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Inactivation of Bovine Trypsinogen and Chymotrypsinogen by Diisopropylphosphorofluoridate

(zymogen activation/active site/serine proteases)

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ABSTRACT Diisopropylphosphorofluoridate reacts with trypsinogen and chymotrypsinogen and inhibits the potential activity of both zymogens. The reactions follow pseudo first-order kinetics and proceed approximately four orders of magnitude slower than diisopropylphosphorylation of the corresponding enzymes. Correlation of initial rates of inactivation with incorporation of the reagent indicates that zymogen inactivation results from incorporation of 1 mol of organic phosphate per mol of protein. Peptides isolated from the active-site region of trypsinogen account for more than 60% of the label originally present in the [^{14}C]diisopropylphosphoryl zymogen. It is concluded that loss of activation of trypsinogen is due to alkylphosphorylation of Ser_{183}. It is proposed that reduced reactivity of the zymogen, as compared to the enzyme, primarily reflects inefficient binding of substrates and inhibitors, and that Ser_{183} of the active site exists in trypsinogen in an activated state.

Diisopropylphosphorofluoridate (DFP) inhibits serine proteases by alkylphosphorylation of a specific serine residue of the active site (1). In contrast, and by definition, the parent zymogens are believed to be unreactive toward this and other active-site reagents (2–4). Recently, Robinson (5) reported that carbamylation of the α-amino group of ϵ-guanidinated trypsin destroys esterase and amidase activity without abolishing reactivity toward DFP. Control experiments with native and guanidinated trypsinogen revealed unexpected reactivity of the zymogens toward DFP. It was concluded, therefore, that in trypsinogen (Robinson, N. C., Neurath, H. & Walsh, K. A., submitted for publication), as in trypsin (6), a serine residue of the active site had been alkylphosphorylated.

The present investigation was undertaken to measure the rate of zymogen inactivation, to confirm the site of interaction between DFP and trypsinogen, and to extend the applicability of these findings to chymotrypsinogen.

METHODS

Reaction of Zymogens with DFP. Trypsinogen or chymotrypsinogen was exposed to [^{14}C]DFP in a jacketed vessel at 25°. A Radiometer TTT-1 autotitrator was used to maintain pH 7.0 by the addition of 0.1 N NaOH. Initially the reaction mixture contained 50 mM $CaCl_2$, 0.25 M KCl, 40–75 mM DFP, and 2% trypsinogen, in a total volume of 5.0 ml. At selected intervals 0.2-ml aliquots were removed and diluted 4-fold with 1.0 M sodium formate (pH 3.0) in order to quench the reaction with DFP. Excess DFP was removed from each aliquot by gel filtration on 1.5 × 6.0-cm columns of Sephadex G-25 equilibrated with 0.1 M sodium formate (pH 3.5). Protein concentration was determined by measuring the absorbance of each aliquot at 280 nm.

Suitable portions of the desalted aliquots were removed, assayed for activatability, and counted on a scintillation counter. Trypsinogen (0.5 mg) in 1.0 ml of 0.1 M sodium formate (pH 3.5) was activated with 50 units of the acid protease from *Aspergillus oryzae* as described by Robinson (5). After 1 hr, the activation mixture was assayed for tryptic activity toward N-α-benzoyl-L-arginine ethyl ester as described by Walsh and Wilcox (7). "Activatability" was expressed in units of tryptic activity per mg of trypsinogen.

Chymotrypsinogen (0.1 mg) was activated with 0.27 mg of trypsin after dilution to 2.0 ml with 1.0 M Tris·HCl (pH 7.6) containing 0.2 M $CaCl_2$, at 0°. After 80 min chymotryptic activity was measured with *N*-α-acetyl-L-tyrosine ethyl ester as described by Wilcox (8). The substrate solution included 0.01 M benzamidine to eliminate tryptic hydrolysis of the ester (7).

Incorporation of ^{14}C was measured with a Packard model 3003 Tri-Carb Scintillation Spectrometer by adding about 1.0 mg of labeled protein in 1.0 ml of aqueous solution to 10 ml of a toluene-based scintillant (5).

Isolation of Peptides from [^{14}C]DIP-Trypsinogen. Trypsinogen (200 mg) was inactivated as described above until 33% activatability remained. The pH was lowered to 2.5 with 88% formic acid and the product was dialyzed exhaustively against 10% acetic acid at 4°. An aliquot was subjected to amino-terminal analysis in a sequenator according to the method of Edman and Begg (9), as modified by Hermodson *et al.* (10). The bulk of the partially modified zymogen was lyophilized and oxidized with performic acid as described by Hirs (11).

