Application of Various Inverse Substrates to Thrombin-Catalyzed Peptide Synthesis

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Thrombin-catalyzed peptide synthesis has been studied using nine series of "inverse substrates," *i.e.*, *p*-amidinophenyl, *p*- and *m*-guanidinophenyl, *p*- and *m*-(guanidinomethyl)phenyl, and four position isomers of guanidinonaphthyl esters derived from N^{α}-(*tert*-butyloxycarbonyl)amino acid as acyl donor components. These substrates were classified into two types with respect to their response to thrombin. One group includes *p*-amidino- and *p*-guanidinophenyl esters, which undergo less enantioselective coupling reaction. Substrates classified into the other group are *m*-guanidinophenyl, *p*- and *m*-(guanidinomethyl)phenyl, and four position isomers of guanidinonaphthyl esters which are involved in the enantioselective coupling reaction. Thus amino acid residues of L-series (in the present case; N^{α}-Boc-L-Ala) are readily coupled to afford peptides by assigning them to either of the inverse substrates. The optimum condition for the coupling reaction was studied by changing organic solvent, pH, and acyl acceptor concentration. It was found that the enzymatic hydrolysis of the resulting product was negligible.

Key words inverse substrate; thrombin; enzymatic peptide synthesis; amidinophthyl ester; guanidinophthyl ester; guanidinonaphthyl ester

Peptide synthesis by protease-catalyzed reverse reaction has been extensively studied with a variety of amino acids and peptide derivatives as coupling components.[1—4] It is known that enzymatic peptide synthesis is more advantageous than chemical synthesis in many respects; it is highly stereoselective and racemization-free, and requires minimal side-chain protection.[1—4] The most serious defect of the enzymatic method, however, is the restrictive substrate specificity. Thus, the application of proteases for peptide synthesis has been limited by the specificity of the enzymes.

In a previous paper, we reported that the *p*-amidinophenyl and *p*-guanidinophenyl esters behave as specific substrates for trypsin[5,6] and trypsin-like enzymes such as thrombin and plasmin, *etc.*[7] In these esters the site-specific groups (charged amidinium and guanidinium) for the enzyme are included in the leaving-group portion instead of being in the acyl moiety. Such a substrate was termed "inverse substrate" by us[8] and "substrate mimetics" by Jakubke *et al.*[9] Inverse substrates allow the specific introduction of an acyl group carrying a non-specific residue into the trypsin active site without recourse to a cationic acyl moiety, which is a characteristic of conventional substrates. These acyl trypsin intermediates are expected to play a key role in trypsin-catalyzed peptide synthesis. We previously reported bovine trypsin-catalyzed peptide synthesis using N^{α}-Boc-amino acid *p*-amidinophenyl (N^{α}-Boc-AA-OAm) (AA=amino acid),[10] *p*-guanidinophenyl (N^{α}-Boc-AA-O*p*Gu),[11] *m*-guanidinophenyl (N^{α}-Boc-AA-O*m*Gu),[12] *p*-(guanidinomethyl)phenyl (N^{α}-Boc-AA-O*p*GM),[13,14] *m*-(guanidinomethyl)phenyl (N^{α}-Boc-AA-O*m*GM),[12] and guanidinonaphthyl esters (N^{α}-Boc-AA-OGN)[15] as acyl donors.

It was reported that thrombin exhibits the greatest geometric adaptability of the substrate among the proteases, thrombin, trypsin and plasmin.[16] It was assumed, therefore, that thrombin-catalyzed coupling reaction using of inverse substrate as acyl donor component is the most promising procedure for peptide synthesis. Studies on the requirements for thrombin-catalyzed reaction with various inverse substrates (Fig. 1) were investigated.

Results and Discussion

Thrombin-catalyzed coupling reaction of N^{α}-*tert*-butyloxycarbonyl-L-alanine *p*-guanidinophenyl ester (N^{α}-Boc-L-Ala-O*p*Gu) (**2a**) and L-alanine *p*-nitroanilide (L-Ala-*p*NA) to give N^{α}-Boc-L-Ala-L-Ala-*p*NA was examined in dimethylsulfoxide (DMSO), *N*,*N*-dimethylformamide (DMF), or acetonitrile as a co-solvent. The reaction was also evaluated under the condition where the pH of the medium was changed and the concentration of acyl acceptor (L-Ala-*p*NA) was changed. The coupling product was obtained in high yield with DMSO, but unsatisfactorily with other organic solvents.

