lacking, we have chosen to follow Young (*10*) in using the symbols Pgd^A and Pgd^B for the alleles at the 6-PGD locus in the cat. This conforms to standard genetic usage, is conveniently similar to the common symbol used for the enzyme (6-PGD), and is consistent with the more general use of Roman (rather than Greek) letter superscripts. With this nomenclature, the genotypes of cats for 6-PGD variants are Pgd^A/Pgd^A (phenotype A), Pgd^B/Pgd^B (phenotype B), and Pgd^A/Pgd^B (phenotype AB).

The manifestations of activity in three bands in heterozygotes is consistent with the hypothesis of molecular hybridization advanced by Schwartz (*11*) to explain the same situation for an esterase of maize. This has been accepted and extended by others (*12*). According to this hypothesis, each allele controls production of a specific protein subunit, the active enzyme being a dimer. In heterozygotes, random association of subunits occurs and therefore gives rise to twice as many hybrid molecules as homologous molecules of each type in the ratio of $A^2 + 2AB + B^2$. The more intense enzymatic activity of the intermediate band on zymograms is presumably related to the greater number of hybrid enzyme molecules expected by this hypothesis.

In our studies of cats, we noted the greater intensity of enzymatic staining for the intermediate band of the AB phenotype. If the following assumptions are accepted, certain conclusions may be drawn. The assumptions are: (i) both alleles at the 6-PGD locus produce subunit molecules of equal enzymatic efficiency when combined in a dimer; (ii) both alleles are equally active with respect to production of subunits; (iii) polymerization of the subunits to an enzymatically active dimer takes place within the producing cell; (iv) the staining reaction in zymograms is stoichiometrically related to the number of active enzyme molecules. The conclusions would be that gene activity is quantitatively expressed by enzyme activity, that the relative staining intensity of zymogram bands depicts quantitative molecular relationships, and that, in the cat 6-PGD system, the evidence is against differential activity of loci on the two autosomes. Our results (Fig. 1) are consistent with this interpretation.

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2. The cats originally studied and used in genetic analysis were members of a colony established at Rainier School.
3. This mixture is used in preference to one without the inosine when a delay between drawing and laboratory procedures is expected.
4. Electrophoresis was performed in a laboratory of the Department of Medicine, Division of Medical Genetics, Univ. of Washington.
5. Stock buffer contained (per liter) 109 g of tris(hydroxymethyl)aminomethane, 30.9 g of boric acid, 7.6 g of EDTA disodium salt. A 1:20 dilution of stock buffer was used for preparing a gel of 33 g of hydrolyzed starch (Connaught Medical Research Laboratories, Toronto) in 300 ml of diluted buffer. The mixture was heated to 75°C with constant stirring and debubbled by decreased pressure; 0.15 ml of a solution (10 mg/ml) of triphosphopyridine nucleotide was added before pouring the gel in trays (14 by 22 cm). Buffer for the filter-paper contact bridges was stock-diluted 1:7.
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7. Staining solution consisted of a mixture of 10 ml of 0.1*M* tris-HCl buffer, *p*H 8.0; 0.1 ml of 0.1*M* $MgCl_2$; 10.0 ml MTT tetrazolium, 2 mg/ml; 0.5 ml of 6-phosphogluconate, 20 mg/ml; 0.2 ml triphosphopyridine nucleotide, 10 mg/ml; 0.05 ml phenazine methosulfate, 5 mg/ml (Sigma Chemical Co.).
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Aminoaciduria Resulting from Cycloleucine Administration in Man

Abstract. *Cycloleucine (1-aminocyclopentanecarboxylic acid) administration in cancer patients resulted in a reversible marked aminoaciduria consisting predominantly of cystine, ornithine, lysine, and arginine, with only minor elevations of other amino acids. Plasma levels of amino acids were essentially unchanged except for slight decreases in ornithine and lysine. This aminoaciduria is most likely due to a blockage by the drug of the renal tubular reabsorption of cystine, ornithine, lysine, and arginine, resulting in an aminoaciduria of the type seen in cystinuria.*