The oxidized protein (150 mg) was incubated with trypsin (3 mg) at 40° while the pH was maintained at 8.0. After base uptake had ceased (2–3 hr), 3 mg of "TPCK-treated" trypsin (Worthington) were again added and digestion was allowed to proceed for an additional 3 hr. The pH was then lowered to 2.3 with 1 M HCl and insoluble material was removed by centrifugation. Radioactivity in the supernatant and in the pellet were determined.

The supernatant was applied to a 2.5 × 25 cm column of Dowex 50-X2 (200–400 mesh), equilibrated at 55° with 0.05 M pyridine acetate (pH 2.4). The column was eluted with the equilibrating buffer at a flow rate of 45 ml/hr until 70 fractions (2 ml each) had been collected. At this point, the column was

Abbreviations: DFP, diisopropylphosphorofluoridate; DIP, diisopropylphosphoryl.

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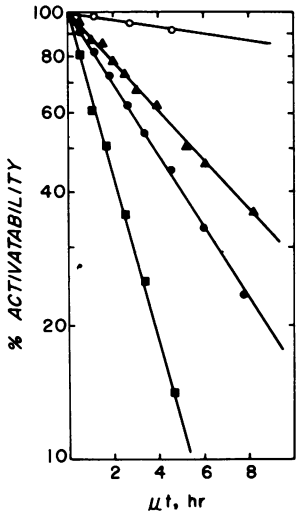

FIG. 1. First-order plots of the inactivation of bovine trypsinogen and chymotrypsinogen by DFP at 25°. Initial salt concentrations for all experiments: 0.05 M $CaCl_2$ and 0.25 M KCl. The pH was maintained at 7.0 by the addition of 0.1 N NaOH. Initial concentrations: 4.8 mM DFP and 50 μM trypsinogen (O——O); 41 mM DFP and 0.83 mM trypsinogen (▲——▲); 75 mM DFP and 0.83 mM trypsinogen (●——●); 67 mM DFP and 0.78 mM chymotrypsinogen (■——■).

stripped by application of 1.5 M pyridine acetate (pH 4.6) until 120 fractions had been collected.

Radioactive fractions were pooled and subjected to high-voltage paper electrophoresis at pH 6.5. Peptides were detected with ninhydrin and by scanning excised strips for radioactivity. Labeled peptides were further purified by preparative high-voltage paper electrophoresis at pH 6.5.

Calculation of Second-Order Rate Constants. Results from zymogen inactivation experiments were interpreted in terms of pseudo first-order kinetics by use of the equation

$$\ln Z_t = -k_1\mu t + \ln Z_0 \qquad [1]$$

This integrated equation corrects for spontaneous hydrolysis of DFP and progressive dilution during the course of the reaction in a pH-stat. Z_t and Z_0 represent the specific activatability of zymogen (units/mg) at time t and at time zero. μ is a time-dependent factor compensating for changes in concentration, and is defined by the equation

$$\mu = 1/kt[N_1/N_2 + 1/1 - \exp(-kt)] \qquad [2]$$

where k is the pseudo first-order rate constant for the spontaneous hydrolysis of DFP, N_1 is the normality of DFP at t_0 with respect to hydrogen ions released upon hydrolysis, and N_2 is the normality of the titrant. The derivation and application of Eq. [1] will be described elsewhere [P. H. Morgan, in preparation]. A plot of $\ln Z_t$ against μt should be linear, with a negative slope equal to k_1, the observed pseudo first-order rate constant for zymogen inactivation.

RESULTS

Kinetics of zymogen inactivation by DFP

The inactivation of trypsinogen and chymotrypsinogen followed pseudo first-order rate laws with respect to zymogen (Fig. 1). The apparent first-order rate constants divided by initial concentrations of DFP yielded the second-order rate constants given in Table 1. According to these data, the zymogens and the model compound *N*-acetyl-L-tyrosinamide exhibit comparable reactivities toward DFP that are about four orders of magnitude slower than those of trypsin and chymotrypsin.

