

On the *Streptomyces albus* G DD Carboxypeptidase Mechanism of Action of Penicillin, Vancomycin, and Ristocetin*

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ABSTRACT: The activity of the D-alanyl-D carboxypeptidase from the penicillin-resistant *Streptomyces albus* G is not or very little affected by penicillins and related antibiotics. The molecular basis for the mechanism of action of penicillin is discussed.

The *Streptomyces albus* G D-alanyl-D carboxypeptidase

appears as a model for the study of a mechanism of penicillin resistance that does not involve the enzymatic degradation of the antibiotic. Vancomycin and ristocetin are shown to inhibit the hydrolysis of sensitive peptides by the *Streptomyces albus* G D-alanyl-D carboxypeptidase and the mechanism of inhibition is discussed.

It has been proposed that the structural analogy between penicillin and C-terminal acyl-D-alanyl-D-alanine of those peptides that are involved in transpeptidation during the last step of bacterial wall peptidoglycan biosynthesis, is the molecular basis for its antibacterial action (Tipper and Strominger, 1965). The idea is further supported by the finding that the action of the DD carboxypeptidase from *Escherichia coli* upon peptides ending in D-alanyl-D-alanine sequence, is competitively inhibited by penicillins and related antibiotics at very low concentrations (Izaki and Strominger, 1968). In a survey carried out on 65 local wild strains (Leyh-Bouille *et al.*, 1970a), only two *Streptomyces* were found sensitive to low concentrations (10 µg/ml) of penicillin G. The penicillin-resistant *Streptomyces albus* G secretes a D-alanyl-D carboxypeptidase (Ghuysen *et al.*, 1970) which appears to be the transpeptidase that has undergone solubilization (Leyh-Bouille *et al.*, 1970b). The main purpose of the present paper is to describe the influence exerted by penicillins on the isolated *S. albus* G enzyme. Since vancomycin and ristocetin are known to combine with peptides ending in acyl-D-alanyl-D-alanine (Perkins, 1969), the influence exerted by these antibiotics on the hydrolysis of such peptides by the *S. albus* G enzyme is also hereby reported.

Materials and Methods

Enzyme. The DD carboxypeptidase from *S. albus* G, a preparation containing 100,000 units/mg, was used (Ghuysen *et al.*, 1970).

Substrates. The following peptides were used (Leyh-Bouille *et al.*, 1970b): (1) $N^{\alpha}(\beta$ -1,4-*N*-acetylglucosaminyl)-*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl); N^{ϵ} (pentaglycyl)-L-lysyl-D-alanyl-D-alanine from *Staphylococcus aureus*

(referred to as disaccharide pentapeptide pentaglycine); (2) UDP-*N*-acetylmuramyl-L-alanyl- γ -D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanyl-D-alanine (= UDP-pentapeptide with *meso*-DAP); (3) N^{α},N^{ϵ} -bisacetyl-L-lysyl-D-alanyl-D-alanine; (4) N^{α},N^{ϵ} -bisacetyl-L-lysyl-D-alanyl-D-leucine; (5) the *Butyribacterium rettgeri* peptide dimer (see Table I, 5 in Leyh-Bouille *et al.*, 1970b); (6) the *E. coli* peptide dimer (see Table I, 9 in Leyh-Bouille *et al.*, 1970b); (7) the *Bacillus subtilis* peptide dimer (see Table I, 10, in Leyh-Bouille *et al.*, 1970b); and (8) the *Corynebacterium poinsettiae* disaccharide peptide dimer (see Table I, G, in Leyh-Bouille *et al.*, 1970b).

