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Metabolism of 9-Ethyl-6-Mercaptopurine by Humans.* (29015)

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It has been reported that 9-ethyl-6-mercaptopurine (9-E-6-MP) will produce remission when administered orally to chronic granulocytic leukemia patients(1). This drug is excreted in the urine of humans primarily as the S-glucuronide but a significant amount of the drug (10-20%) is excreted as a compound that has a U.V. spectrum identical with thiouric acid, (TUA)(2). However, the possibility has not been excluded that this compound is 9-ethyl-TUA since the spectrum of the 9-alkylated derivative would probably be identical with TUA. We have synethesized 9-E-6-MP labeled with C^{14} in the ethyl position (9-E-C^{14})-6-MP, administered it to patients and have also studied the effect of xanthine oxidase and nucleoside phosphorylase *in vitro* on 9-E-6-MP. These results are reported below.

Methods. 9-(E-C^{14})-6-MP was synthesized by published methods using C^{14}-ethylamine (1.85 mc/mmole) which was obtained from New England Nuclear(3,4). Xanthine oxidase (XO) was purchased from Worthington Enzymes. Inosine, hypoxanthine and 6-MP were obtained from Mann Research Laboratory, while 6-MP-ribotide was a generous gift from Dr. George Hitchings of Wellcome Research Laboratory. Spectra were obtained with a Beckman DU Spectrophotometer and enzyme reactions were carried out using the same instruments with attached thermospacer through which 37.5°C water was pumped. Ion exchange chromatography on Dowex-1 $\times$ 8 columns was performed as previously described(5). A Packard Tricarb liquid scintillation counter was used to assay samples for C^{14}.

Partially purified purine nucleoside phosphorylase (PNP) was prepared from rat liver as follows:

Rat liver was perfused with cold saline and 0.25 M sucrose followed by homogenization in 9 volumes of ice cold 0.25 M sucrose. The homogenate was fractionated by differential centrifugation by the method of Schneider and Hogeboom(6). The soluble supernatant obtained after removal of the microsomal fraction was adjusted to pH 5.2 with 10% acetic acid and left in an ice bath for one-half hour. It was then centrifuged at 8000 $\times$ *g*, the supernatant decanted from the precipitate, and the precipitate discarded. The supernatant which contained virtually all of the XO and PNP was dialyzed against pH 7.5 sodium phosphate buffer overnight. This solution was then fractionated with solid amon-

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ium sulphate. The 0-20% fraction was discarded. The fraction that precipitated between 20-40% of saturation was dissolved in sodium phosphate buffer (pH 7.5, 0.1 N) of volume equal to 2 × that of the original liver. This fraction was dialyzed overnight against 100 vol. of phosphate buffer, and any precipitate then removed by centrifuging at 8000 × *g* for 30 minutes. All of the above steps were carried out at 5°C. The supernatant contained about 80% of the PNP and most of the XO activity.

Patients were maintained on a metabolic ward and given the labeled drug orally before breakfast. All urine voidings were collected separately for the next 24 hours, assayed for radioactivity, frozen and stored at —20°C.

Results. Drug was administered to one normal and one leukemic (acute lymphoblastic) patient, both weighing approximately 50 kg. One patient was given 500 mg/kg of 9-E-6-MP which contained 50 μc of C^{14}. After fractionation of the urine on Dowex-1 × 8 the eluent containing the compound with a spectrum similar to TUA was found to contain small amounts of the S-glucuronide of the drug. This was removed by passing this fraction through Dowex-50 × 8 which holds the S-glucuronide but allows passage of TUA. The pH of this eluent was adjusted to 7 with NaOH (1 M), diluted 3-fold with water and again chromatographed on Dowex-1 × 8. The eluent containing the compound with a spectrum similar to TUA had a specific activity which was less than 5% of the specific activity of the administered drug (assuming that the UV extinction of this compound is approximately that of TUA). Similar results were obtained with a second patient. To ascertain that 9-E-6-MP used in these experiments did not contain 6-MP, the following experiments were carried out.

