on a Sepharose 6B column. The 0-50% ammonium sulfate fraction of rat liver cytosol was loaded on a column of Sepharose 6B, and the inhibitory activity of RR and ATP hydrolyzing activity were measured.

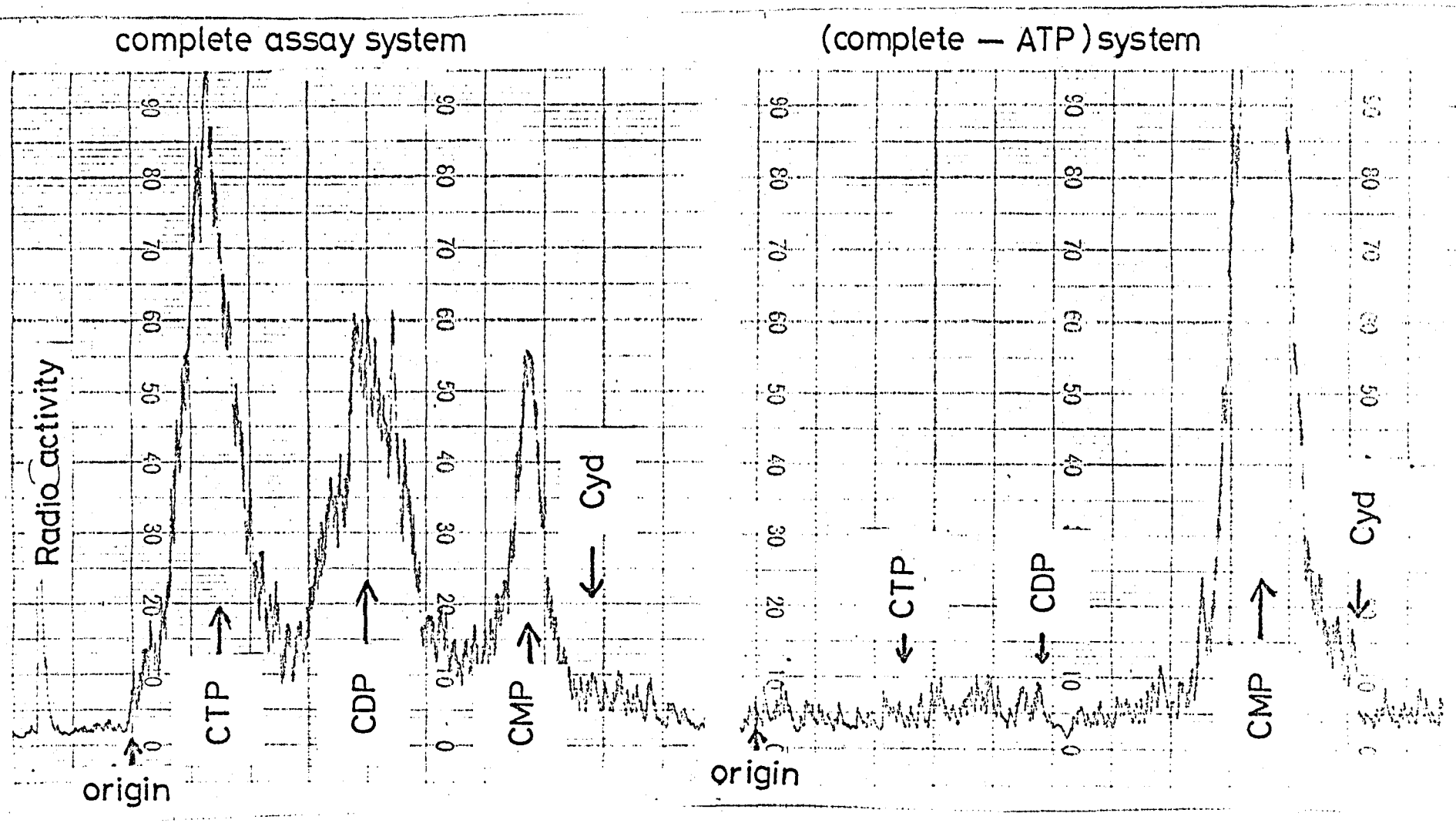

Chart 3 Effect of high molecular weight ribonucleoside diphosphate reductase inhibitor (HRRI) on CDP. HRRI (Sepharose 6B fraction) was incubated for 30 min with CDP and all the cofactors needed for RR assay, but without RR enzyme solution. Then CTP, CDP, CMP, and Cyd were separated by PEI-cellulose TLC and the radioactivity was scanned.

