Biochemistry 3, 329.
Žemlička, J. (1975), *J. Am. Chem. Soc. 97,* 5896.
Žemlička, J., and Chládek, S. (1966), *Collect. Czech. Chem. Commun. 31,* 3775.
Žemlička, J., and Holý, A. (1967), *Collect. Czech. Chem. Commun. 32,* 3154.
Žemlička, J., and Šorm, F. (1965), *Collect. Czech. Chem. Commun. 30,* 1880.

The Mechanism of Action of Dipeptidyl Aminopeptidase. Inhibition by Amino Acid Derivatives and Amines; Activation by Aromatic Compounds†

Robert M. Metrione* and Nancy L. MacGeorge

ABSTRACT: A variety of amino acid and peptide amides have been shown to be inhibitors of dipeptidyl aminopeptidase. Among these compounds derivatives of strongly hydrophobic amino acids are the strongest inhibitors (Phe-NH_2, K_i = 1.0 ± 0.2 m*M*), while amides of basic amino acids were somewhat less effective (Lys-NH_2, K_i = 36 ± 3 m*M*). Short chain amino acid amides are notably weaker inhibitors (Gly-NH_2, K_i = 293 ± 50 m*M*). The interaction of the side chains of compounds with the enzyme appears to be at a site other than that at which the side chain of the amino-penultimate residue of the substrate interacts since the specificity of binding is different. Primary amines have been shown to inhibit, e.g., butylamine, K_i = 340 ± 40 m*M*, and aromatic compounds have been shown to stimulate activity toward Gly-Gly-NH_2 and Gly-Gly-OEt (phenol, 35% stimulation of activity at a 1:1 molar ratio with the substrate). The data suggest that inhibition involves binding at the site occupied by the free α-amino group and the N-terminal amino acid.

Dipeptidyl aminopeptidase (dipeptidyl aminopeptidase I, dipeptidyl peptidase, dipeptidyl-transferase, cathepsin C; EC 3.4.14.1) is a lysosomal exopeptidase which is capable of removing dipeptides sequentially from the amino terminus of a peptide chain (McDonald et al., 1969). The specificity of the enzyme has been studied in some detail (Jones et al., 1952; Izumiya and Fruton, 1956; Fruton and Mycek, 1956; Wiggans et al., 1954; Planta et al., 1964; Voynick and Fruton, 1968). From these studies it appeared that dipeptidyl aminopeptidase had a rather narrow specificity, substrate largely being confined to compounds of the type NH_2-C(R_1)H-CO-NH-C(R_2)H-CO-X, in which R_2 was the side chain of a strongly hydrophobic amino acid. Planta et al. (1964) and Metrione et al. (1966) had reported that the enzyme had no activity toward proteins. However, McDonald et al. (1969) demonstrated that the enzyme possesses a much broader specificity and is capable of hydrolyzing the peptide bonds of proteins in the presence of chloride ions. It is now clear that in the presence of suitable activators the CO–X bond can be hydrolyzed provided that: (1) the α-amino group is free; (2) the amino terminal amino acid (with the side chain R_1) is not basic (Arg or Lys[1]); (3) that the penultimate amino acid (with side chain R_2) is not proline; and (4) that the dipeptide is not linked through a peptide bond to a proline. The usefulness of the enzyme for protein sequence studies has been described (Callahan et al., 1972).

Highly purified dipeptidyl aminopeptidase (Metrione et al., 1966) has been shown to be composed of eight subunits of 25000 molecular weight each (Metrione et al., 1970) and to require sulfhydryl (Fruton and Mycek, 1956) and chloride activation (McDonald et al., 1966). There is some evidence that dipeptidyl aminopeptidase is an allosteric enzyme (Gorter and Gruber, 1970).

Fruton and Mycek (1956) reported on the ability of amino acid derivatives to inhibit dipeptidyl aminopeptidase. Their results indicated that Phe-NH_2 is a much better inhibitor than D-Phe-NH_2; Tyr-NH_2 is a weaker inhibitor than Phe-NH_2; Phe-Phe is an inhibitor; and that Ac-Phe, Phe, β-phenylethylamine, and Leu-NH_2 were not inhibitors. The authors concluded that the requirements for competitive inhibitors included an L-amino acid (stereochemical specificity), a free amino group, and a linked (amidated or esterified) carboxyl group, and that inhibition is favored by an amino terminal aromatic amino acid.

