2-Carboxyl-5-(2-thenyl)-penta-2,4-dienoic Acid.—This substance was prepared by the procedure described above in which 19.5 g. of 2-thiophenaldehyde, 48.5 g. of the ester, 37.8 g. of potassium hydroxide and 200 ml. of methanol was used. Acidification gave the product as a yellow solid which, after recrystallization from acetic acid, melted at 211–212° dec. and weighed 11.1 g.

Anal. Calcd. for $C_{10}H_8O_4S$: C, 53.56; H, 3.60. Found: C, 53.91; H, 3.60.

Reduction of this substance to the corresponding saturated derivative could not be effected under 2 atmospheres of hydrogen pressure using platinum as a catalyst.

2-Carboxyl-5-(2-furyl)-penta-2,4-dienoic Acid.—Application of the above-described reaction conditions to a mixture of 43.0 g. of potassium hydroxide, 250 ml. of methanol, 19.2 g. of furfural, and 55.8 g. of the ester gave 9.53 g. of red-brown solid, m.p. 197–198° dec. Further purification afforded a tan sample having the same melting point. This substance absorbed four mole-equivalents of hydrogen (platinum, ethanol) at 30° and 2 atmospheres pressure.

Anal. Calcd. for $C_{10}H_8O_5$: C, 57.69; H, 3.87. Found: C, 58.29; H, 4.00.

Subsequent reactions with this material proceeded anomalously in several respects and will be described in a later report.

6-(Substituted)-aminopurines.—The appropriate amines were condensed by the same general procedure whereby one part of 6-methylthiopurine was mixed with 2 to 5 parts of the corresponding amine, and the reaction mixture was sealed in a micro Carius tube and heated at 130 to 140° for 15 to 18 hours. At the conclusion of the heating period, the cooled bomb was opened carefully with an oxygen torch. The liberated methylmercaptan was immediately evident as a foul smelling by-product. In several instances a precipitate was present at this stage which could be washed with cold alcohol, dried, and analyzed directly. In other experiments the solvent had first to be removed under reduced pressure to yield a mass of crystals which, after treating with charcoal, and cooling, yielded the desired product. The yields varied considerably among the different amines; however, this may have been due more to solubility factors than to extent of the reaction, since the more soluble products were difficult to isolate in the small scale experiments that were conducted. Specific reaction conditions are itemized in Table I for each of the amines studied. The extent of purity of the reaction product could be ascertained readily by determining its ultraviolet absorption spectrum. 6-Methylthiopurine has a characteristic double peak at about 283 and 290 mμ (10 γ/ml. in 95% ethanol), which is absent in the 6-(substituted)-aminopurines. The 6-(substituted)-aminopurine spectra were almost identical in possessing a λ_{max} from about 267–271 mμ when examined at concentrations of 10 γ/ml. in 95% ethyl alcohol.

AUSTIN, TEXAS

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF IOWA STATE COLLEGE AND THE CHEMISTRY DEPARTMENT AND THE OCEANOGRAPHIC INSTITUTE OF THE FLORIDA STATE UNIVERSITY]

Recoverability of Phenylthiohydantoins from Amino Acids[1]

BY SIDNEY W. FOX,[2] WILLIAM SERAT,[3] THOMAS L. HURST, ONNOLEE UNDERWOOD TRAPP AND DONNA LUTJENS

RECEIVED DECEMBER 8, 1956

Factors in the reaction of phenyl isothiocyanate with amino acids and amino acid residues in the analysis of peptides have been studied. Some of the same phenomena which interfere with quantitation of the extractive procedure contribute to the validity of the subtractive procedure. Use of an excess of phenyl isothiocyanate enhances the decomposition of amino acids to other substances.

In the development of methods of peptide analysis the need for convenient determinations of residue sequence and of characterization has been apparent for well over a decade.[4] The characteristics of an ideal N-terminal reagent for sequence assignment were prescribed in 1945.[4] Many of the attributes of such a reagent were found in fluorodinitrobenzene (FDNB) which has been successfully used by Sanger,[5] du Vigneaud,[6] Craig[7] and others.