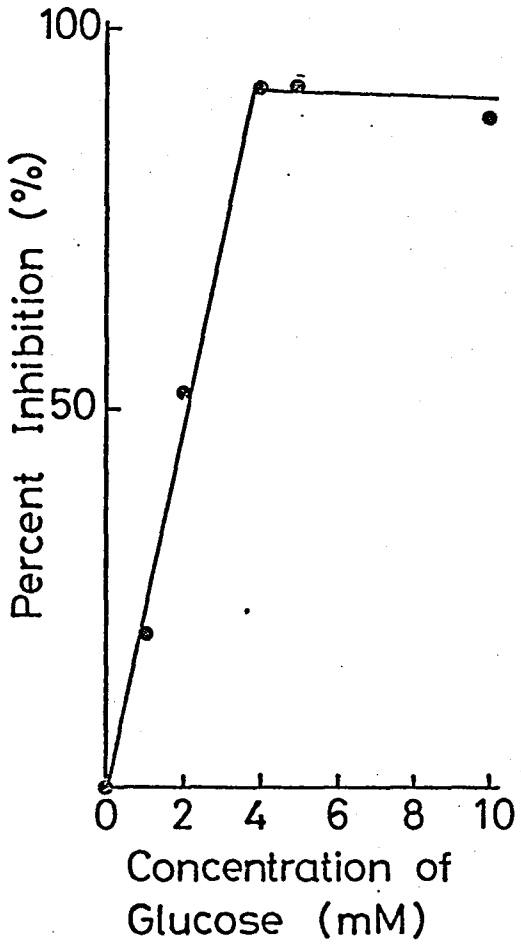

Chart 4 Effect of glucose on ribonucleoside diphosphate reductase in vitro. Glucose (0.1, 1, 2, 4, 5 and 10 mM) was incubated with the 0-35% ammonium sulfate fraction of cytosol of AH-130 tumor cells (RR enzyme solution) and RR assay mixtures for 60 min, and the inhibition of RR was measured.

Part 3

Effect of Uracil on Metabolism of 5-Fluorouracil *in vitro*

SUMMARY

The effect of uracil on the metabolism of 5-fluorouracil (5-FU) in vitro was studied. 5-FU was mainly phosphorylated in intact Yoshida sarcoma cells, whereas it was mainly degraded in liver slices. Uracil inhibited degradation of 5-FU much more than its phosphorylation; incubation of 2,500 μM of uracil with 2.5 μM of 5-FU (molar ratio, 1,000:1) inhibited the degradation of 5-FU by 70%, but did not affect its phosphorylation.

With homogenates of Yoshida sarcoma or liver, uracil inhibited degradation of 5-FU greatly, phosphorylation of 5-FU by α-D-ribose 1-phosphate (RiblP) and ATP to some extent, and phosphorylation by 5-phospho-α-D-ribosyl diphosphate (PPRibP) very little.

The activities of the enzymes involved in the metabolism of 5-FU in various tissues were also determined. Degradation of 5-FU was much faster in liver than in other tissues and was very slow in tumor tissue. Phosphorylation of 5-FU with RiblP and ATP was rapid in Yoshida sarcoma and bone marrow. Phosphoribosyltransferase activity was high in Yoshida sarcoma and thymus, but low in bone marrow.

INTRODUCTION

Regulation of DNA synthesis occurring naturally within the cells were described previously. This part describes the metabolism of antimetabolites, the artificial regulation of DNA synthesis. We chose 5-fluorouracil (5-FU) as an object of our study, because it has been used extensively in the treatment of certain types of cancer[2,4,5]. 5-FU was first synthesized in 1957 by Heidelberger and its active metabolite is generally thought to be 5-fluorodeoxyuridine 5'-monophosphate (FdUMP)[3], which is a potent inhibitor of thymidylate synthase[1]. Usually, the inhibitory activities of antimetabolites on cell growth are reversed by their normal substrates, but Rich et al.[7] demonstrated that uracil (Ura), cytosine and thymine did not reverse the growth inhibition by 5-FU in mammalian cells (H.Ep#1 human strain).

Mukherjee et al.[6] and Schumacher et al.[8], independently, reported that coadministration of uracil with 5-FU to rats enhanced its toxicity. Although Schumacher et al.[8] suggested that this might be due to the inhibition of 5-FU degradation, the precise mechanism was unknown. In this work, metabolism of 5-FU, compared with Ura, and the effects of Ura on 5-FU metabolism, were investigated to determine the reason for these findings.

MATERIALS AND METHODS

Chemicals 5-Fluorouracil (5-FU), 5-fluorouracil [5-^{14}C], and 2-fluoro-3-ureidopropionic acid (F-β-UPA) were from Taiho Pharmaceutical Co., Tokushima. Uracil, 3-ureidopropionic acid, β-alanine, NADPH, α-D-ribose 1-phosphate dicyclohexylammonium salt (Rib1P), adenosine 5'-triphosphate disodium salt, and other reagents were purchased from Sigma Chemical Co., U.S.A. Donryu and Wistar-Kins strain rats weighing ca. 120 g were purchased from Kitayama LABES Co., Kyoto.

Tumor Cells Yoshida sarcoma cells were maintained by intraperitoneal transfer in Donryu strain rats. Cells were collected 7 days after inoculation, and used for experiments after washing them three times with saline.

Preparation of Enzyme Solutions Wistar-King strain rats weighing 150-200 g were decapitated and their liver was rapidly perfused with saline (20 ml) and removed. All subsequent procedures were carried out at 4°. Tissues and tumor cells were freeze-thawed twice and homogenized in 3 volumes of 0.25 M saccharose containing 5 mM 2-mercaptoethanol and 0.5 mM EDTA (for experiments on uracil degradation) or with 50 mM Tris-HCl (pH 8.0), containing 5 mM 2-mercaptoethanol (for other experiments). For kinetic studies, the homogenates were cen-

trifuged at 105,000 x g for 60 min and the supernatant was submitted to ammonium sulfate fractionation (30-50% for dihydrouracil (DHU) dehydrogenase and 35-55% for other experiments) and then dialyzed against 10 mM potassium phosphate buffer (pH 7.6) (for DHU dehydrogenase) for 10 mM Tris-HCl (pH 8.0) (for other experiments).