Experiments in our laboratory intended to develop an affinity chromatographic method for the separation and isolation of intracellular proteolytic enzymes have led us to a reinvestigation of the inhibition of beef spleen dipeptidyl aminopeptidase.

Experimental Procedure

Isolation of Enzyme and Determination of Enzyme Activity. The transamidation assay was employed in the manner described by Metrione et al. (1966). For routine assays, Gly-Phe-NH_2 or Gly-Gly-OEt was the substrate unless otherwise indicated. The hydrolysis assay was the method of

† From the Department of Biochemistry, Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania 19107. *Received June 27, 1975.* This investigation was supported by a grant from the National Institutes of Health, CA-12053.

[1] Abbreviations are those recommended by the IUPAC–IUB Commission on Biochemical Nomenclature Symbols for Amino-Acid Derivatives and Peptides (1972).

Table I: Inhibition of Dipeptidyl Aminopeptidase by Amino Acid and Peptide Derivatives.[a]

Inhibitor	Substrate: Gly-Gly-OEt % Inhibition	Gly-Gly-OEt K_i (mM)[b]	Gly-L-Phe-NH_2 % Inhibition	Gly-L-Phe-NH_2 K_i (mM)[b]
Phe-NH_2	100	1.0 ± 0.2[c]	95	1.25 ± 0.15[c]
Trp-NH_2	95	0.7 ± 0.3[c]	95	1.5 ± 0.4[c]
Leu-NH_2	90	9.2 ± 1[c]	71	10.0 ± 0.2[c]
Bz-Arg-NH_2	82	1.7 ± 0.5[c]	78	1.4 ± 0.5[c]
Arg-NH_2	70	13.5 ± 2.1[c]	36	
Lys-NH_2	42	36 ± 3[d]	10	
Ac-Leu-NH_2	33	40 ± 4[d]	15	
Gly-βAla-NH_2	30	56.2 ± 2.2[d]	38	
Gly-Gly-NH_2	28		33	26.5 ± 50[d]
Glu-αOMe	24		5	
Gly-NH_2	22		35	293 ± 50[d]
βAla-NH_2	22		21	
Gly-D-Phe-NH_2	19		28	
Ac-Gly-NH_2	<5		<5	
Bz-Gly-NH_2	<5		<5	
Arg	<5		<5	
Glu	<5		<5	
Gly	<5		<5	
Bz-Arg	<5		<5	

[a] The assays for the percent inhibition studies were performed as described in the Experimental section with an inhibitor to substrate ratio of 1. [b] The K_i's were determined by plots of rate^{-1} vs. inhibitor concentrations of [c] 0.002–0.1 mM. [d] 0.02–0.1 mM.

Table II: The Effect of Amino and Aromatic Compounds on the Activity of Dipeptidyl Aminopeptidase.

Compound	% Effect[a] Gly-Gly-OEt	Gly-Gly-NH_2	Gly-Phe-NH_2
Pyridine	+44	+38	0
Phenol	+38	+35	0
Dimethylamine	0	0	0
Butylamine	−21	−14	−22
Glycinamide	−22	−27	−35

[a] A positive (+) sign indicates activation, and a negative (−) sign indicates inhibition against the substrate indicated. The concentration of each inhibitor or stimulator tested was 0.05 M.

Table III: Inhibition of Dipeptidyl Aminopeptidase by Amines.

Inhibitor[a]	Chain Length	% Inhibition Gly-Gly-OEt[b]	Gly-Phe-NH_2[c]	K_i (mM)
Methylamine	2	15	5	N.D.
Ethylamine	3	17	20	305 ± 35[b]
Propylamine	4	14	19	430 ± 50[c]
Butylamine	5	21	22	340 ± 40[c]
Pentylamine	6	11	24	205 ± 15[c]
Hexylamine	7	5	27	335 ± 15[c]

[a] The concentration of amines in the reaction mixture was 0.05 M. [b] The substrate was Gly-Gly-OEt. [c] The substrate was Gly-Phe-NH_2. The assay was by the transamidation method. The chain length measured all carbon and nitrogen atoms from the amine to the end of the chain. N.D. indicates not determined.

