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CONTRIBUTION OF HYDROLYZED NUCLEIC ACIDS AND THEIR CONSTITUENTS TO THE APPARENT AMINO ACID COMPOSITION OF BIOLOGICAL COMPOUNDS

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SUMMARY

Acid hydrolysis of protein-free mixtures of nucleotides, nucleosides, and nucleic acids yields amino acids, free bases, and possibly other unidentified fragments when analyzed by thin-layer chromatography and by standard amino acid analysis. Glycine is the predominant amino acid detected, which may constitute 47-97% of the apparent amino acid composition, depending on the type of material subjected to hydrolysis. Obviously, hydrolyzed nucleic acids or their constituents can therefore contribute to the apparent amino acid composition of a supposedly pure peptide or of other more complex mixtures of compounds mistakenly believed to contain only protein. To circumvent this problem, we suggest that nucleotides or nucleic acid moieties should be removed from any product for which the amino acid composition is desired, and that whenever a large glycine peak is noted in a hydrolyzed sample, the presence of nucleic acids or their constituents should be suspected.

INTRODUCTION

Since the original report of Spackman et al. (1) which described a two-column chromatographic procedure for the analysis of amino acids and related compounds normally present in physiological fluids, several modifications of the method have been published (2-10). The various procedures differ with respect to the buffers, number and type of columns, and type of amino acid analyzer employed, but all have certain aspects in common. For example, they all employ ninhydrin or hydrindantin as a chromophore and require that peptides or other polymers be hydrolyzed, usually in concentrated acid under reduced atmospheric pressure for an extended period of time (generally up to 24 hr). In many laboratories the amino acid analyzer is employed to analyze a wide variety of samples, ranging from pure proteins through the spectrum of complex physiological fluids such as human urine, spinal fluid, and plasma. It is known

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that nucleotides, nucleosides, and nucleic acid bases are present in such complex physiological samples. For example, at least 45 different purine and pyrimidine derivatives are found in normal human urine (11).

Previously, when analyzing a chromatographically purified subfraction of the dialyzable portion of human leukocyte extracts (MW <20,000) shown later to contain hypoxanthine as the major component (12), one of us noted that when the fraction was subjected to acid hydrolysis and standard amino acid analysis, more than 90% of the material contained therein eluted as glycine (13). The present study documents that when protein-free mixtures of nucleotides, nucleosides, free bases, tRNA, and/or DNA are hydrolyzed, glycine and several other free amino acids are obtained. This indicates that the results noted previously (12,13) are not unique to hypoxanthine. Our findings have obvious implications in regard to the routine amino acid analysis of supposedly pure peptides or more complex mixtures of compounds.

MATERIALS AND METHODS

Materials. Calf thymus DNA was purchased from Worthington Biochemicals. Nucleotides, nucleosides, purines, pyrimidines, and glycine and other amino acids used were purchased from Sigma Chemical Co. Yeast transfer RNA (tRNA) was obtained from Bethesda Research Laboratories. Cellulose thin-layer chromatography (TLC) plates (plastic backed with or without fluorescent indicator) were purchased from Eastman Kodak.

Methods. Ascending thin-layer chromatography was carried out with methanol:HCl:H_2O (70:20:10 V/V/V) as the solvent. The thin-layer plates were exposed to short-wave (254 nm) ultraviolet light (UV) for photography (Type 665 Polaroid film).

For amino acid analysis, samples were hydrolyzed for 16 hr at 110°C in 6 N HCl with 0.1% phenol *in vacuo* (less than 30 microtorr) as described previously (14). Analyses were carried out in a Durrum D-500 amino acid analyzer. Aliquots of acid-hydrolyzed material were saved in each case for analysis by thin-layer chromatography and amino acid analysis. Compounds with free amino groups were detected by spraying TLC plates with a solution of 0.3% (W/V) triketohydrindene hydrate (ninhydrin) in acetone containing 0.5% (V/V) pyridine and then heating them in an oven for 60 min at 60°C. The color was developed further by incubation of the plates in the dark overnight at 25°C. N-protected compounds were detected by the method of Nitecki and Goodman (15), which involves N-halogenation with chlorine (clorox) followed by staining with O-tolidine in 10% (V/V) glacial acetic acid in water. This method detects all compounds containing NH-CO groups, as well as most compounds containing amines. When the plates were to be sprayed with both ninhydrin and clorox, ninhydrin was always used first.