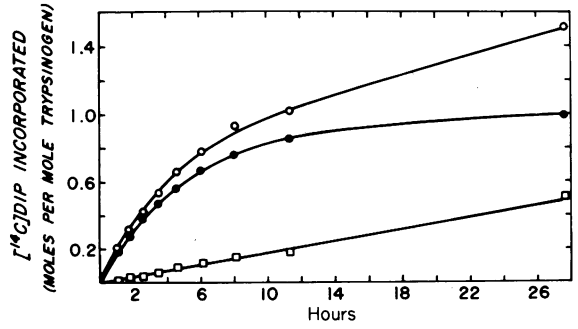

FIG. 2. Incorporation of [14C]diisopropylphosphate into trypsinogen at 25°. Initial concentrations: 0.834 mM trypsinogen, 0.05 M $CaCl_2$, 0.25 M KCl, and 75 mM DFP. The pH was maintained at 7.0 by the addition of 0.1 N NaOH. Total incorporation (O——O); active-site incorporation, calculated on the assumption that inactivation results from the incorporation of one DIP residue per molecule of trypsinogen (●——●); nonactive-site incorporation, calculated by subtracting active-site incorporation from total incorporation (□——□).

Estimation of DIP groups incorporated

The use of [14C]DFP allowed correlation of rates of incorporation and inactivation. A typical time course for DIP incorporation into trypsinogen is shown in Fig. 2 (*open circles*). Assuming that inactivation results from diisopropylphosphorylation of a single active-site residue, the incorporation plot may be resolved into active-site (*closed circles*) and nonactive-site (*open squares*) components. That this assumption is justified can be seen from a plot of percent activatability remaining against [14C]DIP incorporated, as shown in Fig. 3.

TABLE 1. *Second-order rate constants for reaction with diisopropylphosphorofluoridate*

Protein or model compound	k_2 (liter mol^{-1} min^{-1})
Chymotrypsin	2700*
Trypsin	300†
Chymotrypsinogen	0.105‡
Trypsinogen	0.041§
N-acetyl-L-tyrosinamide	0.029¶

* 0.12 μM chymotrypsin, 75 mM sodium phosphate (pH 7.0) 25°, 5% 2-propanol (27).

† 0.257 μM trypsin, 15 mM $CaCl_2$, 75 mM Tris·HCl (pH 7.2) 25°, 5% 2-propanol (27).

‡ Initial concentrations: 0.78 mM chymotrypsinogen, 0.05 M $CaCl_2$, 0.25 M KCl, 67 mM DFP. The pH was maintained at 7.0 by the addition of 0.1 N NaOH at 25°.

§ Initial concentrations: 0.834 mM trypsinogen, 0.05 M $CaCl_2$, 0.25 M KCl, 75 mM DFP. The pH was maintained at 7.0 by the addition of 0.1 N NaOH at 25°.

¶ 2 mM *N*-acetyl-L-tyrosinamide, ionic strength 0.2, sodium potassium phosphate (pH 7.4) 30°, 27.5 mM DFP (13).

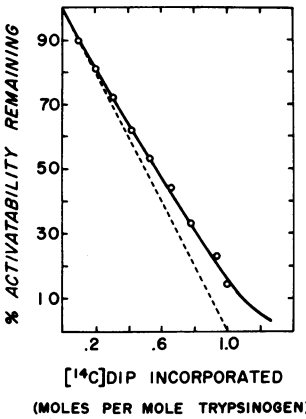

FIG. 3. Percent trypsinogen activatability remaining against [^{14}C]DIP-incorporation. The theoretical relationship between inactivation and the incorporation of one DIP group is illustrated by the *dotted line.*

As the reaction time approaches zero, the experimental curve approaches asymptotically the theoretical line describing an inactivation contingent upon the incorporation of one equivalent of inhibitor. Thus, it may be concluded that loss of activatability results from the modification of a single residue and that this residue reacts at least ten times more rapidly with DFP than do other residues that are not part of the active site. Within the interval required for 99% inactivation of trypsinogen, the incorporation of DIP into these other sites is apparently linear with respect to time (see Fig. 2). This observation is consistent with the hypothesis that extraneous alkylphosphorylation proceeds very slowly at several non-active-site residues. The identity of these additional sites of incorporation has not been investigated, but in view of evidence from other systems, the phenolic hydroxyls of tyrosine seem likely targets for modification by DFP (12–14).

Identification of the major site of diisopropylphosphorylation in trypsinogen

Sequenator analysis of the DIP-trypsinogen used for peptide isolation yielded the amino-terminal sequence expected for intact zymogen. The possibility was thus excluded that trypsinogen had inadvertently become activated and that the active enzyme had subsequently reacted with DFP.