Assay of 5-FU Degradation For assay of 5-FU degradation, the incubation mixture in a final volume of 1 ml contained NADPH (1.0 μmol), ATP (5.0 μmol), nicotinamide (50 μmol), potassium phosphate buffer (pH 7.6) (45 μmol), ^{14}C-5-FU (0.1 μCi), and the enzyme solution (0.4 ml). The mixture was incubated for 10 or 60 min at 37° and the reaction was stopped by immersing the mixture in a boiling water for 2 min. The mixture was centrifuged (3,000 rpm, 10 min) and the supernatant was added to 2 M KOH (0.06 ml, 0.12 mmol), stood at room temperature for at least 30 min to hydrolyze the DHU formed, and then mixed with 60% $HClO_4$ (0.02 ml, 0.12 mmol) and centrifuged (3,000 rpm, 10 min). A 10-μl aliquot of the supernatant was applied to a thin-layer chromatography (TLC) plate (Merck TLC plates with silica gel 60 F_{254} pre-coated, 2 x 20 cm, thickness, 0.25 mm), and developed with a mixture of 99% ethanol and 1 M ammonium acetate (5:1, v/v). Samples of 5-FU, F-β-Ala, and F-β-UPA were applied to the plate before the test sample. 5-FU was

located by measuring UV absorption at 254 nm, while F-β-Ala and F-β-UPA were located by spraying with ninhydrin and Ehrlich reagent, respectively. The spots of each substance were scraped into vials and extracted with 4 M HCl (0.04 ml). Then 10 ml of scintillator [toluene-Triton X-100 system, containing 1,4-bis[2-(5-phenyloxazolyl)]benzene (0.1 g/liter) and 2,5-diphenyloxazole (4.0 g/liter) in toluene] was added and radioactivity was measured in a Beckmann liquid scintillation spectrometer.

Assay of 5-FU Phosphorylation For assay of phosphorylating activity through uracil ribosyltransferase and uridine kinase the incubation mixture in a final volume of 0.5 ml contained RiblP (2.0 μmol), ATP (5.0 μmol), $MgCl_2$ (2.5 μmol), NaF (5.0 μmol), Tris-HCl buffer, pH 8.0 (25 μmol), ^{14}C-5-FU (0.050 μCi), and the enzyme solution (0.20 ml). For kinetic studies on uracil ribosyltransferase, ATP, $MgCl_2$, and NaF were omitted from this mixture. For assay of pyrimidine phosphoribosyltransferase, the mixture contained PPRibP (2.0 μmol), $MgCl_2$ (2.5 μmol), ^{14}C-5-FU (0.05 μCi), and the enzyme solution (0.20 ml) in a final volume of 0.50 ml. Mixtures were incubated at 37° for 10 min, mixed with 60% $HClO_4$ (0.025 ml, 0.15 mmol), centrifuged (3,000 rpm, 10 min), and 10-μl aliquots of the supernatant were subjected to silica gel TLC, with a mixture of chloroform, methanol, and acetic acid (17:3:1,v/v). 5-FU and FUrd, applied before the test sample, were located

by their UV absorption at 254 nm. Subsequent procedures were the same as in the assay of 5-FU degradation.

Assay on Intact Cells and Liver Slices Freshly prepared Yoshida sarcoma cells were used as intact cells. Liver slices were prepared by perfusion of fresh liver with Eagle's MEM, and cutting it up with a razor. The incubation mixture contained 5-fluorouracil[6-^{3}H] (10 μCi) with or without uracil and 0.2 g of intact cells of liver slices in 0.8 ml of Eagle's minimum essential medium (MEM). Preparations were incubated for 60 min, washed three times with saline, and 5.0% $HClO_4$ (0.5 ml) was added. The denatured cells or slices were homogenized and the products were analyzed by TLC as described above.

RESULTS

The metabolism of 5-FU (Chart 1) in the presence and absence of Ura was investigated. First we examined metabolism of 5-FU in intact Yoshida sarcoma cells and liver slices.

Effect of Uracil on 5-FU Metabolism in Intact Yoshida Sarcoma Cells and Liver Slices Intact Yoshida sarcoma cells or rat liver slices were incubated in Eagle's MEM containing ^{14}C-5-FU, with or without uracil. In Yoshida sarcoma cells, 5-FU was mainly phosphorylated and was degraded only slightly, whereas in liver slices, degradation of 5-FU was greater than its phosphorylation, as shown in Table I. Therefore, the effect of uracil on phosphorylation was examined using Yoshida sarcoma cells, while its effect on degradation of 5-FU was examined using liver slices. Various concentrations of uracil were added with three different concentrations of 5-FU, 250, 25, and 2.5 μM.

With the high concentration of 5-FU (250 μM), addition of 2,500 μM uracil (10 times the concentration of 5-FU) decreased phosphorylation by 31% and degradation by 66%; with 25 μM of 5-FU, addition of 250 μM uracil inhibited phosphorylation by 10% and degradation by 39%; with the low concentration of 5-FU (2.5 μM), addtion of 25 μM uracil caused no decrease in phosphorylation, but decreased degradation by 6%, as shown in Chart

2. It seems very interesting that the presence of 2,500 μM uracil with 2.5 μM 5-FU did not inhibit the phosphorylation of 5-FU, but decreased its degradation by 70%.

These findings led us to investigate the metabolism of 5-FU at the enzyme level.

Degradation of 5-FU and Uracil On the basis of the suggestion by Schumacher et al.[8] that uracil might inhibit the degradation of 5-FU, we studied the degradation of 5-FU to 2-fluoro-β-alanine (F-β-Ala) (Pathway 1) in homogenates of tumor and various organs. Chart 3 shows that the degradation of 5-FU and uracil is much more rapid in liver than in other tissues, and that little degradation occurred in the tumor homogenate. 5-FU was degraded more rapidly than uracil in all the tissues tested.

