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-

tivity were seen in the β-APH and γ-GPH peaks as well as in several peptide peaks (Table 1). The radioactivity present in the β-APH and γ-GPH peaks was isolated by preparative chromatography in *n*-butanol-ethanol-water, and further purified by preparative electrophoresis in formic acid at *p*H 2.0. The increment in the β-APH and γ-GPH regions was also seen after electrophoresis at *p*H 2.0 (Table 1). The radioactive material present in the β-APH and γ-GPH peaks isolated by preparative chromatography in *n*-butanol-ethanol-water followed by preparative electrophoresis at *p*H 2.0 was examined in five additional systems (Table 1). The increment coincided with γ-GPH in all systems tested; that is, four chromatographic systems and electrophoresis at three *p*H values (Table 1). There was no increment associated with β-APH in five systems. An example of one of these systems—electrophoresis at *p*H 3.5 for 5 hours at 4000 volts—is shown in Fig. 1. The increment in the β-APH region seen with systems 1 and 2 is presumably due to peptides. Since a radioactive increment, obtained after pronase digestion of ^{3}H-PHA-treated pepsin digests of the phosphorylated and nonphosphorylated forms of the sodium-potassium–activated adenosine triphosphatase, cannot be chromatographically or electrophoretically separated from authentic γ-GPH in a total of seven systems, it is presumably radioactive γ-GPH. This indicates that the acyl phosphate "intermediate" which is present in the phosphorylated form of the sodium-potassium activated adenosinetriphosphatase is an L-glutamyl-γ-phosphate residue.

Table 1. Radioactivities in the β-APH and γ-GPH peaks in various chromatographic and electrophoretic systems. The radioactivities are corrected to the original enzyme preparations. The numbers in parentheses are the increments in the various systems expressed as a percentage above the radioactivity present in nonphosphorylated form of the enzyme. Paper electrophoresis at 4000 volts for varying times, as indicated, was performed (*5*). Electrophoresis in system 2 was with the material in the β-APH and γ-GPH regions isolated in system 1. Electrophoresis in systems 3 and 4 and chromatography in systems 5, 6, and 7 were with the material in the β-APH and γ-GPH regions isolated in system 1 followed by system 2. The positive increment in the β-APH region in systems 1 and 2 is presumably due to peptide. C, chromatography; E, electrophoresis.

System	β-APH region			γ-GPH region		
	Increment		R_F	Increment		R_F
	Count/min	Percent		Count/min	Percent	
1. C in *n*-butanol:ethanol:water (2:1:1 by volume)	196,200	26	0.49	292,680	24	0.56
2. E at *p*H 2.0 for 25 minutes	23,580	8		136,980	48	
3. E at *p*H 3.5 for 3 hours				66,600	59	
4. E at *p*H 5.3 for 3 hours*				73,710	33	
5. C in pyridine:acetic acid:water (10:7:3 by volume)			0.59	76,600	39	0.80
6. C in *t*-butanol:acetic acid:water (70:15:15 by volume)			0.28	65,340	45	0.55
7. C in phenol-ammonia†			0.70	83,160	60	0.81

* β-APH and γ-GPH do not separate at this *p*H. † From (*8*).

The yield of increment running as γ-GPH was only 2 percent of that expected, if all of the acyl phosphate formed in the presence of sodium and determined as described (*3*) had been converted to γ-GPH and degraded completely to γ-GPH on enzymatic digestion. This low yield is primarily due to the fact that the bulk of the increment resisted complete enzymatic digestion and remained with peptides. Furthermore, γ-GPH is fairly unstable, and gradual loss was encountered on repeated chromatography and electrophoresis (10 to 20 percent loss of γ-GPH occurs with each run at 4°C). Although the increment recovered as γ-GPH, as a percentage of blank, was not large, it was always obtained when separate batches of enzyme and ^{3}H-PHA were run through the entire procedure.

