ACIDIC PEPTIDE CONJUGATES IN MAMMALIAN LIVER

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SUMMARY

Examination of the trichloroacetic acid-soluble fraction of several mammalian tissues has revealed the presence of a variety of peptides that have acidic properties and that are closely associated with material showing maximum absorbance at about 268 mμ. These conjugates contained from two to seven different amino acids. Although the peptides and the materials absorbing u.v. were closely associated during the early stages of the purification, they disassociated in the course of further fractionation. The nature of the linkage between these components remains to be established.

INTRODUCTION

Studies[1] of the kinetics of amino acid incorporation into protein, demonstrating non-uniform labeling, indicate the participation of amino acid conjugates as intermediates in protein biosynthesis, but they provide no information regarding the chemical nature or molecular size of these intermediates. Over the past 3 years we have examined a number of tissue fractions for the possible presence of amino acid conjugates and describe below the occurrence of oligo-peptides in the trichloroacetic acid-soluble fraction of the kidney of the rat and the liver of the rat and the rabbit. Because of the very low concentration of these compounds, definitive characterization has not yet been accomplished. The present communication is in the nature of a preliminary report and includes no evidence implicating these compounds in protein biosynthesis.

METHODS

Tissues were removed, rapidly minced into cold 10% trichloroacetic acid (TCA) and then homogenized either in a Potter-Elvehjem apparatus or in a Waring blendor. The protein precipitate was removed by centrifugation. After extraction of the TCA with ether, the supernatant solution was lyophilized. The dry residue was taken up in a small volume of water. This solution (pH 4–5) was placed on a column of Dowex-50-H^+ ion exchange resin and the material eluted with water was collected

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for further fractionation. This material was lyophilized and stored in the cold as a dry powder pending analysis.

Acid-washed Whatman No. 3 filter paper was used for paper chromatography and electrophoresis. The following chromatographic solvent systems were employed: (a) butanol–acetic acid–water (4:2:1); (b) 70% propanol, 30% water (v/v); (c) 90% methyl cellosolve, 10% water, (v/v); (d) saturated ammonium sulfate–isopropanol–water (79:2:19)[2]. High voltage electrophoresis on paper was carried out as described by MICHL[3] with pyridine–acetic acid buffer, pH 6.0. Fractions separated on paper were eluted with distilled water and, when necessary, concentrated under reduced pressure at room temperature.

Columns (1.2 × 40 cm) of ECTEOLA-formate* were washed with water until the pH of the eluate was neutral. The sample (adjusted to pH 4.5) was applied and the column was washed with approx. 400 ml water until the absorbance of the eluate was less than 0.03 at 260 mμ. Gradient elution was begun with a constant-volume mixing chamber containing 1 l of water that was fed from a reservoir containing 0.2 *M* ammonium formate, pH 4.3. The flow rate was 16 to 20 ml/h. Samples of 5 ml were collected. In the experiment, results of which are summarized in Fig. 1, after 800 ml of eluate had been collected, the solution in the mixing chamber was replaced with 1 l of 0.1 *M* ammonium formate, pH 4.3, and the elution was continued with 0.5 *M* ammonium formate of the same pH in the reservoir. KORN[4] has reported the separation from tissue extracts of several unidentified compounds possessing absorption at 260 mμ using a similar system. Most of the mono-, di- and triphosphates of adenosine, guanosine, uridine and cytidine can be separated by this technique and