Phosphorylation of 5-FU Next we investigated phosphorylation of 5-FU (Pathways 2 and 3 in Chart 1) to the active form. We did not investigate Pathway 4, including deoxyribosyltransferase and deoxyuridine (dUrd) kinase, because Wilkinson[9] reported that this is only a minor pathway in mammalian cells. The phosphorylation of 5-FU in homogenates of tumor and various tissues was determined using either α-D-ribose 1-phosphate (Rib1P) and ATP (Pathway 2) or 5-phospho-α-D-ribosyl diphosphate (PPRibP) (Pathway 3) as a cofactor. As shown in Chart 2 and 5, activities for phosphorylation by Pathway 2 were high in Yoshida

sarcoma and bone marrow (Chart 4), and activities for Pathway 3 were high in Yoshida sarcoma and thymus (Chart 5). These results suggest that the two pathways are almost equally important in all tissues except the bone marrow.

Effect of Uracil on 5-FU Metabolism The effect of various concentrations of uracil on 5-FU metabolism was then investigated. Homogenates of rat liver were used in assay of degradation of 5-FU and homogenates of Yoshida sarcoma for the assay of its phosphorylation. Table II shows that degradation of 5-FU (Pathway 1) was markedly inhibited by uracil at concentrations greater than 250 μM, but the phosphorylation of 5-FU with PPRibP (Pathway 3) was not inhibited by uracil. Inhibition of phosphorylation by uracil through FUrd (Pathway 2) was great when the concentration of 5-FU was low, inhibition being less with higher concentrations of 5-FU. Therefore, the FUrd pool in Pathway 2 at high concentration of 5-FU was studied using a homogenate from Yoshida sarcoma cells. As shown in Chart 6, the FUrd pool increased and phosphorylation of 5-FU decreased when uracil was added to the assay system with RiblP and ATP. This finding suggests that uridine formed from uracil inhibited the phosphorylation of FUrd.

K_m and V_{max} Values of the Enzymes Involved in Metabolism of 5-FU The K_m and V_{max} values of DHU dehydrogenase, uracil ribosyltransferase, and pyrimidine 5'-phosphoribosyltransferase

were measured with uracil or 5-FU as a substrate. As shown in Chart 7, the K_m and V_{max} values for degradation of 5-FU and uracil (DHU dehydrogenase) were 4.0×10^{-5} M and 0.51, and 1.0×10^{-5} M and 0.22, respectively; the K_m and V_{max} values for phosphorylation of 5-FU and uracil (uracil ribosyltransferase) were 5.6×10^{-5} M and 1.2, and 4.5×10^{-4} M and 10 (with RiblP and ATP), and 6.3×10^{-4}M and 10, and 9.1×10^{-3} M and 26 (with PPRibP), respectively. The K_m value of DHU dehydrogenase was apparently lower than those of the phosphorylating enzymes, indicating that 5-FU and uracil tend to be used in the degradation pathway, but that this pathway is soon saturated (low V_{max} value). On the other hand, the pathways for phosphorylation have a large capacity and K_m values for 5-FU are lower than those for uracil so that inhibition of 5-FU phosphorylation by uracil is slight.

DISCUSSION

Rich et al.[7] demonstrated that Ura, Cyt, and Thy did not reverse the growth inhibition by 5-FU on H.Ep#1 human strain, even at 100 times the concentration of 5-FU. Schumacher et al.[8] found that uracil enhanced the teratogenicity of 5-FU and suggested that this might be due to inhibition of 5-FU degradation. However, there is a possibility that Ura inhibits the degradation of 5-FU and also the phosphorylation of 5-FU. Therefore, in the present work we studied the effects of Ura on 5-FU metabolism, and found that degradation of 5-FU was highest in the liver and very low in tumor tissues (Chart 3), and that the degradation was markedly inhibited by the addition of uracil (Table I). These findings are consistent with those of Schumacher et al.

On the other hand, phosphorylation of 5-FU was highest in tumor cells, and inhibition of phosphorylation was less than that of degradation. The results of kinetic studies clearly explained the fluctuation of 5-FU and uracil observed in vivo and in experiments on intact cells.

Wilkinson and Crumley[9] found that Pathway 4 (Chart 1), including deoxyribosyltransferase and dUrd kinase, plays only a minor role in phosphorylation of 5-FU in mammalian cells. Using intact Novikoff hepatoma cells, which have high thymidine

kinase activity, they found that inhibition of thymidylate synthetase activity is completely eliminated by hydroxyurea, a potent inhibitor of ribonucleotide reductase. These findings suggest that formation of 5-fluorodeoxyuridine 5'-monophosphate from 5-FU proceeds via FUMP, FUDP, and FdUDP, but does not involve dUrd kinase.

Our results showed that the conversion of 5-FU to FUMP can proceed by either phosphoribosylation (Pathway 3) or through FUrd (Pathway 2) (Charts 4 and 5), and when high concentration of 5-FU was used as a substrate of phosphorylation, Ura inhibited phosphorylation of 5-FU due to the competition of FUrd kinase by Urd, while the phosphorylation of 5-FU at a low concentration was not decreased by Ura. These mechanisms can be explained well from the results of kinetic studies of these enzymes.

As the activity of 5-FU-phosphorylation was much higher in tumor cells than in all the normal tissues examined and this activity was not inhibited by Ura, at a low level of 5-FU (Chart 2), a suitable dose of Ura with 5-FU might increase the levels of 5-FU and the active forms of 5-FU specifically in the tumor, and thus enhancing its antitumor activity without increasing its toxicity.

When Ura was coadministered with 1-(2-tetrahydrofuryl)-5-fluorouracil (FT-207), a masked compound of 5-FU, to mice

bearing sarcoma-180 at the molar ratio of 4:1, the antitumor activity of FT-207 on sarcoma-180 were markedly enhanced without increasing its toxicity. This combination of drugs (FT-207, Ura, 1:4 mol/mol) is showing a good responce also to human cancers.