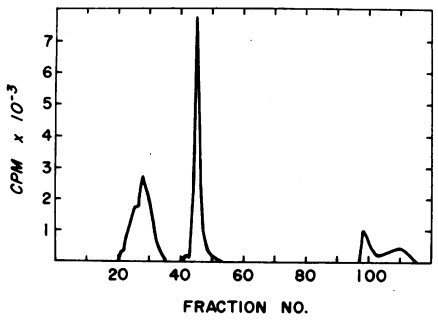

FIG. 4. Chromatography of tryptic peptides derived from oxidized [^{14}C]DIP-trypsinogen. A 2.5 × 25 cm column of Dowex 50-X2 (200–400 mesh) was equilibrated at 55° with 0.05 M pyridine acetate (pH 2.4). Flow rate was 45 ml/hr and fraction size was 2 ml. Elution with equilibrating buffer through fraction 70. Beginning with fraction 71, 1.5 M pyridine acetate (pH 4.6) was applied to remove any remaining radioactivity from the column. The pools containing fractions 23–33 and 44–47 were designated 0-Tg-1 and 0-Tg-2, respectively.

Labeled tryptic peptides from oxidized [^{14}C]DIP-trypsinogen were eluted from Dowex 50-X2 as two major radioactive peaks, 0-Tg-1 and 0-Tg-2, accounting together for more than 60% of the isotope incorporated (Fig. 4, Table 2). The radioactive component of the first peak exhibited an electrophoretic mobility relative to cysteic acid of 0.52 at pH 6.5; hence, the labeled peptide was tentatively identified as the "active site peptide" previously isolated from trypsin by Dixon *et al.* (6) that consists of residues 177–192 in the sequence of trypsinogen (15). Subsequent purification of this component by preparative high-voltage paper electrophoresis did in fact yield a peptide exhibiting the composition unique to the active-site region of trypsinogen (Table 3).

When subjected to electrophoresis at pH 6.5, the second major radioactive peak from Dowex 50-X2 (0-Tg-2) revealed a single labeled peptide, with a mobility of 0.32. Since incomplete tryptic cleavage of the active-site peptide of trypsin had been reported by Dixon *et al.* (6), this entire fraction was subjected to a second tryptic digestion and to electrophoresis at pH 6.5. The labeled peptide from the second digestion of 0-Tg-2 migrated with a mobility of 0.53. After preparative high-voltage electrophoresis and amino-acid analysis, this fraction was shown to originate from the same active-site sequence in trypsinogen as 0-Tg-1 (Table 3). The low values observed for valine in these analyses undoubtedly reflect incomplete hydrolysis of the Val–Val bond in this peptide.

Since DFP reacts with tyrosyl and "activated" seryl residues, the absence of tyrosine in labeled peptides from 0-Tg-1 and 0-Tg-2 permits the site of phosphorylation to be assigned to active-site serine.

Cleavage of the activation peptide from DIP-trypsinogen

[^{14}C]DIP-trypsinogen (1.5 mol DIP per mol of zymogen) and untreated trypsinogen were separately incubated with *A. oryzae* protease under conditions routinely used for zymogen activation (5). After 1 hr, each activation mixture was applied to paper and subjected to electrophoresis at pH 6.5 for 90 min. Ninhydrin analysis revealed identical peptide patterns for native and DFP-treated proteins. In both cases, the major peptide migrated with a mobility relative to cysteic acid of 0.79. Two minor ninhydrin-positive components were also observed in each chromatogram. Amino-acid analysis of

TABLE 2. *Yields of radioactive peptides from tryptic degradation of* [^{14}C]*DIP-trypsinogen*

Fraction	Radioactivity (cpm)	Yield (%)
Oxidized [^{14}C]DIP-trypsinogen (150 mg)	49,035	100.0
Insoluble fraction after acidification of hydrolysate	4,545	9.3
0-Tg-1	16,968	34.6
0-Tg-2	14,172	28.9
Residue eluted with 1.5 M pyridine acetate (pH 4.6)	7,780	15.9
Total	43,465	88.7

the major peptide confirmed the identification of this component as the activation peptide of bovine trypsinogen (16): lysine (1.00), aspartic acid (4.08), and valine (0.90).