The nonmetabolizable α-amino acid, 1-aminocyclopentane-1-carboxylic acid (cycloleucine, NSC-1026) has been reported to inhibit growth of several experimental tumors (*1*, *2*). Clinical trials have generally been unimpressive in all patients with the possible exception of those with leiomyosarcoma (*3*). Studies by Sterling and Henderson (*4*) indicated that the drug inhibited incorporation of certain amino acids into proteins of ascites tumors and that the effect was probably due to inhibition of transport of certain amino acids into the tumor cells; however, Berlinguet *et al.* (*5*) presented evidence that cycloleucine inhibited protein synthesis in vivo and in vitro, presumably by interference with the formation of aminoacyl-transfer RNA complexes. Other studies had shown that cycloleucine did not inhibit transamination of amino acids nor did it inhibit their enzymatic oxidation (*6*). The drug was not incorporated into protein and was not further metabolized or degraded in animals (*6*, *7*). Cycloleucine has been used in studies of amino acid transport and has been shown to act competitively in the active transport of several amino acids (*8*). Recently Clark *et al.* (*9*) reported

Table 1. Urinary amino acid levels (micromoles per day) of patients before and during treatment with cycloleucine.

Patient	Cystine		Ornithine		Lysine		Arginine	
	Before	During	Before	During	Before	During	Before	During
E	156	4,748	36	10,150	184	40,845	37	1,720
T	66	5,507	18	2,050	120	8,240	25	2,894
K	158	4,640		1,685	136	8,894		1,458
Z	161	5,200	27	3,520	106	13,100	14	3,500
W	168	5,450	29	2,880	332	5,530	17	1,670
Average (μmole/day)	142	5,109	28	4,057	175	15,322	23	2,248
Average (mg/day)	34	1,228	3.7	536	26	2,240	4.0	392

that growth inhibition by cycloleucine in rats was relieved by force feeding and concluded that cycloleucine does not affect the distribution of a number of amino acids in rat tissues when food intake is maintained.

The present studies were undertaken to evaluate blood and urine levels of cycloleucine and to determine its effect on plasma and urine amino acid levels in patients undergoing clinical trials of cycloleucine in phase II of the Clinical Drug Evaluation Program (CDEP) of the Cancer Chemotherapy National Service Center, National Cancer Institute.

Patients studied were advanced cancer patients receiving cycloleucine under the protocols of the CDEP. The dosage of the drug was 300 mg/kg per day, given intravenously daily until toxic manifestations were observed. Complete 24-hour urine specimens (preserved with thymol or toluene) and fasting blood samples (drawn in EDTA Vacutainers) were collected before starting treatment, during treatment, and at intervals after cessation of drug treatment.

Urine and plasma samples were analyzed for amino acids and other ninhydrin-reactive substances by an automatic amino acid analyzer, by use of the general procedure of Spackman *et al.* (*10*) as well as that of Piez and Morris (*11*) as modified by Coulson *et al.* (*12*). Plasma was deproteinized with sulfosalicylic acid, and homocitrulline was used as an internal standard.

Cycloleucine readily assayed as a peak emerging between leucine and tyrosine. Administration of the drug resulted in the appearance of large peaks of unmetabolized compound in chromatograms of both plasma and urine, and no other new peaks appeared, confirming other reports that the drug is not metabolized (*6, 7*). After several days of drug administration, patients usually excreted about 90 percent of the daily dose in the urine, at which time urine concentrations ranged from 72.4 to 172 mmole per day for the five patients studied most extensively. At that time plasma levels ranged from 3.73 to 10.8 μmole/ml of plasma.

The rate of excretion of drug from the body following cessation of therapy could be approximated by a straight line on a semilogarithmic plot with a half-time of 2.4 days for both plasma and urinary decay rates. This excretion rate is considerably greater than found in rats and mice, where a half-life of 22 days was reported (*13*). This difference in rate of excretion probably is the reason for the lesser toxicity of the drug in man than in rats and mice.

Table 2. Plasma levels of amino acids (micromoles per milliliter of plasma) before and during treatment of patients with cycloleucine. Uncertainties are standard deviations.

Patient	Ornithine		Lysine		Arginine	
	Before	During	Before	During	Before	During
E	0.192	0.144	0.182	0.131	0.136	0.101
T	.174	.101	.230	.101	.067	.101
Z	.083	.080	.197	.117	.072	.096
W	.142	.079	.278	.229	.060	.057
S	.128	.060	.225	.197	.063	.111
Average	0.144 ±0.042	0.093 ±0.032	0.222 ±0.037	0.155 ±0.055	0.080 ±0.032	0.093 ±0.021

Administration of the drug produced a marked aminoaciduria in all patients studied. This aminoaciduria was characterized chiefly by extremely elevated levels of cystine, ornithine, lysine, and arginine, sometimes reaching several hundred times the pre- or post-treatment levels. Typical levels are shown in Fig. 1. Elevated levels of taurine, threonine, serine, valine, tyrosine, and histidine are also apparent but the most marked changes occur in the levels of cystine, ornithine, lysine, and arginine. Following cessation of drug treatment the urinary levels all returned to pretreatment levels. Also shown in Fig. 1 are the plasma levels determined for the period during and after drug administration. These levels were essentially unchanged by drug treatment.