In the development of more highly quantitative methods, of stepwise methods, and of procedures for characterization, a reagent of choice,[8] however, appears to be phenyl isothiocyanate[9] (PTC). Direct identification of the extracted phenylthiohydantoin (PTH) formed by fission of the reaction product of phenyl isothiocyanate with protein or peptide nevertheless poses procedural difficulties. Recoveries are usually low[10] (typically 20–40%), although higher recoveries have been attained.[11]

Probably the greatest error in the direct extraction procedure is introduced by destruction of the phenylthiohydantoin by the same mineral acid used for its formation. Such an effect was evident in the earliest experiments with phenyl isothiocyanate in this Laboratory.[12] This picture of simultaneous formation and destruction of PTH has been mentioned,[8] confirmed in detail[10,11] and is in accord with the known behavior of hydantoins.[13] The probability that destruction of PTH was occurring in the presence of mineral acid first led, indeed, to use of a subtractive method. The subtractive method consists of amino acid assays of an hydrolyzate followed by subsequent hydrolyses and assays after the terminal residues are successively removed. If the PTH formed decom-

(1) Journal Paper No. J-3087 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project No. 863, supported by Grants G-3025 and G-3025(C) of the National Institutes of Health, Public Health Service and a grant from the General Foods Corporation. Contribution No. 80 of the Oceanographic Institute, The Florida State University.

(2) Chemistry Department and Oceanographic Institute, The Florida State University, Tallahassee.

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poses substantially to substances other than the original amino acid, the subtractive method would nevertheless yield the desired quantitative result. Experiments with synthetic peptides therefore emphasized finding conditions which would completely destroy N-terminal residues. The results indicated especially a need for a relatively high PTC/peptide ratio for quantitative loss of the N-terminal residue.[12]

These considerations emphasized the desirability of obtaining an evaluation of the extent of utilization of the PTHs following typical hydrolytic treatment and of the survival of the amino acids after the full analytical treatment with excess PTC.[12] Data on recovery from peptides have been provided for some of the amino acid residues.[12] In the absence of a roster of peptides representing all types of N-termini, free amino acids were carried through the treatment (Table IV).

Experimental and Results

The utilization of the PTHs from each of thirteen amino acids is reported in Table I. The PTHs were prepared as described by Edman.[9] In order to bring these into solution it was necessary to dissolve the compounds in ethanol and in some cases to autoclave promptly the resultant 5% ethanolic solution containing PTH and basal medium. The control and assay solutions contained the same proportion of ethanol.

TABLE I

UTILIZATION OF THIRTEEN PHENYLTHIOHYDANTOINS FROM AMINO ACIDS IN MICROBIAL ASSAY OF THE CORRESPONDING AMINO ACID

Amino acid as PTH	Assay organism	Assay titration values in ml. 0.05 *N* NaOH: Blank	PTH	Amino acid
Alanine	*L. citrovorum*	4.64	4.63	5.76
Arginine	*S. faecalis*	1.92	1.97	5.28
Aspartic acid	*L. mesenteroides*	1.56	1.59	3.16
Glutamic acid	*L. arabinosus*	5.42	5.52	6.51
Glycine	*L. brevis*	4.97	4.28	7.71
Isoleucine	*L. arabinosus*	1.02	3.00	5.56
Leucine	*L. arabinosus*	3.73	4.80	8.15
Lysine	*L. mesenteroides*	0.70	0.72	2.36
Methionine	*S. faecalis*	0.12	0.04	4.25
Phenylalanine	*L. brevis*	2.38	2.23	7.98
Proline	*L. brevis*	0.70	0.65	5.28
Tyrosine	*L. brevis*	1.13	1.17	6.40
Valine	*S. faecalis*	0.94	1.00	2.80

For the tests, 2.50 ml. of basal medium[14] lacking only the amino acid under study and including 0.25 ml. of the corresponding PTH in ethanol and 2.25 ml. of distilled water were combined in a culture tube. The amount of PTH chosen was that equivalent to 10 μg. of the amino acid in the final solution. In one set of control tubes, no PTH was included. In another set of tubes, 10 μg. of the amino acid was present. All tubes were prepared in triplicate; the figures given in Tables II–IV are averages. Except for glycine assays, individual values seldom exceeded $\pm 5\%$ variation from the average of triplicates. The tubes were covered with a towel, autoclaved for 15 minutes under 15 lb. of steam pressure, inoculated after cooling and incubated at 37° for 72 hr. The organisms used are given in Table I.