Effects of DMSO, DMF, and acetonitrile concentration on coupling yields are shown in Fig. 2. Coupling yields higher than 50% were observed at the DMSO concentration range of 40—60%, and the best yield (63%) was obtained at 50% DMSO. The effect of DMF concentration was similar to that of DMSO, but the coupling yields were much lower. The acetonitrile showed different behavior from those of DMSO and DMF, and the best yield (27%) was obtained at the 20% concentration. Although a high concentration of organic solvent prevents the hydrolysis of the acyl enzyme, it will decrease the enzymatic activity due to the denaturation of thrombin as well as trypsin.[17] Consequently, the coupling yield was decreased at a concentration of organic solvents above 60%.

The effect of pH of the reaction medium on the coupling yields was analyzed. DMSO was mixed with 50 mM solution of 3,3-dimethylglutaric acid (G), tris(hydroxymethyl)-aminomethane (T), and 2-amino-2-methyl-1,3-propanediol (A) (GTA buffer) (containing 20 mM $CaCl_2$) at various pH. The pH values given in Fig. 3 are those of the buffer itself before mixing with organic co-solvent. The pH-dependency of the coupling yield was determined (Fig. 3). The yield was increased with the increase of the pH values. The best yield was obtained at pH 10 even though the optimum pH for thrombin-catalyzed hydrolysis is 7.4.[18]

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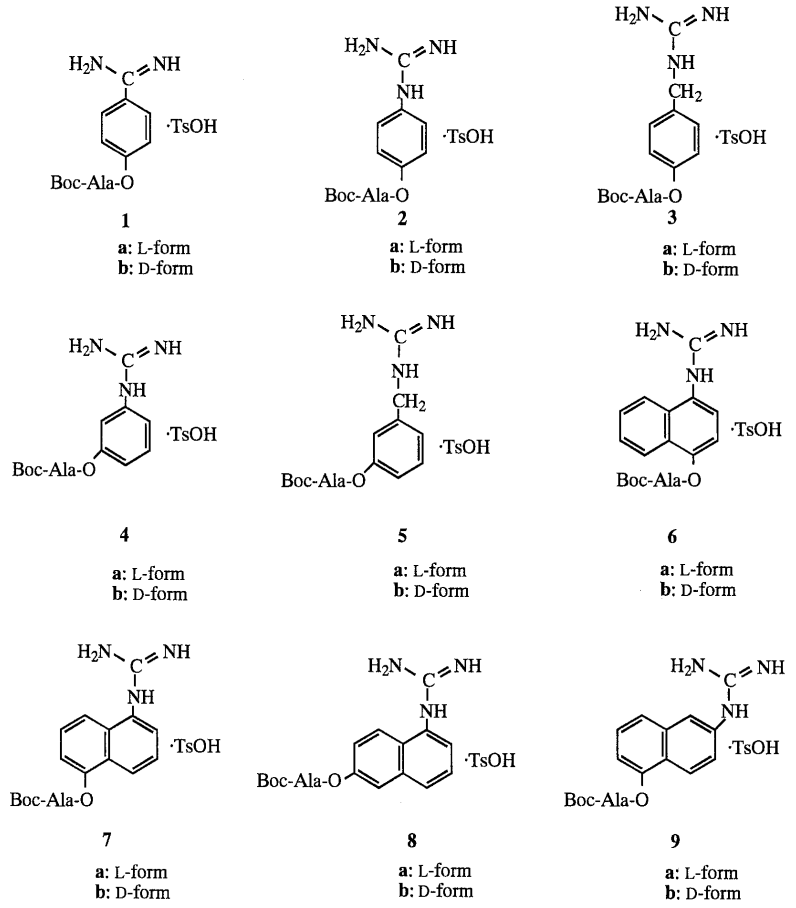