Table 1 shows average urinary excretion values of cystine, ornithine, lysine, and arginine before and during treatment with cycloleucine. The urinary loss of lysine, for example, averaged 2.24 g per day. Plasma levels (Table 2) show decreases in ornithine and lysine but not in arginine.

Since plasma amino acid levels were not elevated, it is possible to rule out an "overflow" type of aminoaciduria. The data suggest an inhibition by cycloleucine of renal tubular reabsorption of specific amino acids. It is of

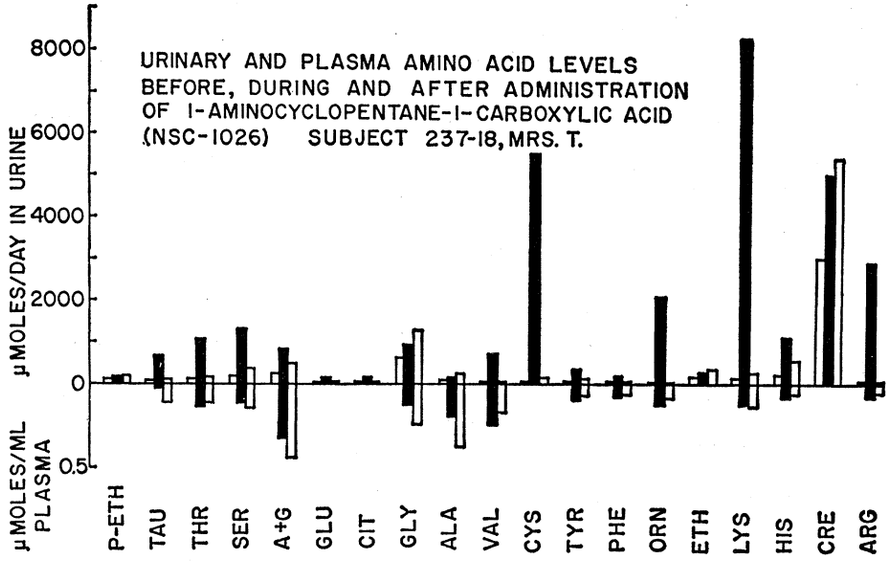

Fig. 1. Urine and plasma amino acid levels before, during, and after administration of cycloleucine to patient 237-18. Values above the horizontal line are urine levels in micromoles per day; values below the horizontal line are plasma levels in micromoles per milliliter of plasma. The solid bars represent levels after 3 days of cycloleucine treatment. The first bar (open) in each triplet represents the level before treatment, and the third bar represents values 21 days after cessation of treatment. Plasma samples before treatment were not available from this particular study. Abbreviations represent, in order of their use, phosphoethanolamine, taurine, threonine, serine, asparagine plus glutamine, citrulline, glycine, alanine, valine, cystine, tyrosine, phenylalanine, ornithine, ethanolamine, lysine, histidine, creatinine, and arginine. Methionine, isoleucine, and leucine were poorly resolved and are not reported, although levels were not strikingly different from normal.

interest that the amino acids most elevated in the urine are also those elevated in cystinuria where a specific genetically controlled defect occurs in the tubular reabsorption of these same amino acids (*14*). Thus, cycloleucine apparently produces a temporary, reversible aminoaciduria of the cystinuria type.

It is tempting to postulate that any antitumor effect of cycloleucine is the result of the large loss of amino acids. However, preliminary studies in rats indicate that aminoacidurias of this magnitude are not observed at drug doses reported to have antitumor effect. Further, attempts at replacing (by feeding or by intraperitoneal injection) a variety of amino acids in treated rats has so far been unsuccessful in preventing the toxicity of the drug, although a recent report (*9*) indicates that normal weight gains in rats given cycloleucine can be achieved by force-feeding of diet. Data were not presented on urine or tissue levels of the basic amino acids found to be affected in the present study, and I am not aware of any previous reports of this type of aminoaciduria resulting from cycloleucine administration.