It may be seen that the assay values in the use of PTH were almost the same as in the absence of the PTH except for three cases. These were glycine, isoleucine and leucine. Glycine–PTH appeared to be inhibitory. Although the individual assay values for glycine were the most variable, repetition of the experiment confirmed the finding that the derivative was truly inhibitory. The PTHs utilized were those of isoleucine and leucine.

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TABLE II

UTILIZATION OF ISOLEUCINE-PTH AND OF LEUCINE-PTH AT VARIOUS CONCENTRATIONS OF THE DERIVATIVES

PTH as molar equiv. of L-amino acid Per tube, γ	Utilization, % Isoleucine	Leucine
10	75	45
15	63	43
25	48	36

TABLE III

RECOVERY OF ISOLEUCINE AND LEUCINE FROM THE CORRESPONDING PTH AFTER HYDROLYTIC TREATMENT

Molar equiv. of L-amino acid Per tube, γ	Recovery, % Isoleucine	Leucine
10	28	15
15	27	13
25	26	13

TABLE IV

RECOVERY OF AMINO ACIDS FOLLOWING N-TERMINAL ANALYTICAL TREATMENT WITH EXCESS PHENYL ISOTHIOCYANATE AND HYDROLYSIS

Amino acid	Recovery, %
Alanine (cx)	4.4, 9.7
Arginine (fr)	5.5, 2.0
Aspartic acid (mq)	6.8, 12
Cystine (aq)	6.8, 2.0
Glutamic acid (aVI)[a]	10.3, 12
Glycine (bt)	2.8, 5.5, 5.7
Histidine (mr)	5.4, 5.7
Isoleucine (aq, fq, fq)	4.4, 1.7, 1.4
Leucine (aq, fq, aq, fq)	4.1, 2.2, 5.7, 4.4
Lysine (mq)	2.6, 2.5
Methionine (fq, fq)	11.6, 5.3
Phenylalanine (bt)	1.5, 2.6
Proline (bt)	21, 20, 20
Serine (my)	2.4, 1.2
Threonine (fz)	1.8, 1.4
Tryptophan (aq)	4.5, 1.6
Tyrosine (bq)	3.5, 3.3
Valine (fq)	1.1, 0.0

[a] VI in B. F. Steele, H. E. Sauberlich, M. S. Reynolds and C. A. Baumann, *J. Biol. Chem.*, **177**, 533 (1949). Letter combinations of organism and medium are given in S. W. Fox, T. L. Hurst and C. Warner, THIS JOURNAL, **76**, 1154 (1954). Multiple symbols correspond to each determination; a single pair of symbols indicates the combination was used in all tests.

Accordingly, the PTHs were tested in a range of concentrations against standard curves for isoleucine and leucine. The results are presented in Table II.

In order to test the effect of the hydrolytic treatment on the PTH, samples of the leucine-PTH and isoleucine-PTH equivalent to 100 μg. of the L-amino acid per ml. of ethanol were each added to an equal volume of 12 *N* hydrochloric acid and autoclaved at 15 lb. steam pressure for 16 hr. These solutions were concentrated to dryness and dissolved in ethanol and prepared for assay as before. Results are given in Table III.

The recoveries of amino acids following full treatment of the type employed in analyses on peptides and proteins are given in Table IV. The procedure employed in these tests was to pipet 2.0 ml. of solution containing 2.00 mg. of standard L-amino acid into a 50-ml. beaker, to add 2.5 ml. of pyridine and 0.025 ml. of phenyl isothiocyanate and to add a small amount of brom thymol blue powder. To this was added sufficient 0.2 *N* sodium hydroxide to give an emerald green color. The beaker was covered and set in a 37° incubator for 6 hr., with maintenance of the original alkalinity by frequent additions of sodium hydroxide. The beakers were evaporated to dryness in evacuated desiccators containing sulfuric acid. When dry, 5.0 ml. of 3 *N* hydrochloric acid was added to each and the sample was auto-

claved at 15 lb. pressure for 16 hr. The hydrochloric acid was driven off over steam, and the beaker was set in a desiccator containing also a beaker of ammonium hydroxide to neutralize any free acid. The contents were then diluted to volume for assay.

The results in Table V were obtained in the same way as for Table IV, except for the quantities used. To 50 mg. of each amino acid were added 1.0 ml. of water, 2.0 ml. of pyridine and the designated volume of PTC.