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Table I. Metabolism of 5-FU in Intact Yoshida Sarcoma and Liver Slices

	Metabolites formed (%)	
	Yoshida sarcoma cells	Liver slices
Phosphorylation	44	3
Degradation	1	80

Intact Yoshida sarcoma cells (0.1 g) or liver slives (0.2 g) were incubated in Eagle's MEM containing 5-FU (25 uM) for 60 min at 37° with shaking. Then metabolites in the cells or slices were analyzed.

Table II. Effect of Uracil on 5-FU Metabolism

	5-FU (uM)	Uracil (uM)	Inhibition (%)
Degradation	10	10	0
		100	44
	25	25	12
		100	47
		250	69
		1000	88
	80	320	86
		1000	92
Phosphorylation	2.5	2.5	7
		10	17
		25	43
		250	85
with RiblP	80	80	3
and ATP		320	38
	250	1000	52
with PPPRibP	2.5	2.5	3
		10	3
		25	0
		250	16
	80	80	0
		320	8

Various concentration of uracil was added to the reaction mixture for assay of degradation of phosphorylation of 5-FU. Liver homogenate was used in assay of degradation and Yoshida sarcoma in assay of phosphorylation.

Fig 1

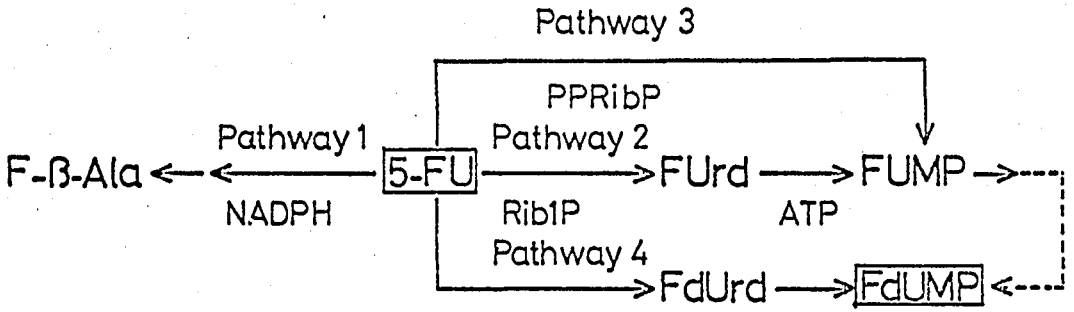

Fig. 1. Possible metabolic pathways of 5-FU

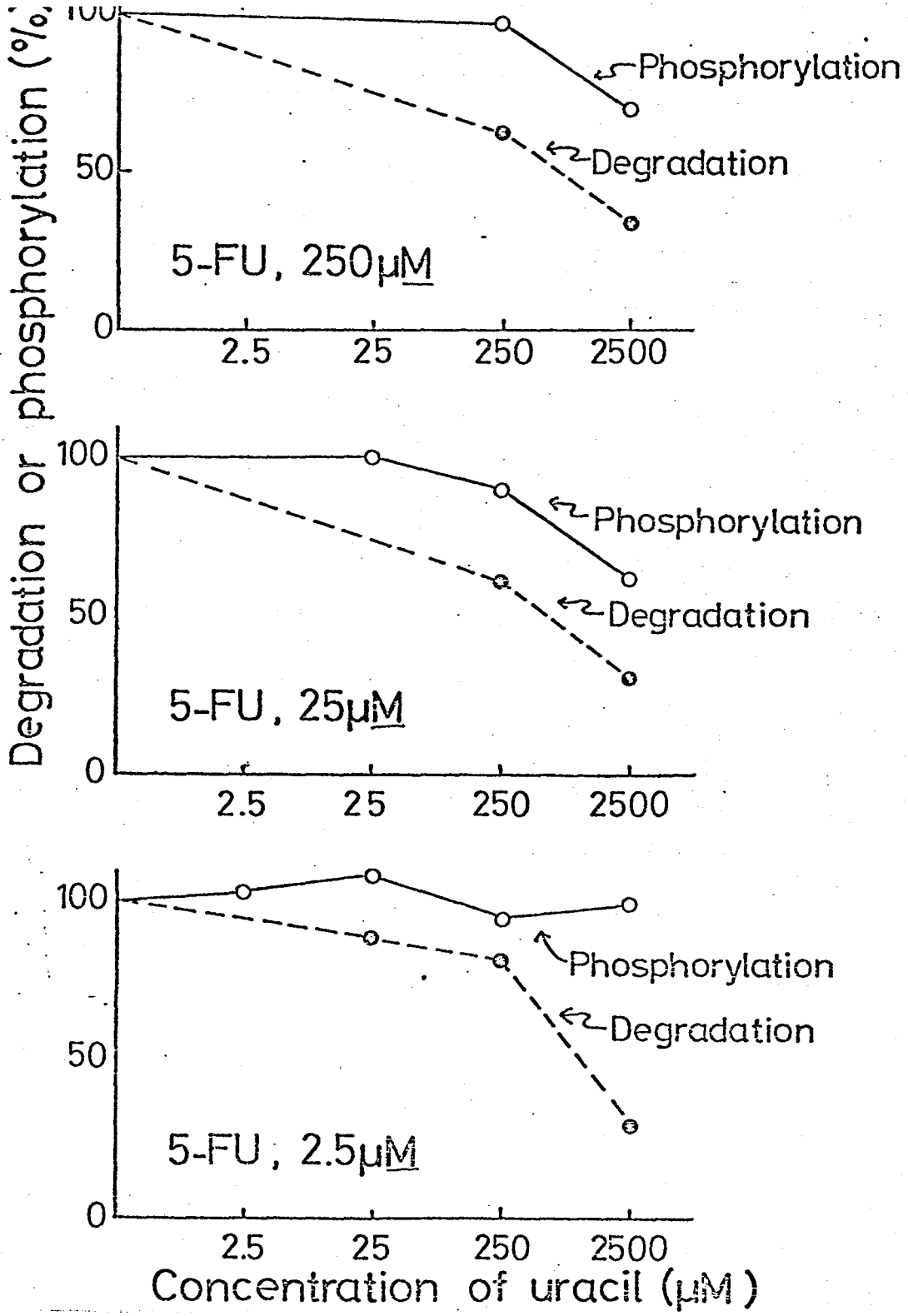

Fig. 2. Effect of uracil on metabolism of 5-FU in intact Yoshida sarcoma cells and liver slices

5-FU (250, 25, 2.5μM) and the indicated concentrations of uracil were incubated with liver slices or intact Yoshida sarcoma cells in Eagle's MEM at 37° for 60 min. Phosphorylation of 5-FU was measured in Yoshida sarcoma cells and degradation of 5-FU in liver slices. Activities in the absence of uracil were taken as 100%.

(o—o) Yoshida sarcoma, (o--o) liver slices

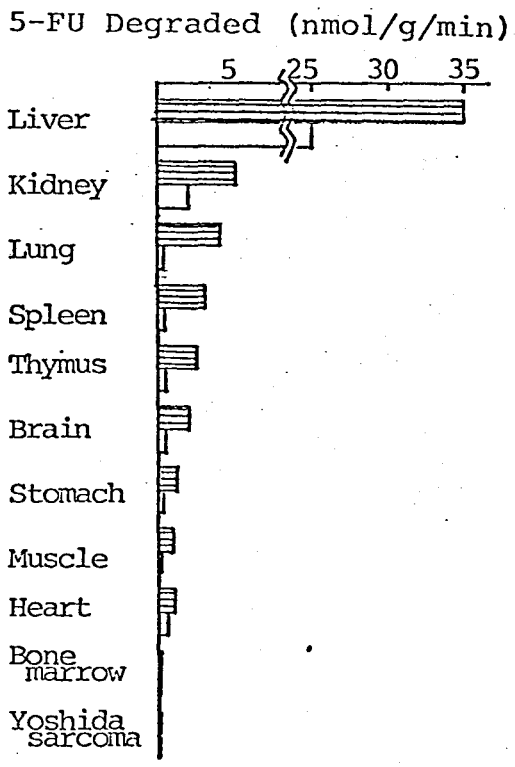

Fig. 3. Degradations of 5-FU and uracil in various tissues

5-FU (80uM) or uracil (80uM) was incubated for 10 min (liver) or 60 min (other tissues) with homogenates of various tissues. The rate of degradation of 5-FU was measured.