Fruton and Mycek (1956). The enzyme was isolated according to the method of Metrione et al. (1966) to yield the CM fraction. The K_m values were determined from Dixon plots (Dixon, 1953), employing two substrate concentrations.

Substrates and Inhibitors. βAla-NH_2, Glu-αOMe, Arg-NH_2, Gly-βAla-NH_2, Leu-NH_2, Ac-Leu-NH_2, and Gly-Arg-NH_2 were obtained from Fox Chemical Company. D-Arg, Bz-Arg-NH_2, Phe-NH_2, Trp-NH_2, Gly-Gly-OEt, and Gly-Phe-NH_2 were from Sigma Chemical Co. Gly-Gly-NH_2 and Gly-NH_2 were from Fluka. Lys-NH_2 and Ac-Gly-NH_2 were from Cyclo Chemical Co. Bz-Gly-NH_2 was from Schwarz/Mann. 4-Phenylbutylamine-1 was from K & K Laboratories. All were tested by thin-layer chromatography (Eastman 13181 silica gel plates; 1-propanol–ammonia, 7:3, and 1-butanol–acetic acid–water, 100:15:37). All compounds used gave a single spot by fluorescence quenching and after spraying with ninhydrin. In addition, the Leu-NH_2 was hydrolyzed in 6 N HCl, 105°, 16 hr and shown to contain Leu by comparison with Leu on thin-layer chromatography in the butanol–acetic acid–water and propanol–ammonia systems. The nitrogen content of 4-phenylbutylamine-1 was determined by Kjeldahl analysis and found to be 9.42%; the calculated content was 9.40%.

Z-Gly-Glu-αOMe was synthesized by coupling Glu-αOMe (200 mg) in 10 ml of water containing 209 mg of sodium bicarbonate with 360 mg of carbobenzoxyglycine-N-hydroxysuccinimide ester (Fox Chemical Co.) in 10 ml of dioxane which was added slowly with stirring. The product was recrystallized from ethyl acetate, yielding 244 mg, and gave a single spot when chromatographed on silica gel in three thin-layer solvent systems (methanol–ethyl acetate, 1:2; 2-butanol–acetic acid–water, 100:15:37; 1-propanol–ammonium hydroxide, 7:3 w/v; Eastman 13181 plates). Deblocking was accomplished by hydrogenation in methanol over 10% palladium on charcoal, yielding Gly-Glu-αOMe.

Ethylamine hydrochloride, propylamine, pentylamine, hexylamine, and dimethylamine were from Eastman. Butylamine was from Fisher and methylamine hydrochloride was from Sigma.

β-Alanine, γ-aminobutyric acid, Δ-aminovaleric acid, and ε-aminocaproic acid were from Sigma. The corresponding ethyl esters were prepared by dissolving the acid in cold ethanol saturated with HCl and allowing the reaction to proceed at 4° overnight. The reaction was checked for completeness by thin-layer chromatography on silica gel plates, in 1-butanol–acetic acid–water (100:15:37).

Results

Inhibition by Amino Acid and Peptide Derivatives. The effectiveness of a number of amino acid and peptide derivatives as inhibitors of dipeptidyl peptidase was tested (Table I). The best inhibitors tested were hydrophobic amino acid amides. Basic amino acid amides are less effective. Glutamic acid α-monomethyl ester is a distinctly poorer inhibitor but is still moderately effective when Gly-Gly-OEt is the substrate. Gly-NH_2 and βAla-NH_2 are as effective as Glu-OMe against Gly-Gly-OEt as the substrate and more effective against Gly-Phe-NH_2. These results indicate that specificity of binding of the inhibitors is different from the specificity of the enzyme toward substrates and suggest that a different binding site may be involved.

The effect of blocking of the α-amino group of the amino acid amide is dependent on both the side chain and the blocking group involved. Blocking of either leucinamide or glycinamide by an acetyl group results in a marked de-

Table IV: Inhibition of Dipeptidyl Aminopeptidase by Ω-Amino Acid Esters.

Inhibitor	Chain Length	% Inhibition Gly-Gly-OEt[a]	% Inhibition Gly-Phe-NH_2[b]	K_i (mM)
Glycine ethyl ester	6	20	11	315 ± 45[a]
β-Alanine ethyl ester	7	11	11	N.D.
γ-Aminobutyryl ethyl ester	8	32	5	105 ± 5[a]
Δ-Aminovaleryl ethyl ester	9	24	7	128 ± 12[a]
ε-Aminocaproyl ethyl ester	10	<5	<5	N.D.