RESULTS AND DISCUSSION

Table 1 shows the "apparent" amino acid content obtained when acid-hydrolyzed nucleic acids or mixtures of nucleotides, nucleosides, and free bases (purines and pyrimidines) were analyzed in the amino acid analyzer. The values shown are the computer-derived percent compositons. The "apparent" compositions varied from run to run, depending on (a) the amount of material put through the column (i.e., very small peaks were not computed and thus did not contribute to the calculated compositions), and (b) the extent of hydrolysis. From Table 1, for example, it can be seen that merely doubling the amount of DNA from 20 μg for the first run to 40 μg for the second run allowed the computer to "see" the additional "amino acid" peaks. In this case the two runs were carried out on material from the same hydrolyzed sample.

Table 1

Apparent Amino Acid Composition of Mixtures Containing Hydrolyzed Nucleic Acid Constituents[a]

Amino acid	Ribonucleo-tides	Ribonucleo-sides	Free bases	Yeast tRNA	Calf thymus DNA	
					1st run	2nd run
CMCS[b]	0.928	--	--	--	--	--
Asp	1.381	2.094	0.708	3.041	5.965	4.056
Thr	1.092	1.050	--	4.570	5.206	3.405
Ser	2.945	3.971	1.402	6.536	8.866	6.094
Glx	0.962	1.107	0.855	2.433	5.703	3.969
Gly	88.53	79.29	97.03	77.47	58.08	47.13
Ala	1.613	2.094	--	3.007	7.133	6.398
Cys	--	2.599	--	--	--	--
Val	--	1.417	--	--	--	--
Ile	--	0.832	--	--	--	1.735
Leu	--	1.184	--	1.094	--	6.766
His	2.543	2.887	--	--	--	--
Lys	--	--	--	--	--	9.976
Arg	--	1.462	--	1.844	9.041	10.47

[a]Acid-hydrolyzed samples analyzed for "apparent" amino acid composition included yeast tRNA, calf thymus DNA (two runs), and mixtures of ribonucleotides (uridine 3'-phosphoric acid, cytidine 3'-phosphoric acid, adenosine 3'-phosphoric acid, guanosine 3'-phosphoric acid), ribonucleosides (uridine, cytidine, adenosine, guanosine), and free bases (uracil. cytosine, adenine, guanine).

[b]Apparent "carboxymethylated cysteine".

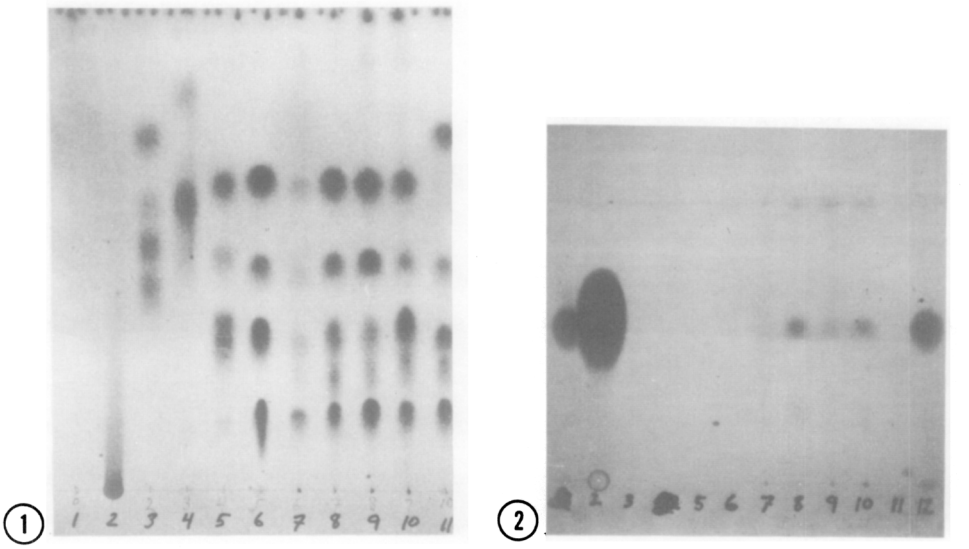

Figure 1. Analysis of acid-hydrolyzed samples by cellulose thin-layer chromatography as observed under short-wave UV. Untreated control samples are numbered 1 through 6: (1) 500 μg glycine; (2) 30 μg yeast tRNA; (3) 5'-nucleoside monophosphates (10 μg G, 20 μg A, 10 μg C, 10 μg U); (4) 3'-nucleoside monophosphates (10 μg G, 20 μg A, 10 μg C 10 ug U); (5) nucleosides (10 μg G, 20 μg A, 10 μg C, 10 μg U); (6) purines and pyrimidines (10 μg G, 20 μg A, 10 μg C, 10 μg U). Acid-hydrolyzed samples (each containing approximately 10 ug per nucleotide moiety) are numbered 7 through 11: (7) yeast tRNA; (8) purines and pyrimidines; (9) nucleosides; (10) 3'-nucleoside monophosphates; (11) calf thymus DNA. In all cases the order of nucleotides, nucleosides, and free bases is G, A, C, and U (in the order of increasing mobility), except that thymine replaces uracil in the case of acid-hydrolyzed DNA. The acid-hydrolyzed adenines appear streaked as two spots in the example shown but usually appear more like the unhydrolyzed control.