DISCUSSION

The present results indicate that reaction of DFP with zymogens of the serine proteases trypsinogen and chymotrypsinogen precludes subsequent activation. The unexpected nature of this finding is emphasized by the fact that DFP is frequently used in the preparation of these proteins to suppress spontaneous activation (1, 8). The possibility was considered that DFP might react with the zymogen at some locus other than the potential active site, possibly by blocking cleavage of the activation peptide. This possibility was excluded by the demonstration that *A. oryzae* protease effectively catalyzes release of the activation peptide from [^{14}C]DIP-trypsinogen. The known reactivity of the phenolic hydroxyl group of tyrosine toward DFP suggested this residue as an alternate site of modification (13). However, isolation of labeled active-site peptides from [^{14}C]DIP-trypsinogen eliminated consideration of tyrosine as a significant site of diisopropylphosphorylation.

The stoichiometry of incorporation (Fig. 3) implies that the observed inactivation results from alkylphosphorylation of a single locus in trypsinogen. This was confirmed by isolation of radioactive peptides from [^{14}C]DIP-trypsinogen. Two peptides, accounting for more than 60% of the counts incorporated, were isolated from the active-site region of the zymogen, i.e., residues 177–192 in the trypsinogen sequence (15). In view of the highly specific reaction of DFP with the active-site serine of these proteases, these results are interpreted as evidence for the alkylphosphorylation of Ser_{183} in trypsinogen. This conclusion is consistent with the identity of labeling patterns observed in peptide maps derived from [^{14}C]DIP-trypsin and [^{14}C]DIP-trypsinogen (5).

The first chemical evidence of a preexisting active site in trypsinogen was provided by Robinson (5), who cited loss of zymogen activatability upon treatment with DFP as evidence for enhanced reactivity of Ser_{183} in trypsinogen. Similarly, the slow reaction of guanidinated trypsinogen and of α-carbamyl-ϵ-guanidinated trypsin (but not DIP-guanidinated trypsin) with the active-site titrant *p*-nitrophenyl,*p'*guanidinobenzoic acid was interpreted as reflecting the activated state of Ser_{183}. These conclusions have not been substantiated by peptide isolation from DIP-trypsinogen and correlation of the rate of inactivation with that of DIP incorporation. Of additional significance is the observation that chymotrypsinogen is similarly inactivated by DFP. In this instance, the chemical evidence of an intact active site in the zymogen agrees with the crystallographic evidence for a preexisting "charge relay system" in chymotrypsinogen (18). The retarded rate of inactivation of chymotrypsinogen is consistent with the distorted substrate binding site noted in the electron density map of the zymogen (18) and with the lower affinity of guanidinated trypsinogen for *p*-nitrophenyl,*p'*-guanidinobenzoic acid (5).

The limited solubility of DFP in aqueous solutions (about 75 mM is saturated) has precluded an extension of the kinetic analysis to a concentration range in which the zymogen becomes saturated by inhibitor. Therefore, it is not possible to compare the catalytic rate constants of the zymogen with those of the enzyme. However, it is of interest to compare, if only qualitatively, second-order rate constants for the reaction of DFP with trypsin, trypsinogen, and *N*-acetyl-L-tyrosinamide (Table 1). It is observed that trypsinogen and *N*-acetyl-L-tyrosinamide exhibit comparable rates of alkylphosphorylation, whereas enzyme and zymogen differ by about four orders of magnitude. This large disparity may be attributed to differences in the reactivity of Ser_{183}, to differences in the binding of DFP, or to both. Inagami and Hatano (19) have shown that integrity of the specificity site of trypsin is required for normal reactivity toward DFP, and Glazer (20) has demonstrated that this site is incompletely developed in trypsinogen. Whether inefficient binding of DFP is alone sufficient to account for the reduced rate of diisopropylphosphorylation remains to be determined. This question may possibly be resolved by probing the reactivity of Ser_{183} toward reagents of low intrinsic affinity for the zymogen.

TABLE 3. *Amino-acid composition of radioactive peptides isolated from tryptic hydrolysate of* [^{14}C]*DIP-trypsinogen*

Amino acid	0-Tg-1*	0-Tg-2†
	g residues/mol of lysine‡	
Cysteic acid	2.03 (2)§	2.04
Aspartic acid	1.81 (2)	2.05
Serine	2.62 (3)	2.69
Glutamic acid	1.00 (1)	1.16
Proline	0.91 (1)	0.94
Glycine	3.91 (4)	4.21
Valine	1.14 (2)	1.21
Lysine	1.00 (1)	1.00

* Composition of Dowex 50-X2 fraction 0-Tg-1 after electrophoretic purification of the radioactive component.

† Composition of Dowex 50-X2 fraction 0-Tg-2 after a second tryptic digestion and electrophoretic purification of the radioactive component.

‡ No other amino acids were present in amounts exceeding 0.05 mol per mol of lysine. Determined on a 20-hr hydrolysate (110°).

