Grossberg, A. L., and Pressman, D. (1968), *Biochemistry 7*, 272.
Harden, A., and Norris, D. (1911), *J. Physiol. 42*, 332.
Holmquist, B. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 888.
Latt, S. A., Auld, D. S., and Vallee, B. L. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 1383.
Lipscomb, W. N., Hartsuck, J. A., Reeke, G. N., Jr., Quiocho, F. A., Bethge, P. H., Ludwig, M. L., Steitz, T. A., Muirhead, H., and Coppola, J. C. (1968), *Brookhaven Symp. Biol. 21*, 24.
Pauly, H., and Sauter, H. (1930), *Ber. 63*, 2063.
Riordan, J. F. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 462 Abstr.
Riordan, J. F., and Hayashida, H. (1970), *Biochem. Biophys. Res. Commun. 41*, 122.
Riordan, J. F., Sokolovsky, M., and Vallee, B. L. (1967), *Biochemistry 6*, 3609.
Riordan, J. F., and Vallee, B. L. (1963), *Biochemistry 2*, 1460.
Riordan, J. F., and Vallee, B. L. (1964), *Biochemistry 3*, 1768.
Roy, G. L., Laferriere, A. L., and Edwards, J. O. (1957), *J. Inorg. Nucl. Chem. 4*, 106.
Schulman, R. G., Navon, G., Wyluda, B. L., Douglass, D. C., and Yamane, T. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 39.
Simpson, R. T., Riordan, J. F., and Vallee, B. L. (1963), *Biochemistry 2*, 616.
Smith, E. L. (1949), *Proc. Nat. Acad. Sci. U. S. 35*, 80.
Snoke, J. E., and Neurath, H. (1949), *J. Biol. Chem. 181*, 789.
Snoke, J. E., Schwert, G. W., and Neurath, H. (1948), *J. Biol. Chem. 175*, 7.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Thiers, R. E. (1957), *Methods Biochem. Anal. 5*, 273.
Toi, K., Bynum, E., Norris, E., and Itano, H. A. (1967), *J. Biol. Chem. 242*, 1036.
Vallee, B. L., and Riordan, J. F. (1968), *Brookhaven Symp. Biol. 21*, 91.
Vallee, B. L., Riordan, J. F., Bethune, J. L., Coombs, T. L., Auld, D. S., and Sokolovsky, M. (1968), *Biochemistry 7*, 3547.
Vallee, B. L., Riordan, J. F., and Coleman, J. E. (1963), *Proc. Nat. Acad. Sci. U. S. 49*, 109.
Waldschmidt-Leitz, E. (1931), *Physiol. Rev. 11*, 358.
Werber, M. M., and Sokolovsky, M. (1972), *Biochem. Biophys. Res. Commun. 48*, 384.
Yang, P. C., and Schwert, G. W. (1972), *Biochemistry 11*, 2218.
Yankeelov, J. A., Jr. (1970), *Biochemistry 9*, 2433.
Yankeelov, J. A., Jr., Kochert, M., Page, J., and Westphal, A. (1966), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 25*, 590.
Yankeelov, J. A., Jr., Mitchell, C. D., and Crawford, T. H. (1968), *J. Amer. Chem. Soc. 90*, 1664.

Synthesis and Biological Evaluation of Poly-γ-glutamyl Derivatives of Methotrexate†

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ABSTRACT: Chemical methods for the synthesis of a family of poly-γ-glutamyl derivatives of methotrexate (*N*-[*p*-[((2,4-diamino-6-pteridyl)methyl)methylamino]benzoyl]glutamic acid) which are also metabolites of the drug, are reported. The lability of the 4-amino group to both acid and base initially presented a major obstacle to these syntheses. Attempts were made to prepare the N^2,N^4-ditrifluoroacetyl-N^{10}-methylpteroic acid derivative by reaction of the appropriate pteroic acid analog with trifluoroacetic anhydride under anhydrous conditions. The desired product was obtained in only 10% yield; the remaining was degraded. These problems were circumvented by the discovery of a suitable solvent system for 2,4-diamino-N^{10}-methylpteroic acid (equal volumes of tetrahydrofuran and Me_2SO). Subsequent studies established that protection for the 2- and 4-amino groups was unnecessary, and a simple direct route to these derivatives was at hand. A comparison of the effectiveness of these derivatives with methotrexate as inhibitors of growth of the folate-requiring bacterium *Streptococcus faecium* ATCC 8043 has shown that these derivatives become increasingly less toxic to *S. faecium* as the γ-glutamyl chain is lengthened. A study of the reactivity of hog kidney conjugase (pteroylglutamyl-γ-glutamyl hydrolase) toward the methotrexate derivative with six additional γ-glutamyl residues has been carried out. Column chromatography of an incomplete enzymic (conjugase) hydrolysis mixture suggests varying affinities of the enzyme for different chain lengths with a pronounced preference for the derivative with a total of seven glutamyl residues. These studies also permit the conclusion that the γ-glutamyl residues are hydrolyzed one at a time.