Figure 2. Analysis of acid-hydrolyzed samples by thin-layer chromatography and staining with ninhydrin. Untreated control samples are numbered 1 through 6, 11, and 12: (1) 10 μg glycine; (2) 500 μg glycine; (3) 5'-nucleoside monophosphates (10 μg G, 20 μg A, 10 μg C, 10 μg U); (4) 3'-nucleoside monophosphates (10 μg G, 20 μg A, 10 μg C, 10 μg U); (5) nucleosides (10 μg G, 20 μg A, 10 μg C, 10 μg U); (6) purines and pyrimidines (10 μg G, 20 μg A, 10 μg C, 10 μg U); (11) 100 μg yeast tRNA; (12) 10 μg glycine. Acid-hydrolyzed samples (each containing approximately 10 μg per nucleotide moiety or 40 μg per sample) are numbered 7 through 10: (7) yeast tRNA; (8) purines and pyrimidines; (9) nucleosides; (10) 3'-nucleoside monophosphates. The TLC was first viewed by short-wave UV, which confirmed the presence of free bases in the hydrolyzed samples. The plate was then stained with ninhydrin, which revealed the presence of glycine only in the hydrolyzed samples.

When the hydrolyzed mixture of free bases was analyzed with the amino acid analyzer, most of the product was recorded as glycine (Table 1). Yet analysis by TLC using short-wave UV absorption (Fig. 1) showed that significant amounts of the four bases (guanine, adenine, cytosine, and uracil) were still present.

Acid-hydrolyzed nucleotides, nucleosides, DNA, and tRNA also showed all four bases when the TLC plates were viewed under short-wave UV. This finding at first led us to believe that the four bases ran unhydrolyzed as a single glycine peak. However, when an unhydrolyzed mixture of the four bases was passed through the amino acid analyzer, no peaks of material were recorded. The ninhydrin dye used to detect amino acids analyzed by the computerized system analyzer does not stain the free bases, as shown by the absence of ninhydrin staining on analysis of the unhydrolyzed free bases by TLC (Fig. 2). Thus it appears that the conditions of hydrolysis used to digest peptides to free amino acids causes partial digestion of nucleic acids and results in free bases, amino acids, and possibly other unidentified fragments.

In an experiment (not shown) in which acid-hydrolyzed ^{32}P-labeled tRNA was analyzed by TLC, 100% of the labeled phosphorus was released as free phosphate. Thus, in agreement with Mangold (16), it would appear that the sugar-phosphate backbone of nucleic acids is extensively hydrolyzed. In another experiment (Fig. 2), acid-hydrolyzed yeast tRNA, free bases, nucleotides, or nucleosides analyzed by TLC showed only the four bases by UV absorption; however, staining with ninhydrin revealed that the most prevalent amino acid, glycine, was also present and easily detected. We have also visualized both amino acids and free bases simultaneously by analyzing hydrolyzed calf thymus DNA on cellulose TLC plates without fluorescent indicator and staining with O-tolidine after chlorination.

In another experiment, acid-hydrolyzed calf thymus DNA and a set of amino acid standards were analyzed with the amino acid analyzer. The amino acid standards included all of the common amino acids at equal molar ratios. When the mixture of hydrolyzed DNA and amino acids was analyzed, only amino acid peaks (i.e., no new peaks) were observed. The peak ratios, however, were different from those observed when only the amino acid mixture was analyzed, reflecting the additional contribution of the hydrolyzed DNA. Thus, the

hydrolyzed products must be either the common amino acids, such as glycine (Table 1), or other unidentified fragments with identical column retention times.

CONCLUSION AND SUMMARY

Acid hydrolysis of nucleotides and nucleic acids yields free bases, amino acids, and possibly other unidentified fragments. These reaction products contribute to the observed amino acid composition of any mixture containing them that is subjected to analysis using an amino acid analyzer. For this reason, any nucleotide or nucleic acid moieties should be removed from any peptide for which the amino acid composition is desired. This may be a problem in particular when the polymer under study is a ribonucleoprotein, deoxyribonucleoprotein, or nucleic acid - protein complex for which the chemical composition is not known. In practice, whenever a large glycine peak is noted in a hydrolyzed sample, the presence of nucleic acids should be suspected.

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