Some question has been raised (*7*) as to whether the acyl phosphate is the true intermediate in the reaction of the sodium-potassium–activated adenosine triphosphatase, because hydroxylamine does not appear to inhibit the enzyme. For this negative finding there are several explanations, including the possibility that the acyl phosphate site is not accessible to hydroxylamine in the native enzyme [the reaction with hydroxylamine in the identification of the phosphorylated "intermediate" as an acyl phosphate was carried out either with denatured enzyme or with pepsin digests of denatured enzyme (*3*, *4*)]. In spite of the doubts raised as to whether the acyl phosphate is the true phos-

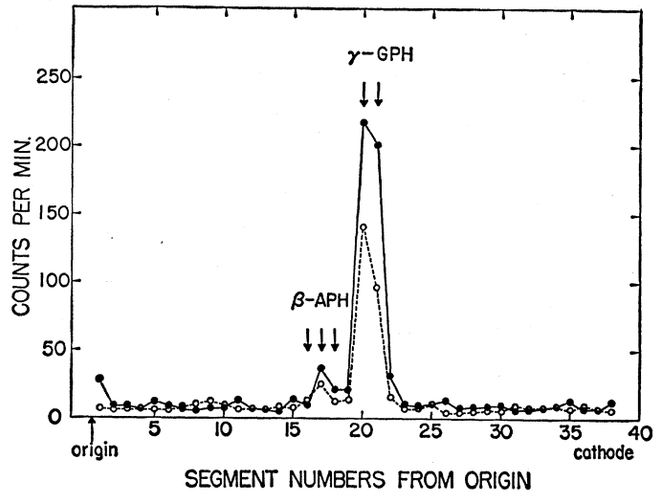

Fig. 1. Electrophoresis at *p*H 3.5 of chromatographically and electrophoretically purified pronase digest of ^{3}H-PHA-treated pepsin digests of phosphorylated and nonphosphorylated forms of a sodium- and potassium-activated adenosine triphosphatase. Electrophoresis for 5 hours was performed as described in Table 1. The phosphorylated (solid line) and the nonphosphorylated (dashed line) forms of the enzyme with sufficient added β-APH and γ-GPH carrier to strain were run in duplicate; one strip was stained with ninhydrin or ferric chloride and the other was assayed for radioactivity as previously described (*5*). The arrows point to the segment numbers in which the carrier β-APH and γ-GPH spots were seen on the adjacent duplicate electrophoretic strip. Each segment was 1 cm wide.

phorylated intermediate in the enzyme reaction, its existence and its response to various transport variables can hardly be questioned.

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Tritiated Thymidine: Effect of Decomposition by Self-Radiolysis on Specificity as a Tracer for DNA Synthesis

Abstract. *Tritiated thymidine undergoes decomposition by self-radiolysis. As shown by beta liquid-scintillation measurement and by autoradiography, the resultant breakdown products did not label DNA, but entered macromolecules, other than nucleic acid, contained in the cytoplasm. Caution should be exercised in using aged tritiated thymidine solutions.*

The use of thymidine labeled with tritium at a high molar specific activity as a tracer in biological research, and especially as a specific label for DNA in cytological investigations, is currently widespread (*1*, *2*). The need to use radiochemically pure thymidine for such research has been stressed (*2*). However, in the case of tritiated thymidine it had not been demonstrated that the products of self-radiation could give misleading results if such products are present in the solutions of the tracer compound. We now report a study of the products of self-radiolysis of tritiated thymidine when it is used as a tracer in cytological investigations.

Two series of experiments were carried out with the following solutions (*3*): (i) thymidine-(*methyl*-^{3}H) freshly prepared at a specific activity of 1000 mc/mmole (radioactive concentration 1 mc/ml); (ii) thymidine-(*methyl*-^{3}H) decomposed by gamma irradiation (2.5 megarad during 1 hour) and having a specific activity of 1000 mc/mmole (radioactive concentration 1 mc/ml); (iii) thymidine-6-^{3}H impurities isolated by preparative chromatography on paper from a solution of the tritiated compound which had been allowed to undergo self-radiolysis over a period of about 3 months. The impurities were separated from the thymidine-6-^{3}H on Whatman No. 1 paper, first in *n*-butanol saturated with water and then in ethyl acetate saturated with phosphate buffer at *p*H 6.0. All impurities were combined, and the solution (radioactive concentration at 0.37 mc/ml) was used in the experiments described below. The specific activity of the tritiated thymidine was 12,000 mc/mmole.