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Sodium-Potassium Adenosine Triphosphatase: Acyl Phosphate "Intermediate" Shown to be L-Glutamyl-γ-Phosphate

Abstract. *Peptides obtained from pepsin digestion of the phosphorylated and nonphosphorylated forms of a preparation of brain microsomal sodium-potassium–activated adenosine triphosphatase were treated at pH 5.4 with* N-(n-*propyl-2,3-*^{3}*H) hydroxylamine of high specific activity, then separated by column chromatography, and further digested with pronase. A compound isolated in higher amounts from the phosphorylated enzyme than from the nonphosphorylated enzyme migrated with authentic* L-*glutamyl-γ-propylhydroxamate in four chromatographic systems and on electrophoresis on paper at three different pH's. The acyl phosphate "intermediate" in the phosphorylated form of the adenosinetriphosphatase therefore appears to be an* L-*glutamyl-γ-phosphate residue.*

There has been considerable interest in a membrane-bound, ouabain-inhibitable, adenosine triphosphatase that is activated by sodium and potassium. This enzyme is believed to be involved in the coupled transport of Na and K (*1*), but the mechanism of its action is largely unknown. However, there is evidence for a phosphorylated "intermediate" in the overall enzyme reaction (*2*); this is based on the fact that the terminal phosphate of adenosine triphosphate (ATP) is transferred to the protein of the enzyme in the presence of Na and that a dephosphorylation of the protein occurs upon the subsequent addition of K. Both the phosphorylation and dephosphorylation are inhibited by ouabain, which also inhibits the sodium-potassium–activated adenosine triphosphatase. Accumulated evidence (*3, 4*) indicates that the phosphorylation of the protein involves the formation of a high-energy acyl phosphate bond. This conclusion is based on the conversion of the phosphorylated "intermediate" to inorganic phosphate by alkali (*3, 4*) and, more importantly, by hydroxylamine at *p*H 5.4 (*3, 4*) or by purified acyl phosphatase (*3*). We now report the identification of the acyl phosphate residue.

If the acyl phosphate residue is not COOH-terminal, it is most likely to be L-aspartyl-β-phosphate or L-glutamyl-γ-phosphate. Since the instability of these compounds limits extensive purification, we made a derivative of the acyl phosphate with a radioactive hydroxylamine and isolated the resulting hydroxamate. Since hydroxylamine cannot be made radioactive in atoms which do not exchange with water we synthesized *N*-(*n*-propyl-2,3-^{3}H)hydroxylamine (^{3}H PHA) (*5*). The *N*-(*n*-propyl)hydroxylamine was found to react with the ^{32}P-labeled acyl phosphate "intermediate" (*5*). We prepared pepsin digests of the phosphorylated and nonphosphorylated forms of the sodium-potassium–activated adenosine triphosphatase from guinea pig brain (approximately 100 mg of protein for each), using unlabeled ATP (*3*). The lyophilized pepsin digests were dissolved in 1 ml of 0.1*M* ^{3}H-PHA (specific activity 666 mc/mmole) in 0.5*M* acetate buffer (*p*H 5.4) and incubated at room temperature for 30 minutes. All steps were carried out with the phosphorylated and nonphosphorylated forms of the enzyme in parallel under identical conditions. The ^{3}H-PHA–treated pepsin digests of the phosphorylated and nonphosphorylated forms of the enzyme were separated from unreacted ^{3}H-PHA by column chromatography on Bio-Gel P-2 which had been equilibrated with water. A fraction which contained peptides with molecular weights above 600 was collected; after lyophilization, it was chromatographed in bands, by descending chromatography on Whatman No. 3 MM paper in a system consisting of *n*-butanol, ethanol, and water (2:1:1, by volume). A peptide fraction (R_F, 0 to 0.24) was eluted with water and further digested with pronase (0.25 mg per milligram of protein) at *p*H 7.4 for 10 hours at 25° C in the presence of carrier L-glutamyl-γ-propylhydroxamate (γ-GPH) and L-aspartyl-β-propylhydroxamate (β-APH), which we have synthesized (*6*). The standard γ-GPH gave a sharp melting point and a correct elemental analysis; staining of chromatograms with either ninhydrin or ferric chloride indicated a single compound. The pronase digests were chromatographed (descending) at 4°C on Whatman No. 3 MM paper in the *n*-butanol-ethanol-water system. If the acyl phosphate residue in the enzyme incubated in the presence of Na had formed a derivative with ^{3}H-PHA, there should be an increment in radioactivity in any compounds containing this derivative. Increments in radioac-