TABLE I. Plate Counts of an *Escherichia coli* Culture at Intervals during Growth, Unfrozen and after 2 Cycles of Freezing and Thawing.

Age of culture (hr)	Unfrozen	Frozen twice	Surviving fraction after freezings (%)
0	33,000	9,300	28.2
1	480,000	238	.05
2	2,500,000	1,320	.05
3	32,000,000	4,070	.01
4	183,000,000	102,000	.06
5	600,000,000	44,000,000	7.3
6	620,000,000	162,000,000	26.1
7	950,000,000	73,000,000	7.7
8	760,000,000	72,000,000	9.5
9	650,000,000	102,000,000	15.7

which occur during the growth cycle are poorly understood it does not seem profitable to speculate on these changes in relation to the mechanism of freezing damage.

Since it is apparent that the phase of growth may have a tremendous influence on the susceptibility of bacteria to freezing damage, it is important to consider this factor in the interpretation of freezing damage data.

Summary. The sensitivity of *Escherichia coli* to freezing damage varies during the growth cycle with maximum susceptibility during the period of logarithmic growth.

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Hydrolysis of Amino Acid Amide Derivatives of 2-Aminofluorene, 4-Aminobiphenyl and 4, 4'-Diaminobiphenyl by Leucine Aminopeptidase.* (22524)

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It has been established that carcinogenic amines, such as 2-aminofluorene, 4-aminobiphenyl and 4,4'-diaminobiphenyl (benzidine), combine *in vivo* with tissue proteins or protein precursors(1-3). Although the exact mode of combination with proteins is unknown, Greenstein, *et al.*(4) have suggested that the binding may involve a peptide bond between the amino group of the amine and a carboxyl group of the protein or protein precursor. Moreover, these investigators have demonstrated that peptide derivatives, in which the carcinogenic amine is linked to the carboxyl group of different amino acids, are susceptible to hydrolysis by tissue homogenates. Although the enzyme or group of enzymes responsible for the hydrolytic action has not been identified, it is reasonable to assume on the basis of

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TABLE I. Rates of Hydrolysis of Certain Amino Acid Amides. 0.05 M substrate was incubated at 40°C for 4 hr in presence of 0.06 M Tris [tris(hydroxymethyl)aminomethane] buffer at pH 8.5, 0.002 M $MnCl_2$, 60% methanol and 1.3 γ protein N/ml of aminopeptidase. The enzyme was activated for 30 min. under the same conditions in absence of substrate(5). AF = 2-aminofluorene, BP = 4-aminobiphenyl, Bz = benzidine.

Substrate	C_1
L-Leucinamide	11.
L-Norvalyl-AF	.14
L-Isoleucyl-AF	.026
L-Valyl-AF	.015
L-Phenylalanyl-AF	.01
Glycyl-AF	.01
L-Norvalyl-BP	.135
D-Norvalyl-BP	0
L-Isoleucyl	.01
L-Valyl-BP	.01
L-Norleucyl-BP	.028
L-Tryptophyl-BP	.015
D-Norleucyl-BP	0
L-Norleucyl-AF*	+++++
L-Phenylalanyl-BP*	++
D-Phenylalanyl-BP*	0
Glycyl-BP*	++
Di-L-Alanyl-Bz*	+++
Di-Glycyl-Bz*	±

* These compounds were too insoluble to permit determination of an approximate C_1 value. Apparent extent of hydrolysis could be estimated qualitatively by observing amount of amino acid detected after chromatographic analysis of the reaction mixtures.

the known requirements for the specificity of leucine aminopeptidase(5), that this enzyme could hydrolyze the carcinogenic amine-peptide bond. All tissue homogenates used by Greenstein, *et al.*(4) are known to contain this enzyme(6). It is the purpose of this paper to present data on the hydrolysis of several amino acid amide derivatives of carcinogenic amines by leucine aminopeptidase.

Methods. The leucine aminopeptidase used in this study was prepared from swine kidney essentially as previously described(7). The derivatives of the carcinogenic amines were prepared by Dr. J. P. Greenstein and his associates(4). We wish to express our thanks to them for their courtesy and generosity in furnishing samples for this study.