Antibiotics. Ristocetin (Spontin) was a gift from Abbott Laboratories, Chicago, Ill., and from the International Center of Information on Antibiotics (I.C.I.A., Liège, Belgium). Vancomycin, cephalothin or 7-(thiophene-2-acetamido)cephalosporanic acid, and penicillin N or (D-4-amino-4-carboxybutyl)penicillanic acid were gifts from I.C.I.A. Cephalosporin C or (D-4-amino-4-carboxybutyl)-7-aminocephalosporanic acid was a gift from Dr. E. P. Abraham, University of Oxford, England. Oxacillin or (5-methyl-3-phenyl-4-isoxazolyl)-6-aminopenicillanic acid was a gift from Bristol Benelux S.A., Brussels. Sodium benzylpenicilloate was prepared as described earlier (Perkins, 1969). Ampicillin or α -aminobenzyl-6-aminopenicillanic acid and penicillin G were commercial compounds. Penicillin G, penicillin N, and ampicillin are sensitive to penicillinase. Cephalothin, cephalosporin C, and oxacillin are resistant to penicillinase. Penicillin N and cephalosporin C have different nuclei but identical D-aminopimelic acid side chains.

Assay of Antibiotic Sensitivity of *S. albus* G. Plates of nutrient broth agar with plugs of agar containing antibiotics (cylinder technique: 100 µl of antibiotic solutions) were seeded with conidia and incubated at 25°. After 48 hr, the minimal concentrations of antibiotics that provided a visible zone of growth inhibition were recorded. Essentially the same results were obtained when a suspension of actively growing mycelium was used instead of conidia and when the antibiotics were allowed to diffuse through the agar gel for 8 hr before the plates were seeded.

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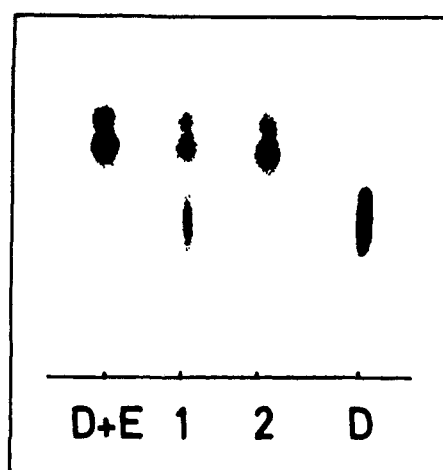


FIGURE 1: Effects of vancomycin and ristocetin on carboxypeptidase action. Substrate: *B. rettgeri* peptide dimer (Table V, 5). Conditions of incubation: peptide dimer (15 μ moles) was incubated for 8 hr at 37°, in 0.01 M Veronal buffer, pH 9 (final volume, 30 μ l), with 10 μ g of enzyme in the absence and in the presence of antibiotics. Mixtures were chromatographed on silica gel thin layer using solvent isobutyric acid-1 N NH_4OH (5:3 v/v); detection = ninhydrin; D = untreated dimer; D + E = carboxypeptidase-degraded dimer; 1 and 2 = D + E, incubated in the presence of ristocetin (molar ratio to peptide: 1 to 1) or of vancomycin (molar ratio to peptide: 2 to 1); 2 = D + E, incubated in the presence of vancomycin (molar ratio to peptide: 1 to 1).

Experimental Section

Effects of Antibiotics on Growth of *S. albus* G. *Streptomyces* growth inhibition requires at least 500 μ g/ml of cephalothin, 1000 μ g/ml of penicillin G, and 10,000 μ g/ml of oxacillin, ampicillin, and cephalosporin C (Table I). No growth inhibition was observed with penicillin N at dose levels of 10,000 μ g/ml. Growth inhibition was observed with

TABLE I: Growth of *Streptomyces albus* G in the Presence of Antibiotics.

Concentration (μ g/ml)	Peni- cillin N	Ampi- cillin and Cephalo- sporin C	Oxa- cillin	Peni- cillin G	Ce- phalo- thin	Risto- cetin or Vanco- mycin
10,000	+	0 ^b	0	0	0	0
7,500	+		±	0	0	0
1,000	+	+	+	0	0	0
750	+	+	+	+	0	0
500	+	+	+	+	±	0
100	+	+	+	+	+	0
50	+	+	+	+	+	0
10	+	+	+	+	+	0

^a + = concentrations of antibiotics providing no visible zone of growth inhibition. ^b 0 = concentrations of antibiotics providing a visible zone of growth inhibition.