5-FU, uracil

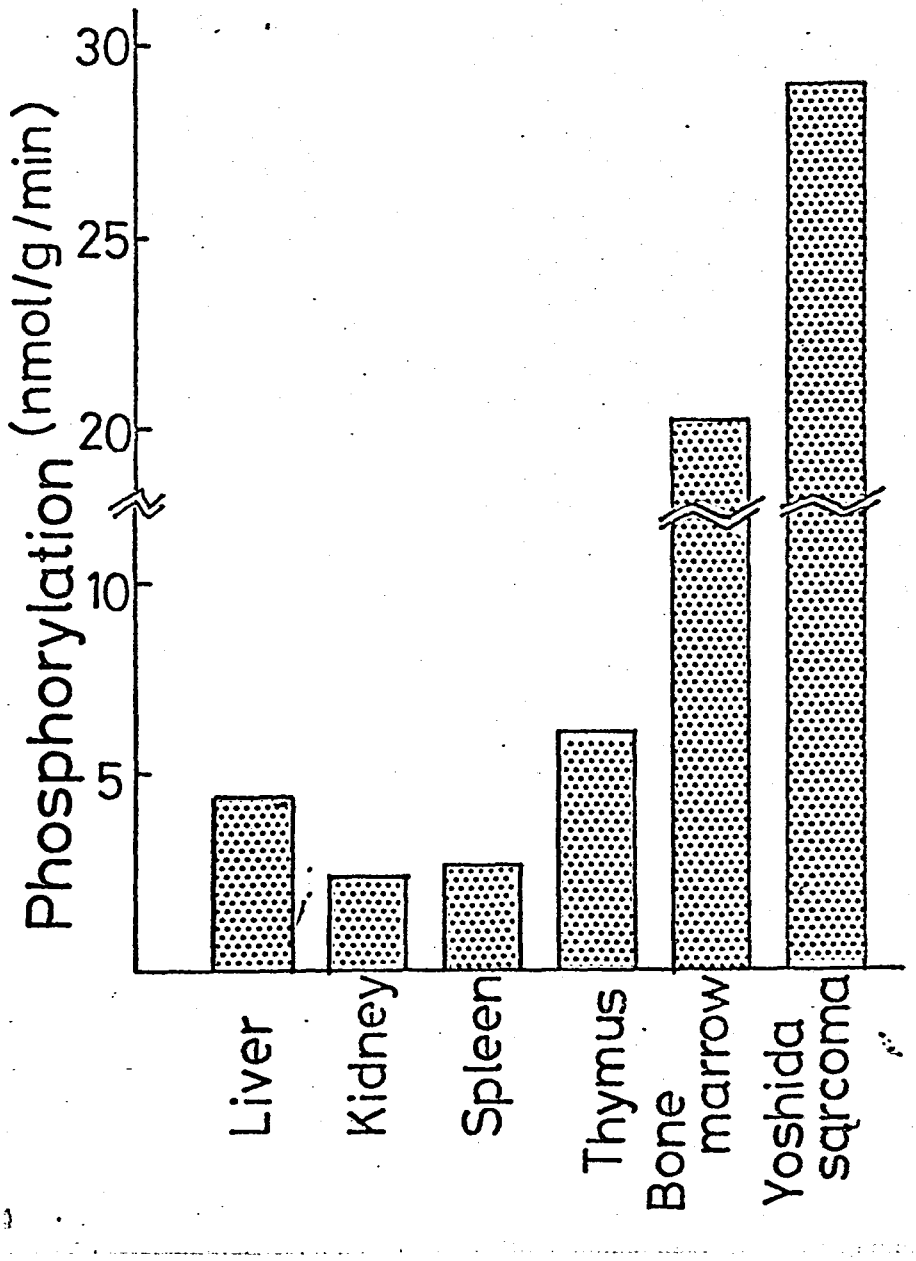

Fig. 4. Phosphorylation of 5-FU in various tissues in the presence of RiblP and ATP

The rate of phosphorylation by Pathway 2 (Fig. 1) was measured by incubating 5-FU (80μM) with various tissues in the presence of RiblP and ATP for 10 min.

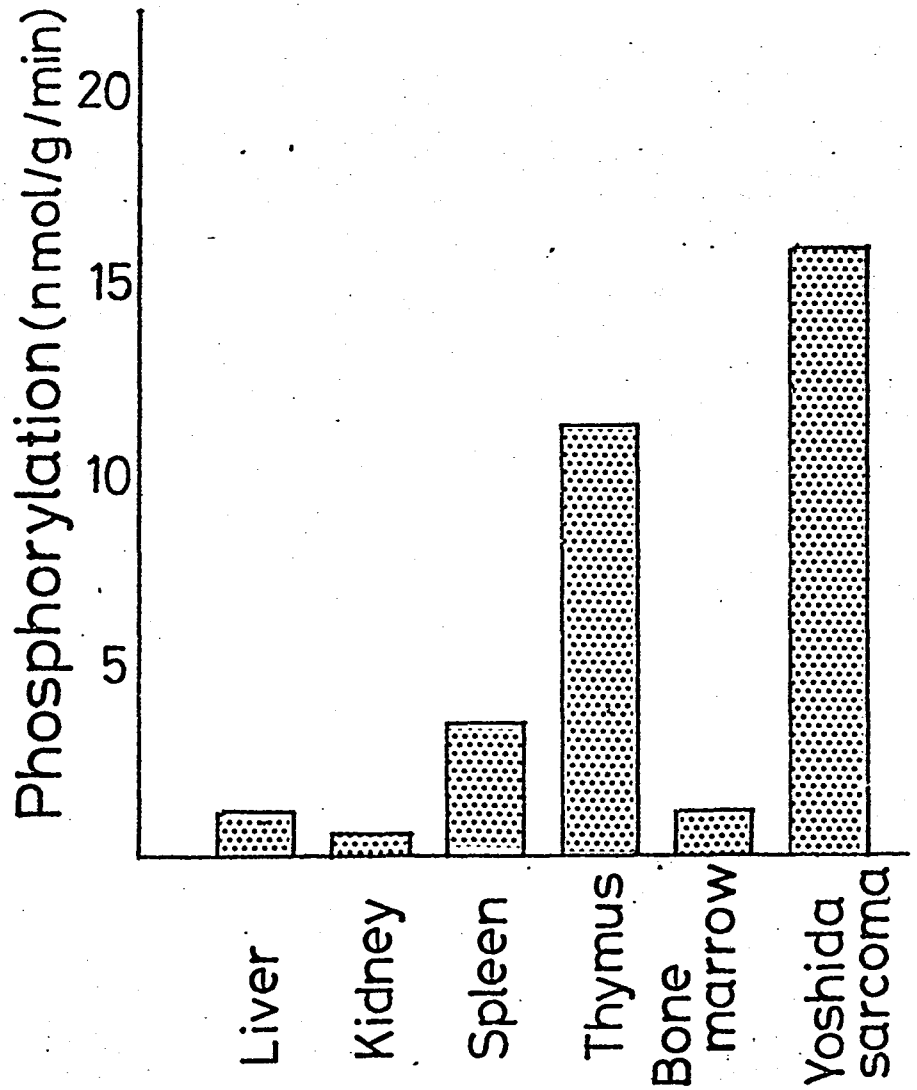

Fig. 5. Phosphorylation of 5-FU in various tissues in the presence of PPRibP

The rate of phosphorylation by Pathway 3 (Fig. 1) was measured by incubating 5-FU (80μM) with various tissues in the presence of PPRibP.