9-E-6-MP was dissolved in 0.1 NaOH (0.5 mg/ml), the solution adjusted to pH 7.5 with H_3PO_4 and a final concentration of 100 μg/ml obtained by addition of 0.1 M sodium phosphate buffer (pH 7.5). Three ml aliquots were pipetted into 2 Beckman cuvettes (1 cm) which were placed in the DU spectrophotometer until the solution temperature reached 37.5°C. Fifty μl of commercial XO were then added to one of the cuvettes, and the change of absorbance at 355 mμ measured. As may be observed in Fig. 1, there was no change over a period of 10 minutes indicating that no TUA was formed. At the end of this time, 3 μg of 6-MP were added which was oxidized to TUA as indicated by an absorbance increase at 355 mμ. The expected increase of absorbance at 355 mμ was again observed when 30 μg of 6-MP was added. This experiment demonstrated that 6-MP contamination of the 9-E-6-MP administered to the patients was less than 0.2%. It is apparent from the above that 9-E-6-MP is not a substrate of commercial XO. From the data presented below, it may be seen that 9-E-6-MP does not serve as substrate for rat liver XO or rat liver nucleoside phosphorylase.

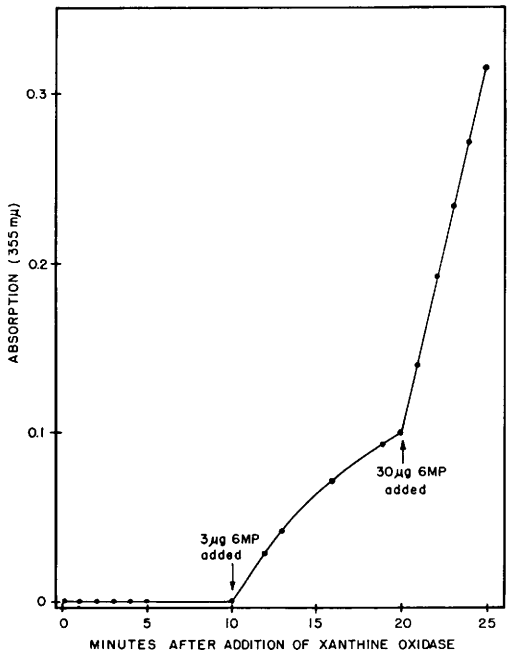

FIG. 1. Failure of commercial xanthine oxidase to metabolize 9-E-6-MP. Cells contained 100 μl/ml of 9-E-6-MP at beginning of incubation. 100 μl of commercial XO added at time 0. See text for other experimental details.

The activity of PNP was measured by coupling this reaction with xanthine oxidase and measuring the amount of uric acid or thiouric acid formed spectrophotometrically (7).

(1) Inosine + PO_4 $\xleftrightarrow{(PNP)}$ hypoxanthine + ribose-1-PO_4

hypoxanthine $\xrightarrow{(XO)}$ uric acid

(2) 6-MP-riboside + PO_4 $\xleftrightarrow{(PNP)}$ 6-MP + ribose-1-PO_4

6-MP $\xrightarrow{(XO)}$ thiouric acid

As may be observed from Table I, the amount of XO present in the preparation of XO-PNP from rat liver was rate limiting. Formation of uric acid was the same whether inosine or hypoxanthine were added as substrate, and thus, the data presented in Table I may be interpreted as a measure of XO activity, not PNP activity. To measure the activity of PNP 100 μl of commercial XO was added to each cuvette with liver XO-PNP. This provided XO in several-fold excess required to make PNP the rate limiting enzyme, when riboside was added as substrate. The activity of PNP is shown in Table II. This preparation hydrolyzed inosine at a rate of 11.2 μmoles/mg of protein/hr. 6-MP-riboside was phosphorylyzed at approximately 3% the rate of inosine. 9-E-6-MP did not serve as substrate, *e.g.*, was not altered.

Discussion. The data presented above leave little doubt that 9-E-6-MP is dealkylated to some extent when administered orally to humans at 10 mg/kg/day. If the dealkylation product is 6-MP then enough of this drug will (1.5-2 mg/kg) be liberated to account for the efficacy of 9-E-6-MP administered to patients with chronic granulocytic leukemia.

TABLE I. Activity of Rat Liver Xanthine Oxidase-Purine Nucleoside Phosphorylase Preparation.*

Substrate	Activity†
Hypoxanthine	.4
Inosine	.41
6-MP	.015
6-MP-riboside	.014
9-E-6-MP	.000

* See text for details of preparation.

† μmoles of uric acid or thiouric acid formed/mg protein/hr. See text for assay conditions.

Measurements made at substrate levels giving maximal activity.