[a] The substrate was Gly-Gly-OEt. [b] The substrate was Gly-Phe-NH_2. The assay was the transamidation method. The chain length measured all carbon and oxygen atoms from the amino group to the end of the ester. N.D. indicates not determined.

crease in inhibitory capacity. The benzoylation of the α-amino group of arginine amide results in a slight increase in inhibitory capacity toward Gly-Gly-OEt as the substrate and a distinct increase toward Gly-Phe-NH_2 as the substrate, but Bz-Gly-NH_2 is not an inhibitor.

All the amino acid amides tested by Lineweaver–Burk or Dixon (1953) plots, as indicated by the presence of a K_i in Table I, were competitive inhibitors in agreement with the results reported by Fruton and Mycek (1956). These authors also reported that Phe is not an inhibitor. We have tested a variety of amino acids (Phe, Leu, Arg, Gly, and Glu) and found that none is an inhibitor.

Interaction of Aromatic and Amino Compounds with Dipeptidyl Aminopeptidase. Some simple amines and aromatic compounds were tested for the ability to affect the catalytic efficiency of the dipeptidyl aminopeptidase (Table II). The effectiveness of primary amines as inhibitors is further evidence for the importance of the binding of the amino group of the substrate. Simple aromatic compounds stimulate the activity of the enzyme toward Gly-Gly-OEt and Gly-Gly-NH_2, but have no effect on the activity toward Gly-Phe-NH_2. Inhibition by butylamine or Gly-NH_2 is effective on all three substrates.

The effect of increasing chain length of amino compounds was investigated. Aliphatic amines, Ω-amino acid esters, amino acid amides, and peptide amides are all effective inhibitors. The aliphatic amines varying in size from ethyl to pentyl are of about equal effectiveness as inhibitors (Table III). Methylamine is without effect on Gly-Phe-NH_2 transamidation, while hexylamine is ineffective against Gly-Gly-OEt transamidation. For each inhibitor the K_i was measured with the substrate against which it was most effective, where one substrate is distinctly more effective than the other.

Among the Ω-amino acid esters (Table IV) the situation seems to be more complex. The butyryl and valeryl derivatives are effective against Gly-Gly-OEt, but relatively ineffective against Gly-Phe-NH_2, while ε-aminocaproyl ethyl ester is not inhibitory against either substrate.

When chain length is compared with inhibitory capacity, it appears that there is some consistency among the three series of compounds among chain lengths of three to seven (Figure 1). All the compounds tested in this range have K_i's of 200–400 mM. Compounds of chain length eight to nine are better inhibitors, as reflected by the lower K_i.

Specificity. Several substrates were tested for activity with the beef spleen enzyme. Gly-Arg-NH_2 was shown to be an excellent substrate, being transamidated at 5.3 times

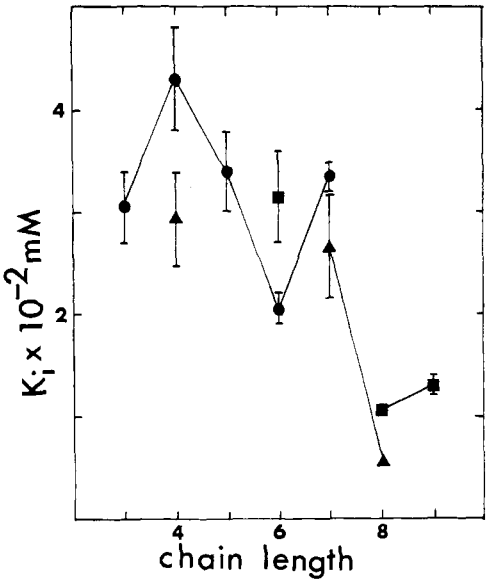

FIGURE 1: The effect of chain length on the K_i of (●) aliphatic amines, (▲) Ω-amino acid esters, and (■) aliphatic amino acid and peptide amides. The K_i's were determined as described in Tables I, III, and IV.

the rate of Gly-Gly-OEt. This is qualitatively consistent with the earlier report by McDonald et al. (1969) that Gly-Arg derivatives are the best substrates for the rat liver enzyme.