Fig. 1. Structure of Inverse Substrates

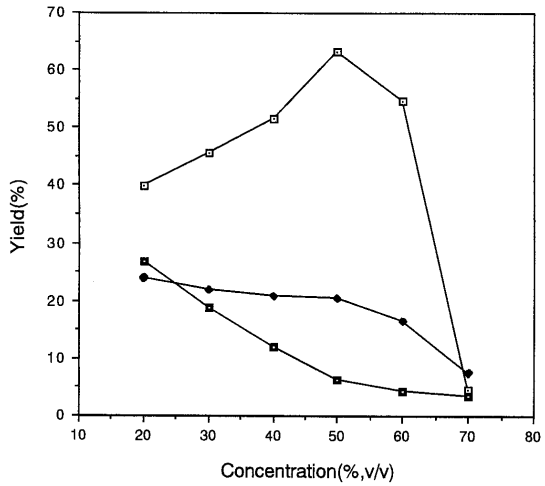

Fig. 2. Effect of Organic Solvent on Thrombin-Catalyzed Condensation of N^{α}-Boc-L-Ala-O*p*Gu with L-Ala-*p*NA

Reaction was carried out in 50 mM GTA buffer (pH 10) containing DMSO (□), DMF (◆), and acetonitrile (■) at 25 °C. Product yield was analyzed after a reaction period of 1.5 h in which the coupling was completed. N^{α}-Boc-L-Ala-O*p*Gu, 1 mM; L-Ala-*p*NA, 20 mM; thrombin, 5 μM.

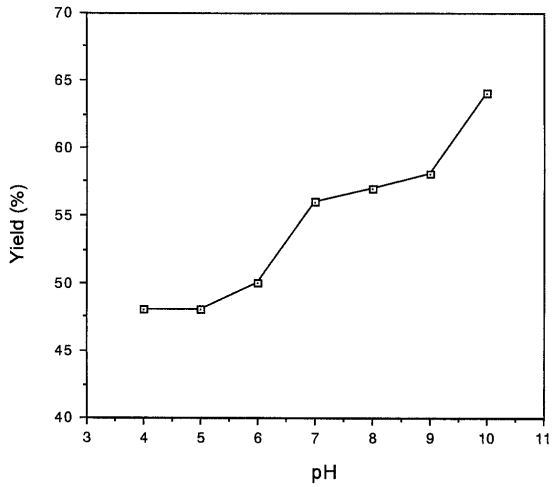

Fig. 3. pH Dependency of Thrombin-Catalyzed Condensation

Reactions were carried out in 50 mM GTA buffer containing 50% DMSO at 25 °C. Product yield was analyzed after a reaction period of 1.5 h in which the coupling was completed. N^{α}-Boc-L-Ala-O*p*Gu, 1 mM; L-Ala-*p*NA, 20 mM; thrombin, 5 μM.

The effect of acyl acceptor concentration on the coupling yields in 50% aqueous DMSO is shown in Fig. 4. The dependency can be explained as being due to the saturation of the enzyme binding site with the acyl acceptor. The reaction yield reached its maximum (63%) at the concentration around 20 mM of acyl acceptor.

Consequently, a standard condition for thrombin-catalyzed peptide coupling reaction was selected as described in Experimental. The time courses of the coupling of N^{α}-Boc-L-Ala-O*p*Gu (**2a**) and N^{α}-Boc-D-Ala-O*p*Gu (**2b**) with L-Ala-*p*NA are shown in Fig. 5. In these cases, the D-acyl donor is a versatile substrate for the enzymatic synthesis as well as the L-acyl donor, even though the reaction is slow.

The results of thrombin-catalyzed coupling reaction were compared with those of trypsin-catalyzed coupling reaction

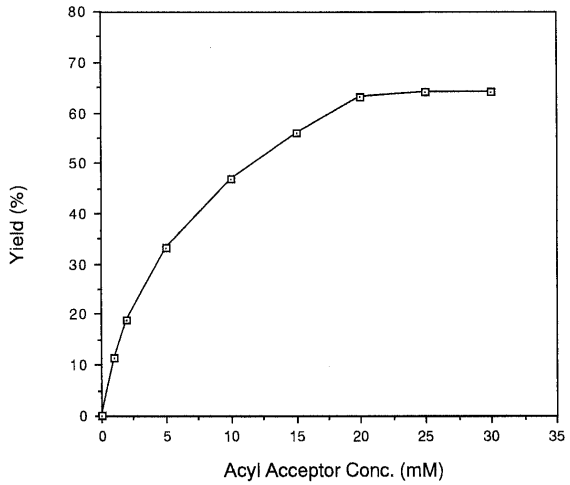

Fig. 4. Effect of Acyl Acceptor Concentration on Thrombin-Catalyzed Condensation

Reactions were carried out in 50 mM GTA buffer (pH 10) containing 50% DMSO at 25 °C. Product yield was analyzed after a reaction period of 1.5 h in which the coupling was completed. N^{α}-Boc-L-Ala-O*p*Gu, 1 mM; thrombin, 5 μM; L-Ala-*p*NA, 1—30 mM.