Activation of the aminopeptidase was performed essentially as described earlier(5). However, the insolubility of most of the amino acid amide derivatives in water prevented studying rates of hydrolysis under the usual conditions. Since the compounds are more soluble in methanol, 60% methanol was used for study of the enzymatic reaction. The effect of methanol on the rate of hydrolysis of amino acid amides by the aminopeptidase has been studied(8).

Results. Table I shows that all of the L-isomers tested are hydrolyzed by the enzyme although the rates are slow when compared with the sensitive substrate, L-leucinamide. When possible, proteolytic coefficients (C_1) were determined for each of the substrates by the titrimetric procedure of Grassmann and Heyde(9) in order to obtain at least a rough comparison of the susceptibility of each amide to the aminopeptidase. (C_1 is the first order velocity constant k_1 per mg protein N per ml calculated in minutes and decimal logarithms). Like the unsubstituted amides, those compounds with aliphatic side chains are more susceptible to hydrolysis than those with aromatic side chains(5). However, some reservation should be made in considering the relative rates of hydrolysis, since the degree of solubility of each compound in 60% methanol is different and would influence the rate of hydrolysis. Furthermore, methanol does not inhibit hydrolysis of all amides to the same extent(8). L-Norvalylaminofluorene appears to be hydrolyzed at a greater rate than the L-norleucine derivative, which is unexpected on the basis of previous specificity studies(5). However, the norvaline compounds are completely soluble in 60% methanol whereas the norleucine compounds are only partly soluble. For the enzyme preparation used, the C_1 for L-leucinamide was 60 in water whereas it was 11 in 60% methanol. In order to confirm the titrimetric results shown in Table I, each reaction mixture was examined for free amino acids after completing the experiment. Excess substrate was removed from 0.75 ml of each reaction mixture by addition of an equal volume of water. The mixture was centrifuged, the supernatant solution acidified to pH 2 and treated with Dowex-50-X5 (20-40 mesh) ion exchange resin in the H^+ cycle for adsorption of the free amino acids(10). After washing the resin thoroughly, the amino acids were eluted with 5N NH_4OH and the eluates con-

TABLE II. Inhibition of Leucine Aminopeptidase by Benzidine, 4-Aminobiphenyl and 2-Aminofluorene. Each reaction mixture contained 0.05 M leucinamide, 0.06 M Tris buffer at pH 8.5, 0.002 M $MnCl_2$, 30% methanol, 0.29 γ protein N/ml of aminopeptidase, and inhibitor at the concentration indicated. The enzyme was activated under the same conditions for 30 min. in absence of substrate.

Inhibitor	Inhibition (%)			
	.001 M	.005 M	.01 M	.05 M
Benzidine	5	8	22	30
4-Aminobiphenyl	20	20	23	25
2-Aminofluorene	6	—	14	25

centrated to dryness *in vacuo* over concentrated sulfuric acid. The eluted amino acids were then chromatographed on Whatman No. 1 filter paper in butanol-acetic acid-water (200-30-75) for 48 hours. The papers were dried and sprayed with a ninhydrin solution. The chromatograms showed only the amino acid present in the original amide derivatives. No free amino acids could be detected in control reaction mixtures incubated without aminopeptidase under the same conditions.

Further confirmation for enzymatic hydrolysis was obtained from the green-blue color which developed on addition of the reaction mixtures to the ion-exchange resin. Because of the oxidation of Mn^{++} to MnO_2 in the reaction mixture during hydrolysis, as well as the presence of free amine, conditions were favorable for the oxidation of the amines to benzidine blue and related compounds(11). Control reaction mixtures did not give any color nor did those compounds containing the D-isomers, which were resistant to enzymatic hydrolysis. Qualitatively, it appeared that those compounds hydrolyzed most rapidly also produced the most color. Under proper conditions it might be feasible to use this reaction for a quantitative estimation of free carcinogenic amine.