The three samples of tritiated material were analyzed by paper and thin-layer chromatography (*4*), the products being as follows: (i) more than 98 percent thymidine-(*methyl*-^{3}H), and less than 1 percent each of labeled thymine and other decomposition products; (ii) about 5 percent thymidine (*methyl*-^{3}H), about 20-percent-labeled thymine, and about 75 percent other decomposition products; (iii) less than 2 percent thymidine (*methyl*-^{3}H), about 25-percent-labeled thymine, and greater than 73 percent other decomposition products.

At least six major decomposition products were present in each of the solutions ii and iii, but because of the complexity of the mixture and the difficulty in separating the products, their quantitative estimation and that of the thymidine and thymine present is only approximate. However, the products of the self-radiolysis and gamma radiolysis of thymidine in aqueous solution have been fully investigated (*5*).

Tetrahymena pyriformis (amicronucleate strain GL) was grown in 2 percent proteose-peptone and 0.1 percent liver extract; the cultures were synchronized for cell division by a series of heat shocks (*6*). Radioactivity in the amount of 20 μc was added to 20-ml (50,000 cells per milliliter) cultures which were agitated in a 100-ml erlenmeyer flask at 28°C. Label was added 100 minutes after the end of the heat shocks, about 25 minutes after the first maximum division activity (Fig. 1). At this time, a maximum (60 percent) of cells has just entered DNA-synthesis. During the ensuing 150 minutes, 1-ml samples were taken from each flask at 20- or 30-minute intervals. Then, 1 ml of 10 percent trichloroacetic acid (TCA) at 0°C was immediately added, and the samples were kept in an ice bath until suction-filtered onto Whatman No. 3 paper filters. The filters had been previously treated with 5 ml of growth medium mixed with an equal volume of 10 percent TCA

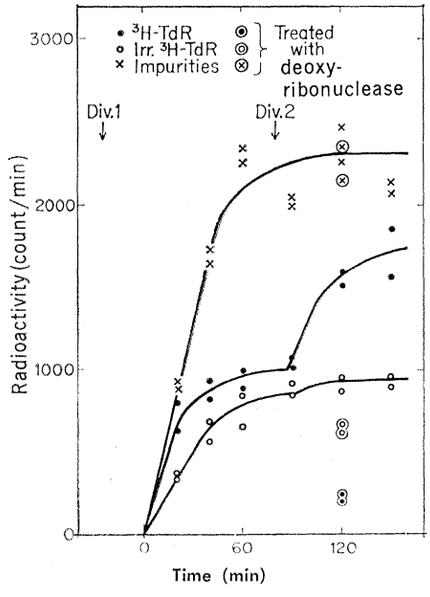

Fig. 1. Beta liquid-scintillation measurements of label incorporation. Freshly made ^{3}H-thymidine gives rapid label incorporation with a leveling off toward 90 minutes; at the maximum of division 2, another period of label incorporation begins. Treatment with deoxyribonuclease at 120 minutes removes almost all the label. Irradiated ^{3}H-thymidine gives a similar, but lower, curve for label incorporation for the first 90 minutes, after which there may be a slight acceleration of new label incorporation. Deoxyribonuclease treatment removes only 30 percent of the label. The impurities give very rapid incorporation for the first 40 minutes and no further incorporation. Deoxyribonuclease treatment did not remove any of this label. With both the irradiated ^{3}H-thymidine and its impurities there is apparently leveling off of incorporation, probably due to the depletion of the label. The counts recorded have been corrected for quenching and background.