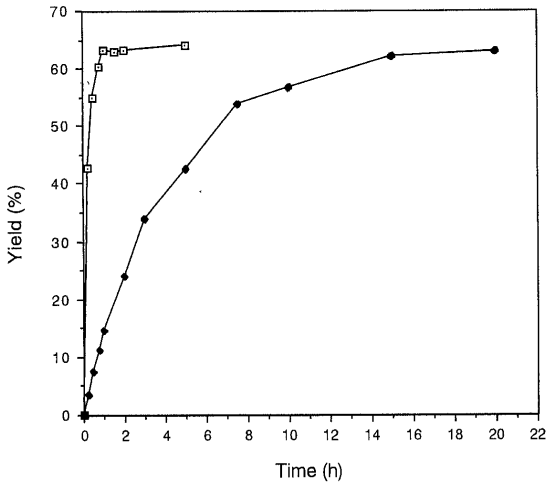

Fig. 5. Time Course of the Peptide Coupling Reaction

Reaction was carried out in 50 mM GTA buffer (pH 10) containing 50% DMSO at 25 °C. Product yield was analyzed after a reaction period of 1.5 h for N^{α}-Boc-L-Ala-O*p*Gu and 24 h for N^{α}-Boc-D-Ala-O*p*Gu in which the coupling were completed. N^{α}-Boc-L-Ala-O*p*Gu (□) or N^{α}-Boc-D-Ala-O*p*Gu (◆), 1 mM; L-Ala-*p*NA, 20 mM; thrombin, 5 μM.

Table 1. Yield of Thrombin-Catalyzed Peptide Synthesis[a]

Entry No.	Acyl donor (No.)	Reaction time (h)[b]	Product	Yield (%)[c]
1	N^{α}-Boc-L-Ala-OAm (**1a**)	0.5 [0.1][d]	N^{α}-L-Ala-L-Ala-*p*NA	73 [77][d]
2	N^{α}-Boc-L-Ala-O*p*Gu (**2a**)	1 [0.2][e]	N^{α}-L-Ala-L-Ala-*p*NA	63 [64][e]
3	N^{α}-Boc-L-Ala-O*p*GM (**3a**)	0.5 [5][g]	N^{α}-L-Ala-L-Ala-*p*NA	76 [95][g]
4	N^{α}-Boc-L-Ala-O*m*Gu (**4a**)	3 [12][f]	N^{α}-L-Ala-L-Ala-*p*NA	51 [58][f]
5	N^{α}-Boc-L-Ala-O*m*GM (**5a**)	5 [12][f]	N^{α}-L-Ala-L-Ala-*p*NA	55 [67][f]
6	N^{α}-Boc-L-Ala-O(1-4)GN (**6a**)	0.75 [1][h]	N^{α}-L-Ala-L-Ala-*p*NA	69 [79][h]
7	N^{α}-Boc-L-Ala-O(1-5)GN (**7a**)	1 [2][h]	N^{α}-L-Ala-L-Ala-*p*NA	63 [72][h]
8	N^{α}-Boc-L-Ala-O(1-6)GN (**8a**)	1.5 [12][h]	N^{α}-L-Ala-L-Ala-*p*NA	53 [75][h]
9	N^{α}-Boc-L-Ala-O(2-5)GN (**9a**)	1 [12][h]	N^{α}-L-Ala-L-Ala-*p*NA	71 [88][h]
10	N^{α}-Boc-D-Ala-OAm (**1b**)	3 [1][d]	N^{α}-D-Ala-L-Ala-*p*NA	50 [65][d]
11	N^{α}-Boc-D-Ala-O*p*Gu (**2b**)	15 [0.3][e]	N^{α}-D-Ala-L-Ala-*p*NA	62 [74][e]
12	N^{α}-Boc-D-Ala-O*p*GM (**3b**)	48 [24][g]	N^{α}-D-Ala-L-Ala-*p*NA	36 [63][g]
13	N^{α}-Boc-D-Ala-O*m*Gu (**4b**)	48 [24][f]	N^{α}-D-Ala-L-Ala-*p*NA	23 [20][f]
14	N^{α}-Boc-D-Ala-O*m*GM (**5b**)	72 [24][f]	N^{α}-D-Ala-L-Ala-*p*NA	10 [36][f]
15	N^{α}-Boc-D-Ala-O(1-4)GN (**6b**)	48 [1][h]	N^{α}-D-Ala-L-Ala-*p*NA	22 [75][h]
16	N^{α}-Boc-D-Ala-O(1-5)GN (**7b**)	48 [6][h]	N^{α}-D-Ala-L-Ala-*p*NA	17 [74][h]
17	N^{α}-Boc-D-Ala-O(1-6)GN (**8b**)	48 [24][h]	N^{α}-D-Ala-L-Ala-*p*NA	13 [46][h]
18	N^{α}-Boc-D-Ala-O(2-5)GN (**9b**)	48 [24][h]	N^{α}-D-Ala-L-Ala-*p*NA	26 [39][h]