The inability of 9-E-6-MP to serve as substrate for rat liver XO or rat liver PNP was not surprising in light of our earlier reports that 9-E-6-MP is not dealkylated by the rat but excreted unaltered or as the S-glucuronide(5). Although it is possible that 9-E-6-MP may serve as substrate for human XO or PNP, this seems unlikely. We have some data which suggest that the dealkylation of 9-E-6-MP may possibly be carried out by leukocytes or bone marrow. In 2 of the patients to whom we have given 9-E-6-MP who had high neutrophil counts, we have identified small amounts of a compound in the urine which appears to be identical with thioxanthine(2). It has been reported that this product is not formed when xanthine oxidase acts on 6-MP but rather that 6-MP is attacked at the 8-position first to yield 8-OH-6-thiopurine(8). We have observed that a tissue culture, originally derived from a patient with chronic granulocytic leukemia, metabolizes 6-MP to a compound which also appears to be thioxanthine(9). While we have been unable absolutely to demonstrate dealkylation of 9-E-6-MP by this culture, we have obtained indirect evidence that suggests dealkylation occurs.

TABLE II. Activity of Rat Liver Purine Nucleoside Phosphorylase.*

Substrate	Activity†
Inosine	11.2
6-MP-riboside	.35
9-E-6-MP	.00

* See text for experimental details.

† μmoles uric acid or thiouric acid formed/mg protein/hr. Excess commercial XO added so that phosphorylysis of riboside is rate limiting. Measurements made at substrate levels giving maximal activity.

Summary. Evidence was presented that 9-E-6-MP is dealkylated by the human in amounts adequate to provide enough 6-MP to explain the effectiveness of this drug when it is administered to chronic granulocytic leukemia patients. 9-E-6-MP does not serve as substrate for xanthine oxidase, nor purine nucleoside phosphorylase. The possibility that the enzyme responsible for dealkylation is present in human bone marrow or neutrophils is discussed.

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Enzyme Studies in Muscular Dystrophy: IV. Acid Deoxyribonuclease in Nutritional and Hereditary Muscular Dystrophy.* (29016)

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A number of hydrolytic enzymes of muscle, with optimum activity in the acid pH ranges, have been reported to be increased in nutritional muscular dystrophy resulting from Vit. E-deficiency(1·2,3), and in hereditary dystrophy in the mouse(4,5) and chicken (5). It has been suggested that these acid hydrolases are segregated, in an inactive state, in cytoplasmic particles called lysosomes and are released under conditions which cause tissue damage(6). Despite the overall loss of muscle mass, the deoxyribonucleic acid (DNA) content of muscle in nutritional dystrophy and in genetically determined dystrophy of the mouse(7) and chicken(8) is also increased. Since acid deoxyribonuclease (DNase II) is considered to be one of the group of lysosomal enzymes(6), we have investigated its activity in these types of muscle wasting.

Materials and methods. Nutritional muscular dystrophy was produced in young rabbits (600-900 g) by feeding them Diet 11 of Goettsch and Pappenheimer(9) which is deficient in Vit. E and which was treated with 1% ethereal Fe Cl_3. Stripped lard† was used instead of commercial lard. Three times a week, the diet of controls was supplemented by oral administration of 25-50 mg of dl-α-tocopherol in peanut oil. After the appearance of symptoms of Stage II(10) of muscular dystrophy, the animals were sacrificed. Dystrophic mice and their normal littermates, obtained from R. B. Jackson Memorial Laboratory, were 129/Re × C57BL/6 hybrids. The mice were 2-3 months old when sacrificed, and muscle from 2 dystrophic animals usually was pooled for each determination. Dystrophic chickens, raised at this Institute, had been hatched from eggs supplied from the flock maintained by the Dept of Genetics, Univ. of Connecticut.‡ Control chickens were purchased from commercial suppliers. The chickens were approximately 3 months of age when sacrificed.

The method of Coleman(11) was used to determine DNase II activity. Water homogenates of rabbit and mouse thigh and leg muscle and chick breast muscle, prepared in a Waring blendor or Virtis "45" homogenizer, were used as the enzyme source. Under these conditions complete activation of lysosomal enzymes should have been obtained (6). Homogenates were assayed at dilutions where activity varied directly with concentration in an almost linear relationship. Enzyme and substrate blanks were included with each

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