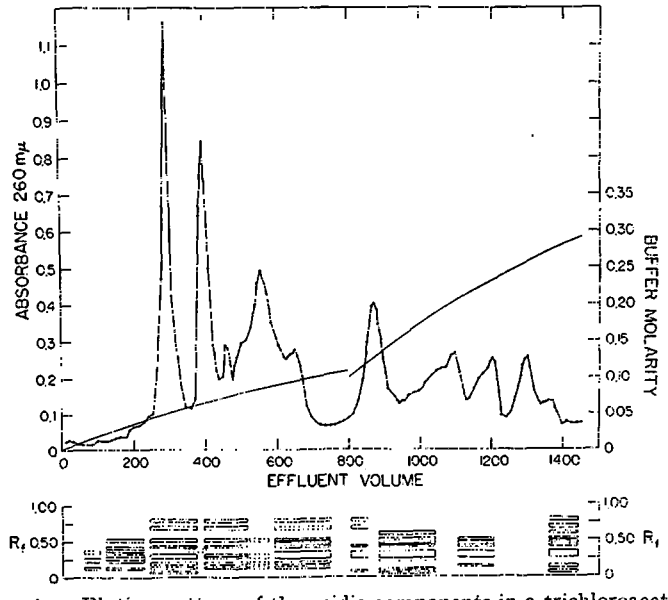

Fig. 1. Upper portion: Elution pattern of the acidic components in a trichloroacetic acid extract of rat liver chromatographed on ECTEOLA-formate and the calculated gradient of buffer molarity. *Lower portion:* Pattern of ninhydrin positive components in paper chromatograms of acid-hydrolyzed aliquots of the column eluate (butanol–acetic acid–water (4:2:1)).

* ECTEOLA-formate was kindly provided by Dr. E. KORN, who prepared it.

at least some of the compounds separated by chromatography of liver extracts and absorbing light at 260 mμ probably represent simple nucleotides.

RESULTS

In a preliminary experiment, data from which are summarized in Table I, the kidneys from a single rat (273 mg protein) were treated as described above. The water eluate from Dowex-50-H^+ was chromatographed on paper using solvent (c). Under u.v. light several quenching bands and several fluorescent bands could be distinguished on the paper. Staining with ninhydrin revealed three bands with distinct blue or purple color which were designated K1, K2 and K3. These three bands and also a ninhydrin-negative band that was fluorescent under u.v. light (K4) were eluted and again chromatographed, this time using solvent (a). K1 and K4 each yielded only a single component. At least 3 distinct bands were identified on the chromatogram of K2. Two of these stained with ninhydrin and one did not. The latter band reacted with the molybdic acid reagent for phosphate and absorbed u.v. light. K3 was resolved into 4 components by chromatography with solvent (a). After acid hydrolysis glutamic acid, glycine, alanine and aspartic acid were identified in all 4 of the components of K3.

TABLE I

CHROMATOGRAPHIC PROPERTIES AND AMINO ACID COMPOSITION OF PEPTIDE CONJUGATES FROM RAT KIDNEY

Band number	*Solvent (c)**	*Solvent (a)**			*Amino acids in hydrolysate***						
	R_F	R_F	*Ninhydrin staining*	*u.v.****	*Glu*	*Gly*	*Ala*	*Asp*	*Leu*	*Val*	*Thr*
K-1	0.13–0.24	0.04–0.12	+	f	±	±					
K-2-1		0.03–0.06	+	f	2+	2+		±			
K-2-2	0.24–0.30	0.06–0.09	+	f	±						
K-2-3		0.09–0.14	—	q	3+	2+		1+			
K-3-1		origin	—	f	3+	3+	2+	2+	±	±	
K-3-2	0.40–0.52	0.02–0.06	+	f	2+	1+	1+	1±	±	±	±
K-3-3		0.06–0.11	+	q	2+	1+	1+	1+			1+
K-3-4		0.24–0.27	—	q	3+	3+	2+	2+	1+	1+	1+
K-4	0.62–0.70	0.20–0.28	—	f	2+	2+	1+	1+			

* See text.

** Glu = glutamic acid; gly = glycine; ala = alanine; asp = aspartic acid; leu = leucine; val = valine; thr = threonine.

*** f = fluorescent band; q = quenching band.

Acid hydrolysis of pure nucleotides is known to give rise to some amino acids, particularly glycine[5], as degradation products. However, the spectrum of amino acids obtained and the yield in relation to the 260 mμ absorbance show that only a small fraction of the amino acids in these hydrolysates could be derived in this way. One of the components contained in addition leucine (or isoleucine), valine and threonine, *i.e.* a total of 7 different amino acids. The hydrolysate of band K4 (after chromatography with solvent (a)) was shown to contain glutamic acid, glycine, alanine and aspartic acid. In addition to these amino acid components there were traces of several others, including some basic amino acids, in the hydrolysates of a number of the fractions.

5 g of rat liver was subjected to a similar fractionation procedure and again paper chromatography revealed a number of components containing conjugated amino acids. An extract of rat spleen yielded a pattern of components resembling that obtained from liver.