a) Conditions: acyl donor, 1 mM; acyl acceptor (L-Ala-*p*NA), 20 mM; thrombin, 5 μM; 50% DMSO–GTA (50 mM, pH 10, containing 20 mM $CaCl_2$); 25 °C. *b*) The values in brackets are reaction time (h) of trypsin-catalyzed peptide synthesis. *c*) The values in brackets are yield (%) of trypsin-catalyzed peptide synthesis. *d*—*h*) See reference 9 (*d*), 10 (*e*), 11 (*f*), 13 (*g*), and 14 (*h*).

previously reported,[10—15] and are summarized in Table 1. In general, toward all inverse substrates derived from N^{α}-Boc-L-Ala (**1a**—**9a**), thrombin behaved as a moderately effective catalyst for the synthesis of the peptides (entry 1—9 in Table 1). As shown in Table 1, thrombin can also be utilized for the synthesis of peptides containing D-amino acid though the reaction requires a long time (entry 10—11).

From the comparative study of thrombin and trypsin as the catalyst of peptide coupling, the substrates in this study were classified into two types. One of them includes *p*-amidino- (**1**) and *p*-guanidinophenyl esters (**2**), which behave as good acyl donor for both thrombin- and trypsin-catalyzed reactions (entry 1, 2, 10 and 11 in Table 1). The reaction rate for the acyl donor (**1b**, **2b**) derived from D-alanine was slower than those of L-enantiomer, but the final coupling yield was nearly equal to that for the corresponding L-enantiomer (**1a**, **2a**). *meta*-Substrate (**4**, **5**) and bulky substrate (**3**, **6**—**9**) are grouped in the other type. The reaction of L-alanine derivatives is more favorable and faster than that by trypsin (entry 4—9 in Table 1). These observations can be explained by the greater geometric adaptability of thrombin active site as discussed in the previous report.[16] However, the thrombin-catalyzed coupling reaction of D-alanine derivatives (**3b**—**9b**) was unexpectedly inefficient. It can be assumed that enantiomeric selectivity of thrombin was pronounced toward such inverse substrates that carry bulky group as naphthalene, though the binding site of thrombin seems spatially less restricted.[16]

In any event, all inverse substrates derived from L-alanine (**1a**—**9a**) result in good peptide coupling yield regardless of their structures. The peptide containing D-amino acid was readily obtainable using small substrates such as *p*-amidino-

and *p*-guanidinophenyl esters.

It appears that secondary hydrolysis of the coupling product can be disregarded in our enzymatic procedure, since this product is not decreased even after 72 h reaction time.

Experimental

All inverse substrates were prepared according to our previous papers.[5,6,10,12—15] Bovine thrombin (EC 3.4.21.5) was purchased from Sigma Chemical Co. (lyophilized, 1000 units, T-6634). DMSO, DMF, and acetonitrile at HPLC grade were used from Kanto Chemical Co., Inc. L-Ala-*p*NA was purchased from Peptide Institute, Inc. 3,3-Dimethylglutaric acid and 2-amino-2-methyl-1,3-propanediol, and tris(hydroxymethyl)aminomethane were obtained from Tokyo Chemical Industry Co., Ltd. and ICN Biomedicals, Inc., respectively.