In the course of attempts to identify certain newly discovered and previously unreported metabolites of methotrexate from the rat (Baugh *et al.*, 1973), it became necessary to obtain authentic samples of a variety of poly-γ-glutamyl derivatives of this drug. The approach to their synthesis utilized the solid-phase peptide synthetic procedures employed earlier to

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prepare the poly-γ-glutamyl derivatives of the vitamin folic acid (Krumdieck and Baugh, 1969; Baugh *et al.*, 1970; Baugh and Krumdieck, 1971). Although solution chemistry has now produced these derivatives (Godwin *et al.*, 1972; Meienhofer and Jacobs, 1970), the great economy of time and expense, and the purity of the products obtained by the solid-phase system strongly favored the continued use of these methods (Tamura *et al.*, 1972; Shin *et al.*, 1972; Leslie and Rowe, 1972; Houlihan *et al.*, 1972).

Materials and Methods

The chloromethylated resin was purchased from Schwarz/Mann, Orangeburg, N. Y., as was the Mannex DEAE-cellulose. Cyclo Chemical of Los Angeles, Calif., supplied the *t*-butyloxycarbonyl-L-glutamate α-benzyl ester. Methotrexate was supplied by the National Cancer Institute. The *Pseudomonas* sp. used to remove the glutamyl residue from methotrexate was a gift from Dr. Carl C. Levy of the National Cancer Institute of National Institute of Health. Solvents and other reagent chemicals were obtained as the best quality available from a variety of suppliers and were either dried over P_2O_5 or with molecular sieve prior to use. Tritiated methotrexate was obtained from Amersham/Searle Corp. and was purified by DEAE-cellulose chromatography prior to use.

Preparation of 2,4-Diamino-N^{10}-methylpteroic Acid. The salt medium used by Levy and Goldman (1967) was supplemented with 0.2% methotrexate and inoculated with 1.0 ml of inocula from a log growing culture. The flasks were aerated on a rotary platform shaker for 4 days at 30°. After this period of incubation no methotrexate remained as evidenced by its absence on paper chromatography. Bacteria were removed along with some precipitated 2,4-diamino-N^{10}-methylpteroic acid by centrifugation for 396,000*g* min. The pellet was extracted with 2% Na_2CO_3 and the solution recombined with the supernatant medium. The medium was adjusted to pH 7.0, diluted 1:5 with water, and passed over a large DEAE-cellulose chloride column until the cellulose was loaded to capacity, as determined by visual observation of the adsorption of the yellow product. The column was washed thoroughly with water, then eluted with 0.5 N NH_4OH. The ammoniacal eluate was evaporated under vacuum to pH 7.0. This procedure was repeated until all the medium had been processed. The pooled pH 7.0 concentrates were made 2% with respect to Na_2CO_3. Dry Ice was then added slowly as small chips to avoid freezing, and the precipitate formed was collected on a sintered glass funnel. The solid was dissolved in a minimum amount of 0.1% Na_2CO_3 and the pH lowered to 3.5 with 2 N HCl. The precipitate formed was collected by filtration, washed sequentially with 50% aqueous EtOH, absolute EtOH, and finally with ether, and dried *in vacuo* over P_2O_5. Material obtained in this manner shows a single uv absorbance peak on analytical DEAE-cellulose chromatography and was used directly for subsequent synthetic work.