To permit more extensive fractionation and characterization, 3 kg of rabbit liver from fasted animals was treated as described above. A sample of the material eluted with water from Dowex 50-H^+, when subjected to electrophoresis for 4 h at approximately 20 V/cm, was found to contain a dozen or more u.v. quenching bands, many of which corresponded, of course, to known nucleoside phosphates present in this fraction. There were in addition, however, a number of acidic components with mobilities greater than those of any of the simple nucleoside phosphates and these components were ninhydrin-positive. One of these fractions with high mobility was eluted for further examination. Paper chromatography with solvent (a) separated 3 components of low R_F, all of which appeared on paper to absorb u.v. light and which stained with ninhydrin. Chromatography with solvent (b) separated 6 bands detected either by absorption of u.v. light or by reaction with ninhydrin. These were eluted and hydrolyzed and the hydrolysates were chromatographed in (solvent (a). Three of these hydrolysates contained a rather broad spectrum of different amino acids, 6, 8, and 9 in number, respectively.

Another portion of the original lyophilized acidic fraction was chromatographed on Dowex-1-formate, using continuously increasing concentrations of formic acid[6]. Thirteen components were separated, all of which possessed high absorbance at 260 mμ. Samples of the material contained in peaks 2, 4, 5, 6 and 8 were chromatographed on paper (solvent (a)). Each yielded one or more components which reacted with ninhydrin or which were detected under u.v. light; fraction 2 contained nine such components. When these were eluted, concentrated to a small volume and rechromatographed with the same solvent, four of the bands exhibited spots which were ninhydrin positive and which absorbed u.v. light. Hydrolysates of each of these contained one or more ninhydrin positive components. Tentative identification was made by comparison with known amino acids run on the same chromatogram. The amino acid composition of these 4 fractions is shown in Table II. Nine components were separated from Fraction 5 by paper chromatography. All of these exhibited strong u.v. absorption and all had similar absorption spectra: maximum absorption at

TABLE II

AMINO ACID COMPOSITION OF ACIDIC PEPTIDE CONJUGATES FROM RABBIT LIVER

Dowex-formate peak	*Chromatographic band (Solvent (a))*	*Amino acids in hydrolysate*
	2–1	Histidine, glycine (lysine)
	2–2	Glycine, alanine
2	2–3	Glycine, proline
	2–9	Glycine, threonine
	5–2	Cysteine, histidine, serine, glutamic acid, threonine, (lysine)
5	5–3	Cysteine, glycine, threonine
	5–4	Glycine, glutamic acid

268 mμ and minimum absorption at 235 mμ. After hydrolysis of an aliquot in 6 *N* HCl, these components were each shown to contain 2 to 7 different ninhydrin positive constituents. These were tentatively identified by comparison with known amino acids chromatographed at the same time. The amino acids found in three of the subfractions of peak 5 are indicated in Table II. Aliquots of a number of these components with both ninhydrin color and u.v. absorption were then rechromatographed using solvent (b)) but the ninhydrin positive moiety now ran with an R_F distinct from that of the u.v. absorbing moiety. Whether this dissociation represents splitting of a labile covalent linkage during processing or whether it indicates that the complex is only loosely associated is not known.

Fig. 1 presents data obtained from chromatography on ECTEOLA-formate of the Dowex-50-H^+ water eluate prepared from a single rat liver (8 g). In this experiment the preliminary steps were carried out as rapidly as possible. Lyophilization was begun less than 2 h after the liver was removed and special care was taken in adjusting the pH to hold it below pH 7.0 at all times. Immediately after drying was complete, the sample was taken up in water and placed on the ECTEOLA column. In the upper portion of Fig. 1 is plotted the absorbance at 260 mμ for each fraction along with the calculated gradient of buffer molarity. After 800 ml of eluate had been collected, the solution in the reservoir was changed to 0.5 *M* ammonium formate, pH 4.3, and the mixing chamber was emptied and refilled with 0.1 *M* ammonium formate of the same pH. At the end of the gradient elution shown in Fig. 1 the column was washed with 200 ml 0.1 *M* ammonium hydroxide and only a very small amount of material absorbing at 260 mμ was eluted, less than in any of the peaks shown in Fig. 1. A 3-ml sample from every third tube was hydrolyzed in 6 *N* HCl for 18 h in an autoclave (110°, 15 lbs.).