Enzymatic Peptide Coupling Reaction Peptide coupling reaction was carried out at 25 °C in 50% DMSO–GTA buffer (pH 10, containing 20 mM $CaCl_2$). Concentrations of acyl donors (**1**—**9**), acyl acceptor (L-Ala-*p*NA), and enzyme were 1 mM, 20 mM, and 5 μM, respectively. Concentration of thrombin was calculated as 1000 units/0.8 mg according to the literature.[16] The progress of the peptide coupling reaction was monitored by HPLC under the following conditions: column (4.6×250 mm, Wakosil 5C18-200), isocratic elution at 1 ml/min, 0.1% trifluoroacetic acid/acetonitrile. An aliquot of the reaction mixture was injected and eluate was monitored at 310 nm (chromophore due to *p*-nitroanilide moiety). Peak identification was made by correlating the retention time with that of authentic samples which were chemically synthesized.[19—21] Observed peak areas were used for the estimation of sample concentration.

Acknowledgments This work was supported in part by Grant-in-Aid for High Technology Research Program from the Ministry of Education, Science, Sports and Culture of Japan, and by a grant from the Japan Private School Promotion Foundation.

References

1) Tsuzuki H., Oka T., Morihara K., *J. Biochem.* (Tokyo), **88**, 669—675 (1980).
2) Nakatuka T., Sasaki T., Kaiser E. T. , *J. Am. Chem. Soc.*, **99**, 4485—4488 (1987).
3) Wong C. -H., *Science*, **244**, 1145—1152 (1989).
4) Schellenberger V., Jakubke H.-D., *Angew. Chem. Int. Ed. Engl.*, **30**, 1437—1449 (1991).
5) Itoh K., Sekizaki H., Toyota E., Tanizawa K., *Chem. Pharm. Bull.*, **43**, 2082—2087 (1995).
6) Sekizaki H., Itoh K., Toyota E., Tanizawa K., *Chem. Pharm. Bull.*, **44**, 1577—1579 (1996).
7) Nozawa M., Tanizawa K., Kanaoka Y., *J. Pharmacobio-Dyn.*, **3**, 213—219 (1980).
8) Tanizawa K., Kasaba Y., Kanaoka Y., *J. Am. Chem. Soc.*, **99**, 4485—4488 (1977).
9) Bordusa F., Ullmann D., Elsner C., Jakubke H.-D., *Angew. Chem. Int. Ed. Engl.*, **36**, 2473—2475 (1997).
10) Itoh K., Sekizaki H., Toyota E., Fujiwara N., Tanizawa K., *Bioorg. Chem.*, **24**, 59—68 (1996).
11) Sekizaki H., Itoh K., Toyota E., Tanizawa K., *Chem. Pharm. Bull.*, **44**, 1585—1587 (1996).
12) Sekizaki H., Itoh K., Toyota E., Tanizawa, K., *Amino Acids*, in press.
13) Sekizaki H., Itoh K., Toyota E., Tanizawa K., *Tetrahedron Lett.*, **38**, 1777—1780 (1997) .
14) Sekizaki H., Itoh K., Toyota E., Tanizawa K., *Chem. Pharm. Bull.*, **46**, 846—849 (1998) .
15) Sekizaki H., Itoh K., Toyota E., Tanizawa K., *Chem. Pharm. Bull.*, **47**, 104—110 (1999) .
16) Chase T., Jr., Shaw E., *Biochemistry*, **8**, 2212—2224 (1969).
17) West J. B., Wong C.-H., *J. Org. Chem.*, **51**, 2728—2735 (1986).
18) Markwardt F., Landmann H., *Acta. Biol. Med. Ger.*, **11**, 720—729 (1963).
19) Kawai M., Nagai U., *Bull. Chem. Soc. Jpn.*, **55**, 1327—1328 (1982).
20) Okada Y., Tsuda Y., Hirata A., Nagamatsu Y., Okamoto U., *Chem. Pharm. Bull.*, **30**, 4060—4068 (1982).
21) Bieth J., Weremath C. G., *Biochem. Biophys. Res. Commun.*, **53**, 383—390 (1970).