In addition, Gly-Glu-OMe was shown to be transamidated at 2.7 times the rate Gly-Gly-OEt. This is the first small synthetic substrate with an acidic residue adjacent to the susceptible bond and confirms earlier findings on the hydrolysis of polypeptides (McDonald et al., 1971).

Discussion

Although the inhibition of dipeptidyl aminopeptidase by amino acid derivatives had been previously reported by Fruton and Mycek (1956), this work was essentially confined to derivatives of hydrophobic amino acids. Since that time this enzyme has been shown to have a broad specificity (McDonald et al., 1969). We have shown (Table I) that a wide variety of amino acid derivatives are capable of inhibiting dipeptidyl aminopeptidase. The specificity of inhibition is unrelated to the specificity of catalysis at the amino acid residue (R_2) adjacent to the amino terminus, the site at which most investigations and discussions of the specificity have been focused (Fruton, 1960; Mycek, 1970; McDonald et al., 1971); i.e., hydrophobic amino acid amides are more effective inhibitors than are basic amino acid amides, although Gly-Arg-NH_2 is a better substrate than Gly-Phe-NH_2.

The importance of the amino group in binding of substrates has been previously noted. The amino group is important in inhibitors as well. The competitive nature of this inhibition suggests that inhibition involves binding of the inhibitor in the R_1 binding site; thus binding involves interactions with both the α-amino group and the amino acid side chain. This is particularly clear in the relatively strong inhibition by Gly-NH_2 and complete lack of inhibition by Ac-Gly-NH_2 and Bz-Gly-NH_2. The sharp decrease in inhibition upon acetylation of Leu-NH_2 further supports this idea, although the amino group is clearly not an absolute requirement for inhibition, as it is for substrate binding.

The blocking of Arg-NH_2 by benzoylation, however, re-

sults in an increased inhibitory capacity. This could result from a misorientation of the inhibitor on the enzyme. The positively charged guanidino group may bind at the α-amino group binding site, while the benzoyl ring may bind at the R_1 binding site. Model building indicates that Bz-L-Arg-NH_2 is capable of folding in such a way as to bring the guanidino group and benzoyl ring into approximately the same spatial relationship as is found between the α-amino group and phenyl ring of Phe-NH_2. Thus, Bz-Arg-NH_2 is able to mimic the best known competitive inhibitor if this orientation does occur. The importance of the α-amino group binding site is, therefore, not negated by the increased inhibition upon blockage of Arg-NH_2 by benzoylation. Bz-Arg has no measurable inhibitory capacity toward dipeptidyl aminopeptidase. That Bz-Gly-NH_2 is not an inhibitor would seem to imply that the binding of the hydrophobic ring is much weaker than the binding of the amino group.

The importance of the free α-amino group in inhibitors is further supported by the finding that primary amines (Tables II and III) and Ω-amino acid esters (Table IV) are inhibitors of dipeptidyl aminopeptidase. The lack of inhibition of hydrolysis of Gly-Tyr-NH_2 reported for β-phenylethylamine by Fruton and Mycek (1956) seems somewhat surprising in view of these results with aliphatic amines. We have tested 4-phenylbutylamine-1 and found that this compound stimulates transamidation of Gly-Gly-OEt 113%, but inhibits activity toward Gly-Phe-NH_2 by 22% when present in the same concentration as the substrate, 50 m*M*. Thus, a single compound is either an inhibitor or a stimulator, depending upon the substrate used. These data suggest that inhibition involves binding of the inhibitor at the α-amino group binding site and the R_1 binding site in the substrate binding area of the enzyme.

Fruton and Mycek (1956) have also reported that Leu-NH_2 does not inhibit dipeptidyl aminopeptidase. These workers concluded that inhibition is favored by the presence of an N-terminal aromatic amino acid. In contrast, we have found that Leu-NH_2 is an excellent inhibitor, and this is more in keeping with the known broad specificity of the enzyme. This apparent disagreement may result from the fact that the preparations available in 1956 were relatively crude when compared with the highly purified preparations available for this investigation.