Preparation of Hog Kidney Conjugase [Pteroylglutamyl-(γ-glutamyl)$_n$-glutamic Acid Hydrolase]. This enzyme was prepared essentially as described by Iwai *et al.* (1964). Briefly, 100 g of frozen hog kidney was minced and homogenized in 300 ml of H_2O for 5 min, at top speed in the Omnimixer. The homogenate was centrifuged for 810,000*g* min. The supernatant was heated to 50° for 10 min and recentrifuged as before. The second supernatant was stirred for 15 min at room temperature with 10 g of wet weight washed Dowex 1-Cl^-, and filtered. The filtrate was assayed using the radioactive method previously described (Krumdieck and Baugh, 1970) with pteroylglutamyl-γ-glutamyl-γ-[U-^{14}C]glutamic acid as substrate. The crude enzyme had a specific activity of 0.20 μmol of [^{14}C]glutamate liberated/mg of protein in 10 min.

DEAE-Cellulose Column Chromatography. A standardized chromatography procedure was employed throughout the work reported here. A column 1.2 × 30 cm of DEAE-cellulose in the chloride form was carefully packed and washed with water. After application of the compound or mixture to be purified (at pH 7.0), the column was again washed with water. All columns were eluted by a linear gradient consisting of 1 l. of 0.005 M phosphate buffer (pH 7.0) in the mixing chamber and 1 l. of 0.5 M NaCl in the same buffer in the reservoir. Fraction volumes were 19 ml in all cases.

Experimental and Results

Synthesis of Poly-γ-glutamyl Peptides. Homoglutamyl peptides bonded through γ-peptide linkage and of varying chain lengths were prepared by the solid-phase procedures previously reported (Krumdieck and Baugh, 1969; Baugh *et al.*, 1970; Baugh and Krumdieck, 1971). After each addition of *t*-butyloxycarbonylglutamate α-benzyl ester to the growing resin-bound peptide chain, and prior to deprotection, unreacted free α-amino groups were terminated by acetylation. This was accomplished by the addition to the CH_2Cl_2 washed resin of 5 ml of triethylamine in 30 ml of CH_2Cl_2, and after mixing, 3 ml of acetic anhydride/mequiv of starting glutamyl resin-ester was added. Reaction was allowed to proceed with mixing, for 2 hr. Acetylation was followed by washing with CH_2Cl_2, selective removal of the *t*-butyloxycarbonyl group with 20% CF_3COOH in CH_2Cl_2, neutralization with triethylamine, and the addition of *t*-butyloxycarbonylglutamyl α-benzyl ester as the mixed anhydride formed with isobutyl chloroformate. In the mixed anhydride formation, *N*-methylmorpholine was used as acid acceptor. All reactions were carried out on 1.0 mmol of initial resin-ester. A single solvent, CH_2Cl_2, was employed throughout. Thus, the family of γ-glutamyl α-benzyl γ-resin-esters of any desired chain length were prepared.

Coupling of 2,4-Diamino-N^{10}-methylpteroic Acid. Although a great deal of experimentation was devoted to the preparation of N^2,N^4-ditrifluoroacetyl-N^{10}-methylpteroic acid as a means of solubilizing and protecting this compound to permit its use in anhydrous organic solvents such as dimethylformamide, degradation of the pteroyl derivative necessitated a different approach. When 2,4-diamino-N^{10}-methylpteroic acid was reacted with trifluoroacetic anhydride under conditions previously employed to prepare N^2,N^{10}-ditrifluoroacetylpteroic acid (Krumdieck and Baugh, 1969; Plante *et al.*, 1967; DeGraw *et al.*, 1965), and the reaction mixture was chromatographed on DEAE-cellulose, three products were obtained. Although no rigorous attempt was made to identify the degradation products, clearly one of them was a substituted *p*-aminobenzoic acid derivative, as determined by its uv spectrum and cochromatography with authentic *p*-(methylamino)-benzoic acid. These results suggested that trifluoroacetylation was occurring at N^{10}, producing an unstable quaternary ammonium ion. Subsequent attack of the trifluoroacetate anion at C_9 results in the cleavage of the C_9–N_{10} bond to form *N*-trifluoroacetyl-*p*-(methylamino)benzoic acid and the trifluoroacetylated pteridine. The proposed mechanism for these reactions may be seen in Figure 1. Please see Discussion for further comments on this point. These problems were resolved by the discovery that 2,4-diamino-N^{10}-methylpteroic acid is soluble

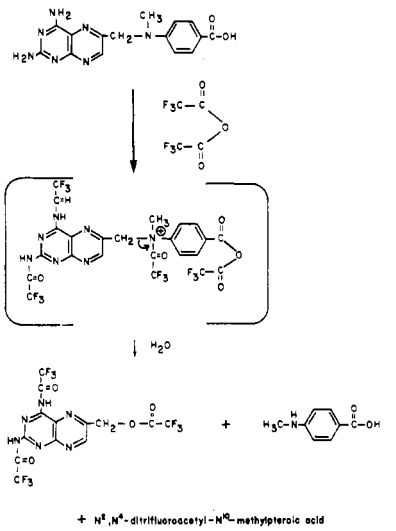

FIGURE 1: Proposed mechanism for the decomposition of 2,4-diamino-N^{10}-methylpteroic acid upon trifluoroacetylation.