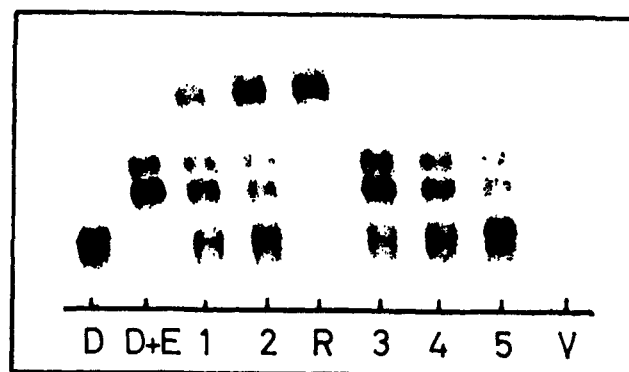


FIGURE 2: Effects of vancomycin and ristocetin on carboxypeptidase action. Substrate: *C. poinsettiae* disaccharide peptide dimer. Conditions of incubation: dimer (19 μ moles) was incubated for 90 min at 37° in Tris- Mg^{2+} buffer pH 7.5 (final volume, 35 μ l), with 0.9 μ g of enzyme in the absence and in the presence of antibiotics. Mixtures were chromatographed on silica gel thin layer using the same solvent as in Figure 1; detection = ninhydrin; D = untreated dimer; D + E = carboxypeptidase-degraded dimer; 1 and 2 = D + E, incubated in the presence of ristocetin (molar ratio to peptide: 1 to 1 and 2 to 1, respectively); 3, 4, and 5 = D + E, incubated in the presence of vancomycin (molar ratio to peptide: 1 to 1, 2 to 1, and 4 to 1, respectively); R = ristocetin alone (40 μ moles); V = vancomycin alone (80 μ moles).

vancomycin and with ristocetin at dose levels of 10 μ g/ml. No significant differences were observed according to the techniques used for the tests (see Materials and Methods).

Effects of penicillins and related antibiotics on carboxypeptidase activity are shown in Table II. Under identical conditions but in the absence of antibiotics, the enzyme concentrations and the incubation times were those required for the reactions almost to reach completion (yield 80–100%). Penicillin G and related antibiotics exerted no or very little inhibition of the D carboxypeptidase activity. Controls were run in which penicillin G (2 and 20 units in 50 μ l of 0.01 M Veronal buffer, pH 8, final volume) was incubated for 1 hr, at 37°, with 1.8–16 μ g of carboxypeptidase. The reaction mixtures were then diluted with 150 μ l of water. The growth inhibitory activities of these solutions upon *S. aureus* strain Y were found to be identical with those of equivalent dilutions of untreated penicillin G, thus demonstrating that the carboxypeptidase is not a penicillinase-like enzyme.

Effects of Vancomycin and Ristocetin on Carboxypeptidase Action. Vancomycin and ristocetin combine with acyl-D-alanyl-D-alanine peptides forming complexes in which peptide and antibiotic occur in the molar ratio of 1 to 1 (Perkins, 1969). In these estimations, one unit of Vancomycin that contains one residue each of glucose, aspartic acid, and N-methylleucine was taken as one molecule (mol wt 1600). Similarly, one unit of ristocetin that contains one residue of arabinose was taken as one molecule (mol wt 4000). As shown in Table III, the amounts of antibiotics required to inhibit hydrolysis by 50% depend not only upon the nature and the amount of the peptide with which the antibiotics combine, but also upon the amount of enzyme present in the incubation mixtures and upon the incubation time. Vancomycin and ristocetin inhibited the enzyme action not only upon acyl-D-alanyl-D-alanine peptides, but also upon C-

TABLE II: Effects of Penicillins and Related Antibiotics on DD Carboxypeptidase.