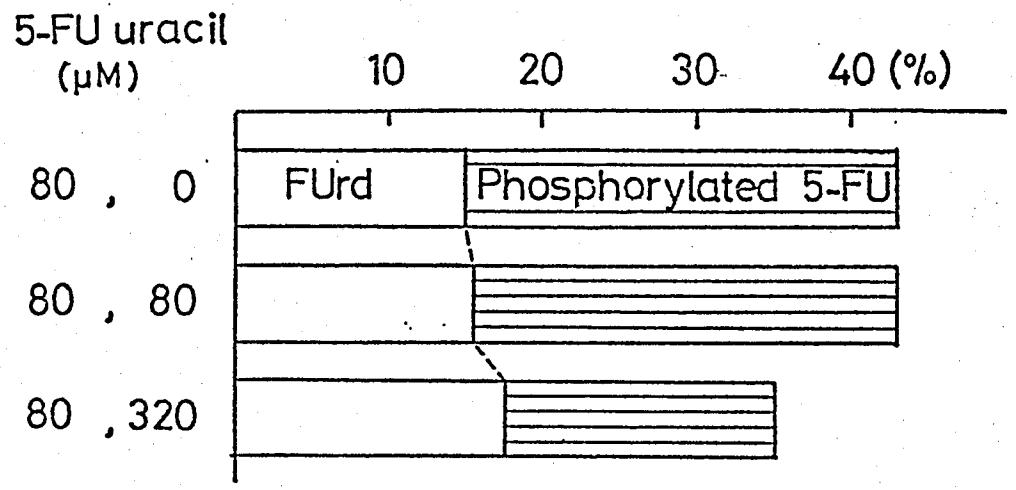

Fig. 6. Effect of uracil on 5-FU ribosyltransferation and phosphorylation

5-FU (80μM) was incubated with liver homogenate in the presence of RiblP, ATP, and uracil (0, 80 or 320μM), and then metabolites of 5-FU were analyzed.

phosphorylated 5-FU, FUrd

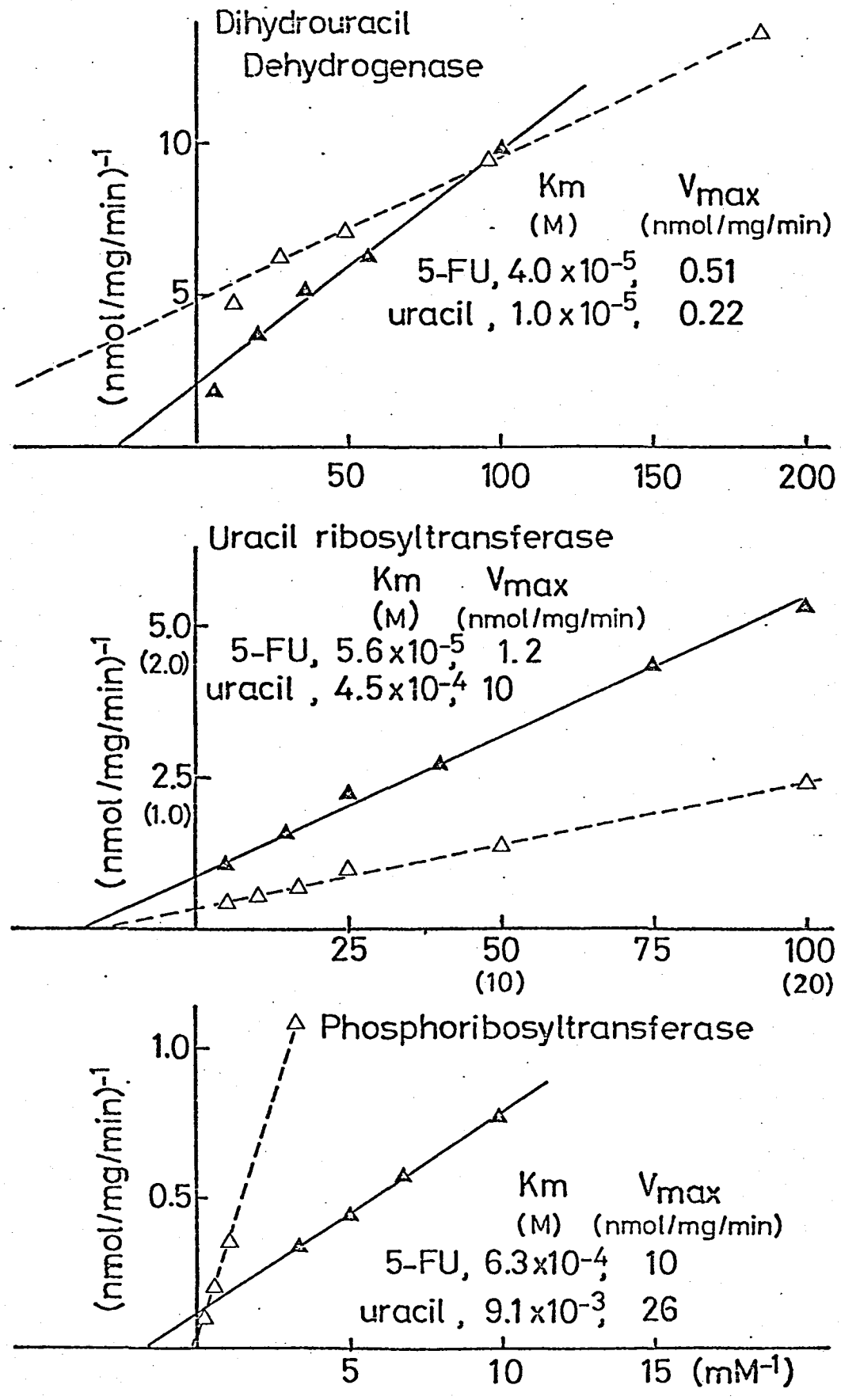

Fig. 7. Km and V_{max} values

The reaction of DHU dehydrogenase, uracil ribosyltransferase, and pyrimidine phosphoribosyltransferase was measure with various concentrations of 5-FU or uracil. Km and V_{max} values, calculated from double reciprocal plots are shown in the graphs.

ACKNOWLEDGEMENT

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