Since product inhibition of the aminopeptidase by the carcinogenic amines could influence the hydrolysis, each amine was tested for its inhibitory properties. The results are shown in Table II. It is seen that only at high concentrations of amine was there any significant inhibition. It is probable that any inhibition caused by free amine did not significantly affect the observed rates of hydrolysis, although the inhibition might account for the fall in velocity constants observed in some cases.

Discussion. The observations of Greenstein, *et al.*(4) have demonstrated that amino acid amide derivatives of certain carcinogenic amines are hydrolyzed by the enzymes of a number of animal tissues. The present study shows that a highly purified peptidase, namely leucine aminopeptidase, can catalyze the hydrolysis of such compounds. Although the present results do not exclude the possibility that other tissue enzymes may also catalyze this reaction, it is obviously unnecessary to assume the presence of novel enzymes to explain the hydrolysis of the amino acid amide derivatives of the carcinogens. Unfortunately, it is difficult to obtain a strictly quantitative comparison of the sensitivity of these compounds to the aminopeptidase, largely because of their insolubility in water. The results obtained in 60% methanol yield only approximations both of the absolute and relative rates of hydrolysis. Studies, to be published elsewhere(8), have demonstrated that the inhibition of the aminopeptidase by methanol and other alcohols varies with different substrates, the greatest degree of inhibition being obtained with the most sensitive compounds. For example, although Table I indicates that the L-norvalyl derivative of aminofluorene is hydrolyzed 14 times more rapidly than the glycyl analog, it is very likely that they differ in susceptibility by a factor which is at least 10 times greater. Nevertheless, the present results are in general accord with the known specificity of the enzyme, derivatives of the large aliphatic amino acids being more sensitive than the aromatic ones or the small aliphatic ones, glycine and alanine. In addition, derivatives of the D amino acids are completely resistant to the action of the enzyme, in agreement with many earlier studies(5,6).

Summary. Amino acid amide derivatives of the carcinogenic amines, 2-aminofluorene, 4-aminobiphenyl and 4,4′-diaminobiphenyl (benzidine) are hydrolyzed by highly purified leucine aminopeptidase of swine kidney. Because of the poor solubility of these com-

pounds in water, the studies were performed in 60% methanol. The results are in general accord with the known specificity of the aminopeptidase.

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Sequential Assays of 4 Amino Acids in Grains of Wheat, Rye, and of Their Hybrid.* (22525)

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The chemical characterization of organisms in ways that may be related to their biological evolution may be taken as a primary objective of comparative biochemistry. A review of peptide analysis(1) listed characterization of proteins for biological differentiation as a principal discernible goal, along with elucidation of amino acid residue sequence. Qualities of an ideal N-terminal agent were also prescribed. The agent with many of these attributes has proved to be fluorodinitrobenzene (FDNB), which has been successfully applied to problems of structure(2). FDNB has received somewhat less use in characterization(3). It has become apparent that a more powerful mode of characterization might be obtained from a method employing quantitative sequential analysis. It therefore proved to be desirable to employ phenylisothiocyanate(4) subtractively(5). Many data have been accumulated by this procedure. A hybrid of wheat and rye(6) afforded an opportunity to investigate chemical characterization as related to biological type. For this purpose, subtractive quantitative sequential peptide analysis was applied to seeds of wheat-rye hybrid (*Triticale*) and to its parents.

For the grains of corn, wheat, and rye, most amino acids have been found not to differ significantly in total content or in proportion in N-terminal and N-penultimate positions (7). As wheat and rye seeds, however, were significantly different in their quantitative contents of total and N-terminal lysine, lysine was chosen for simultaneous assay in wheat, rye, and the hybrid. Assays of leucine were also performed as a basis of comparison inasmuch as the differences in leucine content appeared to be significant. It was furthermore of interest to check on the C-terminality of leucine. Inasmuch as lysozyme is known to contain N-lysine(8,5) and C-leucine(9,10), and the cereals contain N-lysine(7), such analyses permit comparison of whole cereal protein with lysozyme. Methionine and phenylalanine were also studied.

Materials and methods. The parent strains were *Triticum aestivum* and *Secale cereale.* The hybrid, *Triticale,* was generously sup-

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