Substrate (15 μ moles)	Buffer (30 μ l)	Enzyme (μ g)	Incubation Time (hr)	Antibiotic	Molar Ratio of Antibiotic to Peptide	Inhibn (%)
Disacch-pentapeptide-pentaglycine ^c	Veronal ^a	4	1	Cephalothin	15:1	50
	Veronal ^a	4	1	Penicillin G	70:1	50
	Veronal ^a	4	1	Oxacillin	70:1	0
	Veronal ^a	4	1	Penicillin N	70:1	0
	Veronal ^a	4	1	Ampicillin	70:1	0
	Veronal ^a	4	1	Benzylpenicilloate	70:1	0
UDP-pentapeptide ^c (with meso-DAP)	Veronal ^a	4	1	Penicillin G	10:1	0
	Tris-Mg ²⁺ ^b	1.9	1.5	Penicillin G	10:1	10
	Tris-Mg ²⁺ ^b	1.9	1.5	Penicillin G	50:1	40
<i>N</i> ^{α} , <i>N</i> ^{ϵ} -Bisacetyl-L-lysyl-D-alanyl-D-alanine ^c	Tris-Mg ²⁺ ^b	0.8	0.25	Penicillin G	10:1	10
	Tris-Mg ²⁺ ^b	0.8	0.25	Penicillin G	50:1	40
<i>B. rettgeri</i> peptide dimer ^d	Veronal ^a	10	8	Penicillin G	10:1	0
<i>E. coli</i> peptide dimer ^e	Veronal ^a	1.5	1	Penicillin G	1:1	0

^a 0.01 M Veronal buffer, pH 9. ^b 0.02 M Tris-HCl buffer, pH 7.5–0.002 M MgCl₂. ^c Extent of hydrolysis was estimated on the basis of the D-alanine liberated. ^d Extent of hydrolysis was estimated by silica gel chromatography in solvent I. ^e Extent of hydrolysis was estimated on the basis of mono-N-terminal groups of diaminopimelic acid exposed.

terminal D-alanyl-D-R linkages where R is a group of large size. Table III illustrates the action of ristocetin on DD carboxypeptidase activity upon *N* ^{α} ,*N* ^{ϵ} -bisacetyl-L-lysyl-D-alanyl-D-leucine. Moreover, ristocetin and vancomycin inhibited the enzymatic hydrolysis of the *B. rettgeri* peptide dimer and of the *C. poinsettiae* disaccharide peptide dimer into monomers by the carboxypeptidase (Figures 1 and 2). Hydrolysis of the *B. subtilis* heptapeptide diamide into monomers, however, was found not to be inhibited by ristocetin at molar ratio to peptide of 2 to 1, under the following conditions (17 μ moles of heptapeptide; Tris-Mg²⁺ buffer, pH 7.5; final volume 40 μ l; 3.5 μ g of enzyme; 8 hr at 37°).

Discussion

Mechanism of Action of Penicillin. It has been shown that both DD carboxypeptidases from *S. albus* G and from *E. coli* probably have identical catalytic activities (Ghuysen *et al.*, 1970). The *E. coli* DD carboxypeptidase, however, is competitively inhibited by penicillins and related antibiotics at very low concentrations (Izaki and Strominger, 1968; Bogdanovsky *et al.*, 1969) whereas, by contrast, the *Streptomyces* carboxypeptidase is very resistant to these antibiotics. An analogy between penicillins and the conformation of acyl-D-alanyl-D-alanine accepted by the transpeptidase has been invoked, to explain the molecular basis of the antibacterial action of penicillins (Tipper and Strominger, 1965), and has been extended to explain the sensitivity of the *E. coli* DD carboxypeptidase to these antibiotics (Izaki and Strominger, 1968). The isolation from *S. albus* G of a carboxypeptidase which hydrolyzes peptides ending in D-alanyl-D-alanine, but which does not recognize penicillins as structural analogs show that, at least, this analogy is