The stimulation of activity by phenol and pyridine may be due to binding at the site which interacts with the aromatic ring of Gly-Phe-NH_2. Such an interaction could cause a conformational change which results in a more efficient orientation of the active site groups. Such interactions have previously been observed with chymotrypsin (Applewhite et al., 1958; Applewhite and Niemann, 1959) and trypsin (Inagami and Murachi, 1964; Inagami, 1965).

The lack of observed inhibition of Gly-Phe-NH_2 transamidation by aromatic compounds may be a result of the weak interaction of these compounds with the enzyme. Thus, when this substrate is bound, primarily through interaction with the α-amino group, the relatively weakly bound aromatic compound is forced off the enzyme by the aromatic side chain.

The ability of dipeptidyl aminopeptidase to hydrolyze glutamyl bonds has been demonstrated by McDonald et al. (1971) on several polypeptide substrates. These results are confirmed by the finding, presented in this report, that Gly-Glu-OMe is a good substrate for this enzyme. It is now apparent that good substrates can contain either basic, hydrophobic, or acidic residues in the penultimate position and that all these are better substrates than the corresponding glycine compounds, which contain no side chain at this position. It would seem that the binding site for this side chain contains a hydrophobic region, a positive charge, and a negative charge if an attractive force exists for each type of substrate side chain. Such a binding site would appear to be extremely unlikely. This suggests that there must be some other role for these bulky side chains. One possibility is that the primary interaction between enzyme and substrate occurs at the α-amino group; that this interaction forces the bulky side chain on the N-penultimate residue into the enzyme surface causing a shift in the spacing of the catalytic site groups into a spatial relationship which is more effective for catalysis. The role of the N-penultimate side chain is a passive one in this mechanism in that there is little attractive force for the enzyme. This mechanism would explain why phenol results in a 35% stimulation of activity toward Gly-Gly-NH_2, although Gly-Phe-NH_2 is a 1700% better substrate than Gly-Gly-NH_2.

References

Applewhite, T. H., Martin, R. B., and Niemann, C. (1958), *J. Am. Chem. Soc. 80,* 1457.

Applewhite, T. H., and Niemann, C. (1959), *J. Am. Chem. Soc. 81,* 2208.

Callahan, P. X., McDonald, J. K., and Ellis, S. (1972), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 31,* 1105.

Dixon, M. (1953), *Biochem. J. 55,* 170.

Fruton, J. S. (1960), *Enzymes, 2nd Ed. 4,* 233.

Fruton, J. S., and Mycek, M. J. (1956), *Arch. Biochem. Biophys. 65,* 11.

Gorter, J., and Gruber, M. (1970), *Biochim. Biophys. Acta 198,* 546.

Inagami, T. (1965), *J. Biol. Chem. 240,* 3453.

Inagami, T., and Murachi, T. (1964), *J. Biol. Chem. 239,* 787.

IUPAC–IUB Commission (1972), *Biochem. J. 126,* 773.

Izumiya, N., and Fruton, J. S. (1956), *J. Biol. Chem. 218,* 59.

Jones, M. E., Hearn, W. R., Freid, M., and Fruton J. S. (1952), *J. Biol. Chem. 195,* 645.

McDonald, J. K., Callahan, P. X., and Ellis, S. (1971), in Tissue Proteinases, Barrett, A. J., and Dingle, J. T., Ed., New York, N.Y., American Elsevier, p 78.

McDonald, J. K., Reilly, T. J., Zeitman, B. B., and Ellis, S. (1966), *Biochem. Biophys. Res. Commun. 24,* 771.

McDonald, J. K., Zeitman, B. B., Reilly, T. J., and Ellis, S. (1969), *J. Biol. Chem. 244,* 2693.

Metrione, R. M., Neves, A., and Fruton, J. S. (1966), *Biochemistry 5,* 1597.

Metrione, R. M., Okuda, Y., and Fairclough, G. F., Jr. (1970), *Biochemistry 9,* 2427.

Mycek, M. J. (1970), *Methods Enzymology 19,* 285.

Neurath, H., and Hartley, B. S (1959), *J. Cell. Comp. Physiol. 54,* 179.

Planta, R. J., Gorter, J., and Gruber, M. (1964), *Biochim. Biophys. Acta 89,* 511.

Voynick, I. M., and Fruton, J. S. (1968), *Biochemistry 7,* 40.

Wiggans, D. S., Winits, M., and Fruton, J. S. (1954), *Yale J. Biol. Med. 27,* 11.