After removal of HCl these hydrolysates were chromatographed on paper with solvent (a). The lower portion of Fig. 1 is a diagrammatic representation of the ninhydrin staining compounds separated by this means. The R_F scale is only approximate since in some cases the solvent ran off the end of the paper and so all R_F values were adjusted relative to the movement of known amino acids used as markers on each paper. The amino acids have not been definitely identified since there was distortion in the lower portions of the chromatograms. However, the differences in the pattern of ninhydrin staining components in different fractions is readily apparent. Although only every third tube was examined in this way, the fractions of similar composition have been depicted as continuous on the assumption that the material in the intervening tubes would be qualitatively the same. Since the same pattern extended over wide stretches of the chromatogram, this seems very likely. This exploratory survey of the composition of the water eluate from Dowex-50-H^+ indicates that there are a number of different components containing amino acid in the TCA soluble fractions of rat liver. Chromatography on ECTEOLA appears to be a useful tool for their initial separation under mild conditions[4] with good resolution.

Examination of the TCA-soluble fraction of rabbit liver and rat liver has revealed the presence of a number of low molecular weight amino acid conjugates. These conjugates each contained between 2 and 7 different amino acids and the amino acids occurring in one or more components included: glutamic acid, aspartic acid, glycine, alanine, leucine (or isoleucine), valine, threonine, serine, histidine, proline, and cysteine. The acidic properties of the conjugates is not accounted for by their amino

acid composition. In the early stages of fractionation these amino acid conjugates appeared to have an absorption maximum at 268 mμ, but in later chromatographic steps the ninhydrin positive components separated from the u.v. absorbing components. Since this work was initiated several laboratories[7–9] have reported the presence of nucleotide–peptide complexes in microorganisms. The results described above strongly suggest the occurrence of similar compounds in mammalian tissue. Because of the low yields obtained, however, it has not yet been possible to establish conclusively the chemical nature of the complexes.

REFERENCES

1 D. STEINBERG, M. VAUGHAN AND C. B. ANFINSEN, *Science*, 124 (1956) 389.
2 R. MARKHAM AND J. O. SMITH, *Biochem. J.*, 49 (1951) 401.
3 H. MICHL, *Monatsh. Chem.*, 82 (1951) 489.
4 E. D. KORN, *Biochim. Biophys. Acta*, 32 (1959) 554.
5 D. SHEMIN AND D. RITTENBERG, *J. Biol. Chem.*, 167 (1947) 875.
6 R. B. HURLBURT, H. SCHMITZ, A. F. BRUMM AND V. R. POTTER, *J. Biol. Chem.*, 209 (1954) 23.
7 V. V. KONINGSBERGER, C. O. VAN DER GRINTEN AND J. T. G. OVERBEEK, *Koninkl. Ned. Akad. van Wetenschappen., Proc. Ser. B.*, 60 (1957) 144.
8 A. D. BROWN, *Biochim. Biophys. Acta*, 30 (1958) 447.
9 E. HASE, S. MIHARA, H. OTSUKA AND H. TAMIYA, *Biochim. Biophys. Acta*, 32 (1959) 297.

Biochim. Biophys. Acta, 40 (1960) 225–230

BIOSYNTHETIC INCORPORATION OF FLUOROPHENYLALANINE INTO CRYSTALLINE PROTEINS*

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SUMMARY

1. [^{3}H]OFP and [^{3}H]PFP are incorporated into ovalbumin and into lysozyme, as well as into a mixed tissue protein fraction by minced hen's oviduct incubated *in vitro*. In these purified proteins, the analogue is present as an integral part of the molecule and has been found to be distributed among several peptides separated after partial degradation of the respective proteins.

2. The significance of these and of similar studies demonstrating the incorporation of amino acid analogues into otherwise apparently complete and normal proteins is discussed.

* Preliminary reports of these studies have been published[1,2].

The abbreviations used are: PFP, *p*-fluorophenylalanine; OFP, *o*-fluorophenylalanine; TCA, trichloroacetic acid.