not universal among bacteria. The study of the substrate requirements of the *Streptomyces* enzyme has shown that both the C-terminal D-alanyl-D sequence and the side chain of the preceding L-amino acid residue in the peptides are important for its activity (Leyh-Bouille *et al.*, 1970b). To all appearances, the *E. coli* enzyme exhibits the same property (Izaki and Strominger, 1968).¹ Any compound which fulfills the substrate requirements for one of the two sites but not for both of them, is not accepted by any of these enzymes. For example, *N* ^{α} ,*N* ^{ϵ} -bisacetyl-L-lysyl-D-leucyl-D-alanine and *N* ^{α} -acetyl-L-lysyl-D-alanyl-D-alanine which present, respectively, a C-terminal dipeptide and a R₃ side chain that do not fit the conditions required for sensitivity, are not inhibitors of the *Streptomyces* carboxypeptidase (Leyh-Bouille *et al.*, 1970b). Similarly, 6-aminopenicillanic acid which is equivalent to penicillin as a possible analog of the D-alanyl-D-alanine site only, has no action on the *E. coli* carboxypeptidase (Izaki and Strominger, 1968).

One explanation which could accommodate the results of the present study to the structural analogy hypothesis of Tipper and Strominger (1965) is that each of the *E. coli* and the *Streptomyces* carboxypeptidase would recognize one specific configuration in the C-terminal L-D-alanyl-D-alanine sequence and that penicillin would be structurally analogous only to the configuration recognized by the *E. coli*

¹ Replacement of meso-diaminopimelic acid by L-lysine in the substrate UDP-N-acetylmuramyl-L-alanyl- γ -D-glutamyl-(L)-meso-diaminopimelyl-(L)-D-alanyl-D-alanine strongly depresses the rate of hydrolysis of the D-alanyl-D-alanine linkage by the *E. coli* DD carboxypeptidase (Izaki and Strominger, 1968), as observed with the *Streptomyces* enzyme (Leyh-Bouille *et al.*, 1970b).

TABLE III: Effects of Ristocetin and Vancomycin on DD Carboxypeptidase.

Substrate (15 μ moles)	Buffer (30 μ l)	Enzyme (μ g)	Incubation Time (hr)	Antibiotic	Molar Ratio of Antibiotic to Peptide	D-Alanine Liberated (μ moles)
Disacch-pentapeptide-pentaglycine	Veronal ^a	4	1	None		12
	Veronal ^a	4	1	Ristocetin	2:1	1
	Veronal ^a	4	1	or	1:1	2
	Veronal ^a	4	1	Vancomycin	0.5:1	5
<i>N</i> ^{α} , <i>N</i> ^{ϵ} -Bisacetyl-L-lysyl-D-alanyl-D-alanine	Tris-Mg ²⁺ ^b	1	0.5	None		15
	Tris-Mg ²⁺ ^b	1	0.5	Ristocetin	1:1	2.6
	Tris-Mg ²⁺ ^b	4.7	0.5		1:1	7
	Tris-Mg ²⁺ ^b	11	0.5		1:1	12.4
<i>N</i> ^{α} , <i>N</i> ^{ϵ} -Bisacetyl-L-lysyl-D-alanyl-D-alanine	Tris-Mg ²⁺ ^b	0.8	0.25	None		15
	Tris-Mg ²⁺ ^b	0.8	0.25	Ristocetin	1:1	1.9
	Tris-Mg ²⁺ ^b	0.8	0.5		1:1	3.7
	Tris-Mg ²⁺ ^b	0.8	1		1:1	5.5
	Tris-Mg ²⁺ ^b	0.8	2		1:1	8.4
	Tris-Mg ²⁺ ^b	0.8	4		1:1	9.4
UDP-pentapeptide (with <i>meso</i> -DAP) ^d	Veronal ^a	4	4	None		15
	Veronal ^a	4	4	Vancomycin	1:1	8
<i>N</i> ^{α} , <i>N</i> ^{ϵ} -Bisacetyl-L-lysyl-D-alanyl-D-leucine ^c	Tris-Mg ²⁺ ^b	1.5	1	None		15
	Tris-Mg ²⁺ ^b	1.5	1	Ristocetin	1:1	3
	Tris-Mg ²⁺ ^b	1.5	1		2:1	2