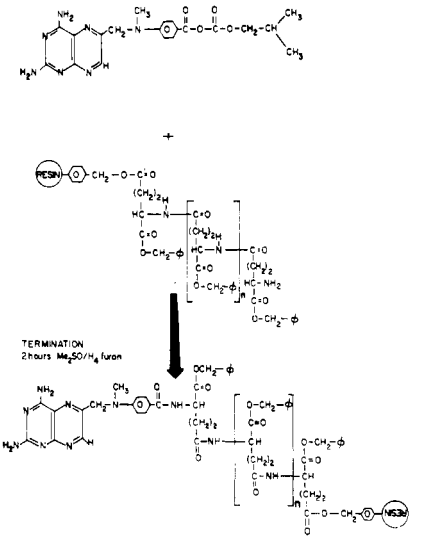

FIGURE 2: Schematic representation of the termination of the resin-bound γ-glutamyl polypeptide by the coupling of the mixed anhydride of 2,4-diamino-N^{10}-methylpteroic acid and isobutyl chloroformate.

in a mixture of equal volumes of anhydrous tetrahydrofuran and Me_2SO. The pteroic acid analog, carefully dried over P_2O_5 *in vacuo* at 75°, was dissolved by the addition of 25 ml of Me_2SO with warming. When the solid had dissolved, 25 ml of tetrahydrofuran was added and the solution chilled to 0° in an ice bath. Then 1.25 equiv of *N*-methylmorpholine was added, followed by 1.0 equiv of isobutyl chloroformate. After 15 min to permit mixed anhydride formation to occur, the solution was added to the desired resin-bound peptide and the reaction mixed at room temperature for 2 hr.

Cleavage from the Resin and Final Deprotection. The completed resin-bound products were washed sequentially with the tetrahydrofuran–Me_2SO solution, CH_2Cl_2, and finally with *p*-dioxane. The solid was transferred to a 150-ml screw cap glass centrifuge tube and 100-ml of a mixture of equal volumes of *p*-dioxane and degassed 2 N NaOH were added. The mixture was vigorously shaken for 1 hr by hand, then placed in a 50° water bath for 20 min with intermittent shaking. The resin was removed by filtration and washed on the funnel. The combined supernatant and washings were immediately neutralized on the pH meter.

This solution at pH 7.0 was diluted 1:10 with water and its purity ascertained by chromatography on DEAE-cellulose as described. The crude products were consistently found to be pure insofar as single peaks were eluted from the DEAE columns. Yields were calculated spectrophotometrically (Seeger *et al.*, 1949) and ranged from 35 to 40% calculated on the basis of initial resin-ester. The termination of the resin-bound peptides by the coupling of 2,4-diamino-N^{10}-methylpteroic acid is schematically summarized in Figure 2.

Microbiological Activity of Certain Poly-γ-glutamyl Derivatives of Methotrexate. The amounts of various glutamyl derivatives of methotrexate required to inhibit the growth of *Streptococcus faecium* ATCC 8043 by 50% are summarized in Table I. These assays were performed using Difco folic acid assay medium supplemented with 2.5×10^{-9} g of folic acid/10-ml assay tube, in duplicate. The assay tubes, complete except for inhibitor, were autoclaved 10 min at 15 psi. After the tubes were cooled, the inhibitors, sterilized by Millipore filtration, were added aseptically prior to inoculation.

Enzymatic Hydrolysis of 2,4-Diamino-N^{10}-methylpteroyl-glutamyl-(γ-glutamyl)$_5$-γ-glutamic Acid [MTX(G_6)]. Sufficient enzyme to hydrolyze 6.5 μmol of [U-^{14}C]glutamate from the radioactive substrate in 30 min at 37° was incubated with 10 μmol of MTX(G_6) at pH 4.5 in 0.5 M acetate buffer. At the end of the incubation period, the reaction was terminated with 10% CCl_3COOH and the protein removed by centrifugation The supernatant was adjusted to pH 7.0 and diluted 1:10 with water. [^{3}H]Methotrexate (300,000 total cpm), (9 μg) was added as marker and the reaction mixture purified on DEAE-cellulose as described. The elution pattern may be seen in Figure 3.