§ Numbers in parentheses represent ratios expected for residues 177–192 in the sequence of trypsinogen (15).

The postulation of an active site in trypsinogen is corroborated by the recently reported intrinsic activity of this zymogen to catalyze its own activation. This observation was confirmed by use of acetyltrypsinogen, which itself cannot be activated but activates chymotrypsinogen and hydrolyzes the trypsin substrate *p*-toluenesulfonyl arginine methyl ester (21).

Catalytic activity of zymogens is not restricted to precursors of serine proteases. Dogfish and bovine procarboxypeptidases A provide the most extensively characterized examples of intrinsic zymogen activity studied to date. Lacko and Neurath (22) have examined the activity of dogfish procarboxypeptidase A toward peptide and ester substrates and have presented unequivocal evidence of a true active site in this zymogen. More recently the inherent catalytic activity of bovine procarboxypeptidase A S_5 has been probed with substrates and active site-directed reagents (23, 24). The zymogens of rennin (25) and pepsin (26) exhibit a propensity for self-activation by limited proteolysis. It is apparent, therefore, that zymogens are neither inactive nor inert, but rather constitute a chemically and conformationally defined class of "biologically modified" enzymes. It may be possible to study

the mechanism of zymogen activation and enzyme catalysis by probing the nature of the latent activity of zymogens.

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1. Hartley, B. S. (1960) *Annu. Rev. Biochem.* **29**, 45–72.
2. Neurath, H. & Dixon, G. H. (1957) *Fed. Proc.* **16**, 791–801.
3. Neurath, H. (1964) *Fed. Proc.* **23**, 1–7.
4. Jansen, E. F., Nutting, M.-D. F. & Balls, A. K. (1949) *J. Biol. Chem.* **179**, 201–204.
5. Robinson, N. C. (1971) Ph.D. Thesis, University of Washington.
6. Dixon, G. H., Kauffman, D. L. & Neurath, H. (1958) *J. Biol. Chem.* **233**, 1373–1381.
7. Walsh, K. A. & Wilcox, P. E. (1970) *Methods Enzymol.* **19**, 31–63.
8. Wilcox, P. E. (1970) *Methods Enzymol.* **19**, 64–112.
9. Edman, P. & Begg, G. (1967) *Eur. J. Biochem.* **1**, 80–91.
10. Hermodson, M. A., Ericsson, L. H., Titani, K., Neurath, H. & Walsh, K. A. (1972) *Biochemistry*, in press.
11. Hirs, C. H. W. (1967) *Methods Enzymol.* **11**, 197–199.
12. Murachi, T., Inagami, T. & Yasui, M. (1965) *Biochemistry* **4**, 2815–2825.
13. Inagami, T. & Murachi, T. (1970) *J. Biochem.* **68**, 419–421.
14. Kato, K. & Murachi, T. (1971) *J. Biochem.* **69**, 725–737.
15. Walsh, K. A. & Neurath, H. (1964) *Proc. Nat. Acad. Sci. USA* **52**, 884–889.
16. Davie, E. W. & Neurath, H. (1955) *J. Biol. Chem.* **212**, 515–529.
17. Tietze, F. (1953) *J. Biol. Chem.* **204**, 1–11.
18. Freer, S. T., Kraut, J., Robertus, J. D., Wright, H. T. & Xuong, Ng. H. (1970) *Biochemistry* **9**, 1997–2009.
19. Inagami, T. & Hatano, H. (1969) *J. Biol. Chem.* **244**, 1176–1182.
20. Glazer, A. N. (1967) *J. Biol. Chem.* **242**, 3326–3331.
21. Kay, J. & Kassell, B. (1971) *J. Biol. Chem.* **246**, 6661–6665.
22. Lacko, A. G. & Neurath, H. (1970) *Biochemistry* **9**, 4680–4690.
23. Neurath, H. & Walsh, K. A. (1970) *Abstracts, 8th International Congress of Biochemistry*, Interlaken, Switzerland.
24. Uren, J. R., Neurath, H. & Walsh, K. A. (1972) *Fed. Proc.* **31**, Abstr. 916.
25. Foltmann, B. (1969) *Biochem. J.* **115**, 3P.
26. Bustin, M. & Conway-Jacobs, A. (1971) *J. Biol. Chem.* **246**, 615–620.
27. Fahrney, D. E. & Gold, A. M. (1963) *J. Amer. Chem. Soc.* **85**, 997–1000.