^a 0.01 M Veronal buffer, pH 9. ^b 0.02 M Tris-HCl buffer, pH 7.5–0.002 M MgCl₂. ^c Despite the bulky side chain of the D-leucine residue at the C-terminal position in the peptide (M. Nieto and H. R. Perkins, in preparation) a good combination occurred between the peptide and ristocetin with an overall depression in differential ultraviolet absorption at 240 m μ which was about half that observed with *N* ^{α} ,*N* ^{ϵ} -bisacetyl-L-lysyl-D-alanyl-D-alanine. To obtain an independent assessment of the combination reaction, a mixture of peptide and Ristocetin B was submitted to paper electrophoresis in collidine-acetate buffer, pH 7. The free peptide spot was decreased almost to nothing by the presence of ristocetin B, all the complex running nearly in the ristocetin B position. ^d See Materials and Methods.

enzyme.² With regard to the mechanism of action of penicillin whatsoever its precise molecular basis, the *Streptomyces* carboxypeptidase offers the means to study a mechanism of penicillin resistance which does not involve the enzymatic degradation of the antibiotic (by penicillinase or penicillin amidase). The molecular basis for this resistance would reside in a peculiar structure of the enzyme that does not modify its catalytic activities but makes it incapable of recognizing penicillin. Such a resistance may be the result of an alteration affecting the gene which controls the carboxypeptidase synthesis. Similar mechanisms are known to be involved in the resistance to sulfonamides of the synthetase responsible for the synthesis of folic acid (Wolf and Hotch-

kiss, 1963) and in the resistance to rifampicin of the RNA polymerase from resistant strains of *E. coli* and *S. aureus* (Staehelin and Wehrli, 1969).

Mechanism of Action of Vancomycin and Ristocetin. As expected from studies of complex formation between vancomycin or ristocetin and acyl-D-alanyl-D-alanine peptides (Perkins, 1969), these two antibiotics inhibit the action of both the *Streptomyces* (Table III) and *E. coli* carboxypeptidases (Izaki and Strominger, 1968; Bogdanovsky *et al.*, 1969). According to the results reported by Izaki and Strominger (1968), the action of the *E. coli* carboxypeptidase upon UDP-*N*-acetylmuramyl-L-alanyl- γ -D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanyl-D-alanine is only 50% inhibited by vancomycin at a molar ratio antibiotic to peptide of 3 to 1. Under certain conditions of enzyme concentration and of incubation time, vancomycin at a molar ratio to peptide of 1 to 1, inhibited by 65% the action of the *Streptomyces* carboxypeptidase. This apparent discrepancy may result from the fact that inhibition at a given molar ratio antibiotic to substrate, depends upon the amount of enzyme present in the mixture and upon the time of incubation (Table III). It may be expected that enzyme with a high affinity for the substrate will compete more successfully with vancomycin or ristocetin than one with low affinity. With a long incubation the free peptide that is in equilibrium