Discussion

The marked instability of the N^{10}-methylpteroic acid analog to trifluoroacetylation, the lack of necessity for protection of the amino functions at positions 2 or 4, and the fortuitous discovery of a suitable solvent system for 2,4-diamino-N^{10}-methylpteroic acid have led to a simple chemical route to a large number of new analogs of methotrexate. Among these derivatives are recently discovered metabolites of the drug (Baugh *et al.*, 1973).

Substantiation for the mechanism proposed to account for the degradation of 2,4-diamino-N^{10}-methylpteroic acid upon trifluoroacetylation, in addition to the detection of *p*-(methyl-

TABLE I: Inhibition of Growth of *Streptococcus faecium* ATCC 8043 by Derivatives of Methotrexate (MTX) with Increasing Numbers of γ-Glutamyl Residues.

Compound	Amt Reqd for 50% Inhibn of Growth[a] g/Tube	μmol/Tube
MTX	3×10^{-10}	6.6×10^{-7}
MTX(G_1)	4×10^{-9}	6.9×10^{-6}
MTX(G_2)	1×10^{-6}	1.4×10^{-3}
MTX(G_6)	9×10^{-6}	7.3×10^{-3}

[a] Each assay tube contained 2.5×10^{-9} g of folic acid.

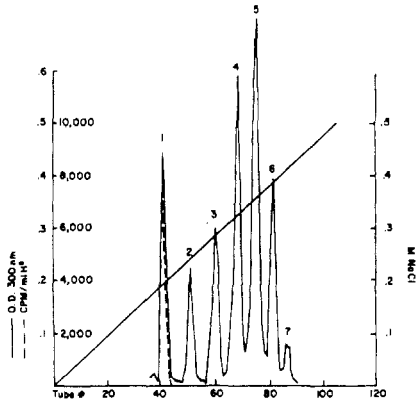

FIGURE 3: Elution pattern from a standard DEAE-cellulose column of an incomplete enzymic hydrolysis reaction mixture. Initial substrate was 10 μmol of MTX(G_6). The broken line (- - - - - -) under peak 1 indicates radioactivity from [^{3}H]MTX added as marker; (——) indicates absorption at 300 nm. See *text* for additional details. The peak numbers also correspond to the *total* number of glutamyl residues for each compound, *e.g.*, MTX(G_6) = 7 total glutamyl residues. Control experiments, less enzyme, lead to quantitative recovery of the initial substrate, MTX(G_6).

amino)benzoic acid, may also be derived from related trifluoroacetylation reactions. For example, acetylation of 2,4-diamino-10-deazapteroic acid, an analog which could not form the quaternary ammonium intermediate, proceeds in excellent yield to the corresponding N^2,N^4-ditrifluoroacetyl derivative. The synthesis of N^2-acetyl-N^{10}-trifluoroacetyl-8-deazapteroic acid has also been accomplished (J. I. DeGraw, personal communication). Samples of N^2-acetyl-N^{10}-trifluoroacetyl-8-deazapteroic acid were received from Dr. DeGraw and converted to the corresponding folic acid derivatives in good yield. These acetylated derivatives have proven to be highly suitable for incorporation into the solid phase peptide synthesis system, and 2,4-diamino-10-deazapteroic acid may be deprotected without significant deamination at the 4 position.

The base cleavage and final deprotection of the completed peptides, in the light of the known lability of the 4-amino group, required special attention to assure the correctness of the structural assignments in the final products. The small scale of the syntheses reported, and the difficulties in obtaining accurate elemental analyses of pteridines in general, required other approaches to prove that 4-deamination does not occur. Methotrexate and N^{10}-methylfolic acid are well separated by the DEAE-cellulose chromatographic methods employed here and may be differentiated spectrophotometrically (Seeger *et al.*, 1949). Therefore the close agreement between the radioactivity and uv absorbance for methotrexate after enzymatic hydrolysis (see Figure 3, peak 1) indicates no loss of the 4-amino group in these procedures. These findings have been confirmed by the synthesis of methotrexate *per se* and comparison of the synthetic product with authentic methotrexate. No N^{10}-methylfolic acid was detected.