TABLE V

RECOVERY FROM 50 MG. OF LEUCINE OR LYSINE AFTER TREATMENT WITH VARIOUS AMOUNTS OF PHENYL ISOTHIOCYANATE (PTC)

Amino acid	Amount of PTC 0.1 ml., %	0.5 ml., %	1.0 ml., %	Lysozyme standard assay L-amino acid
DL-Leucine	6.6 ± 0.4	3.5 ± 0.3	2.1 ± 0.2	6.5 ± 0.3
	6.3 ± .1	3.2 ± .2	2.0 ± .2	6.3 ± .3
L-Lysine	1.9 ± .1		1.0 ± .1	5.9 ± .1
	1.6 ± .1		1.2 ± .1	5.8 ± .2
	2.0 ± .1		1.3 ± .1	5.9 ± .2

Discussion

The results of Table I show that direct utilization does not occur for most amino acid PTHs. A more extensive study with benzoylamino acids[15] revealed a similarly highly selective pattern for an acylamino acid series. These results are of interest for comparison in that *L. arabinosus* was able to use benzoylleucine but no other benzoylamino acid in substantial proportion, with the partial exception of cystine. The study with benzoylamino acids showed also that *S. faecalis* could not make use of benzoylleucine. In any analytical situation in which it might be significant, utilizability of leucine-PTH or isoleucine-PTH might thus be circumvented by employing another assay microbe. As will be seen, this need probably does not exist in the present type of application (Table IV).

The results of Table II reveal a commonly observed phenomenon, that of decreasing utilization with increasing concentration of a limiting nutrient. Tables II and III taken together demonstrate that in those few cases in which a derivative is utilizable most of this is lost by hydrolytic treatment. These results also help to explain low recoveries recorded in the literature for the extractive Edman procedure.

In evaluating effects in the steps of the subtractive procedure, it should be emphasized that this differs particularly in employing a large excess of PTC. The fact that higher ratios of PTC to glycylvaline result in more complete subtraction of N-terminal glycine[12] demonstrates that these considerations apply to peptides as well as to unsubstituted amino acids (Table IV). In addition such results support the conclusion that excess PTC contributes to destruction of terminal residue.

The ideal type of experiment in which to estimate the quantitative non-recoverability of an N-terminal amino acid residue would involve reaction with PTC of a variety of peptides for each of the residues in the N-terminal position. Some such experiments have been performed,[11,12] but the comprehensive completion of such a study is at present infeasible. Accordingly, each of the amino acids has been carried through such a process, as has been done for the companion C-terminal method,[16] to gain further insight into the reliability of the method. In addition, extended studies comparing concordance of C-terminal exposures and N-terminal exposures during proteolysis provide an independent type of check.[17] When the values of Table II are compared with the low recovery of valine following treatment with *excess* PTC and then hydrolysis as in Table V, it becomes evident that the treatment itself can lead to degradation of the N-terminal residue. Although the mechanism is not understood, this difference in behavior can explain how recoveries of extracted PTH can be incomplete whereas the subtractive method can yield quantitative results within the limits of error of assay.

With the partial exception of proline all of the recoveries in Table IV are essentially negligible within the limits of error of microbial assay. The loss of amino acid is more nearly complete in the N-terminal method than in the companion C-terminal procedure.[16] (In addition, the large difficulties encountered with glutamic acid, aspartic acid and proline at the C-terminus are not observed with the PTC procedure.) The destruction of N-proline in a peptide is more complete than in the amino acid.[12] The greater destructibility in a peptide, when superposed on incomplete utilizability of whatever PTH is formed (Table I) imparts to the subtractive method maximum validity.

The effect of varying ratio of PTC/amino acid also has been studied for lysine and leucine. The results of Table V show a trend similar to that observed with peptides.[12] The results in this table were controlled by simultaneous assay of lysozyme. The ± figures are standard deviations calculated from quadruplicate determinations at each of five levels of assay.

Among principal advantages of the subtractive PTC method are (1) that it functions by virtue of some factors which incidentally interfere with the quantitation of the extractive method and (2) the applicability to studying unfractionated mixtures of peptides[17] in a stepwise manner.[18] The stepwise nature of the method permitted distinguishing unfractionated and unfragmented cereal proteins containing a dominant lysine–arginine sequence[19] from lysozyme containing a lysylvalyl sequence.[8] This quality is particularly needed in a method employed for characterization of unfractionated proteins. A number of such applications have been reported[17–21] as have other advantages and limitations of the method.[17,22]

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