² The fact that penicillin N and cephalosporin C (Table II) do not inhibit the *Streptomyces* carboxypeptidase although their D-5-amino-5-carboxyvaleramido side chains are structurally identical with the side chain of acyl-(L)-*meso*-diaminopimelyl-(L)-D-alanyl-D-alanine—a peptide which is readily recognized by the *Streptomyces* enzyme (Leyh-Bouille *et al.*, 1970b)—would indicate that the nuclei of these antibiotics do not possess the configuration that the *Streptomyces* enzyme recognizes in a C-terminal D-alanyl-D-alanine sequence. Following this suggestion, the two configurations supposed to be specifically recognized by the penicillin-resistant enzyme and by the penicillin-sensitive enzyme, would reside in the D-alanyl-D-alanine sequence of the substrate rather than in its acyl substituent.

with the antibiotic complex will be attacked by the enzyme and removed, so that the final apparent extent of antibiotic inhibition will be reduced. Vancomycin and ristocetin inhibited the *Streptomyces* carboxypeptidase action upon *B. rettgeri* peptide dimer (Figure 1) and upon *C. poinsettiae* disaccharide peptide dimer (Figure 2) but not upon the *B. subtilis* heptapeptide diamide in which latter case the terminal carboxyl group is amidated. Previous experiments (M. Nieto and H. R. Perkins, in preparation) had shown that a bulky side chain at the C-terminal position of the peptide greatly decreased combination with vancomycin but combination with ristocetin was relatively unaffected. The *C. poinsettiae* disaccharide peptide dimer showed good combination with ristocetin and somewhat less with vancomycin, as indicated by difference spectroscopy and paper electrophoresis of the complexes at pH 7. Thus the inhibition of carboxypeptidase action by the antibiotics was consistent with their combination with the substrate in question. According to the results reported by Bogdanovsky *et al.*, (1969), the action of the *E. coli* carboxypeptidase upon the *E. coli* peptide dimer (Table I, 8 in Ghuysen *et al.*, 1970) was not inhibited by ristocetin. However, ristocetin was used at such low concentrations (25–200 μM for a 250 μM peptide

solution) that further experiments are needed to verify the conclusion.

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Transient State Phosphate Production in the Hydrolysis of Nucleoside Triphosphates by Myosin*

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ABSTRACT: The course of presteady-state phosphate liberation in the hydrolysis of nucleoside triphosphates by myosin was studied using a chemical-quench flow apparatus. Phosphate was liberated at rates greatly exceeding the steady-state rates for MgATP, MgITP, and CaATP as substrates at 20°, pH 8.0, 0.5 M KCl. Similar results were found for MgATP as substrate at 0°, and for hydrolysis of MgATP at 20° by heavy meromyosin in 0.05 M KCl. The time course of the transient state hydrolysis could be fitted by a single rate

constant under all conditions. The rate constant varied with substrate concentration. For MgATP and CaATP at 20°, the rate constant approached a limiting value of about 50–100 sec^{-1} for substrate concentrations greater than 10^{-4} M. The size of the presteady-state burst varied with substrate for both MgATP and CaATP, extrapolating to 1.8 moles of $P_i/5 \times 10^5$ g of myosin. In the presence of EDTA, the mechanism of hydrolysis was completely different, exhibiting a lag before the state-state rate was reached.

The hydrolysis of nucleoside triphosphates by myosin and actomyosin has been the subject of a large number of studies, but several important features of the mechanism, notably the nature of the early burst and of activation by actin, are poorly understood. Recently, attempts have

been made to clarify the problem, by examining the presteady-state behavior (Finlayson and Taylor, 1969; Finlayson *et al.*, 1969; a series of papers by Tonomura and collaborators, most recently Kinoshita *et al.*, 1969a,b; Imamura *et al.*, 1968).

We have previously observed an early rapid proton liberation phase by using a stopped-flow apparatus and monitoring protons by means of an indicator dye. Tonomura reported both a rapid absorption and liberation of protons (Tokiwa and Tonomura, 1965). Since the early phase was obtained in presence of Ca or Mg ion, while the stoichiometric early phosphate burst had been found only with Mg ion, it was not clear whether the proton step could be equated with

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