Early in these studies it was felt that the unprotected 2- and 4-amino moieties in the pteroic acid analog would react with the isobutyl chloroformate and that for optimum yields in the mixed anhydride forming steps a minimum of 3 equiv of isobutyl chloroformate would be required. Subsequent studies did not substantiate this point. Indeed, the use of 3 equiv of isobutyl chloroformate produced no coupling of the pteroic acid analog to the resin-bound peptide, presumably because of the reaction of the excess with the free α-amino groups.

Previous reports from this laboratory (Baugh *et al.*, 1970) indicated folic acid with one additional γ-glutamyl residue was equally active for *S. faecium* as folic acid *per se*, and that the triglutamate (pteropterin) was inactive, presumably because of the lack of penetration of the cell. The results reported here regarding the inhibition of bacterial growth suggests that other factors must be considered. Since this organism does not possess enzymes capable of hydrolyzing γ-glutamyl residues, these results must be explained by either reduced penetration with increasing numbers of glutamyl residues or reduced inhibition of dihydrofolic reductase by the longer chain length derivatives. The finding that thymidylate synthetase from a methotrexate-resistant strain of *Lactobacillus casei* is potently inhibited by both folyl- and homofolyl-poly-γ-glutamates suggests a third alternative explanation (Kisliuk, *et al.*, 1971; Y. Gaumont, C. M. Baugh, and R. L. Kisliuk, in preparation).

It was reported earlier that conjugase from human liver shows a distinct preferential reactivity toward substrates of longer chain lengths. Thus the action of the enzyme toward the pteroyltriglutamate was completely inhibited in the presence of equimolar concentrations of the pteroylpentaglutamate, until the longer chain lengths were hydrolyzed (Baugh and Krumdieck, 1971). The results reported here indicate a preferential reactivity toward the initial substrate with a total of seven glutamyl residues, and this substrate was very nearly used up. The disproportionate accumulation of products with a total of four and five glutamates would suggest that these derivatives are not as good substrates as those with seven or six. These observations assume the presence of a single γ-glutamyl peptidase in the reaction mixture. Obviously, the kinetics employing six substrates with continuously varying concentrations are too complex to unravel. Perhaps this problem could be approached by studying initial velocities and determining K_m for each individual substrate. This experiment demonstrates the fact that γ-glutamyl residues are hydrolyzed sequentially by the hog kidney conjugase, and that the family of polyglutamyl derivatives of methotrexate can be cleanly separated by ion-exchange chromatography.

The isolation and identification of poly-γ-glutamyl derivatives as natural metabolites of methotrexate in the rat and their detection in red blood cells from a patient suffering from acute leukemia prompted the studies herein reported (Baugh *et al.*, 1973). The availability of these metabolites by synthesis will permit their study as chemotherapeutic agents and conceivably could provide an understanding of the pharmacological role of these metabolites.

Acknowledgment

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References

Baugh, C. M., and Krumdieck, C. L. (1971), *Ann. N. Y. Acad. Sci. 186*, 7.

Baugh, C. M., Krumdieck, C. L., and Nair, M. G. (1973), *Biochem. Biophys. Res. Commun. 52*, 27.

Baugh, C. M., Stevens, J. C., and Krumdieck, C. L. (1970), *Biochim. Biophys. Acta 212*, 116.

DeGraw, J. I., Marsh, J. P., Jr., Acton, E. M., Crews, O. P., Mosher, C. W., Fujiwara, A. N., and Goodman, L. (1965), *J. Org. Chem. 30*, 3404.

Godwin, H. A., Rosenberg, I. H., Ferenz, C. R., Jacobs,

P. M., and Meienhofer, J. (1972), *J. Biol. Chem. 247*, 2266.
Houlihan, C. M., Scott, J. M., Boyle, P. H., and Weir, D. G. (1972), *Gut 13*, 189.
Iwai, K., Juttner, P. M., and Toennies, G. (1964), *J. Biol. Chem. 239*, 2365.
Kisliuk, R. L., Gaumont, Y., Krumdieck, C. L., and Baugh, C. M. (1971), *Pharmacologist 13*, 208.
Krumdieck, C. L., and Baugh, C. M. (1969), *Biochemistry 8*, 1568.
Krumdieck, C. L., and Baugh, C. M. (1970), *Anal. Biochem. 35*, 123.
Leslie, G. I. and Rowe, P. B. (1972), *Biochemistry 11*, 1696.
Levy, C. C. and Goldman, P. (1967), *J. Biol. Chem. 242*, 2933.
Meienhofer, J., and Jacobs, P. M. (1970), *J. Org. Chem. 35*, 4137.
Plante, L. T., Crawford, E. J., and Friedkin, M. (1967), *J. Biol. Chem. 242*, 1466.
Seeger, D. R., Cosulich, D. B., Smith, J. M., Jr., and Hultquist, M. E. (1949), *J. Amer. Chem. Soc. 71*, 1753.
Shin, Y. S., Buehring, K. U., and Stokstad, E. L. R. (1972), *J. Biol. Chem. 247*, 7266.
Tamura, T., Buehring, K. U., and Stokstad, E. L. R. (1972), *Proc. Soc. Exp. Biol. Med. 141*, 1022.

Interaction of Actin with Analogs of Adenosine Triphosphate†

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ABSTRACT: We have studied the binding to actin of an analog of adenosine triphosphate, adenylyl imidodiphosphate (AMP-PNP), in which a NH replaces the terminal bridge oxygen. This analog will bind to a number of ATPases without being hydrolyzed. The ability of AMP-PNP to bind to G-actin was assayed by three methods: competitive binding with a spin-labeled nucleotide, protection against denaturation, and direct isolation and characterization by chromatography of the nucleotide bound to polymerized actin. All three methods indicated that AMP-PNP will bind to actin, and that the affinity constant was severalfold weaker than that of ADP. G-actin which contains bound AMP-PNP can polymerize to F-actin without the nucleotide dephosphorylation which commonly occurs during polymerization. G-actin·AMP-PNP polymerizes at the same rate as G-actin·ATP, and both polymerize faster than G-actin·ADP. Sonication of the F-actin polymer showed that the stability of its structure was not altered when the bound ADP was replaced with AMP-PNP. The above results suggest that the energy of dephosphorylation, liberated during the polymerization of actin, is not used to facilitate the polymerization.

Actin is a globular protein of molecular weight 45,000 which is capable of polymerizing into a double-stranded polymer. This polymer forms the backbone of the thin filaments in a muscle fiber, and its interaction with myosin in the presence of ATP is known to produce the force of contraction. The actin monomer, known as G-actin, binds one nucleotide and one divalent cation. In G-actin the nucleotide can be either a di- or triphosphate and rapidly exchanges with unbound nucleotides in the medium. The nucleotide of F-actin exchanges with external nucleotides at a rate which is many orders of magnitude smaller than the rate seen in the case of G-actin. When G-actin with bound ATP is polymerized to F-actin, the ATP is dephosphorylated; and the nucleotide found in the polymer, or in the thin filament of a muscle, is always ADP. Although it has been the center of a number of studies (for a recent review, see Oosawa and Kasai, 1971), the only known role of the actin nucleotide is to stabilize the structure of G-actin. G-actin, but not F-actin, will denature quickly if the nucleotide is removed.

When G-actin·ATP polymerizes, the dephosphorylation of the ATP should provide about 7 kcal/mol of useful energy. There have been two popular theories about the actin nucleotide and the fate of the energy of dephosphorylation. One theory contends that the nucleotide is used during muscle contraction, while the other holds that it is used in the regulation of the polymerization process, possibly to stabilize the polymer. The contraction hypothesis rests largely on the data of Szent-Gyorgyi and Prior (1966) who showed that some exchange of the F-actin nucleotide occurred when actin interacted with myosin in the presence of ATP to superprecipitate. However, the significance of this result has been questioned by other workers who claim that the nucleotide exchange is not related to superprecipitation (Moos *et al.*, 1967). It has also been shown that nucleotide-free actin can interact with myosin, both in the activation of myosin ATPase and in superprecipitation (Barany *et al.*, 1966). The second theory, which holds that the nucleotide is present to regulate or promote polymerization, is very attractive since the nucleotide dephosphorylation occurs during polymerization. Although the nucleotide is not necessary for polymerization as shown by the fact that nucleotide-free G-actin can form a polymer (Barany *et al.*, 1966), G-actin·ATP polymerizes faster than G-actin·ADP (Hayashi and Rosenbluth, 1960). This result has been interpreted as evidence that the energy of the ATP dephosphorylation is used to facilitate the polymerization.

To investigate the role of the actin nucleotide further we have used an analog of ATP in which an NH grouping re-

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